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Benoît Gaudineau, Marjorie Fougère, Frédéric Guaddachi, Frédéric Lemoine, Pierre de La Grange, et al.. Lipocalin 2, the TNF-like receptor TWEAKR and its ligand TWEAK act downstream of NFAT1 to regulate breast cancer cell invasion.: NFAT1/LCN2/TWEAKR/TWEAK regulate motility. *Journal of Cell Science*, 2012, 125 (Pt 19), pp.4475-86. 10.1242/jcs.099879 . inserm-00715876

HAL Id: inserm-00715876

<https://inserm.hal.science/inserm-00715876>

Submitted on 5 Jul 2013

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

4
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15 Running title: NFAT1/LCN2/TWEAKR/TWEAK regulate motility

16
17 Words count: 7442

18 **SUMMARY**

19
20 **NFAT1 is a transcription factor that elicits breast carcinoma cells to become invasive,**
21 **contributing thus to formation of metastasis. The molecular mechanisms by which**
22 **NFAT1 operates in this respect are still poorly known. Here, we report that NFAT1**
23 **increases Lipocalin 2 (LCN2) mRNA and protein expression by binding to specific sites**
24 **in the *LCN2* gene promoter region. We show that the LCN2 protein is required**
25 **downstream of NFAT1 to increase breast cancer cell invasion. We demonstrate that the**
26 **NFAT1/LCN2 axis is sufficient to regulate expression of the TNF-like receptor**
27 **TWEAKR at the RNA level and of its ligand, TWEAK, at the protein level. We show,**
28 **however, that TWEAKR mediates an anti-invasive effect in breast cancer cells whereas,**
29 **depending on LCN2 expression, TWEAK has either anti- and pro-invasive capacities.**
30 **Thus, we identify LCN2 and TWEAKR/TWEAK as critical downstream effectors of**
31 **NFAT1 to regulate breast cancer cell motility and invasive capacity.**
32
33

34 Introduction

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

41 The family of NFAT transcription factors comprises five genes (*NFAT1* to 5). NFAT1,
42 NFAT2, NFAT3 and NFAT4 were first identified as T cell transcription factors that bind *IL-2*
43 promoter after cell activation (Shaw et al. 1988), while NFAT5 is induced by osmotic stress
44 (Lopez-Rodriguez et al. 1999). Because of their role in critical signalling pathways that
45 control cell fate (Baksh et al. 2002; Chuvpilo et al. 2002; Xanthoudakis et al. 1996), one
46 would expect that disturbing NFAT signalling could impact carcinogenesis. Indeed, beside
47 their role in the migratory and invasive capacities of breast cancer cells (Fougère et al. 2010;
48 Jauliac et al. 2002; Yiu and Toker 2006; Yoeli-Lerner et al. 2005, Germann et al; 2012), there
49 is growing evidence for an active role of NFAT factors in carcinogenesis (Buchholz et al.
50 2006; Dejmek et al. 2006; Foldynová-Trantírková et al. 2010; Holzmann et al. 2004; Jönsson
51 et al. 2002). However, beside these reports of a “pro-cancer” role, others are showing the
52 “anti-cancer” capacities of NFAT factors (Glud et al. 2005; Lee et al. 2005; Wu et al. 2010).
53 In line with this duality of function, we have reported that NFAT3 is specifically expressed in
54 oestrogen receptor α -positive breast cancer cells and, contrary to NFAT1 and NFAT5,
55 inhibits these cells' motility by blocking *LCN2* (*Lipocalin 2*) gene expression (Fougère et al.
56 2010).

57 *LCN2* is a secreted protein of the Lipocalin family (Flower 1996), the high expression
58 of which is associated with malignancy in different types of cancers (Bartsch and Tschesche
59 1995; Furutani et al. 1998; Missiaglia et al. 2004; Nielsen et al. 1996), including breast cancer
60 (Stoesz et al. 1998), and is a predictor of breast cancer progression (Hu et al. 2008). Actually,
61 *LCN2* regulates cell migration (Fougère et al. 2010; Leng et al. 2008; Playford et al. 2006)
62 and formation of metastases (Shi et al. 2008; Yang et al. 2009). *LCN2* is induced under
63 diverse inflammatory conditions (Nielsen et al. 1996), and it is known that inflammation is
64 strongly linked to tumorigenesis (Balkwill and Mantovani 2001; Iliopoulos, Hirsch, and
65 Struhl 2009; Pierce et al. 2009) and metastasis formation.

66 Apart from *LCN2*, other inflammatory molecules, such as those of the
67 TWEAK/TWEAKR cytokine receptor axis, have been linked to cancer. TWEAK (TNF-
68 related Weak Inducer of Apoptosis) belongs to the TNF family and is involved in many
69 diseases (Winkles 2008) including breast cancer (Willis et al. 2008). TWEAKR (TNF-related
70 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.

84

85 **Results**

86

87 **NFAT1 up-regulates LCN2 expression in breast cancer cells**

88 Having recently shown that NFAT3 inhibits *LCN2* transcription in breast cancer cells
89 (Fougère et al., 2010), we evaluated here the effect of NFAT1 on *LCN2* expression. However,
90 since NFAT1 and *LCN2* proteins are essentially not expressed in oestrogen receptor-positive
91 (ERA⁺) cancer cells (Fougère et al., 2010), this was done only using ERA-negative (ERA⁻)
92 cells in which, contrary to NFAT3, NFAT1 and *LCN2* are highly expressed. First, we used
93 NFAT1-specific siRNA to silence endogenous NFAT1 in MDA-MB-231, which reduced its
94 expression by 90% (Fig. 1A, right panel) without affecting that of NFAT5 or NFAT4 (data
95 not shown). NFAT1 silencing (which did not result in cell apoptosis as assayed by annexin V
96 labelling data; not shown) reduced by 5-fold *LCN2* mRNA expression relative to control cells
97 (Fig. 1A, left panel), and abrogated both intracellular (Fig. 1B, left panel) and secreted (Fig.
98 1B, middle panel) *LCN2* protein expression.

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

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

107

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

109 *LCN2* plays a critical role in regulating breast cancer cell motility (Fougère et al.
110 2010). In order to evaluate the physiological relevance of *LCN2* modulation by NFAT1, we
111 tested whether *LCN2* was involved in NFAT1-regulated chemoinvasion. Transiently
112 transfecting MDA-MB-231 cells with HA epitope-tagged *LCN2* (Fig. 1C, left panel)
113 increased breast cancer cell chemoinvasion as compared with empty vector-transfected cells
114 (Fig. 1C, right panel), indicating *LCN2* role in the process. To evaluate whether *LCN2*
115 expression downstream of NFAT1 was needed to regulate chemoinvasion, we transiently co-
116 transfected the cells with T7 epitope-tagged NFAT1 together with either control or *LCN2*-
117 targeted siRNA (Fig. 1D, right panel) and in combination with a betagalactosidase (β -gal)

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

123

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

125 To more thoroughly analyse the relationship between LCN2 expression and NFAT1
126 signalling, we investigated the *LCN2* promoter region where 6 potential NFAT-binding sites
127 (-881, -522, -501, -441, -409, -142) (Fig. 2A) have been identified (Fougère et al. 2010).
128 Using a *Luciferase* gene-fused *LCN2* promoter, we found that NFAT1 ectopic expression
129 doubled the promoter activity relative to empty vector-transfected cells (Fig. 2B). To assess
130 whether endogenous NFAT1 interacted with *LCN2* promoter region, chromatin
131 immunoprecipitation (ChIP) was performed in cells treated by control or NFAT1-specific
132 siRNAs: indeed, endogenous NFAT1 bound to *LCN2* promoter whereas no significant signal
133 enrichment was noted when NFAT1 was silenced (Fig. 2C, si ctrl versus si NFAT1). To
134 confirm NFAT1 binding to *LCN2* promoter region and determine whether NFAT1 putative
135 binding sites are functional *in vivo*, we individually mutated each of these. Measurement of
136 Luciferase activity after transient co-transfection of MDA-MB-231 cells with NFAT1 of
137 mutated *LCN2* promoter revealed that the -409 binding site was required for NFAT1 to
138 increase transcriptional activity, the -501 and -142 sites being required for *LCN2* promoter
139 basal activity (Fig. 2D). Electrophoretic mobility shift assays were then performed to confirm
140 that endogenous NFAT1 bound to these identified sites and, indeed, NFAT1 bound to the -
141 501, -409, -142 wild-type, but not mutated, binding sites (Fig. 2E). NFAT1-specific binding
142 was confirmed by pre-incubating nuclear extracts with an anti-NFAT1 antibody that
143 supershifts the complex NFAT1/probe (Fig. 2E, arrows). As control, no supershift was
144 induced by pre-incubating nuclear extracts with control IgG.

145 These data demonstrate that endogenous NFAT1 binds directly to the *LCN2* promoter
146 -501, -142 sites to modulate its basal expression, and to the -409 site to regulate its inducible
147 expression.

148

149 **The NFAT1/LCN2 axis modulates TWEAKR expression**

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

152 cells in order to identify genes regulated by LCN2 potentially involved in the invasive
153 capacity. LCN2 silencing impacted 95 genes by at least 1.5 fold (95% confidence level) (Fig.
154 3A, and Supplementary Table 1), which was confirmed by RT-QPCR for most of these (data
155 not shown). Because we have shown that NFAT1 regulates *LCN2* expression, we examined
156 whether modifying NFAT1 expression affected any of the 95 *LCN2*-regulated genes. Indeed,
157 siRNA silencing of NFAT1 reduced expression of two of them: *TWEAKR* (TNFSFR12A)
158 (Fig. 3B) and *C6ORF55* (VTA1) (data not shown). Thus, out of the 95 identified genes that
159 putatively could be involved in the invasion process of breast cancer cells, only these two
160 were regulated in the same manner by NFAT1 and LCN2. Silencing VTA1 with siRNA had
161 no effect on cancer cell invasive capacity (data not shown). Because *TWEAKR* was already
162 known to participate in the motility of different cell types (Meighan-Mantha et al. 1999;
163 Willis et al. 2008) and was regulated in the same manner by NFAT1 and LCN2, we further
164 investigated this regulation of *TWEAKR* expression by LCN2 and NFAT1 and its role in the
165 invasive behaviour of breast cancer cells. Flow cytometry analysis confirmed that siRNA
166 silencing of either LCN2 or NFAT1 down-regulated *TWEAKR* protein expression in MDA-
167 MB-231 (Fig. 3C) and SUM-159-PT (Supplementary Fig. S1) cells relative to control cells.

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

170

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

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

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

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

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

197

198 **TWEAK increases breast cancer cell invasion independently of the TWEAKR**

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

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

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

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

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

233

234 **Depending on LCN2 expression TWEAK displays either anti- and pro-invasive activities** 235 **in breast cancer cells**

236 Inasmuch as TWEAK availability appeared to be regulated by TWEAKR, LCN2 and
237 NFAT1, we more precisely examined the role of TWEAK protein on breast cancer cell
238 invasion. MDA-MB-231 cells were transiently transfected with control siRNA or with
239 siRNAs targeting TWEAKR, LCN2, NFAT1 or TWEAK, alone or in combination (Fig. 6A).
240 As shown in Fig. 5C, TWEAKR silencing led to increased chemoinvasion, and either NFAT1
241 (Fig. 6A, si TWR si NFAT1) or LCN2 (Fig. 6A, si TWR si LCN2) depletion prevented this
242 increase, demonstrating that LCN2 increased expression elicited by TWEAKR knockdown
243 was responsible for this effect. Indeed, when NFAT1 was silenced, and by consequence
244 LCN2 too, again the increase of chemoinvasion induced by TWEAKR down-regulation was
245 abrogated (Fig. 6A, si TWR si NFAT1). Since we have shown that TWEAKR depletion
246 induces TWEAK protein up-regulation (Supplementary Fig. S3), we examined whether the
247 latter was involved in this increased invasion. Indeed, when TWEAK was silenced by siRNA
248 together with TWEAKR (Fig. 6A, si TWR si TW, -), increased invasion was abolished but it
249 was rescued by adding back recombinant TWEAK (Fig. 6A, si TWR si TW, +). The same
250 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

275 Discussion

276 The role of NFAT transcription factors in breast cancer cell motility has been
277 identified recently (Jauliac et al. 2002; Mancini and Toker 2009; Yoeli-Lerner et al. 2005) but
278 it is still poorly understood. Apart from studies indicating that either *COX2* (Yiu and Toker
279 2006) or the *autotaxin* gene (Chen and O'Connor 2005) are targeted by NFAT1 to increase
280 invasion and migration, we do not know which other specific signalling pathways these
281 factors use to modulate breast cancer cell motility.

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

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

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

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

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

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

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

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

353 In summary, we have shown that signalling through NFAT1 increases invasion
354 through a previously unknown LCN2/TWEAKR/TWEAK axis. We demonstrate that
355 TWEAK can have opposite effects on breast cancer cell invasion: anti-invasive via
356 TWEAKR, independently of LCN2 (Fig.7B, Without LCN2); pro-invasive via another
357 unidentified receptor (X^R) or directly in association with LCN2 (Fig. 7B, Without TWEAKR).
358 Therefore, increased NF A T1 activation leads to up-regulation of LCN2 protein expression
359 that consequently enables TWEAK pro-invasive activity in breast cancer cells. These
360 findings underscore the importance of dissecting the mechanisms by which TWEAK and
361 LCN2 regulate breast cancer cell invasion, downstream of NFAT1, in order to be able to
362 attempt to therapeutically target this pathway to limit the dissemination of metastasis.

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365 **Materials and Methods**

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367 **Cell culture**

368 The MDA-MB-231 cell line was from the American Type Culture Collection, The
369 SUM-159-PT cell line was provided by Alex Toker (Harvard Medical School). MDA-MB-
370 231 and SUM-159-PT cells were maintained in Dulbecco Modified Eagle Medium (DMEM),
371 low glucose (1 g/L D-glucose), 10% Foetal Calf Serum. NIH-3T3 cells were maintained in
372 DMEM, high glucose (4.5 g/L D-glucose), 10% Newborn Calf Serum. All media were
373 supplemented with 2 mM L-Glutamine, 100 U/mL Penicillin and 100 μ g/mL Streptomycin.

374

375 **Antibodies and reagents**

376 The following antibodies were used: Anti-T7 (Novagen; #69522), anti-FLAG (Sigma;
377 #F1804), anti-HA (Roche; #11867423), anti-NFAT1 from ABR Affinity BioReagent (MAI-
378 025) or from Abcam for ChIP assays (ab2722), anti-Actin (Santa-Cruz Biotechnology; SC-
379 1616), anti-LCN2 (Sigma-Aldrich; HPA002695), and anti-TWEAKR (Abcam; ab21359) for
380 flow cytometry. The mouse IgG was from Santa-Cruz Biotechnology (SC-2025) and the anti-
381 mouse phycoerythrin-labelled antibody (F0102B) was from R&D systems. The blocking anti-
382 TWEAKR antibody was from R&D systems (#AF1199).

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

389

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

391 NFAT1 was cloned by PCR in the pcDNA3 vector expressing a N-terminal T7-tag.
392 LCN2 promoter and the pCS4-(n)- β -galactosidase were previously described (Fougère et al.
393 2010). The potential NFAT-binding sites of LCN2 promoter were mutated by PCR using the
394 primers described in Supplementaray Table 2. LCN2 was cloned by PCR in the pcDNA3.1+
395 vector with a HA tag from RNA isolated from MDA-MB-231 cells. The FLAG-TWEAK
396 expression vector was from Origene. All constructions were verified by sequencing. To
397 transiently silence TWEAKR, TWEAK, NFAT1 or LCN2, we used specific siRNAs

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

400 LCN2, TWEAKR and TWEAK mRNA expression was determined by RT-QPCR.
401 Total RNA was extracted using the RNeasy mini kit (QIAGEN) according to the
402 manufacturer's instructions, and cDNA synthesis was prepared using the SuperScript Reverse
403 Transcriptase (RT) (Invitrogen). RT-QPCR was performed using SYBR Green PCR Master
404 Mix on Roche LightCycler as directed by the manufacturer. Cycling parameters were 10
405 minutes at 95°C followed by 45 cycles of 10 seconds at 95°C (denaturation) and 4 seconds at
406 68°C (annealing/extension). Primers are accessible in the Supplementary Information,
407 Supplementary Table 2.

408 **Proteins detection**

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

418 **ELISA for assessing LCN2 and TWEAK**

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

423 **Invasion assays (chemoinvasion and random invasion assays)**

424 The chemoinvasion assay was performed essentially as described, using Transwell chambers
425 (Becton Dickinson) with 8- μ m pore membranes coated with Matrigel (Becton Dickinson).
426 Cells, co-transfected with the relevant expression plasmids and the pCS2-(n)- β -gal reporter
427 plasmid, were resuspended after 24 hours in serum-free medium containing 0.1% BSA, and
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431 cells were added to each well. Conditioned NIH-3T3 medium was added to the bottom wells
432 of the chambers. After 6 hours, cells that had not invaded were removed from the upper face
433 of the filters using cotton swabs, and cells that had invaded to the lower surface of the filters
434 were fixed for 30 min in 4% paraformaldehyde and then stained with PBS containing 1 mg/ml
435 bluo-gal, 2 mM MgCl₂, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide.
436 All cells in each Transwell were counted. All the counts were normalised by the efficiency of
437 transfection. The numbers of cells that invaded in each condition were compared with
438 the empty vector-transfected condition arbitrary set as a ratio of 1. When the assay was
439 performed with cells transiently transfected by siRNA, cells were stained with crystal violet
440 since 95% of the cells were effectively transfected. In some cases, 200 ng/mL recombinant
441 TWEAK was added for 6 hours to the cells during the assay.

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

443 444 **Luciferase assay**

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

451 452 **Electrophoretic mobility shift assay**

453 Harvested cells were washed in ice-cold PBS, 0.5 mM DTT, and lysed in buffer with
454 10 mM HEPES, 10 mM KCl, 15 mM MgCl₂. Nuclei were isolated by centrifugation, and
455 nuclear proteins were extracted in buffer with 20 mM HEPES, 25% glycerol, 420 mM NaCl,
456 15 mM MgCl₂, 0.2 mM EDTA, DTT (0.5 mM) and PMSF (0.2 mM). Binding of nuclear
457 extracts to 5'-end-IRDye700-labeled probes (Eurofins MWG Operon) was carried out by
458 incubating the extracts with 50 fmol of labelled probes in 20 μ L at room temperature for 20
459 min in binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM KCl, 3 mM DTT, 0.25% Tween20
460 and 50 ng/ μ L poly(dI-dC)). Unbound probes were separated from DNA-protein complexes in
461 a 5% polyacrylamide gel, and detected after migration on an infrared imaging system
462 ODYSSEY (Li-Cor Biosciences). Super-shift was obtained by incubating nuclear extracts
463 with 0.5 μ g antibodies (anti-NFAT1 or IgG control) at room temperature for 20 min before
464 adding the labelled probes. Probe sequences are reported in Supplemental Table 2.

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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 μ g anti-NFAT1 antibody or control IgG and 20 μ L of 50% suspension blocked beads in 300 μ L 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 μ L 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 μ L 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.

499 **Array hybridization, data analysis and clustering**

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

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517 **Supplementary online data**

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

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522 **Acknowledgements**

523 We thank H. de Thé and members of his laboratories for insightful discussions, J.C.
524 Gluckman and M. Rajaud (Genosplice) for revising the manuscript. We are grateful to N.
525 Setterblad and the Service Commun d'Imagerie Cellulaire et Moléculaire (supported by grants
526 from the Conseil Régional d'Ile-de-France and the Ministère de la Recherche). B. Gaudineau
527 was supported by a grant from the Cancéropole Ile-de-France and an ARC fellowship. M.
528 Fougère was supported by a doctoral grant from INSERM Région Ile-de-France and an ARC
529 fellowship. This work was supported by the INSERM AVENIR Program and grants from
530 Ligue Nationale contre le Cancer, the Comité de Paris of Ligue Nationale contre le Cancer,
531 ARC, and the Comité Tumeurs de la Fondation de France and the Cancéropole Ile-de-France.
532

533 **Figure Legends**

534

535 **Figure 1.** NFAT1 up-regulates LCN2 expression to increase the invasive capacity of breast
536 cancer cells. (A) MDA-MB-231 cells were transiently transfected with control (si ctrl) or
537 NFAT1-specific (si NFAT1) siRNAs and assayed by RT-QPCR to quantify LCN2 and β -2
538 microglobulin mRNAs as arbitrary units relative to si ctrl-transfected cells. Immunoblots with
539 anti-NFAT1 or anti-actin antibodies are shown. (B) Cells were transfected as in (A). Left
540 panel: Immunoblots with anti-NFAT1, anti-LCN2 or anti-actin antibodies of total cell lysates.
541 Middle panel: LCN2 protein amount was assessed by ELISA in 50 μ l conditioned 48-hr-
542 culture medium from the cells. Right panel: Cells were transiently co-transfected with a GFP-
543 expressing vector and either a control (vector) or a T7 epitope-tagged NFAT1 (NFAT1)
544 vector. After 24 hr, cell lysates of bulk unsorted cells were immunoblotted to probe for
545 NFAT1 (anti-T7) and actin (anti-actin) to avoid a too strong signal, while endogenous LCN2
546 (anti-LCN2) and actin (anti-actin) were assessed in lysates of their GFP⁺ FACS-sorted
547 counterparts. (C) Cells were transiently transfected with a control (vector) or a HA epitope-
548 tagged LCN2 (LCN2) vector in combination with a β -gal-expressing vector, and tested after
549 24 hr for the ability to invade using the chemoinvasion assay. Immunoblots with anti-HA
550 or anti-actin antibodies are shown. (D) Cells were transiently co-transfected with a T7
551 epitope-tagged NFAT1 (NFAT1) or control (vector) vector and either control (si ctrl) or
552 LCN2-specific (si LCN2) siRNAs in combination with a β -gal-expressing vector, and tested
553 after 48 hr in the chemoinvasion assay. Immunoblots with anti-T7 (NFAT1), anti-LCN2
554 or anti-actin antibodies are shown. Statistical analyses were performed relative to si ctrl/
555 vector-transfected cells. All data are representative of three independent experiments. *Bars*:
556 SE. *, $P < 0.05$.

557

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

566 Recruitment of endogenous NFAT1 on LCN2 promoter region was quantified by QPCR after
567 ChIP using an anti-NFAT1 antibody and comparing si NFAT1- and si ctrl-treated cells using
568 primers encompassing the -881 or -522 or -501, -441, -409 and -142 potential NFAT1-
569 binding sites. Immunoblots with anti-NFAT1 or anti-actin antibodies are shown. (D) MDA-
570 MB-231 cells were cotransfected with the pCS4-(n)- β -gal and wild-type or mutated (^x) LCN2
571 promoter (pLCN2) plasmids, and cotransfected with the NFAT1 (+) or control (-) vector.
572 Cells were analysed 48 hr later for Luciferase and β -gal activities. Quantification of LCN2
573 promoter Luciferase activity was normalized relative to that of β -gal. Immunoblots with anti-
574 T7 (NFAT1) or anti-actin antibodies are shown. (E) Nuclear extracts from MDA-MB-231
575 cells were incubated with IRDye700-labeled probes containing the wild-type or mutated (*) -
576 142, -409, -501 predicted NFAT-binding sites. The NFAT1/probe complexes were separated
577 on a 5% polyacrylamide gel. A specific anti-NFAT1 antibody or control IgG were pre-
578 incubated with nuclear extracts before incubation with the IRDye700-labeled probes. Arrows
579 indicate positions of the super-shifted NFAT antibody-probe complex and asterisk position of
580 the non-specific bands. All data are representative of three independent experiments. *Bars*:
581 SE. *, $P < 0.05$.

582
583 **Figure 3.** The NFAT1/LCN2 axis modulates TWEAKR expression. (A) The distance from
584 the gene signal in a given sample to the corresponding average in the 6 samples (MDA-MB-
585 231 cells transfected with si ctrl or si LCN2) was calculated for each LCN2-regulated gene.
586 Corresponding values were displayed and clusterised with MeV4.6.2 from The Institute of
587 Genome Research using Euclidean distance and complete linkage clustering. (B) MDA-MB-
588 231 cells were transiently transfected with control (si ctrl), NFAT1-specific (si NFAT1) or
589 LCN2-specific (si LCN2) siRNAs, and assayed by RT-QPCR to quantify LCN2 and β -2
590 microglobulin mRNAs as arbitrary units relative to si ctrl-transfected cells. Immunoblots with
591 anti-NFAT1, anti-LCN2 or anti-actin antibodies are shown. (C) Cells, transfected as in (B),
592 were stained after 48 hr with an anti-TWEAKR antibody and analysed by flow cytometry. As
593 control, cells were stained only with the secondary phycoerythrin-labelled antibody, omitting
594 the anti-TWEAKR antibody. All data are representative of three independent experiments.
595 *Bars*: SE. *, $P < 0.05$.

596
597 **Figure 4.** Reciprocal regulation of the TWEAKR/TWEAK axis and LCN2 expression. (A)
598 TWEAK and LCN2 (grey line) were assessed by ELISA in 50 μ l concentrated conditioned

599 48-hr culture medium from MDA-MB-231 cells transiently transfected with control (si ctrl),
600 NFAT1-specific (si NFAT1) or LCN2-specific (si LCN2) siRNAs. Immunoblots with anti-
601 NFAT1, anti-LCN2 or anti-actin antibodies are shown. (B) Both proteins were also assessed
602 in medium from the cells transiently transfected with increasing amounts of HA epitope-
603 tagged LCN2 (LCN2) and a fixed amount of FLAG epitope-tagged TWEAK (TWEAK),
604 alone or in combination, or an empty vector control (vector). Immunoblots with anti-HA,
605 anti-FLAG or anti-actin antibodies are shown. (C) Upper panel: LCN2 was assessed by
606 ELISA in 50 μ l conditioned 48-hr culture medium from the cells transiently transfected with
607 either a control (si ctrl) or TWEAKR-specific (si TWEAKR) siRNAs, and a TWEAK-specific
608 (si TWEAK) siRNA, alone or in combination. Lower panel: LCN2 expression was evaluated
609 in cell extracts with an anti-LCN2 or an anti-actin antibody as loading control. (D) LCN2
610 mRNA quantification in the cells transiently transfected with control (si ctrl) or TWEAKR-
611 and TWEAK-specific (si TWEAKR/si TWEAK) siRNAs; 36 hr post-transfection (t=0),
612 vehicle (-) or 2 μ g/mL actinomycin D (+) were added to the cells. After 8-hr treatment, total
613 RNA was obtained and RT-QPCR was performed as described earlier to quantify LCN2 and
614 β -2 microglobulin mRNAs. All data are representative of three independent experiments.
615 *Bars*: SE. *, $P < 0.05$.

616
617 **Figure 5.** TWEAKR is an anti-invasive receptor in breast cancer cells. (A) MDA-MB-231
618 cells were pre-incubated with a TWEAKR-specific neutralizing antibody (α -TWEAKR) or
619 control IgG (Ig ctrl), and tested in the invasion assay in the presence of TWEAK (+) or
620 vehicle (-). (B) Cells were transiently transfected with a T7 epitope-tagged NFAT1 (NFAT1)
621 or control (vector) vector. After 24 hr, cells were pre-incubated with the TWEAKR-specific
622 neutralizing antibody (α -TWEAKR) or control IgG (Ig ctrl) and tested in the invasion assay
623 as in (A). Immunoblots with anti-T7 (NFAT1) or anti-actin antibodies are shown. (C) Cells
624 were transiently transfected with control (si ctrl) or TWEAKR-specific (si TWEAKR)
625 siRNAs. After 48 hr, cells were tested in the invasion assay as in (A). Cell membrane
626 TWEAKR expression monitored by flow cytometry is shown in the right panel. (D) Cells
627 were transiently transfected with control (si ctrl) or LCN2-specific (si LCN2) siRNAs, and
628 tested in the invasion assay after 48 hr as above. All data are representative of three
629 independent experiments. *Bars*: SE. *, $P < 0.05$.

630

631 **Figure 6.** Depending on LCN2 expression TWEAK displays both anti- and pro-invasive
632 activities in breast cancer cells. (A) MDA-MB-231 cells were transiently transfected with
633 control (si ctrl) or TWEAKR- (si TWR), TWEAK- (si TW), NFAT1- (si NFAT1) or LCN2-
634 specific (si LCN2) siRNAs, alone or in combination. Cells were tested 48 hr later in the
635 invasion assay in the presence of TWEAK (+) or vehicle (-). Cell membrane TWEAKR was
636 monitored by flow cytometry: geometric means of TWEAKR expression intensities are
637 indicated above the graph. TWEAK amounts in concentrated conditioned media of
638 transfected cells was monitored by ELISA: values (TWEAK (ng/L) are indicated below the
639 immunoblots with anti-NFAT1, anti-LCN2 and anti-actin antibodies shown at the bottom of
640 Fig. 6. (B) The cells were transiently transfected with control (si ctrl), TWEAK- (si TW) or
641 LCN2-specific (si LCN2) siRNAs, alone or in combination. After 48 hr, cells were tested in
642 the invasion assay as in (A). Expression of TWEAKR, TWEAK and LCN2 is presented as
643 described in (A). (C) The cells were transiently transfected with control (si ctrl), TWEAK- (si
644 TW) or NFAT1-specific (si NFAT1) siRNAs, alone or in combination. After 48 hr, cells were
645 tested in the invasion assay as in (A). Expression of TWEAKR, TWEAK and NFAT1 is
646 presented as in (A). All data are representative of three independent experiments. Bars: SE. *,
647 $P < 0.05$.

648
649 **Figure 7.** A working model of the interactions between NFAT1, LCN2, the TWEAKR and
650 TWEAK. (A) Reciprocal regulatory loops between TWEAK/TWEAKR/LCN2 and NFAT1.
651 NFAT1 up-regulates LCN2 mRNA and protein expression (1). LCN2 protein regulates
652 TWEAKR expression (2) and modulates TWEAK protein expression (3). As a retro-control,
653 TWEAKR and TWEAK inhibit transcription of LCN2 mRNA (4 and 5). (B). In breast cancer
654 cells there is an equilibrium between inhibitory signalling mediated by TWEAK via the
655 TWEAKR independently of LCN2 (1) and activating signalling mediated by TWEAK
656 through binding to an unknown receptor (X^R) in cooperation with LCN2 (2). In the absence of
657 TWEAKR (Without TWEAKR), LCN2-dependent, TWEAK-mediated activating signalling
658 is promoted and invasion increases (left panel). On the contrary, in the absence of LCN2
659 (Without LCN2) LCN2-independent inhibitory signalling mediated by TWEAK via
660 TWEAKR is promoted and invasion is prevented (right panel).

661

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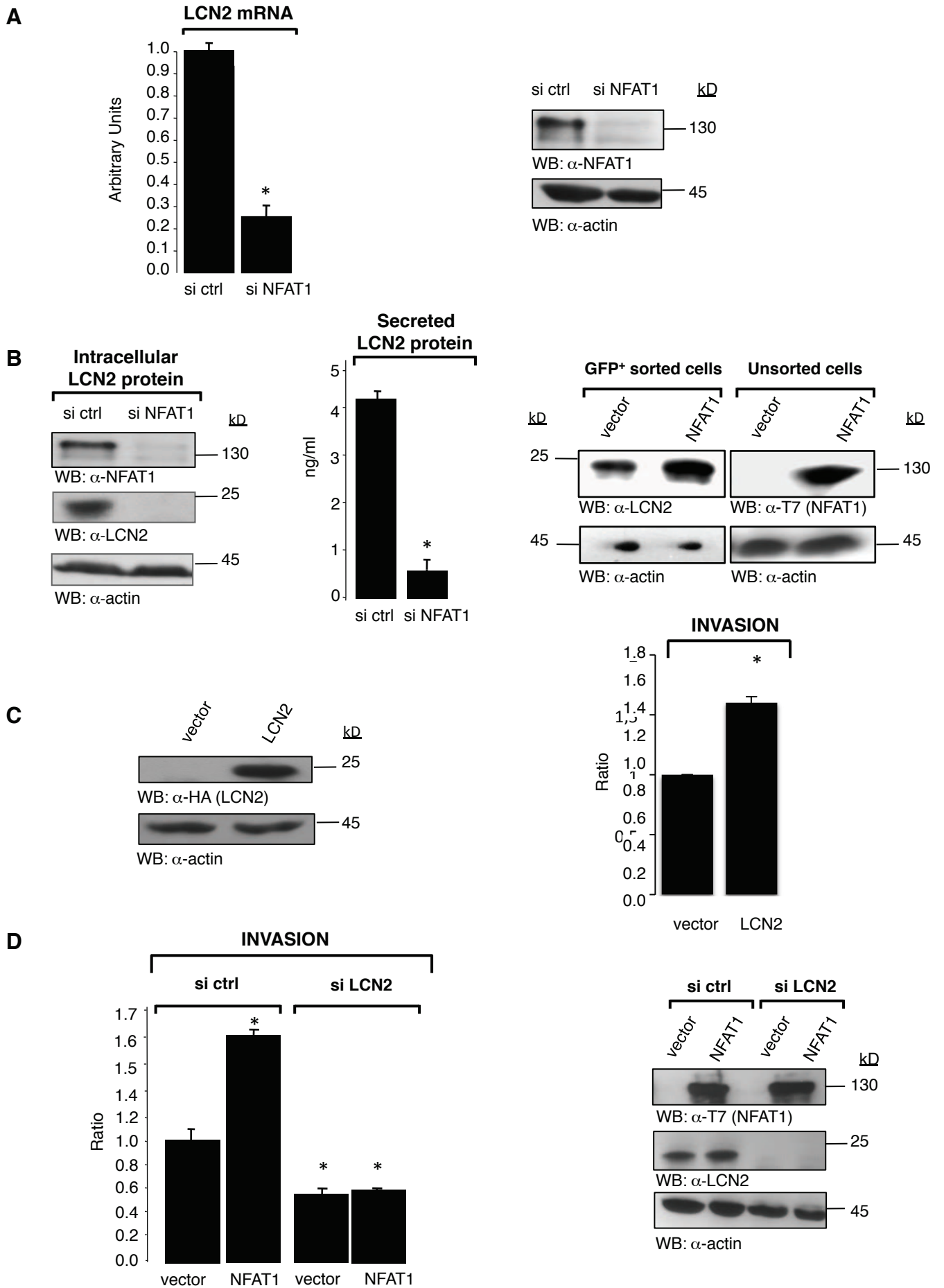


Figure 1 Gaudineau et al.

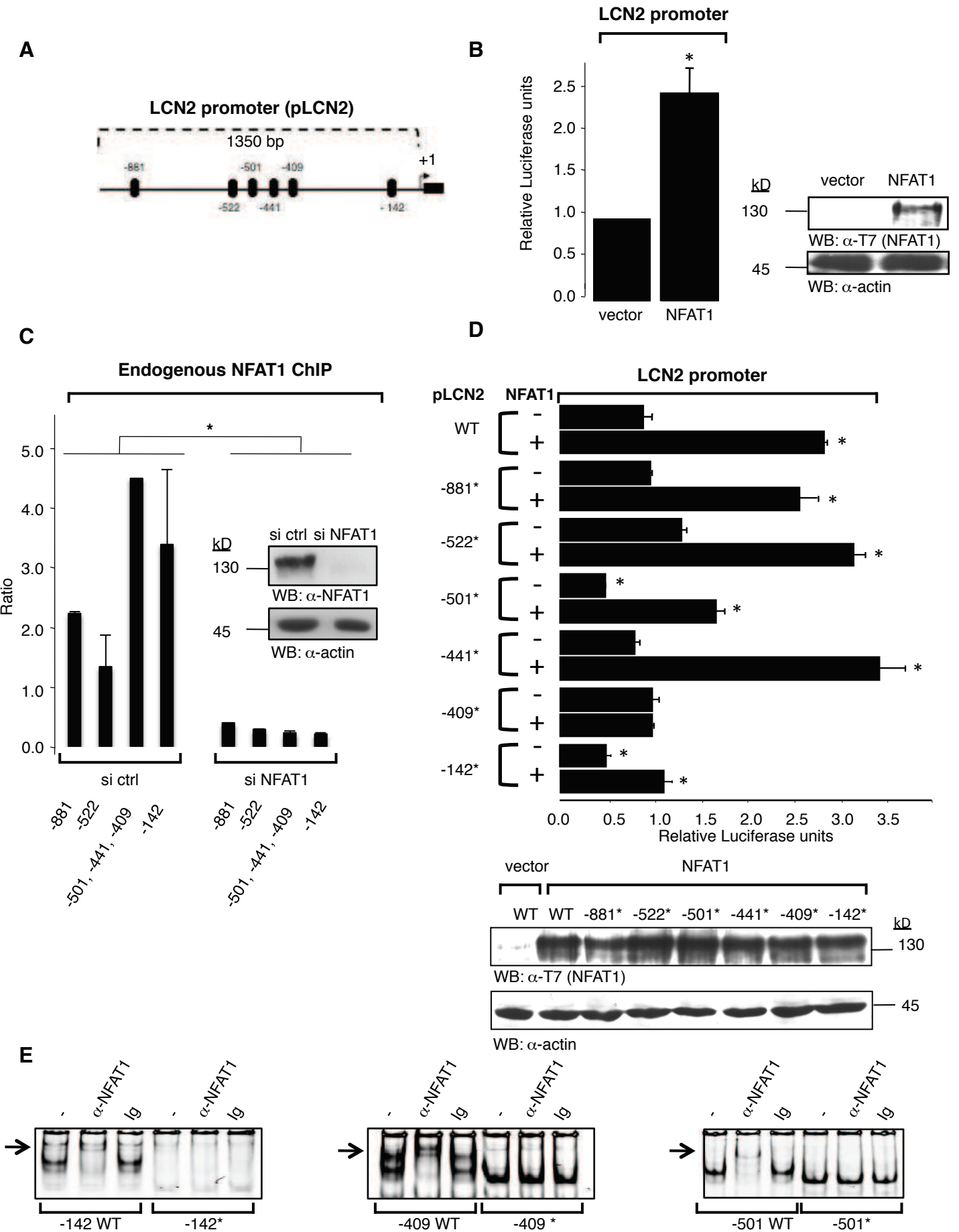


Figure 2 Gaudineau et al.

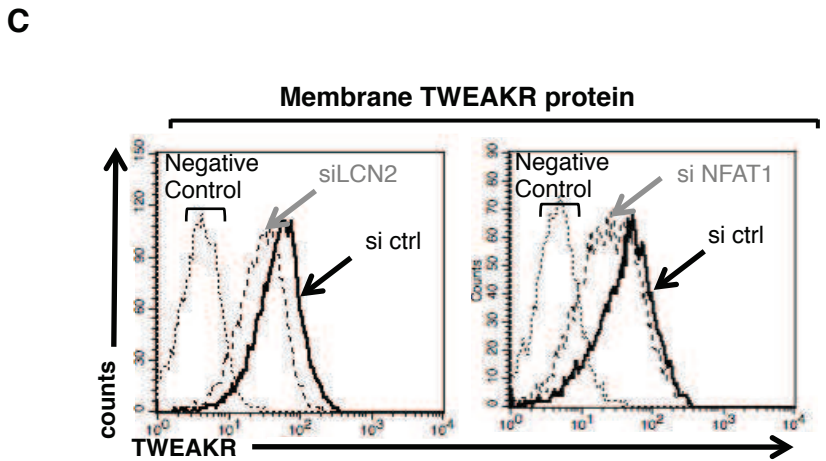
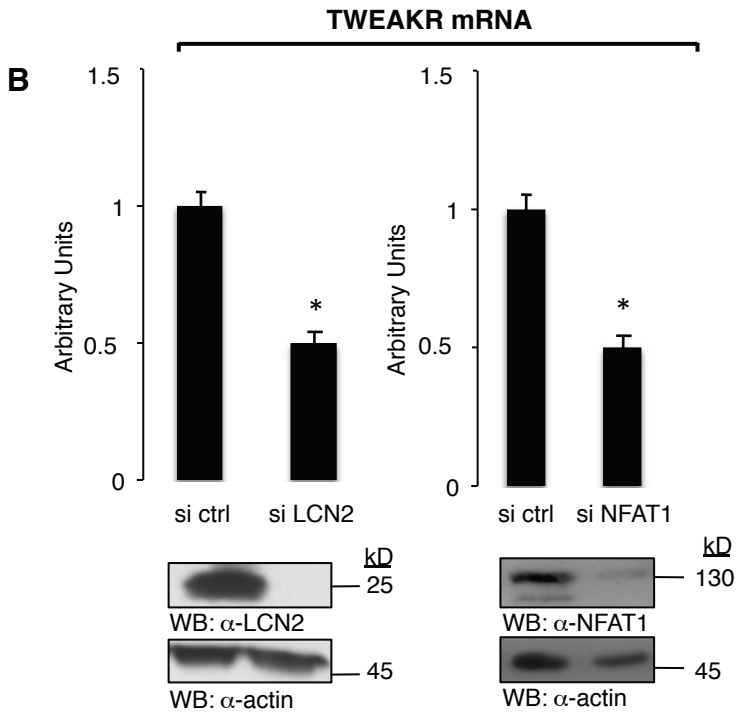
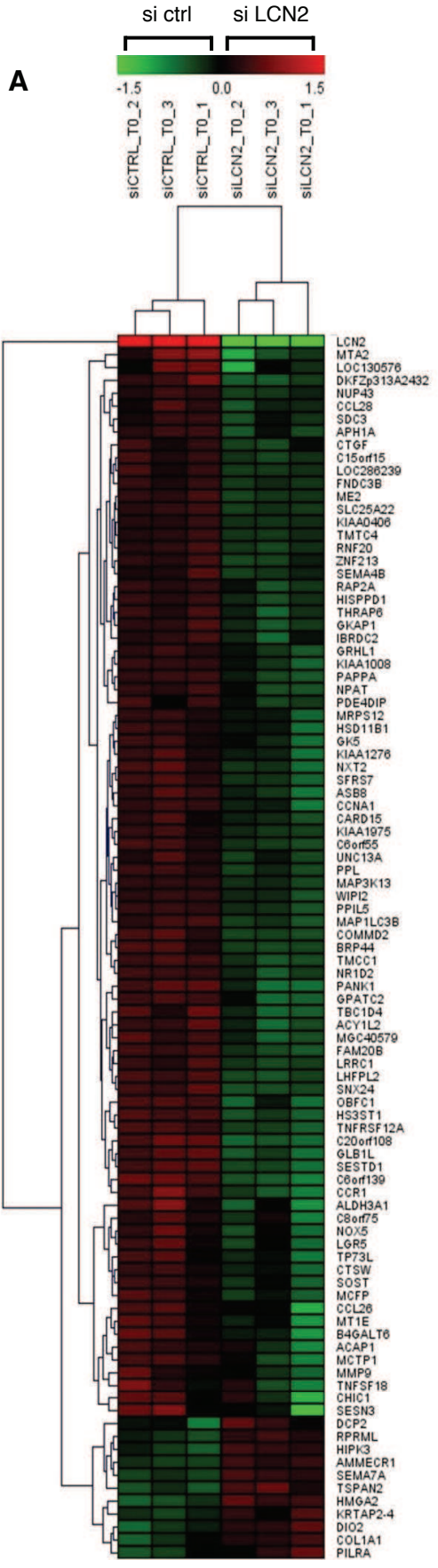


Figure 3 Gaudineau et al.

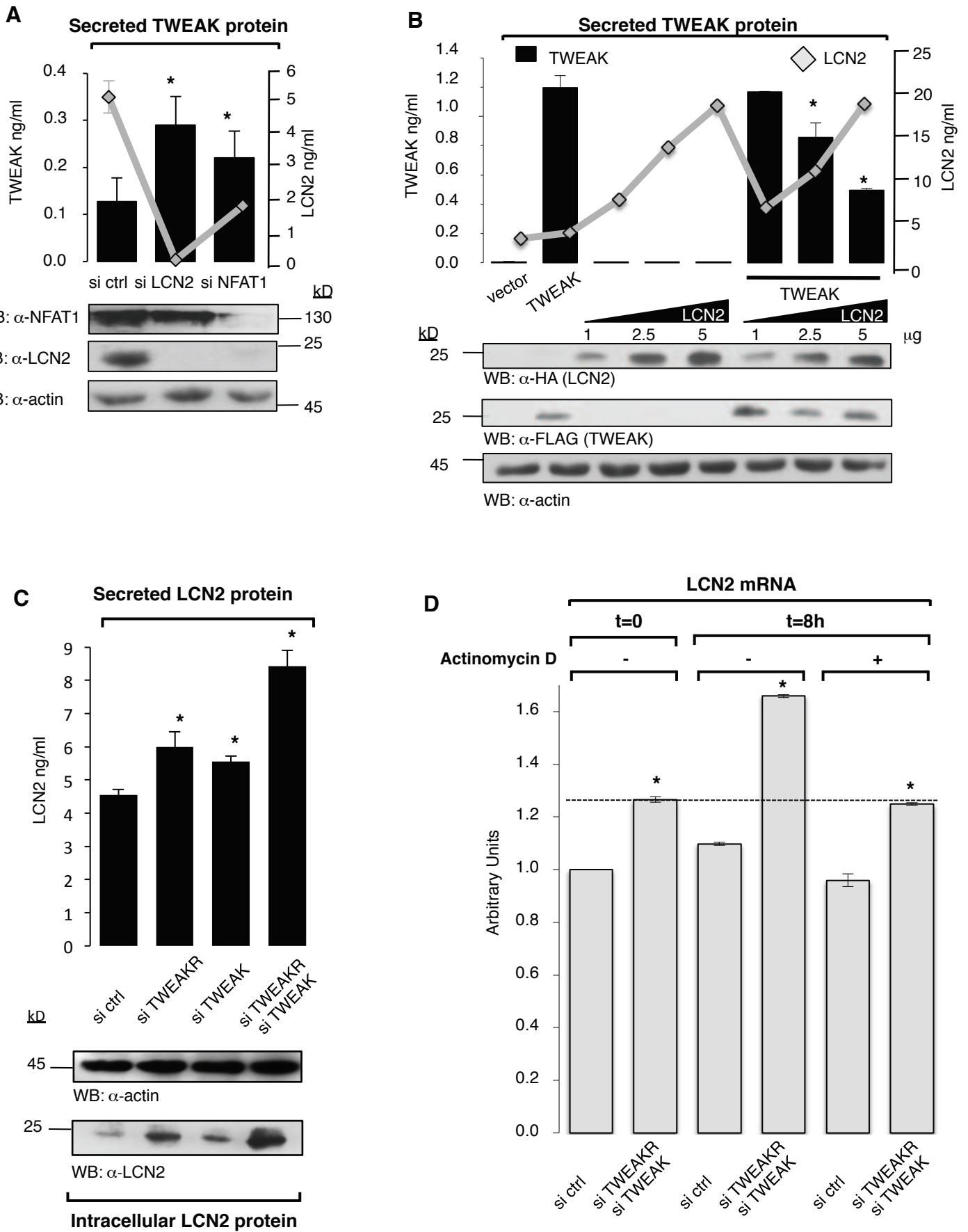


Figure 4 Gaudineau et al.

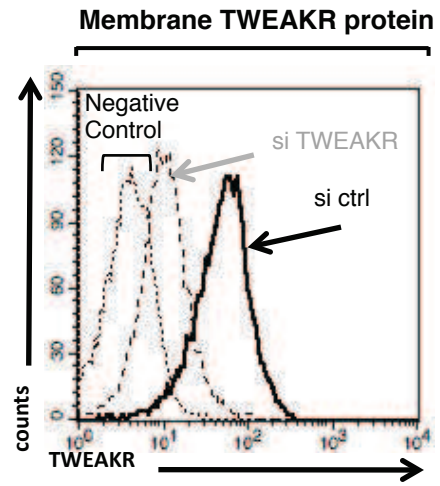
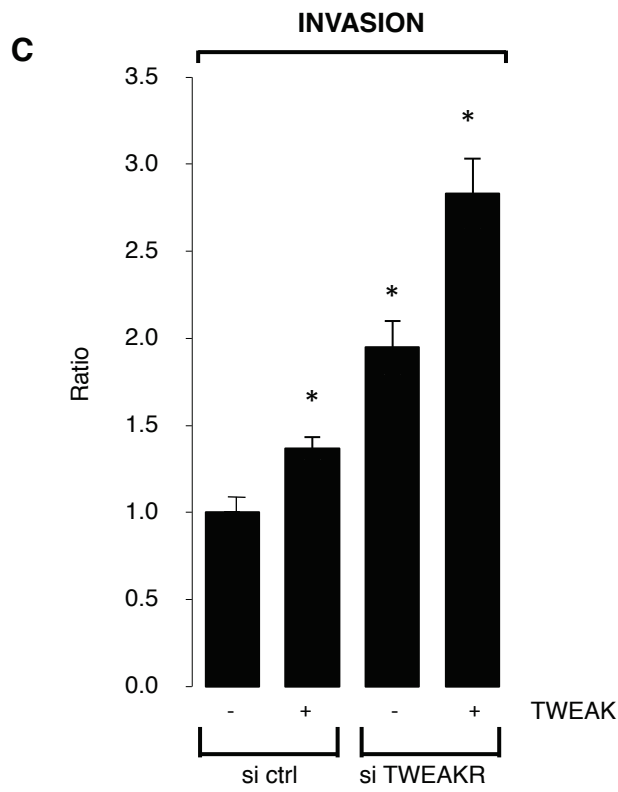
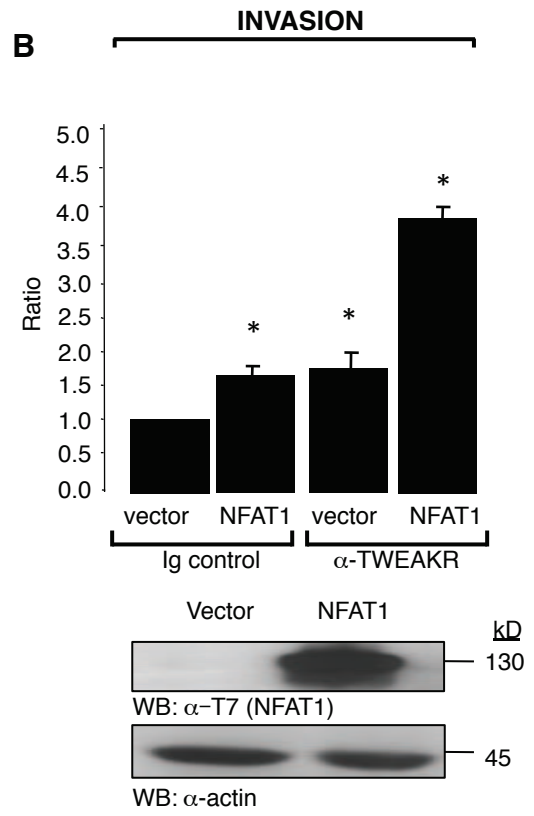
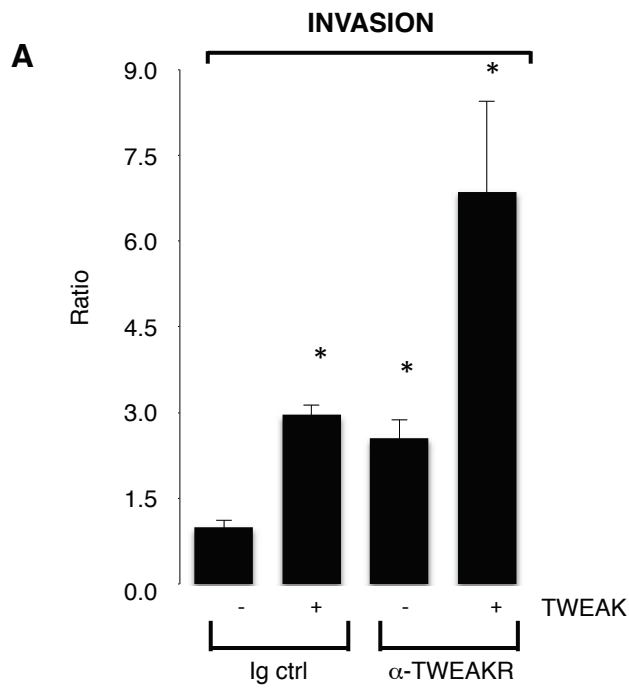


Figure 5 Gaudineau et al.

Figure 6 Gaudineau et al.

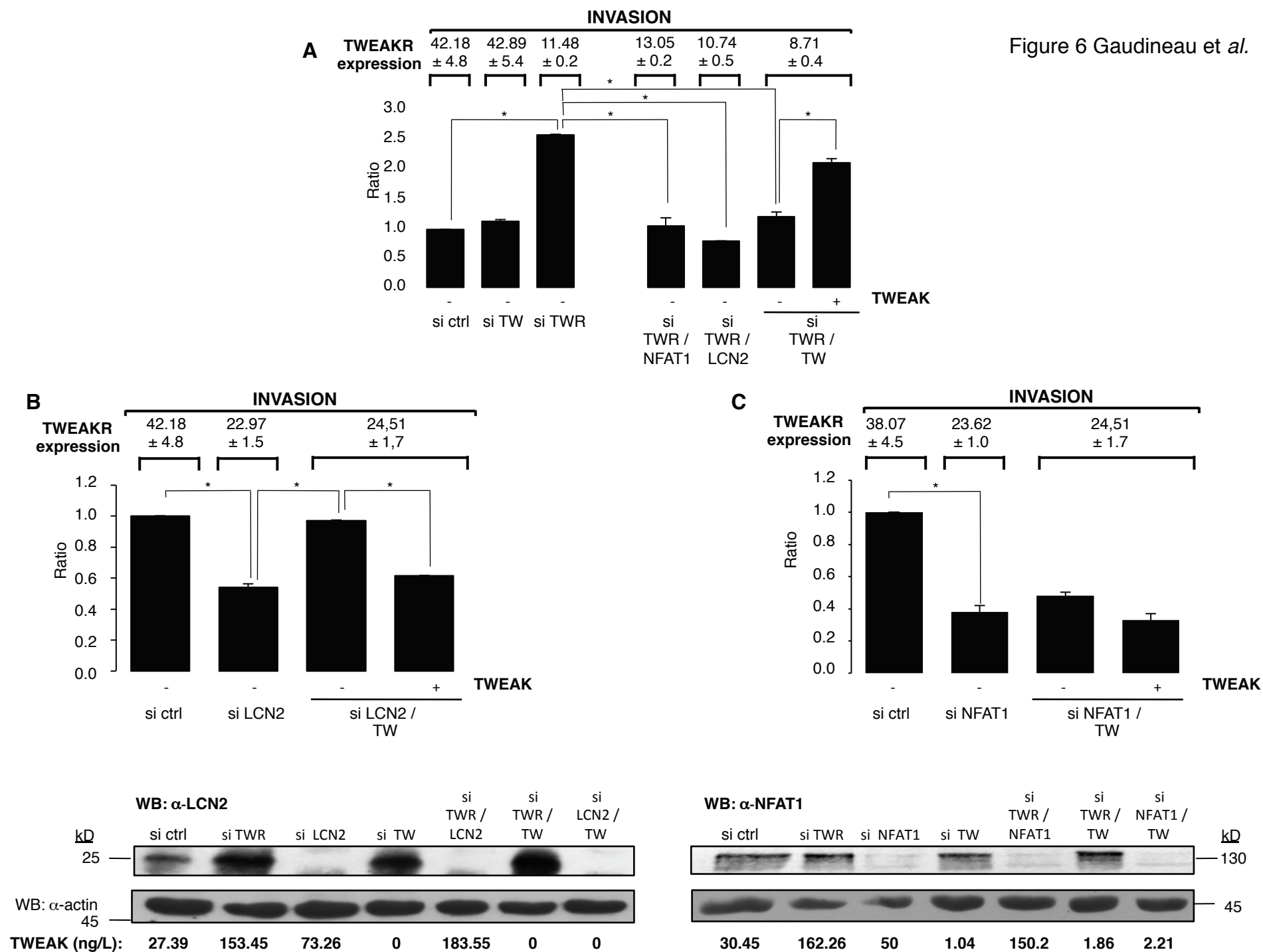


Figure 7 Gaudineau et al.

