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## Accepted Manuscript

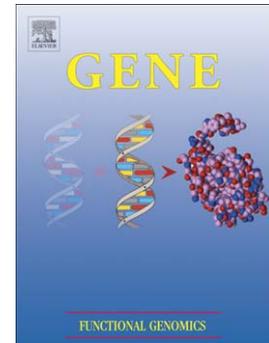
Identification of NF-kappaB responsive elements in follistatin related gene (*FLRG*) promoter

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#### Abbreviations:

ATCC: American type culture collection

B-CLL: B-cell chronic lymphocytic leukemia

Bp: base pair(s)

Ch: chromosome

ECM: extracellular matrix

EMSA: electrophoresis mobility-shift assay

FLRG: follistatin related gene

FSRP: follistatin-related protein

FSTL3: follistatin-like-3

IκB: inhibitor of kappaB

i.e.: *id est*

IKK: IκB kinase

kb: kilobase(s) or 1000 bp

MAPK: mitogen-activated protein kinases

MLP: adenovirus major late promoter

NF-κB: nuclear factor kappaB

s.d.: standard deviation

SBE: smad binding element

TGFβ: transforming growth factor beta

TNFα: tumor necrosis factor alpha

TNFR: TNFα receptor

**Abstract:**

Follistatin related gene (*FLRG*) has been previously identified from a chromosomal translocation observed in a B-cell chronic lymphocytic leukemia (B-CLL). *FLRG* (alternative names: follistatin-related protein, *FSRP* / follistatin-like-3, *FSTL3*) is a secreted glycoprotein highly similar to follistatin. Like follistatin, *FLRG* is involved in the regulation of various biological effects through its binding to members of the transforming growth factor beta ( $TGF\beta$ ) superfamily such as activin A and myostatin. We have previously shown that  $TGF\beta$  and activin A are potent inducers of *FLRG* transcriptional activation through the Smad proteins. Using a biochemical approach, we investigated whether tumor necrosis factor alpha ( $TNF\alpha$ ) could regulate *FLRG* expression since  $TNF\alpha$  plays a critical role in hematopoietic malignancies. We demonstrate that  $TNF\alpha$  activates *FLRG* expression at the transcriptional level. This activation depends on a promoter region containing four 107-108 bp DNA repeats evolutionary conserved in primates. These repeats carry a strong phylogenetic signal, which is not very common among non-coding sequences. Each DNA repeat contains one  $TNF\alpha$  responsive element (5'-GGGAGAG/TTCC-3') able to bind nuclear factor kappaB ( $NF-\kappa B$ ) transcription factors. We also show that  $TGF\beta$ , through the Smad proteins, potentiates the effect of  $TNF\alpha$  on *FLRG* expression. This cooperation is unexpected since  $TGF\beta$  and  $TNF\alpha$  usually have opposite biological effects. In all, this work brings new insights in the understanding of *FLRG* regulation by cytokines and growth factors. It opens attractive perspectives of research that should allow us to better understand the role of *FLRG* during tumorigenesis.

## 1. Introduction

We previously reported the molecular characterization of a t(11;19)(q13;p13) translocation in a B-cell chronic lymphocytic leukemia (B-CLL) (Hayette et al 1998). At the breakpoint on chromosome 19, we identified a new evolutionarily-conserved gene, *FLRG* (follistatin-related gene, also named Follistatin-related protein, *FSRP* / follistatin-like-3, *FSTL3*). It encodes a secreted glycoprotein highly similar to follistatin. As described for follistatin, FLRG physically interacts with members of the TGF $\beta$  (transforming growth factor beta) family such as activin A and myostatin to neutralize their biological effects (Bartholin et al 2002; Hill et al 2002; Maguer-Satta et al 2001; Maguer-Satta et al 2003; Sidis et al 2006; Tsuchida et al 2000). In addition, TGF $\beta$  and activin A are potent inducers of *FLRG* and *follistatin* transcriptional activation through the Smad proteins (Bartholin et al 2001; Maguer-Satta et al 2001; Maguer-Satta and Rimokh 2004). This observation, together with the antagonistic effect of FLRG and follistatin on activin A signaling, indicates that these two secreted proteins participate in a negative feedback loop that regulates activin A function. *In vivo*, FLRG would be involved in the development of gonads (Xia et al 2004), placenta (Ciarmela et al 2003) and virtually everywhere activin A has a role. More specifically, FLRG has been involved in the regulation of human hematopoiesis (Maguer-Satta and Rimokh 2004). The physical interaction between FLRG and fibronectin regulates the adhesion properties of hematopoietic primary cells, and in particular, increases the adhesion of functional hematopoietic progenitors (Maguer-Satta et al 2003; Maguer-Satta et al 2006). In addition, FLRG protein may contribute to bone formation by inhibiting osteoclast differentiation from hematopoietic precursors (Bartholin et al 2005).

Tumor necrosis factor alpha (TNF $\alpha$ ) has been characterized as a serum factor causing necrosis of tumors (Carswell et al 1975; Green et al 1976). TNF $\alpha$  has been involved in numerous biological processes such as apoptosis, inflammation, viral replication and blood cells homeostasis (Aggarwal 2003; MacEwan 2002). TNF $\alpha$  binds TNFR1 and TNFR2 (TNF $\alpha$  receptor one and two) to activate various signaling pathways such as NF- $\kappa$ B (nuclear factor kappaB), MAPK (mitogen-activated

protein kinases) or caspases pathways. The first characterized NF- $\kappa$ B member, NF- $\kappa$ B<sub>1</sub> (p50; p105), is a nuclear protein initially found as a kappa immunoglobulin enhancer interacting protein in cells that transcribed immunoglobulin light chain genes (Sen and Baltimore 1986a; Sen and Baltimore 1986b). Other NF- $\kappa$ B family members have been isolated such as Rel-A (p65), NF- $\kappa$ B<sub>2</sub> (p52; p100), c-Rel and Rel-B. These factors are structurally related by an amino-terminal motif of about 300 amino acids, the Rel-homology domain. In absence of ligand (TNF $\alpha$  or interleukin-1, for instance), the inhibitor of kappaB (I $\kappa$ B) sequesters the Rel factors in the cytoplasm. Activation of TNF $\alpha$  receptors stimulates I $\kappa$ B kinases (IKK1/ $\alpha$  and IKK2/ $\beta$ ). Activated IKKs then phosphorylate I $\kappa$ B to trigger its degradation. In the absence of I $\kappa$ B, released Rel family members migrate into the nucleus to activate transcription.

Using a biochemical approach, we investigated whether TNF $\alpha$  and NF- $\kappa$ B could regulate *FLRG* expression. We demonstrate that *FLRG* is activated at the transcriptional level by TNF $\alpha$  and NF- $\kappa$ B. This activation depends on a promoter region that contains four 107-108 bp evolutionary conserved repeats. Each repeat contains one functionally active NF- $\kappa$ B binding element able to interact with NF- $\kappa$ B<sub>1</sub>(p50) and Rel-A(p65) after TNF $\alpha$  stimulation. We also demonstrate that TGF $\beta$  cooperates with TNF $\alpha$  to activate *FLRG* expression.

## 2. Material and methods

### 2.1. Cell culture

The human hepatocellular carcinoma cell line HepG2, and the mink lung epithelial cell line Mv1Lu were obtained from the American Type Culture Collection (ATCC), and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 0.03% L-glutamine, 100 mg/ml penicillin and 100 mg/ml streptomycin sulfate.

### 2.2. Plasmids and deletion constructs

Various *FLRG* promoter fragments were cloned into the luciferase reporter plasmid pGL3-MLP containing a minimal promoter consisting of a TATA box and the initiator sequence of the adenovirus major late promoter (MLP) provided by JM Gauthier (Glaxo, Wellcome laboratory, Les Ullis, France) as described in (Bartholin et al 2001). *FLRG* promoter fragments specifically used in this study, (-3293/+6) $\Delta$ ( $\kappa$ Bx4), ( $\kappa$ Bx4) and ( $\kappa$ Bx1), were generated by PCR using pGL3-*FLRG*(-3293/+6)-MLP as a template and further cloned into pGL3-MLP.  $\Delta$ ( $\kappa$ Bx4) deletion encompasses nucleotides -1925 to -1492 and  $\kappa$ Bx4 promoter fragment covers nucleotides -1940 to -1470.  $\kappa$ Bx1 fragment covers *FLRG* promoter from nucleotides -1599 to -1706 (H3 repeat, Fig. 3A). In the  $\kappa$ Bx1-mut fragment, the  $\kappa$ B site was mutated as described in paragraph 2.5. Mammalian expression vectors for Smad3 and Smad4 were provided by P ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden). Mammalian expression vectors encoding NF- $\kappa$ B<sub>1</sub>(p50) and Rel-A(p65) were provided by P Jalinot (Ecole Normale Supérieure, Lyon, France). I $\kappa$ B(A32-A36) expression vector was a gift from A Israel (Institut Pasteur, Paris, France). Expression vector encoding CA-IKK2 was obtained from B Baumann (Ulm University, Ulm, Germany).

### 2.3. Transient transfections and reporter assays

HepG2 cells were cotransfected (Exgen 500, Euromedex, France) with the appropriate firefly luciferase reporter and pRL-CMV (Promega) as previously described (Bartholin et al 2001). TNF $\alpha$  (10-20 ng/ml, R&D Systems) and TGF $\beta$  (10 ng/ml, R&D Systems) were added 24 hours after

transfection and luciferase activity was assayed 48 hours after transfection (“Dual luciferase assay kit”, Promega). The same amount of plasmid DNA was kept constant by the addition of “empty” vectors. All the experiments were performed in triplicate, and the luciferase activity was normalized with regard to the renilla luciferase activity expressed by the pRL-CMV vector.

#### 2.4. Northern blots

Total cellular RNA was purified by the acid guanidium thiocyanate-phenol-chloroform method. For northern blot analysis, 10 mg of total RNA were size-fractionated in formaldehyde-1.2% agarose gels and transferred onto nylon filters. The probes were radiolabeled ( $^{32}$ P-dCTP) using the Rediprime labeling kit (Amersham Pharmacia Biotech) and hybridized according to standard procedures. RNA integrity and equal loading was assessed by ethidium bromide staining of ribosomal 18S and 28S RNAs.

#### 2.5. Electrophoresis mobility-shift assay (EMSA)

Nuclear proteins from Mv1Lu cells were treated for 30 minutes in the presence of TNF $\alpha$  (20 ng/ml) and/or TGF $\beta$  (10 ng/ml) and gel-shift assays were performed as previously described (Bartholin et al 2001). Specifically, the wild type (wt- $\kappa$ Bx1) and mutant (mut- $\kappa$ Bx1) double-stranded DNA probes cover *FLRG* promoter from nucleotides -1599 to -1706 (H3 repeat, Fig. 3A). In mut- $\kappa$ Bx1 probe, the  $\kappa$ B site was mutated (USE Mutagenesis kit, Amersham Pharmacia Biotech) into a Spe-I restriction enzyme site (wt 5'-GGGGAGATTCCC-3' to mut 5'-ACTAGT3-') to facilitate further identification of the mutant clones. The SBE (smad binding element) probe (5'-GGTCGAGAGCCAGACAAAAGCCAGACATTTAGCCAGACAATCCCGCAAGAGGGCCGGGG-3') contains three Smad Binding Elements (underlined) (Dennler et al 1998). For supershifts analysis, we used commercial rabbit polyclonal antibodies (Santa Cruz Biotechnology) directed against NF- $\kappa$ B<sub>1</sub>(p50) (#sc-114x), Rel-A(p65) (#sc-109x), Smad4 (#sc-7154x) and Oct1 (#sc-232x).

#### 2.6. Sequence alignment and phylogenetic tree estimation

The four human repeats were first aligned using Clustal W (Thompson et al 1994). Each repeat was then used as query in a BLAST search (Altschul et al 1990) to identify similar sequences among complete genomes. Three matches were found among *Pan troglodytes* (chimpanzee) genome and one among *Macaca mulatta* (rhesus macaque) genome. The eight sequences (4 in human + 3 in chimpanzee + 1 in macaque) were then aligned using Clustal W. A phylogenetic tree was estimated from this alignment using a Bayesian approach (software: MrBayes (Huelsenbeck and Ronquist 2001) and maximum likelihood approach (software: PHYML (Guindon and Gascuel 2003)).

### 3. Results

#### 3.1. *TNF $\alpha$ up-regulates FLRG expression at the transcriptional level*

To investigate the ability of TNF $\alpha$  to modulate *FLRG* expression, we performed a northern-blot analysis using total RNA prepared from HepG2 cells treated or not with TNF $\alpha$  for 24 hours. This approach revealed a large increase in the level of the two *FLRG* RNA transcripts (2.5 and 1.2 kb) in response to TNF $\alpha$  (Fig. 1A). We previously reported the cloning of a reporter construct containing *FLRG* promoter elements located between nucleotides -3293 and +6 (plasmid pGL3-*FLRG*(-3293/+6)-MLP) and driving the expression of a luciferase reporter gene (Bartholin et al 2001). To test whether increased *FLRG* expression in the presence of TNF $\alpha$  resulted from increased transcription, we transfected HepG2 cells with the reporter construct pGL3-*FLRG*(-3293/+6)-MLP. TNF $\alpha$  treatment produces a 4-fold induction of luciferase activity, indicating an increase in *de novo* transcription of the *FLRG* promoter (Fig. 1B). In contrast, the empty vector (pGL3-MLP) is not sensitive to TNF $\alpha$  (Fig. 1B). In all, these results demonstrate that TNF $\alpha$  up-regulates *FLRG* expression at the transcriptional level.

#### 3.2. *Identification of TNF $\alpha$ -responsive elements in FLRG promoter*

Identification of TNF $\alpha$ -responsive elements was allowed by the cloning of various *FLRG* promoter fragments into the pGL3-MLP reporter plasmid as previously described (Bartholin et al 2001). TNF $\alpha$  sensitivity was assayed into HepG2 cells. Deletions up to position -1920 do not significantly change the induction of luciferase activity after TNF $\alpha$  treatment. In contrast, deletion up to nucleotides -1523 completely abrogates this response (Fig. 2A). This result suggests the existence of TNF $\alpha$  responsive elements between nucleotides -1920 and -1523. To validate the presence of such elements in this region, we deleted nucleotides -1925 to -1492 (pGL3-*FLRG*(-3293/+6) $\Delta$ ( $\kappa$ Bx4)-MLP reporter construct). This deletion abolishes TNF $\alpha$ -mediated *FLRG* transcriptional activation (Fig. 2B). To explore whether this ~400 bp long region was sufficient to

drive TNF $\alpha$  activation, we cloned the promoter region located between nucleotides -1940 and -1470 (pGL3-FLRG( $\kappa$ Bx4)-MLP reporter construct). With this *FLRG* promoter fragment, we observe a 4-fold induction of luciferase activity (Fig. 2B). These results are consistent with the presence of TNF $\alpha$ -responsive elements between nucleotides -1920 and -1523 inside *FLRG* promoter.

### 3.3. Evolutionary history of the TNF $\alpha$ -responsive elements found within *FLRG* promoter

Computer assisted sequence analysis revealed that the *FLRG* promoter region (nucleotides 1920-1523) sensitive to TNF $\alpha$ , was composed of four highly conserved 107-108 bp tandem repeats (Human genome, build 36.1, chrom19: 625,498 to 625,924) (Fig. 3A). Very interestingly, these four conserved domains contain short sequences known to bind NF- $\kappa$ B transcription factors (5'-GGGAGAG/TTCC-3'). This observation strongly suggests that *FLRG* expression could be activated by NF- $\kappa$ B factors in response to TNF $\alpha$ . Before testing this hypothesis, we attempted to shed light on the evolutionary origin of these four 107-108 bp tandem repeats. We first searched for homologous sequences among a wide range of genomes. Each of the four human repeats was used as query in a BLAST search against various complete genomes (Altschul et al 1990). Highly similar sequences were only found among *Pan troglodytes* (chimpanzee) and *Macaca mulatta* (rhesus macaque) genomes. Macaque's genome displays a single repeat located within telomeric regions of chromosome 19. Chimpanzee's genome contains three repeats, also located on chromosome 19 (Fig. 3B). The alignment of the *FLRG* promoter sequences from human, chimpanzee and macaque, suggests that early primate genomes had only one repeat while unequal recombination increased the number of repeats during the course of evolution (Fig. 3B). We next aligned these sequences and estimated a phylogenetic tree in order to better understand the origin of the four human repeats. Two phylogenetic estimation methods that rely on sound statistical basis converged to the same tree, which is compatible with the well-known phylogeny of these primates (Fig. 3C). The analysis of this tree suggests that three duplication events (d1, d2 and d3) occurred after the first speciation

event (s1: macaque vs. (human, chimpanzee)) and before the second speciation event (s2: human vs. chimpanzee). The position of C4 and C5 in the tree indicates that these two repeats appeared after s2 (i.e., C4 and C5 are paralogous and both are orthologous to H4). C2 and C3 are absent in chimpanzee. According to the estimated phylogeny, these sequences were lost after s1. Interestingly, this phylogeny is also compatible with the positions of the human and chimpanzee repeats on their chromosomes, i.e., the position of the tips in the tree is the same as the positions of the corresponding sequences on the genomes. Hence, while the method that is used to build the phylogeny ignores the position of the repeats on the chromosome, this information can be recovered from the phylogenetic signal carried by these sequences. The chance that a random phylogeny with the same number of taxa displays such features is small. In all, it is very likely that the proposed phylogeny accurately describes the succession of duplications and speciation events that governed the evolution of these sequences.

#### *3.4. Involvement of NF- $\kappa$ B transcription factors NF- $\kappa$ B1(p50) and Rel-A(p65) in TNF $\alpha$ -mediated FLRG activation*

Having determined that *FLRG* promoter sensitivity to TNF $\alpha$  depended on a genomic DNA region containing four NF- $\kappa$ B binding sites, we then asked whether NF- $\kappa$ B transcription factors NF- $\kappa$ B1(p50) and Rel-A(p65) could regulate *FLRG* transcription. To this end, we cotransfected HepG2 cells with pGL3-*FLRG*(-3293/+6)-MLP reporter construct along with NF- $\kappa$ B1(p50) and Rel-A(p65) expression vectors. Co-expression of NF- $\kappa$ B1(p50) and Rel-A(p65) induces a 2-fold induction of luciferase activity (Fig. 4A). Further evidence for the involvement of NF- $\kappa$ B transcription factors in the TNF $\alpha$ -mediated activation of *FLRG* promoter was obtained by the use of an I $\kappa$ B mutant named I $\kappa$ B(A32-A36). This mutant cannot be degraded by the proteasome due to the lack of induced phosphorylation on serines 32 and 36. I $\kappa$ B(A32-A36) is considered as a “super-repressor” since interaction with NF- $\kappa$ B transcription factors cannot be released after TNF $\alpha$  treatment, thereby preventing nuclear accumulation of NF- $\kappa$ B and transcriptional activation. Transfection of I $\kappa$ B(A32-

A36) expression vector significantly reduces the TNF $\alpha$ -mediated induction of luciferase activity from pGL3-FLRG(-3293/+6)-MLP (Fig. 4B). In order to mimic NF- $\kappa$ B-mediated transcriptional activation, we used a constitutively active IKK2 mutant (CA-IKK2) able to stimulate NF- $\kappa$ B transcription factors in the absence of ligand (Azoitei et al 2005). We cotransfected HepG2 cells with CA-IKK2 along with luciferase reporters consisting of the H3 domain containing either a wild type or a point mutated NF- $\kappa$ B binding site (pGL3-FLRG( $\kappa$ Bx1)-MLP and pGL3-FLRG( $\kappa$ Bx1-mut)-MLP). Expression of CA-IKK2 is sufficient to produce a 5-fold induction of luciferase activity from the pGL3-FLRG( $\kappa$ Bx1)-MLP strengthening the crucial role of NF- $\kappa$ B in *FLRG* transcriptional activation (Fig. 4C). Interestingly, CA-IKK2 fails to activate pGL3-FLRG( $\kappa$ Bx1-mut)-MLP reporter (Fig. 4C). This latter result proves the functional relevance of one NF- $\kappa$ B binding site identified inside *FLRG* promoter.

Taken together, our results demonstrate that NF- $\kappa$ B transcription factors, NF- $\kappa$ B<sub>1</sub>(p50) and Rel-A(p65), are involved in the activation of *FLRG* promoter after TNF $\alpha$  treatment.

### 3.5. Binding of NF- $\kappa$ B1(p50) and Rel-A(p65) to *FLRG* promoter

We next investigated whether TNF $\alpha$  induced the binding of nuclear proteins to the NF- $\kappa$ B binding sites found within *FLRG* promoter. We performed a gel-shift assay using nuclear proteins prepared from cells treated or not with TNF $\alpha$ . We used radiolabeled DNA probes containing either wild-type (wt- $\kappa$ Bx1) or mutated (mut- $\kappa$ Bx1) NF- $\kappa$ B binding sites. These probes correspond to the H3 repeat (covering promoter region from nucleotides -1706 to -1599) (Fig. 3). The migration of the wt- $\kappa$ Bx1 probe appears to be shifted in the presence of nuclear proteins prepared from TNF $\alpha$ -treated cells (Fig. 5A, lines 1 and 2). Intensity of this shifted band is dramatically reduced if we use the mutant probe (mut- $\kappa$ Bx1) (Fig. 5A, lines 3 and 4). This result shows that NF- $\kappa$ B binding sites found within *FLRG* promoter bind nuclear proteins after TNF $\alpha$  treatment. To further identify these nuclear proteins, we performed gel-supershift assays (Fig. 5B). Antibodies against NF- $\kappa$ B<sub>1</sub>(p50) and Rel-

A(p65) were added to the nuclear proteins prior incubation with the radiolabeled wt- $\kappa$ Bx1 probe. Gel separation revealed that NF- $\kappa$ B<sub>1</sub>(p50) and Rel-A(p65) antibodies respectively abrogates (Fig. 5B, line 5) and supershifts (Fig. 5B, line 6) the DNA-protein complex that is normally observed after TNF $\alpha$  treatment (Fig. 5B, line 2). In contrast, antibodies against Smad4 and Oct1 used as negative controls do not impair the formation of this complex (Fig. 5B, lines 3 and 4). These results indicate that TNF $\alpha$  controls the formation of an DNA-protein complex containing endogenous NF- $\kappa$ B transcription factors NF- $\kappa$ B<sub>1</sub>(p50) and Rel-A(p65).

### 3.6. TNF $\alpha$ cooperates with TGF $\beta$ to up-regulate *FLRG* expression

In a previous work, we demonstrated that TGF $\beta$  was a potent inducer of *FLRG* expression via the Smad proteins (Bartholin et al 2001). Here, we examined whether TGF $\beta$  and TNF $\alpha$  had a synergistic effect on *FLRG* expression. Northern-blot analysis reveals that accumulation of *FLRG* transcripts after TGF $\beta$ +TNF $\alpha$  treatment is more pronounced than the accumulation observed in cells treated with TGF $\beta$  or TNF $\alpha$  alone (Fig. 6A). Reporter tests reveal that TNF $\alpha$  and TGF $\beta$  alone produce a 5-fold induction of luciferase activity, whereas in combination, these two cytokines induce a 15-fold induction of luciferase activity (Fig. 6B). These results indicate that TGF $\beta$  and TNF $\alpha$  act in synergy to activate *FLRG* transcription. We next assessed a possible functional interaction between NF- $\kappa$ B and Smads proteins (Fig. 6C). Luciferase assays showed that overexpression of Smads (Smad3+Smad4) and NF- $\kappa$ B (p50+p65) respectively induce a 10-fold and a 2.5-fold induction of *FLRG* promoter. Interestingly, combined overexpression of Smad and NF- $\kappa$ B (Smad3+Smad4+p50+p65) proteins induces a 15-fold induction of *FLRG* promoter. This observation suggests that NF- $\kappa$ B and Smads act in cooperation to activate *FLRG* transcription. To explore the molecular mechanism supporting this cooperation, we performed gel-shift assays by using the wt- $\kappa$ Bx1 probe and nuclear proteins prepared from cells cultured with TGF $\beta$  and/or TNF $\alpha$  (Fig. 6D, left panel). Our results indicate that TGF $\beta$  neither induces the formation of a DNA-protein

complex nor disrupts the DNA-protein complex that is normally observed after TNF $\alpha$  treatment. Another EMSA was performed in the same conditions with a probe containing three canonical Smad Binding Element or SBE (Fig. 6D, right panel). As expected, TGF $\beta$  induces the formation of a DNA-protein complex on the SBE probe. This complex is not disrupted by TNF $\alpha$ . All together, these gel-shift experiments indicate that 1) TGF $\beta$  and TNF $\alpha$  induce the formation of DNA-protein complexes only on their respective responsive elements, 2) TGF $\beta$  does not modulate the TNF $\alpha$ -mediated recruitment of NF- $\kappa$ B onto the  $\kappa$ B sites found within *FLRG* promoter and 3) reciprocally, that TNF $\alpha$  does not modulate the TGF $\beta$ -mediated recruitment of Smad proteins onto canonical SBE sites.

#### 4. Discussion

In this study, we developed a biochemical approach to determine whether *FLRG* expression was regulated by  $\text{TNF}\alpha$ . We clearly demonstrate that *FLRG* transcription is activated after  $\text{TNF}\alpha$  treatment through the NF- $\kappa$ B transcription factors. This activation relies on a promoter region that contains four 107-108 bp repeats (H1, H2, H3 and H4), which are evolutionary conserved in primates. Each repeat contains one NF- $\kappa$ B binding element that interacts with NF- $\kappa$ B transcription factors after  $\text{TNF}\alpha$  stimulation. We have previously shown that  $\text{TGF}\beta$  induces transcriptional activation of *FLRG* through the Smad proteins. Here, we demonstrate that  $\text{TNF}\alpha$  and  $\text{TGF}\beta$  cooperate to activate *FLRG* expression.

NF- $\kappa$ B transcription factors (Rel-A (p65), NF- $\kappa$ B1 (p50), NF- $\kappa$ B2 (p52), c-Rel and Rel-B) bind DNA consensus site (5'-GGGRNNYYCC-3', N = any base, R = G or A, and Y = C or T) (Hayden and Ghosh 2004). The three  $\kappa$ B sites (5'-GGGAGATTCC-3') respectively found within the H2, H3 and H4 repeats perfectly match with the consensus sequence described above whereas the  $\kappa$ B site found within the H1 repeat (5'-GGGAGAGTCC-3', degenerated base is underlined) contains one degenerated base (Fig. 3A). Ancestors of these four human repeats are only found among chimpanzee and macaque genomes. These repeats carry a strong phylogenetic signal, which is not very common among non-coding sequences. This signal helped us to decipher the evolutionary origin of the human *FLRG* promoter. The presence of tandem repeats suggests that the structure of *FLRG* promoter is likely to be subject to frequent rearrangements (most probably due to unequal recombination events). The phylogenetic analysis of these primate sequences seems to confirm this hypothesis: losses and duplications observed in closely related primates clearly suggest fast rates of evolution. At the functional level, it will be of particular interest to investigate if there is a correlation between the number of tandem repeats and sensitivity to  $\text{TNF}\alpha$ , and if we can observe polymorphisms affecting the number of tandem repeats in patients with malignant blood diseases.

*FLRG* and *follistatin* are similar in many respects. Secreted *FLRG* and *follistatin* glycoproteins both interact with members of the TGF $\beta$  family such as activin A to neutralize their biological effects. *FLRG* and *follistatin* also share common regulation. Indeed, both genes are transcriptionally activated by TGF $\beta$  and activin A through the Smad proteins (Bartholin et al 2001; Bartholin et al 2002). Northern blots and reporter tests realized with a 2 kb *follistatin* promoter fragment revealed that *follistatin* was not activated by TNF $\alpha$  (data not shown). The absence of tandem repeats and NF- $\kappa$ B binding motifs within the *follistatin* promoter probably explain this observation. The functional significance of *FLRG* activation by TNF $\alpha$  is still unknown. Interestingly, activation of NF- $\kappa$ B factors has been reported in various hematological malignancies (Braun et al 2006). More specifically, it has been shown that B cells from B-cell chronic lymphocytic leukemia (B-CLL) patients exhibit higher levels of NF- $\kappa$ B activity than B cells from healthy donors (Cuni et al 2004; Furman et al 2000). Increased NF- $\kappa$ B activity in malignant cells up-regulates anti-apoptotic genes to promote their survival (c-FLIP, Bcl-2, Bcl-XL...). In that context, NF- $\kappa$ B inhibitors are efficient anti-cancer drugs (Braun et al 2006). *FLRG* has been identified in a t(11;19)(q13;p13) translocation in a B-CLL (Hayette et al 1998). The chromosomal breakpoint on chromosome 19 is located about 7 kb upstream of *FLRG* suggesting that this rearrangement could impair the regulation of *FLRG* expression (*FLRG* coding region is maintained on the derivative chromosome 19). *FLRG* was highly expressed in B cells from this patient. Normally, *FLRG* expression is not detected in the B cell compartment. These observations suggest that *FLRG* up-regulation in B-CLL cells could result from the constitutively high NF- $\kappa$ B activity in these malignant cells. However, whether *FLRG* expression in these malignant B-cells is directly involved in the tumor formation remains to be clarified. To address this question carefully, additional work is ongoing in our lab, in particular in blood cells. Besides, *FLRG* locus was found to be rearranged in one case of non-Hodgkin's lymphoma B-cells (Hayette et al 1998). This observation strengthens the idea that *FLRG* locus rearrangements are probably not random events in malignant blood disorders. The presence of

tandem repeats suggests that the region of the chromosome 19, containing *FLRG* promoter, may experience frequent recombination events. The relation between such instability of the *FLRG* promoter and the progression of certain cancers needs to be scrutinized more thoroughly. Finally, activin A is known to block the proliferation of hematopoietic cells (Zipori and Barda-Saad 2001). So, it is tempting to speculate that in some malignancies, increased NF- $\kappa$ B activity would result in *FLRG* up-regulation to block the anti-proliferative effect of activin A.

We showed in a previous work that *FLRG* was a direct target gene of the Smad proteins in response to TGF $\beta$  (Bartholin et al 2001). Here, we demonstrate that TGF $\beta$  cooperates with TNF $\alpha$  to activate *FLRG* transcription. The molecular mechanism of this cooperation is still unclear. Our results suggest that it neither involves the formation of a Smad/NF- $\kappa$ B protein complex at the NF- $\kappa$ B binding sites nor an increased DNA recruitment of NF- $\kappa$ B transcription factors. Indeed, in EMSA experiments, TGF $\beta$  does not impair the TNF $\alpha$ -induced complex that forms at the  $\kappa$ B sites. However, we cannot rule out the possibility that other NF- $\kappa$ B members (p52, c-Rel, Rel-B) may be involved in a Smad/NF- $\kappa$ B protein complex. It is also possible that NF- $\kappa$ B/Smad interaction complex would occur through the previously characterized Smad binding element (SBE) present within *FLRG* promoter (Bartholin et al 2001). Further experiments will address these questions as well as the possible indirect mechanisms that could explain the synergic effect of TNF $\alpha$  and TGF $\beta$  to activate *FLRG* expression. From literature, several hypotheses can be proposed. The simplest explanation would be to consider that TGF $\beta$  and TNF $\alpha$  activate two very distinct signaling pathways (respectively Smad and NF- $\kappa$ B pathways) without a real biochemical connection. In contrast, Smad and NF- $\kappa$ B proteins, bound to their respective and distinct responsive elements, could physically interact at the chromatin level through the recruitment of shared transcriptional co-activators such as p300 (Kon et al 1999). Alternatively, TGF $\beta$  could stimulate post-translational modifications of NF- $\kappa$ B factors to potentate their transcriptional activity (Schmitz et al 2001). TGF $\beta$  and TNF $\alpha$  usually have opposite effects. Antagonistic properties of TGF $\beta$  and TNF $\alpha$  are

commonly observed in various biological processes such as inflammation, immunosuppression or extracellular matrix (ECM) remodeling.  $TNF\alpha$  and  $TGF\beta$  inverse functions result from the transcriptional activation of genetic programs with opposite outcomes.  $TNF\alpha$  inhibits fibrosis by reducing ECM deposition whereas  $TGF\beta$  activates fibrosis by increasing ECM deposition. At the molecular level, in contrast to  $TNF\alpha$ ,  $TGF\beta$  down-regulates the transcription of collagenases (involved in ECM degradation) and up-regulates the transcription of type I collagen (a major structural component of the ECM) (Verrecchia and Mauviel 2004). The cooperative effect of  $TGF\beta$  and  $TNF\alpha$  to activate *FLRG* transcription appears in contradiction with these observations. Interestingly, other genes have also been reported to be co-activated by  $TNF\alpha$  and  $TGF\beta$  such as type VII collagen (Kon et al 1999) and PAI-1 (Hou et al 2004). Unfortunately, the functional significance of the co-activation of these genes by  $TGF\beta$  and  $TNF\alpha$  remains unclear as well. In conclusion, this work brings new insights in the understanding of the modulation of *FLRG* expression by cytokines and growth factors. It opens attractive perspectives of research that should allow us to better understand the role of *FLRG* during tumorigenesis.

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ACCEPTED MANUSCRIPT

**Figure legends**

Fig. 1. TNF $\alpha$  activates *FLRG* transcription. (A) Total RNA from untreated and TNF $\alpha$ -treated cells (24 hours treatment) was subjected to northern blot. The filter was assayed for hybridization to radiolabeled *FLRG* cDNA (upper panel). Equal amounts of RNA were visualized by ethidium bromide staining of 28S and 18S ribosomal RNA (lower panel). (B) HepG2 cells were cotransfected either with the pGL3-MLP empty vector or the *FLRG* promoter-luciferase construct pGL3-*FLRG*(-3293/+6)-MLP and cultured in the presence of TNF $\alpha$ . The luciferase activity normalized with renilla luciferase activity expressed from a pRL-CMV cotransfected vector (internal control), is given as the mean+s.d. (standard deviation) of a representative experiment performed in triplicate. MLP, adenovirus major late promoter.

Fig. 2. Identification of TNF $\alpha$ -responsive elements within *FLRG* promoter. (A) and (B). HepG2 cells cultured in the presence or the absence of TNF $\alpha$  were transiently cotransfected with the indicated *FLRG* promoter reporter constructs. The luciferase activity normalized with renilla luciferase activity expressed from a pRL-CMV cotransfected vector (internal control), is given as a fold induction (mean+s.d. of three experiments) in the presence of TNF $\alpha$ . MLP, adenovirus major late promoter.

Fig. 3. Sequence alignment and phylogenetic tree estimation of the TNF $\alpha$  sensitive region present within *FLRG* promoter. (A) TNF $\alpha$  sensitive region present in *FLRG* promoter is composed of four tandem repeats of a 107-108 bp motif. The four repeats were aligned using a standard procedure. Each repeat contains one NF- $\kappa$ B binding site ( $\kappa$ B site, underlined). Bp, base pairs;  $\kappa$ B, NF-kappaB binding site; MLP, adenovirus major late promoter. (B) Schematic view of the alignment of macaque, chimpanzee and human *FLRG* promoter sequences. Position of the human repeats on chromosome 19 is annotated (build 36.1). Bases that are identical are shaded black. The dotted lines

correspond to gaps in the sequences. Ch, chromosome. (C) Phylogenetic tree estimated from the alignment of the eight conserved domains. 's1' corresponds to the speciation event that separates macaque from human and chimpanzee. 's2' is the speciation event that separates human and chimpanzee. 'd1', 'd2', 'd3' and 'd4' designate duplication events.

Fig. 4. Activation of *FLRG* promoter by NF- $\kappa$ B transcription factors. (A) HepG2 cells were cotransfected with pGL3-*FLRG*(-3293/+6)-MLP together with expression plasmid for NF- $\kappa$ B transcription factors NF- $\kappa$ B<sub>1</sub>(p50) and Rel-A(p65). (B) HepG2 cells were cotransfected either with pGL3-MLP or pGL3-*FLRG*(-3293/+6)-MLP, in the presence of an I $\kappa$ B(A32-A36) mutant expression vector, and cultured in the presence or not of TNF $\alpha$ . (C) HepG2 cells were cotransfected with the indicated reporters (pGL3-MLP, pGL3-*FLRG*( $\kappa$ Bx1)-MLP or pGL3-*FLRG*( $\kappa$ Bx1-mut)-MLP) along with an IKK2 mutant expression vector (constitutively active mutant, CA-IKK2). In B, the luciferase activity normalized with renilla luciferase activity expressed from a pRL-CMV cotransfected vector (internal control), is given as a fold induction (mean+s.d. of three experiments) in the presence of TNF $\alpha$ . In A and C, the luciferase activity normalized with renilla luciferase activity expressed from a pRL-CMV cotransfected vector (internal control), is given as the mean+s.d. of experiments performed in triplicate. MLP, adenovirus major late promoter.

Fig. 5. Binding of NF- $\kappa$ B transcription factors to *FLRG* promoter after TNF $\alpha$  treatment. (A) A gel shift assay (electrophoretic mobility shift assay) was performed using labeled probes containing the H3 repeat (Figure 3A) and nuclear proteins prepared from cells treated or not with TNF $\alpha$ . wt- $\kappa$ Bx1 and mut- $\kappa$ Bx1 probes respectively contain a wild-type and a mutated NF- $\kappa$ B binding site. (B) Antibodies against NF- $\kappa$ B<sub>1</sub>(p50) and Rel-A(p65) were used in supershift assays to detect the presence of NF- $\kappa$ B transcription factors in the DNA/protein complex that forms in response to TNF $\alpha$ . Smad7 and Oct1 antibodies were used as negative control.

Fig. 6.  $\text{TNF}\alpha$  and  $\text{TGF}\beta$  cooperate to activate *FLRG* expression. (A) HepG2 cells were treated with  $\text{TNF}\alpha$  and/or  $\text{TGF}\beta$  for 24 hours. Total RNA from untreated cells and  $\text{TNF}\alpha$ -treated cells were subjected to northern blot. The filter was assayed for hybridization to a radiolabeled *FLRG* cDNA probe (upper panel). Equal amounts of RNA were checked by ethidium bromide staining of 28S and 18S ribosomal RNAs (lower panel). (B) HepG2 cells were transfected with pGL3-*FLRG*(-3293/+6)-MLP reporter construct and cultured for 24 hours with or without  $\text{TGF}\beta$  and  $\text{TNF}\alpha$  before luciferase assaying. (C) HepG2 cells were cotransfected with pGL3-*FLRG*(-3293/+6)-MLP and expression vectors for  $\text{NF-}\kappa\text{B}_1$ (p50), Rel-A(p65), Smad3 and Smad4 in different combination 48 hours before luciferase assaying. In B and C, the luciferase activity normalized with renilla luciferase activity, is given as the mean+s.d. of representative experiments performed in triplicate. MLP, adenovirus major late promoter. (D) EMSAs were performed using a labeled probe corresponding to the H3 repeat containing either a  $\text{NF-}\kappa\text{B}$  binding element (wt- $\kappa\text{Bx1}$ , left panel) or a labeled probe containing 3 Smad Binding Element (SBE, right panel) and nuclear proteins prepared from cells treated with  $\text{TNF}\alpha$  or  $\text{TGF}\beta$  as indicated.

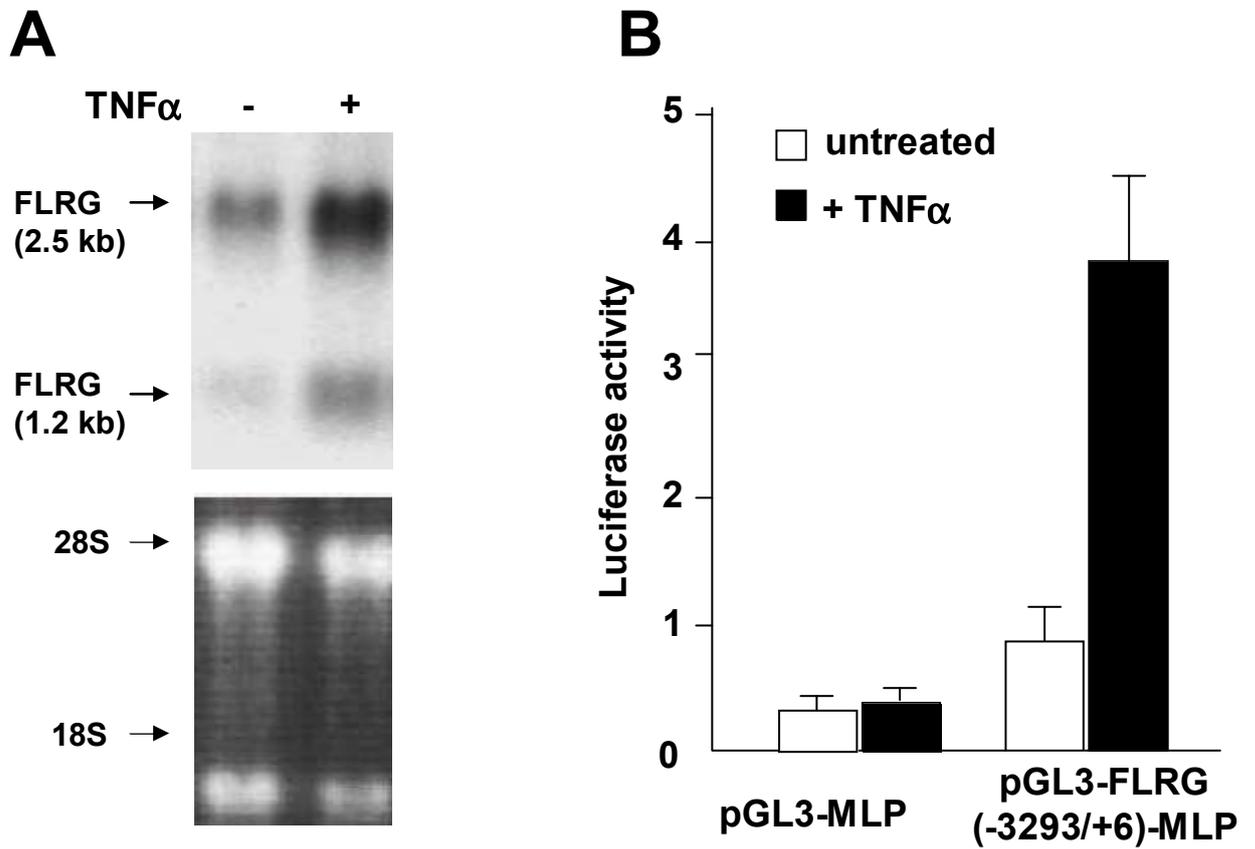


Figure 1, Bartholin et al.

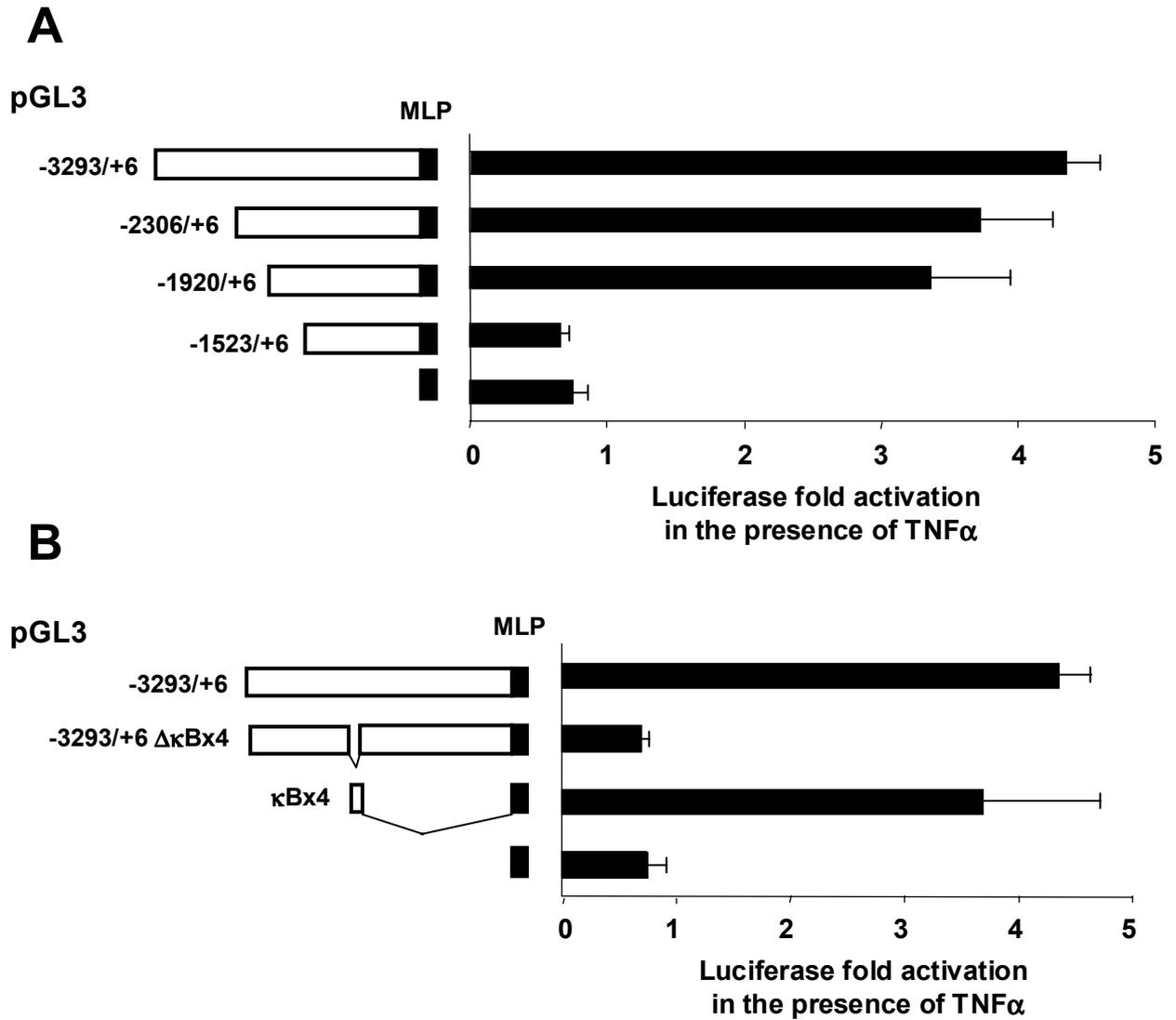
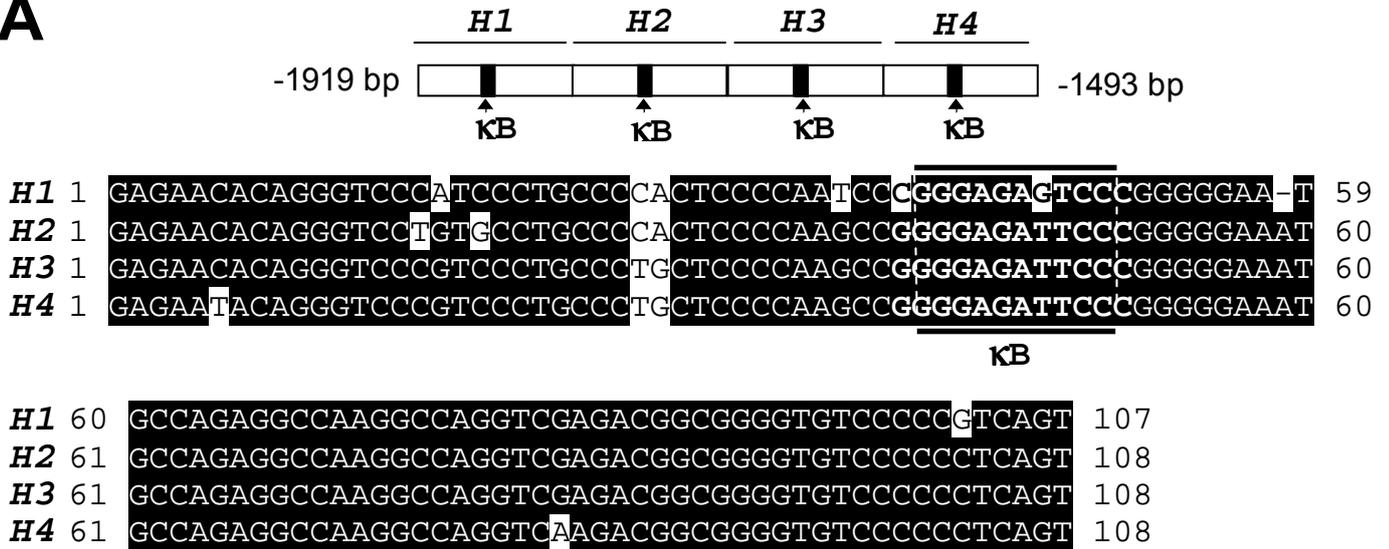
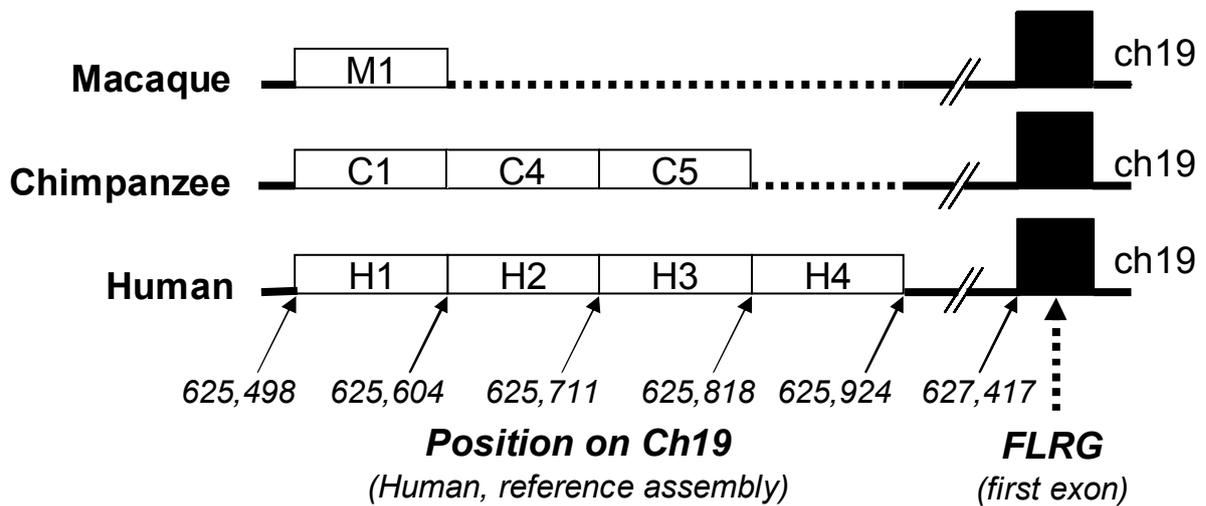
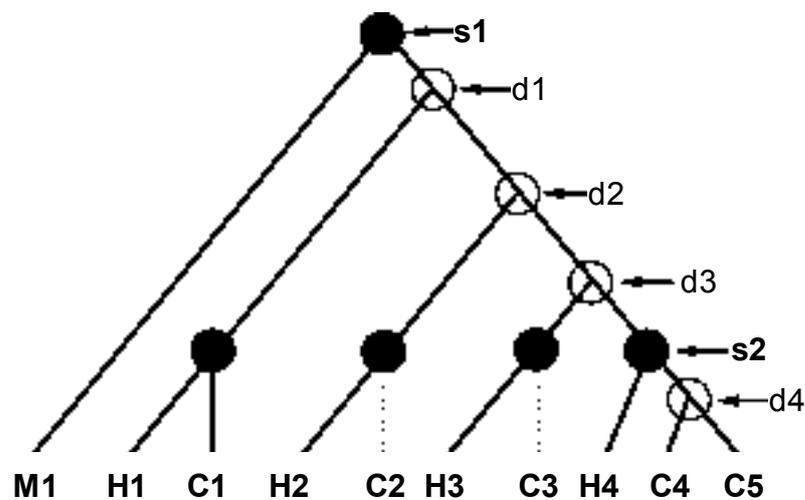


Figure 2, Bartholin et al.

**A****B****C****Figure 3, Bartholin et al.**

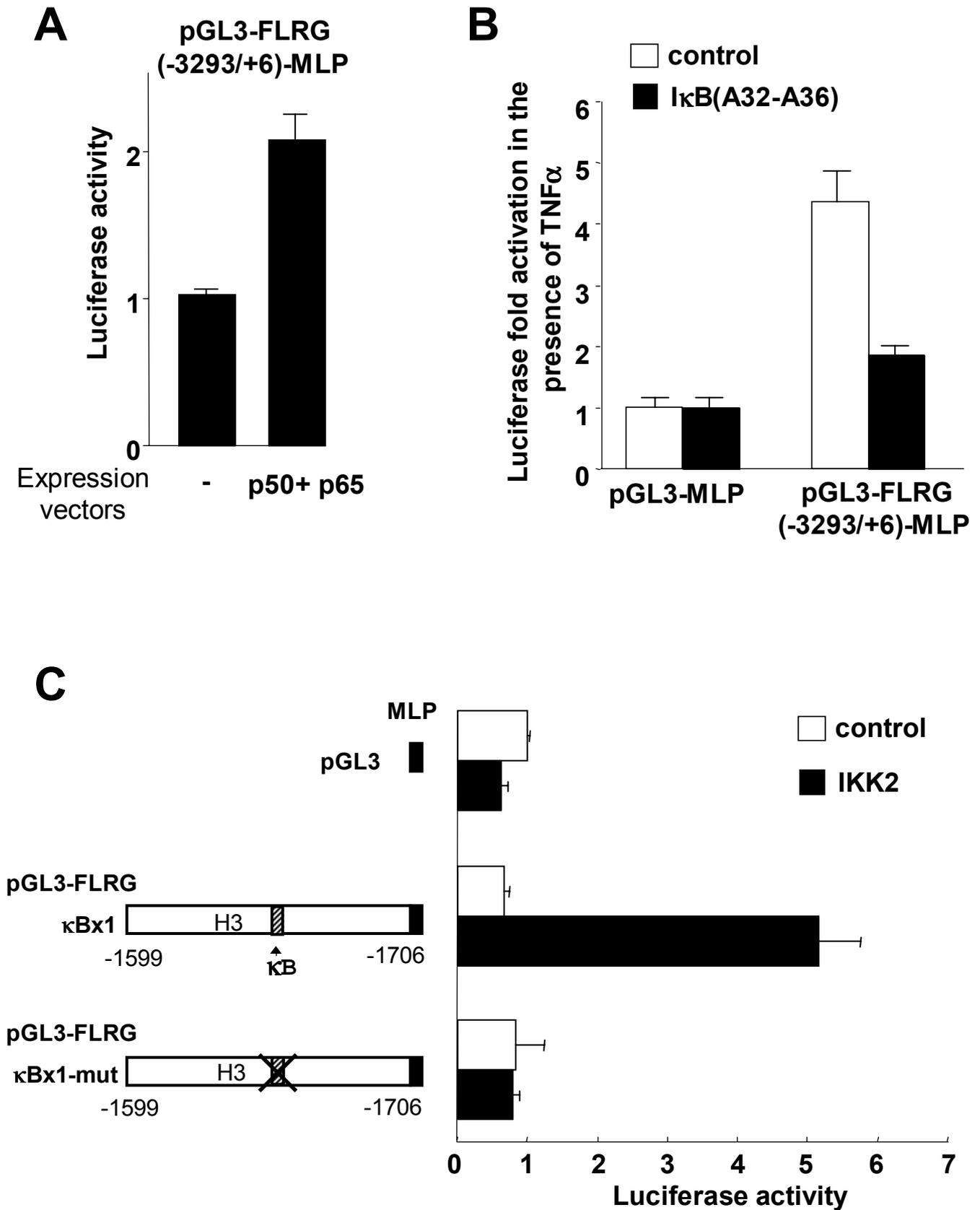


Figure 4, Bartholin et al.

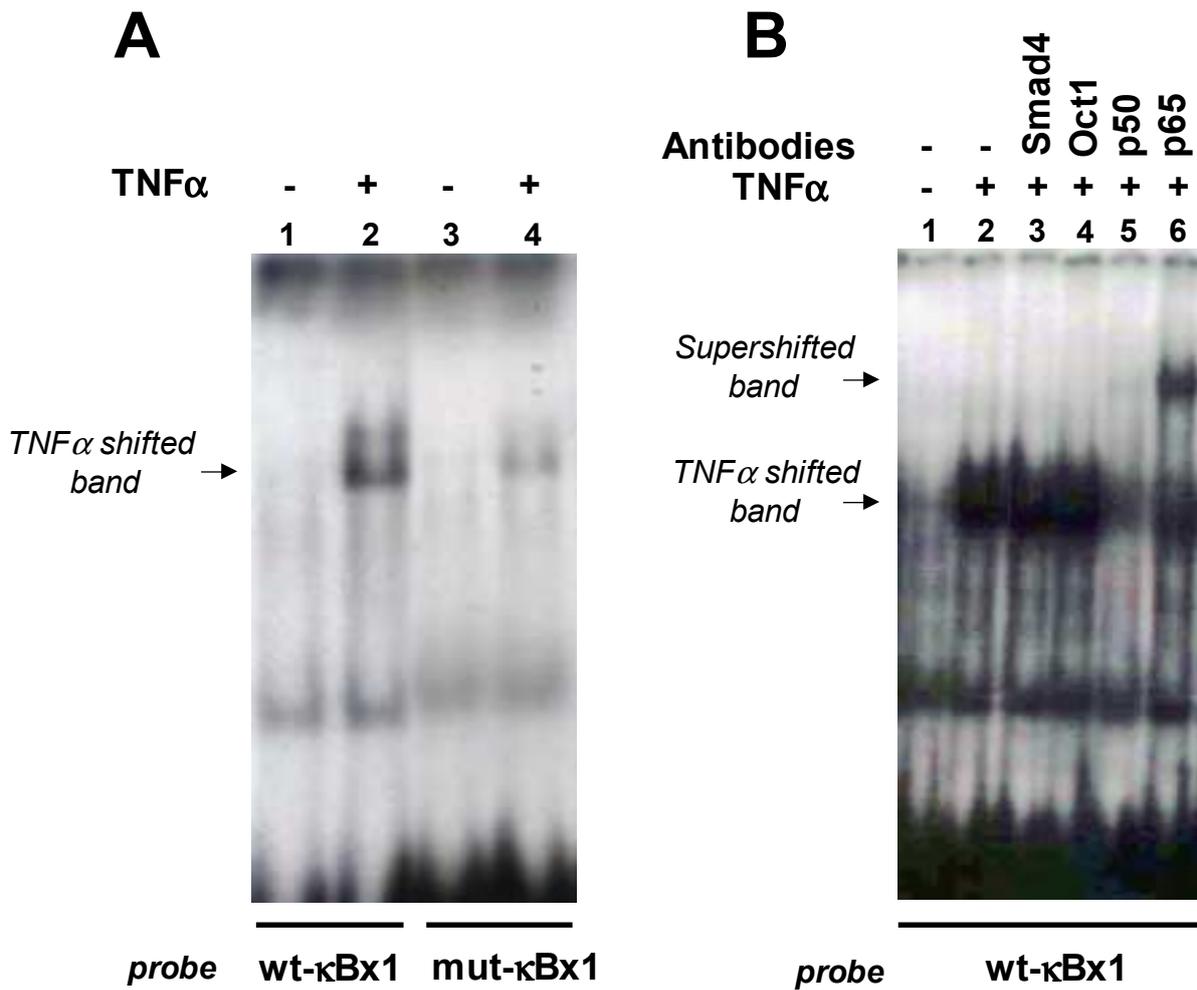
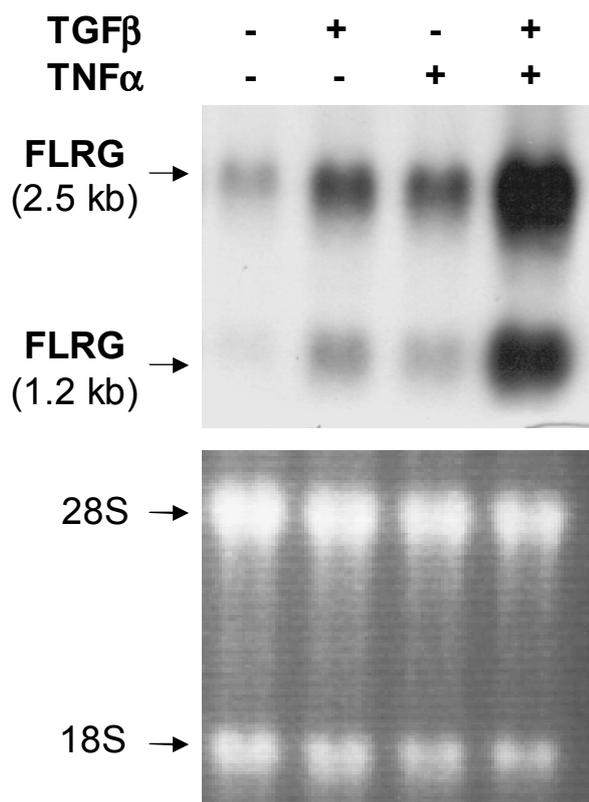
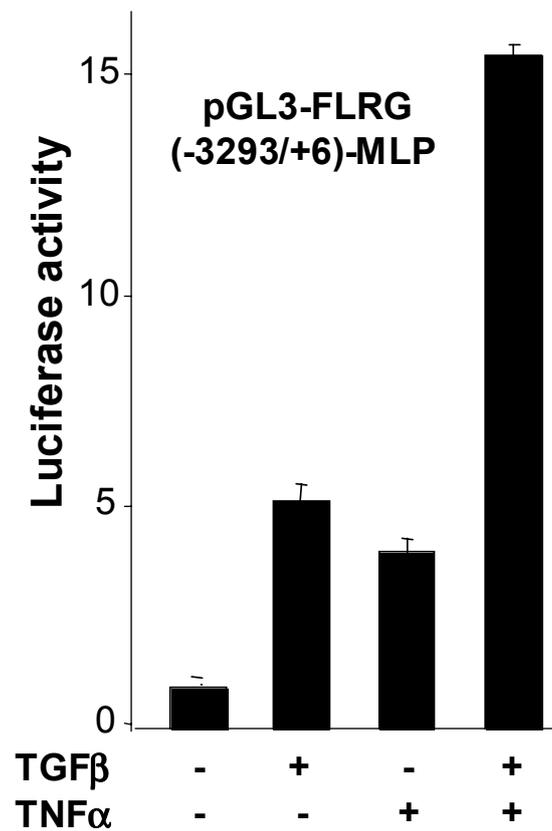
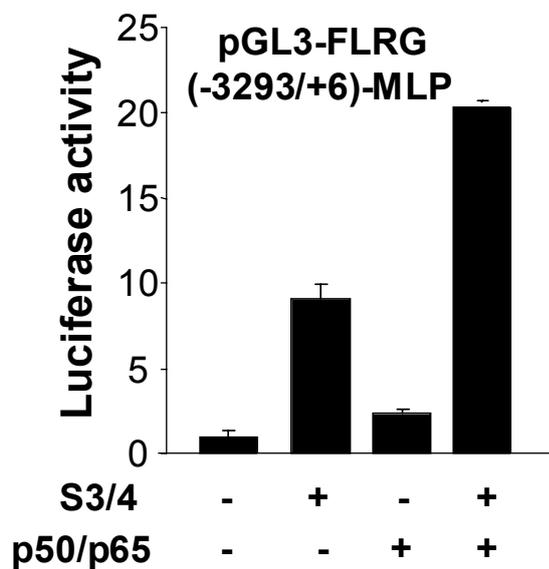
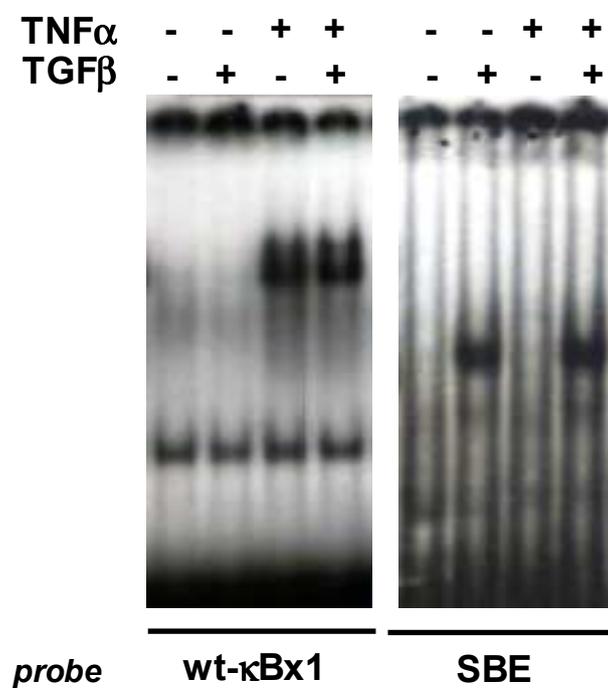


Figure 5, Bartholin et al.

**A****B****C****D****Figure 6, Bartholin et al.**