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**Lipocalin 2 (LCN2), the TNF-like receptor TWEAKR and its
ligand TWEAK act downstream of NFAT1 to regulate breast
cancer cell invasion.**

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SUMMARY

NFAT1 is a transcription factor that elicits breast carcinoma cells to become invasive, contributing thus to formation of metastasis. The molecular mechanisms by which NFAT1 operates in this respect are still poorly known. Here, we report that NFAT1 increases Lipocalin 2 (LCN2) mRNA and protein expression by binding to specific sites in the *LCN2* gene promoter region. We show that the LCN2 protein is required downstream of NFAT1 to increase breast cancer cell invasion. We demonstrate that the NFAT1/LCN2 axis is sufficient to regulate expression of the TNF-like receptor TWEAKR at the RNA level and of its ligand, TWEAK, at the protein level. We show, however, that TWEAKR mediates an anti-invasive effect in breast cancer cells whereas, depending on LCN2 expression, TWEAK has either anti- and pro-invasive capacities. Thus, we identify LCN2 and TWEAKR/TWEAK as critical downstream effectors of NFAT1 to regulate breast cancer cell motility and invasive capacity.

Introduction

Metastases are the main cause of morbidity and mortality in breast cancer patients. Their efficient dissemination is directly linked to the invasive behaviour of cancer cells (Friedl and Wolf 2003), which requires the cells to destroy and reorganize the extra-cellular matrix as well as the capacity to migrate. This is a complex process involving many players among which NFAT transcription factors, especially NFAT1, are critical (Jauliac et al. 2002; Yoeli-Lerner et al. 2005).

The family of NFAT transcription factors comprises five genes (*NFAT1* to 5). NFAT1, NFAT2, NFAT3 and NFAT4 were first identified as T cell transcription factors that bind *IL-2* promoter after cell activation (Shaw et al. 1988), while NFAT5 is induced by osmotic stress (Lopez-Rodriguez et al. 1999). Because of their role in critical signalling pathways that control cell fate (Baksh et al. 2002; Chuvpilo et al. 2002; Xanthoudakis et al. 1996), one would expect that disturbing NFAT signalling could impact carcinogenesis. Indeed, beside their role in the migratory and invasive capacities of breast cancer cells (Fougère et al. 2010; Jauliac et al. 2002; Yiu and Toker 2006; Yoeli-Lerner et al. 2005, Germann et al. 2012), there is growing evidence for an active role of NFAT factors in carcinogenesis (Buchholz et al. 2006; Dejmek et al. 2006; Foldynová-Trantírková et al. 2010; Holzmann et al. 2004; Jönsson et al. 2002). However, beside these reports of a “pro-cancer” role, others are showing the “anti-cancer” capacities of NFAT factors (Glud et al. 2005; Lee et al. 2005; Wu et al. 2010). In line with this duality of function, we have reported that NFAT3 is specifically expressed in oestrogen receptor α -positive breast cancer cells and, contrary to NFAT1 and NFAT5, inhibits these cells' motility by blocking *LCN2* (*Lipocalin 2*) gene expression (Fougère et al. 2010).

LCN2 is a secreted protein of the Lipocalin family (Flower 1996), the high expression of which is associated with malignancy in different types of cancers (Bartsch and Tschesche 1995; Furutani et al. 1998; Missiaglia et al. 2004; Nielsen et al. 1996), including breast cancer (Stoesz et al. 1998), and is a predictor of breast cancer progression (Hu et al. 2008). Actually, LCN2 regulates cell migration (Fougère et al. 2010; Leng et al. 2008; Playford et al. 2006) and formation of metastases (Shi et al. 2008; Yang et al. 2009). LCN2 is induced under diverse inflammatory conditions (Nielsen et al. 1996), and it is known that inflammation is strongly linked to tumorigenesis (Balkwill and Mantovani 2001; Iliopoulos, Hirsch, and Struhl 2009; Pierce et al. 2009) and metastasis formation.

Apart from LCN2, other inflammatory molecules, such as those of the TWEAK/TWEAKR cytokine receptor axis, have been linked to cancer. TWEAK (TNF-related Weak Inducer of Apoptosis) belongs to the TNF family and is involved in many diseases (Winkles 2008) including breast cancer (Willis et al. 2008). TWEAKR (TNF-related Weak Inducer of Apoptosis Receptor, Fn14) expression has been observed in breast tumour

71 samples, where it might regulate cancer cell migration (Michaelson et al. 2005; Willis et al.
72 2008). Indeed, the positive role of the TWEAK/TWEAKR axis on cancer cell migration has
73 been reported (Dai et al. 2009; Tran et al. 2006), but there are also reports that TWEAK
74 inhibits migration (Meighan-Mantha et al. 1999). Recently, a preclinical study has shown that
75 a humanized antibody directed against the TWEAKR can mediate anti-tumour effects by
76 signalling through TWEAKR (Culp et al. 2010).

77 Here, we report that signalling through NFAT1 up-regulates LCN2 expression by
78 NFAT1 direct binding on *LCN2* promoter and that LCN2 is required for NFAT1 to foster
79 breast cancer cell invasion. Downstream of NFAT1 and LCN2, we identify the
80 TWEAKR/TWEAK axis as a key player for regulating the invasive process, and show that,
81 depending on LCN2 expression level, TWEAK displays both anti-invasive and pro-invasive
82 functions. Thus, we uncover a novel NFAT1/LCN2/TWEAKR/TWEAK axis critical to
83 regulate breast cancer cell invasion.

Results

NFAT1 up-regulates LCN2 expression in breast cancer cells

Having recently shown that NFAT3 inhibits *LCN2* transcription in breast cancer cells (Fougère et al., 2010), we evaluated here the effect of NFAT1 on *LCN2* expression. However, since NFAT1 and *LCN2* proteins are essentially not expressed in oestrogen receptor-positive (ERA⁺) cancer cells (Fougère et al., 2010), this was done only using ERA-negative (ERA⁻) cells in which, contrary to NFAT3, NFAT1 and *LCN2* are highly expressed. First, we used NFAT1-specific siRNA to silence endogenous NFAT1 in MDA-MB-231, which reduced its expression by 90% (Fig. 1A, right panel) without affecting that of NFAT5 or NFAT4 (data not shown). NFAT1 silencing (which did not result in cell apoptosis as assayed by annexin V labelling data; not shown) reduced by 5-fold *LCN2* mRNA expression relative to control cells (Fig. 1A, left panel), and abrogated both intracellular (Fig. 1B, left panel) and secreted (Fig. 1B, middle panel) *LCN2* protein expression.

We next examined whether transiently transfected ectopic NFAT1 affected *LCN2* protein expression. Because only 10% of cell were actually transfected, empty vector control (Fig. 1B, right panel, vector) and NFAT1 (Fig. 1B, right panel, NFAT1) were cotransfected with a GFP-expressing vector in order to identify transfected cells. Thus, NFAT1 over-expression increased *LCN2* protein expression relative to control cells as assessed by Western blot analysis (Fig. 1B, right panel).

Thus, in contrast to NFAT3 (Fougère et al. 2010), NFAT1 up-regulates both *LCN2* mRNA and protein expression in breast cancer cells.

Downstream of NFAT1, LCN2 is key to increase breast cancer cell invasion

LCN2 plays a critical role in regulating breast cancer cell motility (Fougère et al. 2010). In order to evaluate the physiological relevance of *LCN2* modulation by NFAT1, we tested whether *LCN2* was involved in NFAT1-regulated chemoinvasion. Transiently transfecting MDA-MB-231 cells with HA epitope-tagged *LCN2* (Fig. 1C, left panel) increased breast cancer cell chemoinvasion as compared with empty vector-transfected cells (Fig. 1C, right panel), indicating *LCN2* role in the process. To evaluate whether *LCN2* expression downstream of NFAT1 was needed to regulate chemoinvasion, we transiently co-transfected the cells with T7 epitope-tagged NFAT1 together with either control or *LCN2*-targeted siRNA (Fig. 1D, right panel) and in combination with a betagalactosidase (β -gal)

expressing vector. NFAT1 increased chemoinvasion in the presence of LCN2, whereas silencing LCN2 precluded NFAT1 pro-invasive effect (si LCN2 / NFAT1), as compared with cells transfected with both control and NFAT1 siRNAs (si ctrl / NFAT1), and was inhibitory by itself (si LCN2) (Fig. 1D, left panel). These results indicate that LCN2 expression downstream of NFAT1 is required to regulate breast cancer cell invasion.

NFAT1 binds to the LCN2 promoter region to up-regulate LCN2 expression

To more thoroughly analyse the relationship between LCN2 expression and NFAT1 signalling, we investigated the *LCN2* promoter region where 6 potential NFAT-binding sites (-881, -522, -501, -441, -409, -142) (Fig. 2A) have been identified (Fougère et al. 2010). Using a *Luciferase* gene-fused *LCN2* promoter, we found that NFAT1 ectopic expression doubled the promoter activity relative to empty vector-transfected cells (Fig. 2B). To assess whether endogenous NFAT1 interacted with *LCN2* promoter region, chromatin immunoprecipitation (ChIP) was performed in cells treated by control or NFAT1-specific siRNAs: indeed, endogenous NFAT1 bound to *LCN2* promoter whereas no significant signal enrichment was noted when NFAT1 was silenced (Fig. 2C, si ctrl versus si NFAT1). To confirm NFAT1 binding to *LCN2* promoter region and determine whether NFAT1 putative binding sites are functional *in vivo*, we individually mutated each of these. Measurement of Luciferase activity after transient co-transfection of MDA-MB-231 cells with NFAT1 of mutated *LCN2* promoter revealed that the -409 binding site was required for NFAT1 to increase transcriptional activity, the -501 and -142 sites being required for *LCN2* promoter basal activity (Fig. 2D). Electrophoretic mobility shift assays were then performed to confirm that endogenous NFAT1 bound to these identified sites and, indeed, NFAT1 bound to the -501, -409, -142 wild-type, but not mutated, binding sites (Fig. 2E). NFAT1-specific binding was confirmed by pre-incubating nuclear extracts with an anti-NFAT1 antibody that supershifts the complex NFAT1/probe (Fig. 2E, arrows). As control, no supershift was induced by pre-incubating nuclear extracts with control IgG.

These data demonstrate that endogenous NFAT1 binds directly to the *LCN2* promoter -501, -142 sites to modulate its basal expression, and to the -409 site to regulate its inducible expression.

The NFAT1/LCN2 axis modulates TWEAKR expression

To assess the mechanisms by which the NFAT1/LCN2 axis up-regulates breast cancer cell invasion, we used transcriptome analysis of LCN2-specific siRNA-treated MDA-MB-231

cells in order to identify genes regulated by LCN2 potentially involved in the invasive capacity. LCN2 silencing impacted 95 genes by at least 1.5 fold (95% confidence level) (Fig. 3A, and Supplementary Table 1), which was confirmed by RT-QPCR for most of these (data not shown). Because we have shown that NFAT1 regulates *LCN2* expression, we examined whether modifying NFAT1 expression affected any of the 95 *LCN2*-regulated genes. Indeed, siRNA silencing of NFAT1 reduced expression of two of them: *TWEAKR* (TNFSFR12A) (Fig. 3B) and *C6ORF55* (VTA1) (data not shown). Thus, out of the 95 identified genes that putatively could be involved in the invasion process of breast cancer cells, only these two were regulated in the same manner by NFAT1 and LCN2. Silencing VTA1 with siRNA had no effect on cancer cell invasive capacity (data not shown). Because *TWEAKR* was already known to participate in the motility of different cell types (Meighan-Mantha et al. 1999; Willis et al. 2008) and was regulated in the same manner by NFAT1 and LCN2, we further investigated this regulation of *TWEAKR* expression by LCN2 and NFAT1 and its role in the invasive behaviour of breast cancer cells. Flow cytometry analysis confirmed that siRNA silencing of either LCN2 or NFAT1 down-regulated *TWEAKR* protein expression in MDA-MB-231 (Fig. 3C) and SUM-159-PT (Supplementary Fig. S1) cells relative to control cells.

These data indicate that *TWEAKR* is regulated by NFAT1 and LCN2 at the mRNA and protein levels in breast cancer cells.

Reciprocal regulation of the *TWEAKR/TWEAK* axis and *LCN2* expression

In contrast to *TWEAKR*, silencing or overexpressing *LCN2* in MDA-MB-231 cells did not modify mRNA levels of its ligand, *TWEAK* (Supplementary Fig. S2A and B), which was indeed not found among the 95 genes identified in Fig. 3A (see Supplementary Table 1). Nonetheless, when *LCN2* or *NFAT1* were independently silenced with siRNA directed to either, which reduced their expression by 90%, *TWEAK* protein levels in cell supernatants increased relative to the control condition (Fig. 4A). To validate that *TWEAK* protein is regulated by *LCN2*, cells were transiently transfected with a fixed amount of a *TWEAK*-expressing vector and increasing amounts of a *LCN2*-expressing vector, which resulted in dose-dependent decrease of *TWEAK* levels (Fig. 4B). Moreover, *TWEAKR* depletion by siRNA led to increased *TWEAK* protein levels without modifying its mRNA expression in MDA-MB-231 and SUM-159-PT cells (Supplementary Fig. S3).

Because *LCN2* regulated *TWEAK* protein amount, we verified whether *TWEAK* or *TWEAKR* per se affected *LCN2* protein levels by transiently transfecting MDA-MB-231

cells with siRNA targeting TWEAKR or TWEAK. Indeed, silencing of either led to LCN2 level increase and this effect was cumulative (Fig. 4C). Moreover, TWEAK and TWEAKR depletion up-regulated LCN2 mRNA expression (Fig. 4D), suggesting a transcriptional link. To confirm this link, the cells were pre-treated by a transcriptional inhibitor (actinomycin D), which inhibited LCN2 mRNA increase elicited by TWEAK and TWEAKR depletion. We verified here that silencing TWEAKR and/or TWEAK did not modify cell apoptosis or proliferation (Supplementary Fig. S4A). No effect of TWEAK and TWEAKR depletion on NFAT1 transcriptional activity was noted, suggesting that other unidentified transcription factors are involved in up-regulating LCN2 expression (data not shown).

Altogether, these results point to a novel axis by which LCN2 modulates TWEAKR at the mRNA level and its cognate ligand TWEAK at the protein level, both of which conversely regulate LCN2 expression at the transcriptional level.

TWEAK increases breast cancer cell invasion independently of the TWEAKR

TWEAKR signalling has already been implicated in the invasive process in different cell models. To validate that TWEAKR was involved in the chemotactic invasive capacity of MDA-MB-231 cells, chemoinvasion assays were performed in the presence or not of recombinant TWEAK, whose presence was thus shown to increase invasion (Fig. 5A). However, blocking TWEAKR with a specific neutralizing antibody also increased invasion as compared to control IgG, and adding recombinant TWEAK potentiated this effect (Fig. 5A), neither of which modifying cell apoptosis or proliferation (Supplementary Fig. S4B, C). When we repeated the experiment in NFAT1-transfected cells, again pre-treatment with the TWEAKR neutralising antibody enhanced chemoinvasion elicited by NFAT1 (Fig. 5B), suggesting that TWEAKR mediates an anti-invasive effect in breast cancer cells in this assay. As a confirmation, we repeated the experiment presented in Fig. 5A by using a siRNA targeting TWEAKR. After verifying that the siRNA actually reduced TWEAKR expression (Fig. 5C, right panel) and did not affect apoptosis or proliferation (Supplementary Fig. S4A), we found again that chemoinvasion increased when TWEAKR was silenced and that this was potentiated by adding recombinant TWEAK (Fig. 5C, left panel).

These findings were surprising in light of the report that, in breast cancer cells, TWEAKR silencing led to loss of invasion potential (Willis et al. 2008). But in that case the assay (Hauck et al. 2002; Hsia et al. 2003) assessed random cell invasion, no chemotactic gradient being used, whereas ours is chemoinvasion assay using conditioned medium from

NIH3T3 cells in order to create a chemotactic gradient (Albini and Benelli 2007). Therefore, we examined if the different assays accounted for the discrepancy. To this end MDA-MB-231 cells, transiently transfected with either control or TWEAKR-specific siRNAs, were comparatively assessed in both assays. Data from Fig. S5 indicate that cells were actually less invasive and that TWEAKR silencing inhibited invasion in the random invasion assay, whereas increased invasion in presence was noted as expected in the chemoinvasion assay. Thus, TWEAKR would differently influence random and directed cell invasion.

To evaluate the role of LCN2 in regulating invasion downstream of TWEAK, MDA-MB-231 cells were transiently transfected with control or LCN2-specific siRNAs and exposed to recombinant TWEAK. In this case, when LCN2 was silenced, TWEAK no longer increased chemoinvasion (Fig. 5D).

These data identify TWEAKR as a receptor that antagonizes the chemotactic invasion process of breast cancer cells. Importantly, they indicate that LCN2 is required for TWEAK to increase chemotactic invasion, either directly or via another as yet unidentified receptor different from TWEAKR.

Depending on LCN2 expression TWEAK displays either anti- and pro-invasive activities in breast cancer cells

Inasmuch as TWEAK availability appeared to be regulated by TWEAKR, LCN2 and NFAT1, we more precisely examined the role of TWEAK protein on breast cancer cell invasion. MDA-MB-231 cells were transiently transfected with control siRNA or with siRNAs targeting TWEAKR, LCN2, NFAT1 or TWEAK, alone or in combination (Fig. 6A). As shown in Fig. 5C, TWEAKR silencing led to increased chemoinvasion, and either NFAT1 (Fig. 6A, si TWR si NFAT1) or LCN2 (Fig. 6A, si TWR si LCN2) depletion prevented this increase, demonstrating that LCN2 increased expression elicited by TWEAKR knockdown was responsible for this effect. Indeed, when NFAT1 was silenced, and by consequence LCN2 too, again the increase of chemoinvasion induced by TWEAKR down-regulation was abrogated (Fig. 6A, si TWR si NFAT1). Since we have shown that TWEAKR depletion induces TWEAK protein up-regulation (Supplementary Fig. S3), we examined whether the latter was involved in this increased invasion. Indeed, when TWEAK was silenced by siRNA together with TWEAKR (Fig. 6A, si TWR si TW, -), increased invasion was abolished but it was rescued by adding back recombinant TWEAK (Fig. 6A, si TWR si TW, +). The same results were obtained with SUM-159-PT cells (Supplementary Fig. S6).

251 These data indicate that TWEAK protein up-regulation is critical to increase invasion
252 and entails the presence of LCN2.

253 Interestingly, we have shown that, like TWEAKR down-regulation, that of either
254 LCN2 or NFAT1 induced increased TWEAK protein levels, albeit without increasing but
255 rather inhibiting chemoinvasion. Therefore, we hypothesized that, beside its pro-invasive role,
256 TWEAK might be anti-invasive in the absence of LCN2 or NFAT1. To clarify this
257 possibility, either LCN2 or NFAT1, alone or in combination with TWEAK, were silenced in
258 MDA-MB-231 cells. Depletion of endogenous LCN2 inhibited invasion (Fig. 6B, si LCN2)
259 and up-regulated TWEAK protein (from 27.4 to 73.3 ng/L; Fig. 6B lower panel). TWEAK
260 up-regulation was critical to blunt invasion when LCN2 was silenced since co-silencing
261 TWEAK with LCN2 abolished the inhibition of invasion induced by LCN2 knockdown (Fig.
262 6B, si LCN2 si TW, -), which could be rescued by adding recombinant TWEAK (Fig. 6B, si
263 LCN2 si TW, +). These results demonstrate that TWEAK, beside its pro-invasive role in the
264 presence of LCN2, possesses an anti-invasive function in the absence of LCN2. We repeated
265 the same experiment by depleting NFAT1 and obtained almost the same results as with LCN2
266 depletion (Fig. 6C), the sole difference being the lack of reversion of the inhibition of
267 invasion by depleting TWEAK and NFAT1 together (Fig. 6B, si NFAT1 si TW, -). This last
268 result is not surprising since NFAT1 might certainly target other genes with LCN2 and
269 TWEAK to regulate breast cancer cell chemoinvasion. Comparable data were obtained using
270 SUM-159-PT cells (Supplementary Fig. S6).

271 Altogether, these data demonstrate that, beside its pro-invasive action, TWEAK has an
272 anti-invasive action in breast cancer cells in the absence of LCN2. Thus, TWEAK is a pro-
273 invasive factor in breast cancer cells in presence of LCN2 and anti-invasive in its absence.
274

Discussion

The role of NFAT transcription factors in breast cancer cell motility has been identified recently (Jauliac et al. 2002; Mancini and Toker 2009; Yoeli-Lerner et al. 2005) but it is still poorly understood. Apart from studies indicating that either *COX2* (Yiu and Toker 2006) or the *autotaxin* gene (Chen and O'Connor 2005) are targeted by NFAT1 to increase invasion and migration, we do not know which other specific signalling pathways these factors use to modulate breast cancer cell motility.

Recently, a new layer of complexity has been added by the observation that among the members of the NFAT family, NFAT3 –expressed in less aggressive oestrogen receptor α -positive breast cancer cells– has anti-migratory and anti-invasive actions in contrast to NFAT1 and NFAT5. One mechanism by which NFAT3 blunts migration is by inhibiting *LCN2* gene expression (Fougère et al. 2010).

Here, we show that *LCN2* expression is required for NFAT1 to increase the invasive capacity of breast cancer cells. We demonstrate that, contrary to NFAT3, NFAT1 binds to *LCN2* promoter to up-regulate *LCN2* mRNA and, therefore, protein expression. Together with our recent report (Fougère et al. 2010), this highlights the differing effects of NFAT isoforms, despite their high sequence homology, which has already been indicated in specific knockout murine models (Graef et al. 2001; Ranger et al. 1998; Xanthoudakis et al. 1996) or by studies on tumour cell transformation (Buchholz et al. 2006; Neal and Clipstone 2003; Robbs et al. 2008; Wu et al. 2010). These observations suggest that each NFAT isoform regulates independent set of genes as well as, as demonstrated here for *LCN2*, differentially modulates overlapping gene subsets critical for cell fate, and they underscore the idea that depending on the cell type where they are expressed NFAT isoforms either suppress or promote oncogenic transformation.

We also evaluated the mechanisms by which *LCN2* modulates breast cancer cell invasion downstream of NFAT1. To this end, we examined the genomic effects of *LCN2* depletion in order to identify the genes that are similarly regulated by NFAT1 and *LCN2*. We found that, among 95 genes affected by *LCN2* down-regulation, two –*TWEAKR* (*TNFSFR12A*) and *C6ORF55* (*VTA1*)– were regulated in the same manner by NFAT1. We focused on *TWEAKR* because this gene has already been implicated in the motility of different cell types (Dai et al. 2009; Tran et al. 2006; Wiley et al. 2001), and silencing *VTA1* had no effect in this respect. We found that, at both the mRNA and protein levels, either NFAT1 or *LCN2* down-regulation inhibited *TWEAKR* expression. We therefore reasoned that *TWEAKR* disappearance was responsible for the inhibition of breast cancer cell invasion

in light of reports showing its role in migration and invasion (Dai et al. 2009; Michaelson et al. 2005; Tran et al. 2006; Willis et al. 2008). Indeed, treating the cells with recombinant TWEAK elicited increase of their chemoinvasive capacity. Surprisingly, when we silenced TWEAKR, invasion was increased and TWEAK could still potentiate this increase. We showed that, in contrast to chemoinvasion, TWEAKR down-regulation inhibited random invasion as reported (Willis et al., 2008). Therefore, we concluded that, in our chemoinvasion model, TWEAKR was a receptor that mediated an anti-invasive effect and that its cognate ligand, TWEAK, could promote invasion via another, as yet unidentified, receptor the presence of which has already been suggested in RAW264.7 cells (Polek et al. 2003). Hence, we hypothesised that TWEAK binding to its cognate receptor (TWEAKR) can inhibit invasion and that blocking this association with a TWEAKR-specific neutralising antibody enables TWEAK binding to the other receptor that promotes invasion, but it cannot be ruled out that TWEAK directly enters the cells. Further studies are needed to identify this potential second TWEAK receptor if it exists. We cannot rule out that NFAT1 and LCN2 participate in the regulation of this potential unknown TWEAK receptor.

When we examined TWEAK expression in the absence of either LCN2 or NFAT1, we found that it was up-regulated at the protein, but not the mRNA, level. This demonstrates that NFAT1, via LCN2, regulates TWEAK protein expression or stability. In the same manner as for LCN2, inhibiting or silencing TWEAKR elicited up-regulated TWEAK protein expression or availability. This highlights the tight regulation between LCN2 and the TWEAKR/TWEAK axis, all the more so that we found also that TWEAKR and TWEAK can both modulate LCN2 expression at the mRNA and protein levels. Therefore, in breast cancer cells, there exists a subtle equilibrium between LCN2, TWEAKR and TWEAK to modulate the availability of these key factors for the regulation of cell invasion (Fig. 7A).

Since we showed that adding recombinant TWEAK increased the cells' invasive capacity, it was puzzling that elevated TWEAK levels induced by depleting either LCN2 or NFAT1 correlated with a decrease rather than an increase of invasion. Indeed, our study shows that presence of LCN2 is necessary for TWEAK to promote its pro-invasive effect (Fig. 5D). Therefore, in the absence of LCN2, TWEAK can only signal by the TWEAKR, and inhibits invasion (Fig. 7B, Without LCN2). Indeed, when TWEAKR is silenced, TWEAK expression is required to increase invasion and needs the presence of LCN2 (Fig. 6A and 7B, without TWEAKR). In contrast, in the absence of LCN2, when LCN2 is silenced, TWEAK is an anti-invasive factor (Fig. 6B). Importantly, this indicates that TWEAK can be either pro-tumorigenic or anti-tumorigenic as reported (Kaduka et al. 2005; Maecker et al. 2005;

Meighan-Mantha et al. 1999), but in our study these opposite effects occur in the same cells, suggesting an equilibrium between the two functions (Fig. 7B, Equilibrium). Critically, we show that LCN2 expression up-regulation by NFAT1 is necessary for TWEAK to increase breast cancer cell invasion. Therefore, disturbing this tight equilibrium may be a new entry to inhibit breast cancer cell invasion and ultimately metastasis formation.

Both LCN2 and TWEAK are able to activate the ERK pathway (Gwira et al. 2005; Peternel et al. 2011; Vincent et al. 2009), ERK apparently being an important actor of breast cancer cell migration (Irie et al. 2005; Krueger et al. 2001). One possibility would be that promoting invasion downstream of TWEAK requires modulation of the ERK pathway in association with LCN2, but this has yet to be determined.

In summary, we have shown that signalling through NFAT1 increases invasion through a previously unknown LCN2/TWEAKR/TWEAK axis. We demonstrate that TWEAK can have opposite effects on breast cancer cell invasion: anti-invasive via TWEAKR, independently of LCN2 (Fig. 7B, Without LCN2); pro-invasive via another unidentified receptor (X^R) or directly in association with LCN2 (Fig. 7B, Without TWEAKR). Therefore, increased NFAT1 activation leads to up-regulation of LCN2 protein expression that consequently enables TWEAK pro-invasive activity in breast cancer cells. These findings underscore the importance of dissecting the mechanisms by which TWEAK and LCN2 regulate breast cancer cell invasion, downstream of NFAT1, in order to be able to attempt to therapeutically target this pathway to limit the dissemination of metastasis.

Materials and Methods

Cell culture

The MDA-MB-231 cell line was from the American Type Culture Collection, The SUM-159-PT cell line was provided by Alex Toker (Harvard Medical School). MDA-MB-231 and SUM-159-PT cells were maintained in Dulbecco Modified Eagle Medium (DMEM), low glucose (1 g/L D-glucose), 10% Foetal Calf Serum. NIH-3T3 cells were maintained in DMEM, high glucose (4.5 g/L D-glucose), 10% Newborn Calf Serum. All media were supplemented with 2 mM L-Glutamine, 100 U/mL Penicillin and 100 µg/mL Streptomycin.

Antibodies and reagents

The following antibodies were used: Anti-T7 (Novagen; #69522), anti-FLAG (Sigma; #F1804), anti-HA (Roche; #11867423), anti-NFAT1 from ABR Affinity BioReagent (MA1-025) or from Abcam for ChIP assays (ab2722), anti-Actin (Santa-Cruz Biotechnology; SC-1616), anti-LCN2 (Sigma-Aldrich; HPA002695), and anti-TWEAKR (Abcam; ab21359) for flow cytometry. The mouse IgG was from Santa-Cruz Biotechnology (SC-2025) and the anti-mouse phycoerythrin-labelled antibody (F0102B) was from R&D systems. The blocking anti-TWEAKR antibody was from R&D systems (#AF1199).

The protein G Plus/Protein A Agarose suspension was from Calbiochem (IP05). Recombinant human TWEAK (#1090TW/CF) was from R&D Systems. The ELISA used to assess TWEAK (#DY1090) and LCN2 (#DLCN20) were from R&D systems. SiRNA were from Dharmacon and their sequences are reported in Supplemental Table 2. Cells were transiently transfected with the appropriate plasmids using Lipofectamine 2000 (Invitrogen) or DharmaFECT (Dharmacon) for siRNA according to the manufacturer's instructions.

Plasmids, siRNA and quantitative real time reverse transcriptase PCR (RT-QPCR)

NFAT1 was cloned by PCR in the pcDNA3 vector expressing a N-terminal T7-tag. LCN2 promoter and the pCS4-(n)-β-galactosidase were previously described (Fougère et al. 2010). The potential NFAT-binding sites of LCN2 promoter were mutated by PCR using the primers described in Supplementarary Table 2. LCN2 was cloned by PCR in the pcDNA3.1+ vector with a HA tag from RNA isolated from MDA-MB-231 cells. The FLAG-TWEAK expression vector was from Origene. All constructions were verified by sequencing. To transiently silence TWEAKR, TWEAK, NFAT1 or LCN2, we used specific siRNAs

(Dharmacon) at 30 nM, and transfected cells with DharmaFECT for 48 hr. Sequences of the different siRNAs used are accessible in the Supplementary Table 2.

LCN2, TWEAKR and TWEAK mRNA expression was determined by RT-QPCR. Total RNA was extracted using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions, and cDNA synthesis was prepared using the SuperScript Reverse Transcriptase (RT) (Invitrogen). RT-QPCR was performed using SYBR Green PCR Master Mix on Roche LightCycler as directed by the manufacturer. Cycling parameters were 10 minutes at 95°C followed by 45 cycles of 10 seconds at 95°C (denaturation) and 4 seconds at 68°C (annealing/extension). Primers are accessible in the Supplementary Information, Supplementary Table 2.

Proteins detection

For all assays using transfected cells, expression or silencing of the protein were verified by immunoblotting. Harvested cells were washed twice and resuspended in cold PBS. Cells were lysed in SDS sample buffer containing β -mercaptoethanol for 20 minutes at 95°C. The lysates were resolved by SDS-PAGE and immunoblotted with the appropriate antibodies. For flow cytometry, MDA-MB-231 or SUM-159-PT cells were stained with an anti-TWEAKR antibody and a secondary phycoerythrin-labelled antibody. As controls, MDA-MB-231 or SUM-159-PT cells were stained only by the secondary phycoerythrin-labelled antibody, omitting the anti-TWEAKR antibody. Analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson).

ELISA for assessing LCN2 and TWEAK

The ELISA for LCN2 was performed as directed by the manufacturer on 50 μ l of cell supernatants. For TWEAK, cell supernatants were concentrated using the Centricon (Millipore) prior to performing the ELISA as directed by the manufacturer on 50 μ l of concentrated supernatant.

Invasion assays (chemoinvasion and random invasion assays)

The chemoinvasion assay was performed essentially as described, using Transwell chambers (Becton Dickinson) with 8- μ m pore membranes coated with Matrigel (Becton Dickinson). Cells, co-transfected with the relevant expression plasmids and the pCS2-(n)- β -gal reporter plasmid, were resuspended after 24 hours in serum-free medium containing 0.1% BSA, and

cells were added to each well. Conditioned NIH-3T3 medium was added to the bottom wells of the chambers. After 6 hours, cells that had not invaded were removed from the upper face of the filters using cotton swabs, and cells that had invaded to the lower surface of the filters were fixed for 30 min in 4% paraformaldehyde and then stained with PBS containing 1 mg/ml bluo-gal, 2 mM MgCl₂, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide. All cells in each Transwell were counted. All the counts were normalised by the efficiency of transfection. The numbers of cells that invaded in each condition were compared with the empty vector-transfected condition arbitrary set as a ratio of 1. When the assay was performed with cells transiently transfected by siRNA, cells were stained with crystal violet since 95% of the cells were effectively transfected. In some cases, 200 ng/mL recombinant TWEAK was added for 6 hours to the cells during the assay.

The random invasion assay was performed as reported (Willis et al. 2008).

Luciferase assay

Cells were cotransfected with the appropriate LCN2 Luciferase reporter construct, pCS2-(n)- β -galactosidase and NFAT1 expression vector or control vector using Lipofectamine 2000. After 48 hr, cells were lysed with the Reporter Lysis Buffer (Promega) and Luciferase and β -gal activities were measured using the Luciferase Assay System (Promega) and Galacton-plus (Tropix) on a luminometer. Luciferase activities were normalized relative to the corresponding β -gal activities.

Electrophoretic mobility shift assay

Harvested cells were washed in ice-cold PBS, 0.5 mM DTT, and lysed in buffer with 10 mM HEPES, 10 mM KCl, 15 mM MgCl₂. Nuclei were isolated by centrifugation, and nuclear proteins were extracted in buffer with 20 mM HEPES, 25% glycerol, 420 mM NaCl, 15 mM MgCl₂, 0.2 mM EDTA, DTT (0.5 mM) and PMSF (0.2 mM). Binding of nuclear extracts to 5'-end-IRDye700-labeled probes (Eurofins MWG Operon) was carried out by incubating the extracts with 50 fmol of labelled probes in 20 μ L at room temperature for 20 min in binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM KCl, 3 mM DTT, 0.25% Tween20 and 50 ng/ μ L poly(dI-dC)). Unbound probes were separated from DNA-protein complexes in a 5% polyacrylamide gel, and detected after migration on an infrared imaging system ODYSSEY (Li-Cor Biosciences). Super-shift was obtained by incubating nuclear extracts with 0.5 μ g antibodies (anti-NFAT1 or IgG control) at room temperature for 20 min before adding the labelled probes. Probe sequences are reported in Supplemental Table 2.

ChIP assay

Crosslinking, 48 hr after siRNA transfection, was performed by incubating cells in 1% formaldehyde for 5 min, and stopped by adding 1:7 volume of 1 M glycine for 5 min. After washing, cells were scraped with 1 mL cold PBS. After cell lysis, nuclei were isolated in a first buffer (20 mM HEPES, 10 mM EDTA, 0.5 mM EGTA, 0.25% TritonX100) and then a second (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA), each for 10 min at 4°C followed by centrifugation. Nuclei pellets were suspended in buffer with 20 mM HEPES, 1 mM EDTA, 0.5 mM EGTA, protease inhibitors, and 0.05% SDS. Nuclei were sonicated for 15 min in a Bioruptor (Diagenode). Efficient sonication was verified on Agarose gel. Protein A/G beads were blocked overnight at 4°C in incubation buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.15% SDS, 1% TritonX100, 0.1% BSA and protease inhibitors). For each condition, the same amount of sonicated chromatin was incubated overnight at 4°C with 4 µg anti-NFAT1 antibody or control IgG and 20 µL of 50% suspension blocked beads in 300 µL final. Afterward, beads were washed twice in buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% SDS, 0.1% DOC, 1% TritonX100), once in the same buffer but with 500 mM NaCl, twice in a third buffer (10 mM Tris, 1 mM EDTA, 0.5 mM EGTA, 0.25 M LiCl, 0.5% DOC, 0.5% NP-40), and last with the final buffer (10 mM Tris, 1 mM EDTA, 0.5 mM EGTA). Chromatin was eluted in 400 µL elution buffer (1% SDS, 0.1 M NaHCO₃), and incubated for 30 min at room temperature. Immunoprecipitated chromatin was reverse-crosslinked with 200 mM NaCl overnight at 65°C, and purified by phenol/chloroform extraction and ethanol precipitation. The pellet was resuspended in 30 µL water. RT-QPCR was performed as described. The relative proportions of coimmunoprecipitated gene fragments were determined based on the threshold cycle (Ct) for each PCR product. Data sets were normalized according to $2^{Ct(\text{unspec. Ab = Ig}) - Ct(\text{spec. ab = anti-NFAT1})}$. The fold difference over background obtained for gene regions was further normalized relative to the value obtained with a primer pair amplifying an intergenic region on chromosome 10. Each sample was quantified in duplicate and from ≥3 independent ChIPs. SEM was determined for each fold difference above the IgG control and intergenic control region. Primer sequences are reported in Supplemental Table 2.

Array hybridization, data analysis and clustering

RNA was purified on Qiagen columns, and its integrity was verified using a Bioanalyzer. Total RNA was processed following Roche Nimblegen's instructions to produce double-strand DNA and was sent to Roche Nimblegen. Labelling, hybridization, data collection, and normalization were carried out according to NimbleGen protocols. The array used was the human 2006-08-03_HG18_60mer_expression array. Normalised data were then filtered according to their expression level: for a given comparison, average expression of at least one of the two compared experimental conditions had to be ≥ 100 . For genes targeted by several Nimblegen's probes, the average of probe-normalised intensities was calculated to estimate a single gene signal intensity. We then performed paired Student's t-tests to compare gene expression intensities. Genes were considered significantly differentially regulated when fold-change was ≥ 1.5 and p-value ≤ 0.05 . The distance from the gene signal in a given sample to the corresponding average in the 6 samples (MDA-MB-231 cells transfected with si ctrl or si LCN2) was calculated for each LCN2-regulated gene. Corresponding values were displayed and clusterised with MeV4.6.2 from The Institute of Genome Research using Euclidean distance and complete linkage clustering. Full array data are shown in Supplemental Table 1.

Supplementary online data

Supplementary data are available at *Journal of Cell Science* online.

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Figure Legends

Figure 1. NFAT1 up-regulates LCN2 expression to increase the invasive capacity of breast cancer cells. (A) MDA-MB-231 cells were transiently transfected with control (si ctrl) or NFAT1-specific (si NFAT1) siRNAs and assayed by RT-QPCR to quantify LCN2 and β -2 microglobulin mRNAs as arbitrary units relative to si ctrl-transfected cells. Immunoblots with anti-NFAT1 or anti-actin antibodies are shown. (B) Cells were transfected as in (A). Left panel: Immunoblots with anti-NFAT1, anti-LCN2 or anti-actin antibodies of total cell lysates. Middle panel: LCN2 protein amount was assessed by ELISA in 50 μ l conditioned 48-hr-culture medium from the cells. Right panel: Cells were transiently co-transfected with a GFP-expressing vector and either a control (vector) or a T7 epitope-tagged NFAT1 (NFAT1) vector. After 24 hr, cell lysates of bulk unsorted cells were immunoblotted to probe for NFAT1 (anti-T7) and actin (anti-actin) to avoid a too strong signal, while endogenous LCN2 (anti-LCN2) and actin (anti-actin) were assessed in lysates of their GFP⁺ FACS-sorted counterparts. (C) Cells were transiently transfected with a control (vector) or a HA epitope-tagged LCN2 (LCN2) vector in combination with a β -gal-expressing vector, and tested after 24 hr for the ability to invade using the chemoinvasion assay. Immunoblots with anti-HA or anti-actin antibodies are shown. (D) Cells were transiently co-transfected with a T7 epitope-tagged NFAT1 (NFAT1) or control (vector) vector and either control (si ctrl) or LCN2-specific (si LCN2) siRNAs in combination with a β -gal-expressing vector, and tested after 48 hr in the chemoinvasion assay. Immunoblots with anti-T7 (NFAT1), anti-LCN2 or anti-actin antibodies are shown. Statistical analyses were performed relative to si ctrl/vector-transfected cells. All data are representative of three independent experiments. *Bars:* SE. *, $P < 0.05$.

Figure 2. NFAT1 binds to *LCN2* promoter region and up-regulates LCN2 expression. (A) Schematic representation of the *LCN2* promoter. Six potential NFAT-binding sites have been found using the TESS software: <http://www.cbil.upenn.edu/cgi-bin/teess/teess>; positions relative to the +1 initiation site are indicated. (B) MDA-MB-231 cells were cotransfected with the pCS4-(n)- β -gal and the *LCN2* promoter plasmids, and with the NFAT1 (NFAT1) or control (vector) vectors. After 48 hr, cells were analysed for Luciferase and β -gal activities. Quantification of LCN2 promoter-mediated Luciferase activity was normalized relative to that of β -gal. Immunoblots with anti-T7 (NFAT1) and anti-actin antibodies are shown. (C)

Recruitment of endogenous NFAT1 on LCN2 promoter region was quantified by QPCR after ChIP using an anti-NFAT1 antibody and comparing si NFAT1- and si ctrl-treated cells using primers encompassing the -881 or -522 or -501, -441, -409 and -142 potential NFAT1-binding sites. Immunoblots with anti-NFAT1 or anti-actin antibodies are shown. (D) MDA-MB-231 cells were cotransfected with the pCS4-(n)- β -gal and wild-type or mutated (^x) LCN2 promoter (pLCN2) plasmids, and cotransfected with the NFAT1 (+) or control (-) vector. Cells were analysed 48 hr later for Luciferase and β -gal activities. Quantification of LCN2 promoter Luciferase activity was normalized relative to that of β -gal. Immunoblots with anti-T7 (NFAT1) or anti-actin antibodies are shown. (E) Nuclear extracts from MDA-MB-231 cells were incubated with IRDye700-labeled probes containing the wild-type or mutated (*) -142, -409, -501 predicted NFAT-binding sites. The NFAT1/probe complexes were separated on a 5% polyacrylamide gel. A specific anti-NFAT1 antibody or control IgG were pre-incubated with nuclear extracts before incubation with the IRDye700-labeled probes. Arrows indicate positions of the super-shifted NFAT antibody-probe complex and asterix position of the non-specific bands. All data are representative of three independent experiments. Bars: SE. *, $P < 0.05$.

Figure 3. The NFAT1/LCN2 axis modulates TWEAKR expression. (A) The distance from the gene signal in a given sample to the corresponding average in the 6 samples (MDA-MB-231 cells transfected with si ctrl or si LCN2) was calculated for each LCN2-regulated gene. Corresponding values were displayed and clusterised with MeV4.6.2 from The Institute of Genome Research using Euclidean distance and complete linkage clustering. (B) MDA-MB-231 cells were transiently transfected with control (si ctrl), NFAT1-specific (si NFAT1) or LCN2-specific (si LCN2) siRNAs, and assayed by RT-QPCR to quantify LCN2 and β -2 microglobulin mRNAs as arbitrary units relative to si ctrl-transfected cells. Immunoblots with anti-NFAT1, anti-LCN2 or anti-actin antibodies are shown. (C) Cells, transfected as in (B), were stained after 48 hr with an anti-TWEAKR antibody and analysed by flow cytometry. As control, cells were stained only with the secondary phycoerythrin-labelled antibody, omitting the anti-TWEAKR antibody. All data are representative of three independent experiments. Bars: SE. *, $P < 0.05$.

Figure 4. Reciprocal regulation of the TWEAKR/TWEAK axis and LCN2 expression. (A) TWEAK and LCN2 (grey line) were assessed by ELISA in 50 μ l concentrated conditioned

48-hr culture medium from MDA-MB-231 cells transiently transfected with control (si ctrl), NFAT1-specific (si NFAT1) or LCN2-specific (si LCN2) siRNAs. Immunoblots with anti-NFAT1, anti-LCN2 or anti-actin antibodies are shown. (B) Both proteins were also assessed in medium from the cells transiently transfected with increasing amounts of HA epitope-tagged LCN2 (LCN2) and a fixed amount of FLAG epitope-tagged TWEAK (TWEAK), alone or in combination, or an empty vector control (vector). Immunoblots with anti-HA, anti-FLAG or anti-actin antibodies are shown. (C) Upper panel: LCN2 was assessed by ELISA in 50 μ l conditioned 48-hr culture medium from the cells transiently transfected with either a control (si ctrl) or TWEAKR-specific (si TWEAKR) siRNAs, and a TWEAK-specific (si TWEAK) siRNA, alone or in combination. Lower panel: LCN2 expression was evaluated in cell extracts with an anti-LCN2 or an anti-actin antibody as loading control. (D) LCN2 mRNA quantification in the cells transiently transfected with control (si ctrl) or TWEAKR- and TWEAK-specific (si TWEAKR/si TWEAK) siRNAs; 36 hr post-transfection (t=0), vehicle (-) or 2 μ g/mL actinomycin D (+) were added to the cells. After 8-hr treatment, total RNA was obtained and RT-QPCR was performed as described earlier to quantify LCN2 and β -2 microglobulin mRNAs. All data are representative of three independent experiments. Bars: SE. *, $P < 0.05$.

Figure 5. TWEAKR is an anti-invasive receptor in breast cancer cells. (A) MDA-MB-231 cells were pre-incubated with a TWEAKR-specific neutralizing antibody (α -TWEAKR) or control IgG (Ig ctrl), and tested in the invasion assay in the presence of TWEAK (+) or vehicle (-). (B) Cells were transiently transfected with a T7 epitope-tagged NFAT1 (NFAT1) or control (vector) vector. After 24 hr, cells were pre-incubated with the TWEAKR-specific neutralizing antibody (α -TWEAKR) or control IgG (Ig ctrl) and tested in the invasion assay as in (A). Immunoblots with anti-T7 (NFAT1) or anti-actin antibodies are shown. (C) Cells were transiently transfected with control (si ctrl) or TWEAKR-specific (si TWEAKR) siRNAs. After 48 hr, cells were tested in the invasion assay as in (A). Cell membrane TWEAKR expression monitored by flow cytometry is shown in the right panel. (D) Cells were transiently transfected with control (si ctrl) or LCN2-specific (si LCN2) siRNAs, and tested in the invasion assay after 48 hr as above. All data are representative of three independent experiments. Bars: SE. *, $P < 0.05$.

Figure 6. Depending on LCN2 expression TWEAK displays both anti- and pro-invasive activities in breast cancer cells. (A) MDA-MB-231 cells were transiently transfected with control (si ctrl) or TWEAKR- (si TWR), TWEAK- (si TW), NFAT1- (si NFAT1) or LCN2-specific (si LCN2) siRNAs, alone or in combination. Cells were tested 48 hr later in the invasion assay in the presence of TWEAK (+) or vehicle (-). Cell membrane TWEAKR was monitored by flow cytometry: geometric means of TWEAKR expression intensities are indicated above the graph. TWEAK amounts in concentrated conditioned media of transfected cells was monitored by ELISA: values (TWEAK (ng/L) are indicated below the immunoblots with anti-NFAT1, anti-LCN2 and anti-actin antibodies shown at the bottom of Fig. 6. (B) The cells were transiently transfected with control (si ctrl), TWEAK- (si TW) or LCN2-specific (si LCN2) siRNAs, alone or in combination. After 48 hr, cells were tested in the invasion assay as in (A). Expression of TWEAKR, TWEAK and LCN2 is presented as described in (A). (C) The cells were transiently transfected with control (si ctrl), TWEAK- (si TW) or NFAT1-specific (si NFAT1) siRNAs, alone or in combination. After 48 hr, cells were tested in the invasion assay as in (A). Expression of TWEAKR, TWEAK and NFAT1 is presented as in (A). All data are representative of three independent experiments. Bars: SE. *, $P < 0.05$.

Figure 7. A working model of the interactions between NFAT1, LCN2, the TWEAKR and TWEAK. (A) Reciprocal regulatory loops between TWEAK/TWEAKR/LCN2 and NFAT1. NFAT1 up-regulates LCN2 mRNA and protein expression (1). LCN2 protein regulates TWEAKR expression (2) and modulates TWEAK protein expression (3). As a retro-control, TWEAKR and TWEAK inhibit transcription of LCN2 mRNA (4 and 5). (B). In breast cancer cells there is an equilibrium between inhibitory signalling mediated by TWEAK via the TWEAKR independently of LCN2 (1) and activating signalling mediated by TWEAK through binding to an unknown receptor (X^R) in cooperation with LCN2 (2). In the absence of TWEAKR (Without TWEAKR), LCN2-dependent, TWEAK-mediated activating signalling is promoted and invasion increases (left panel). On the contrary, in the absence of LCN2 (Without LCN2) LCN2-independent inhibitory signalling mediated by TWEAK via TWEAKR is promoted and invasion is prevented (right panel).

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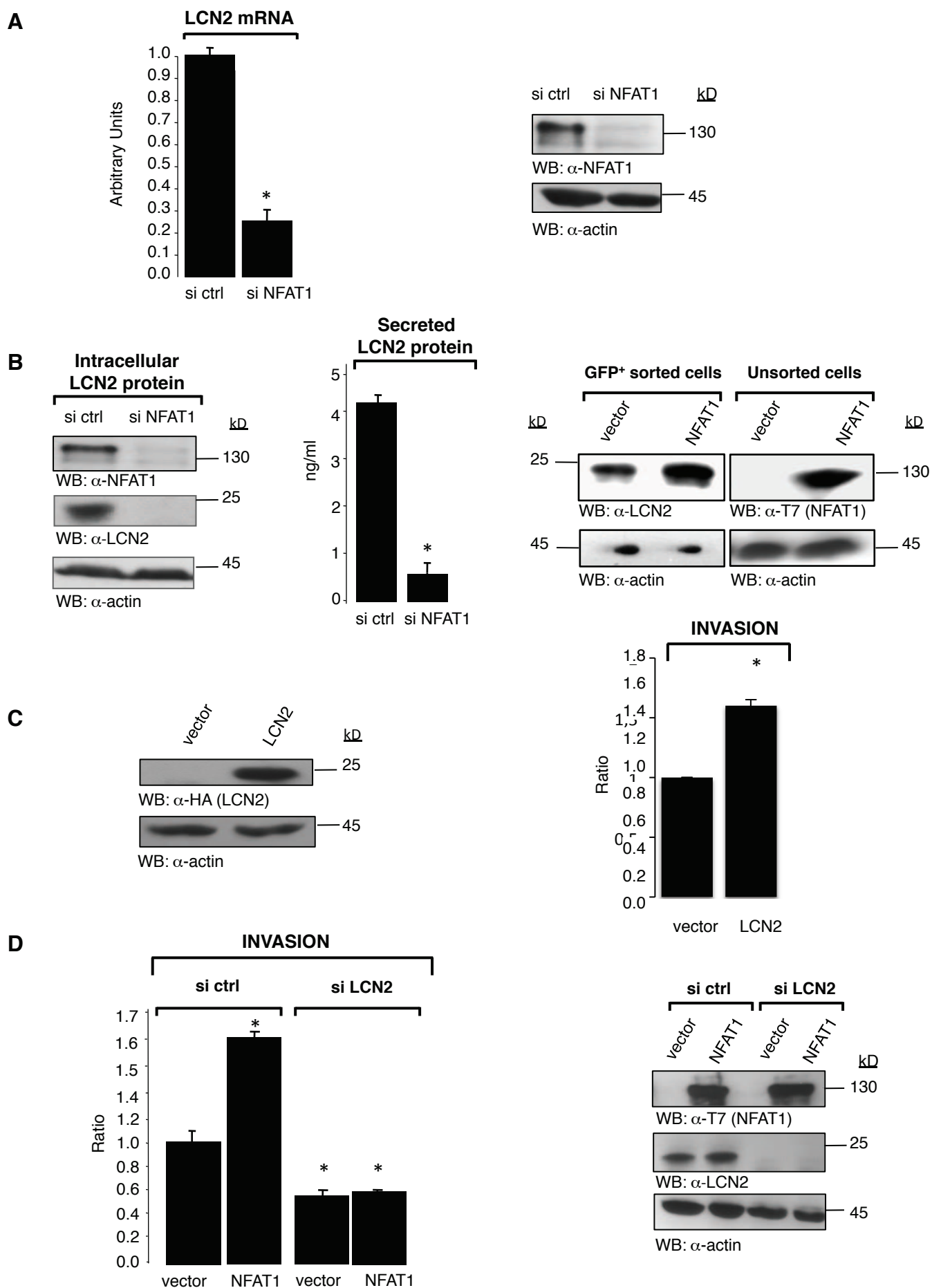


Figure 1 Gaudineau et al.

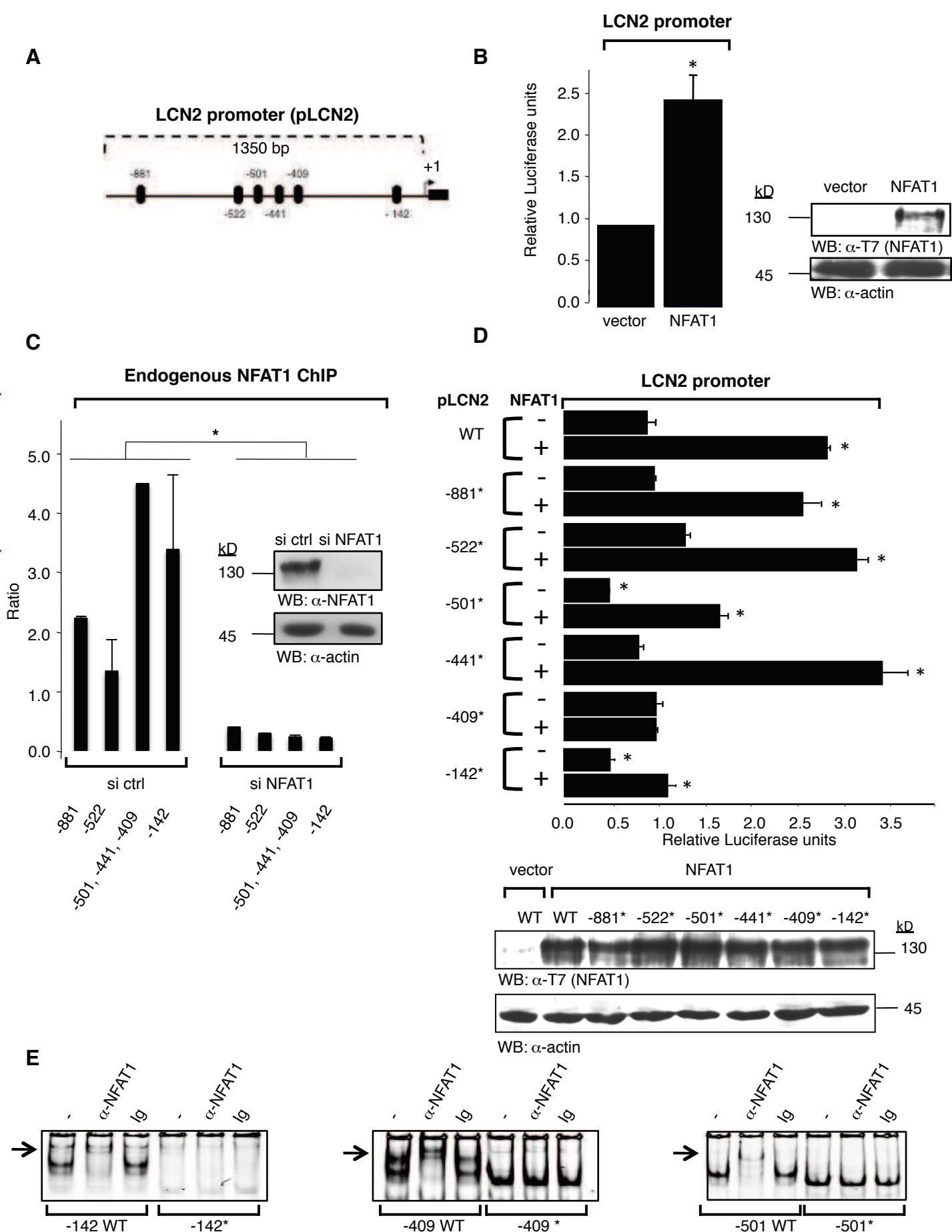


Figure 2 Gaudineau et al.

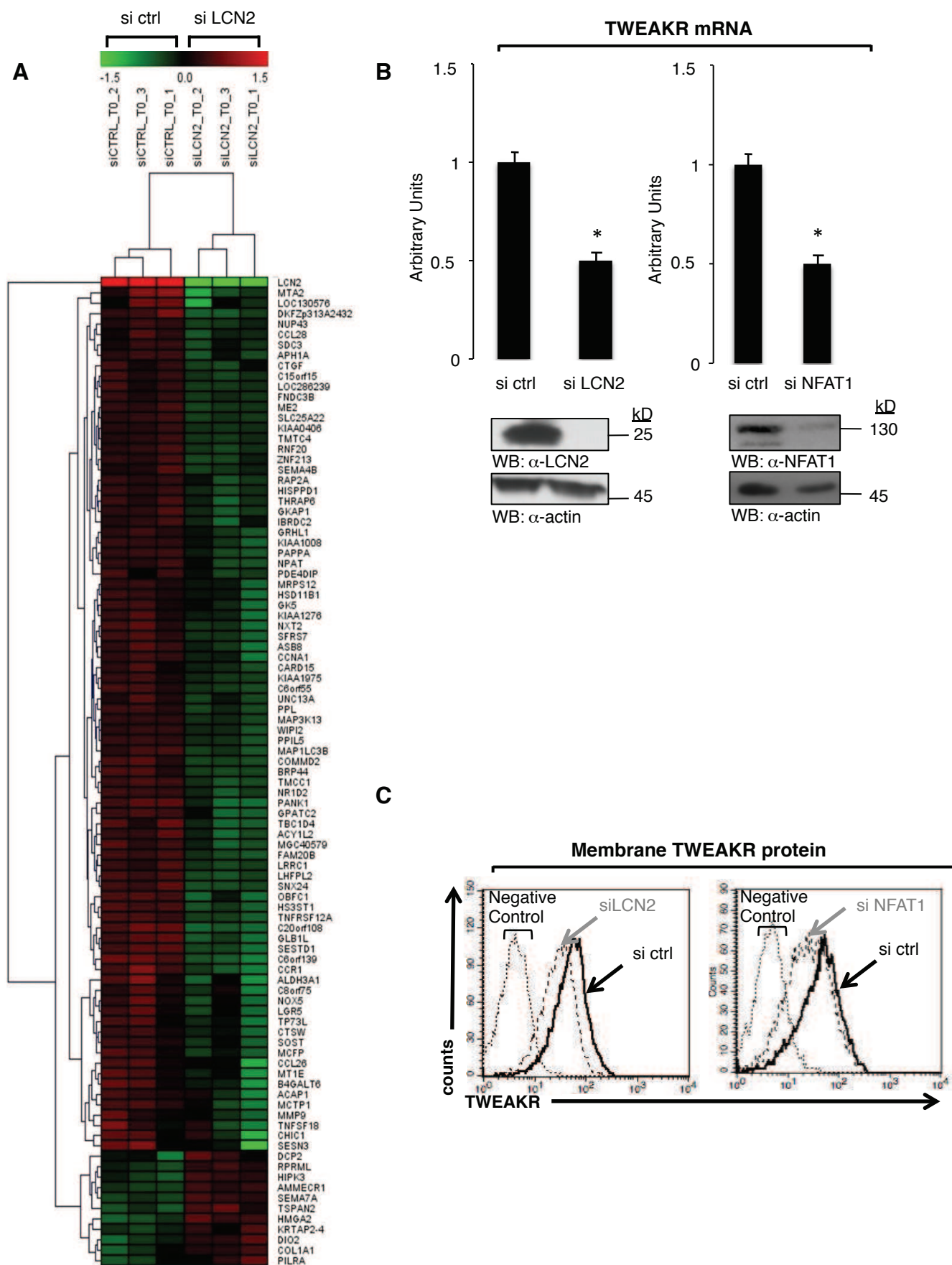


Figure 3 Gaudineau et al.

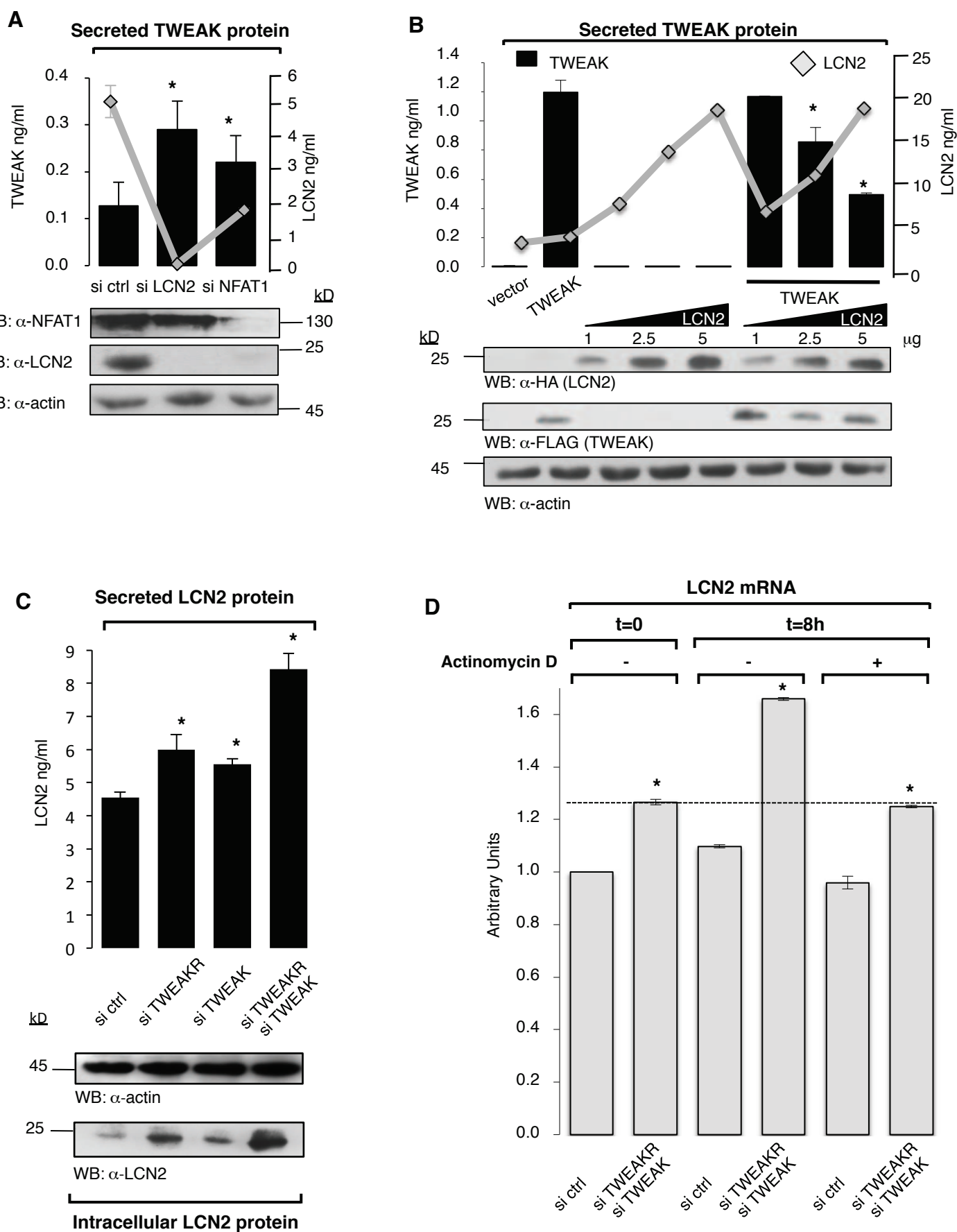


Figure 4 Gaudineau et al.

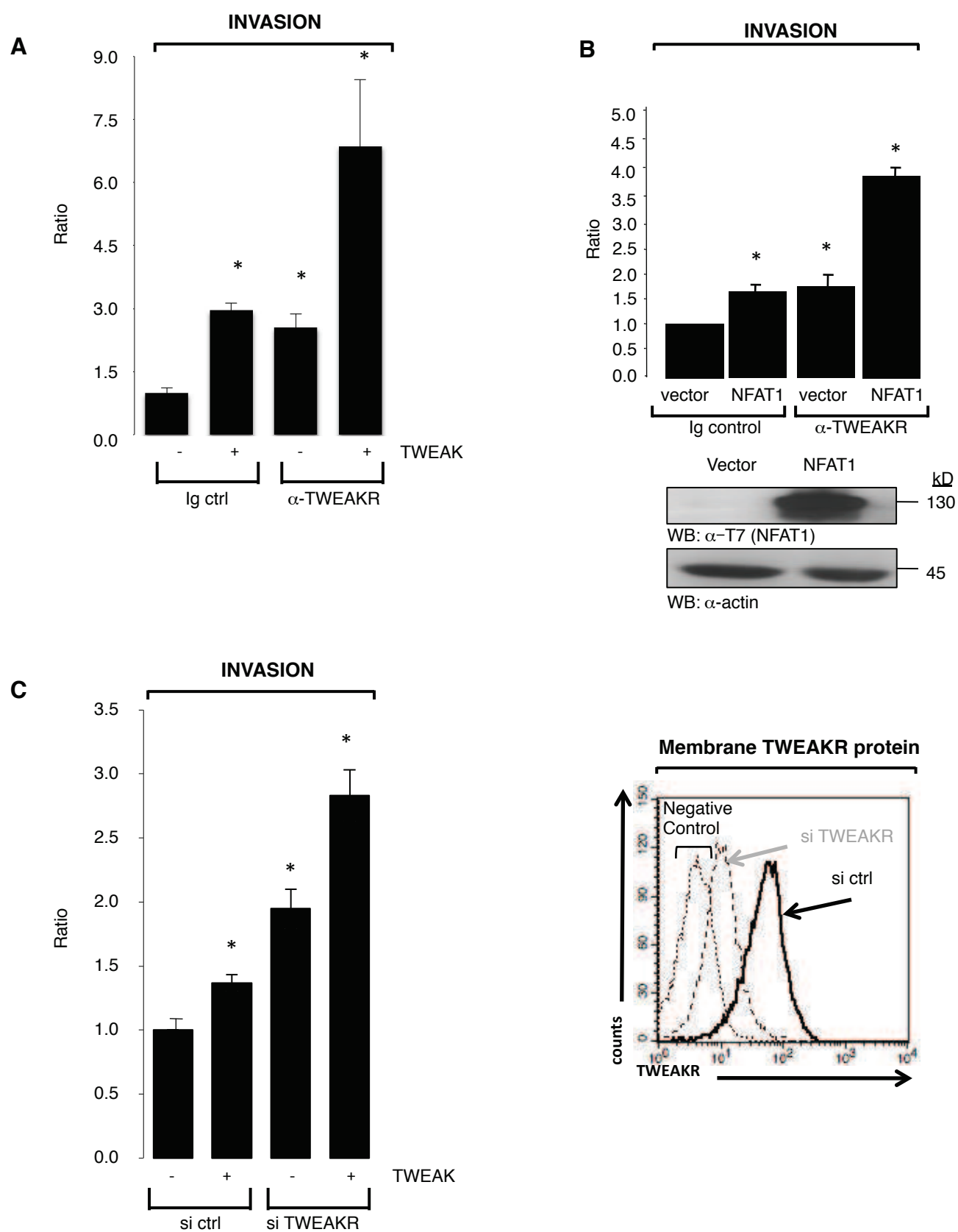
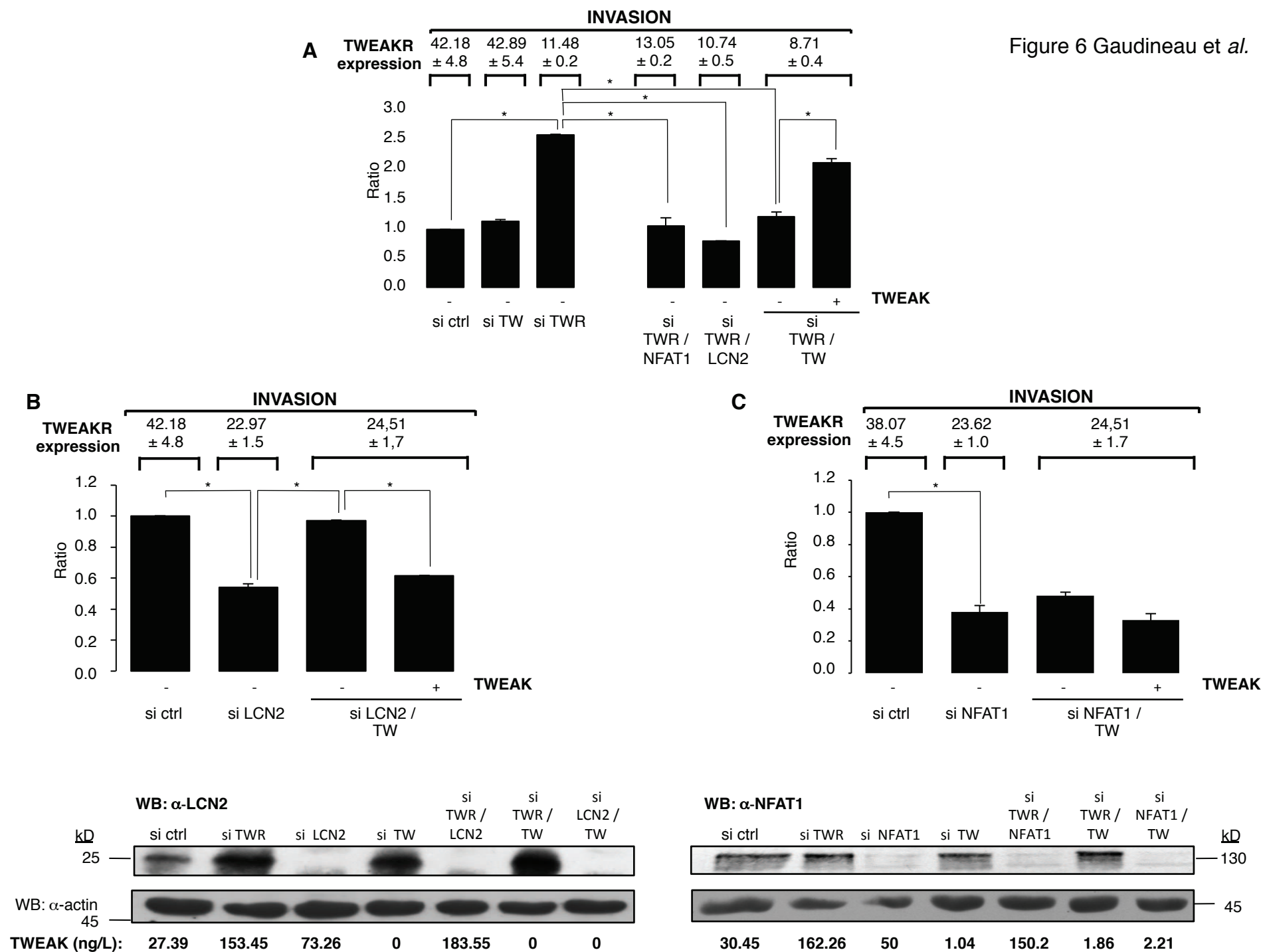


Figure 5 Gaudineau et al.

Figure 6 Gaudineau et al.



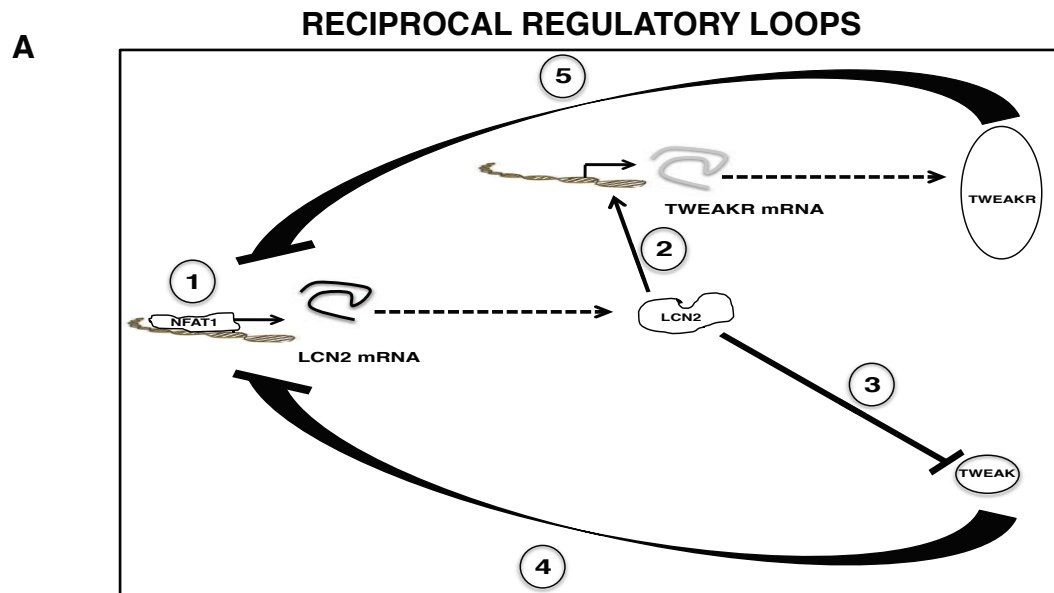


Figure 7 Gaudineau et al.

