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CIKS/DDX3X interaction controls the stability of Zc3h12a mRNA induced by IL-17

Somma Domenico

Università degli Studi di Napoli Federico II
Dipartimento di Medicina Molecolare e Biotecnologie Mediche

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Giuseppe Viglietto
Mario Vitale

CIKS/DDX3X interaction controls the stability of Zc3h12a mRNA induced by IL-17

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LIST OF PUBLICATIONS

This dissertation is based upon the following manucript in preparation:

Domenico Somma, Paola Mastrovito, Maria Neve Grieco, Anna Maria Salzano, Andrea Scaloni, Antonio Leonardi. *CIKS/DDX3X interaction controls the stability of Zc3h12a mRNA induced by IL-17*

Moreover I also contributed to the following publication:

Volpe V, Raia Z, Sanguigno L, **Somma D**, Mastrovito P, Moscato F, Mellone S, Leonardi A, Pacifico F. *NGAL controls the metastatic potential of anaplastic thyroid carcinoma cells.* J Clin Endocrinol Metab. 2013 Jan;98(1):228-35. doi: 10.1210/jc.2012-2528.

ABSTRACT

Interleukin 17 (IL-17) promotes the expression of cytokines and proteins involved in inflammation response via the induction of gene transcription and post-transcriptional stabilization of mRNA.

We show here that IL-17 enhanced the stability of *zc3h12a* mRNA through the RNA binding protein DDX3X. After receptor triggering DDX3X binds the ubiquitin ligase CIKS trough the helicase domain and stabilize *zc3h12a* mRNA by directly binding the mRNA. This process involved CIKS (but the E3 ubiquitin ligase function is dispensable) the adaptors TRAF2 and TRAF5, and the kinase IKKε.

Zc3h12a is an endonuclease involved in controlling inflammatory responses by degrading mRNA of some inflammatory protein such as IL-6. This effect is important to correctly switch-off the IL-17-dependent inflammation.

BACKGROUND

T_H17 linfocites

T helper 17 cells (Th17) are a subset of T helper cells producing interleukin 17 (IL-17) developmentally distinct from Th1 and Th2 cells(Harrington et al., 2005; Park et al., 2005)

Originally IL-17 was thought to be produced exclusively by T cells, but it is now known to be secreted by a variety of innate cells including macrophages, mast cells, dendritic cells, and natural killer(Korn et al., 2009).

Whereas it was known for decades that IL-12 induces Th1 cells (IFN-γ producers) and IL-4 induces Th2 cells (IL-4, IL-5 and IL-13 producers), it was only recently demonstrated that Th17 cells differentiate upon exposure to combinations of IL-6 and transforming growth factor-β (TGF-β).

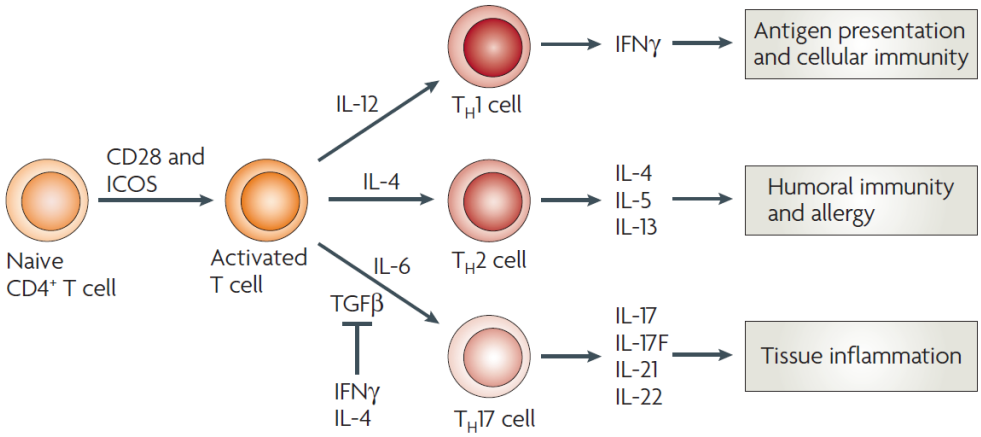

Figure 1: ***General scheme of T-helper-cell differentiation.*** *Nat Rev Immunol*. 2008 May; **8**(5):337-48.

Initial differentiation fase of mouse TH17 cells requires the presence of transforming growth factor-β (TGFβ) and IL-6 as well as STAT3 signalling(Bettelli et al., 2007). Analogous to the differentiation of TH1 and TH2 cell lineages, TH17 cells require additional lineage-specific transcription factors. Both retinoid-related orphan receptor-α (RORα) and RORγt are required for TH17 cell differentiation(Dong, 2008b), and these transcription factors can be induced by the combined presence of TGFβ and IL-6 in naive T cells(Chung et al., 2009; Martin et al., 2009; Sutton et al., 2009).

It has been argued that differentiation of human TH17 cells could

potentially occur independently of TGFβ, as a combination of IL-23 and IL-1β has been shown to be sufficient to induce TH17 cell differentiation. However, murine and human TH17 cells are very similar; both cell types express IL-17A, IL-17F, IL-21, IL-22, IL-23R and IL-1R1, and require RORα and RORγt as crucial transcription factors for their differentiation(Ghoreschi et al., 2010).

IL-17 is essential for host defence against many microbes, particularly extracellular bacteria and fungi, in particular for mucosal epithelia(O'Quinn et al., 2008). Interleukin-17 and other Th17 cytokines are linked to the pathogenesis of diverse autoimmune and inflammatory diseases. The IL-17 receptor is expressed ubiquitously, and hence most cells can potentially respond to this cytokine(Yao et al., 1997).

It has been noted that IL-17A expression can be induced within hours after infection or immunization with an adjuvant and before the differentiation of TH17 cells from native progenitors, which takes several days. This suggests that cells other than TH17 cells can produce IL-17.

Mast cells also produce several members of the IL-17 family. Following Fcε receptor I-mediated activation, mast cells produce substantial amounts of IL-17E, which can influence TH2-type responses(Ikeda et al., 2003). Mast cells also produce IL-17A in response to their activation by Toll-like receptor 2(Mrabet-Dahbi et al., 2009); this has been shown in several diseases, including asthma, psoriasis(Lin et al., 2011), rheumatoid arthritis(Hueber et al., 2010; Moran et al., 2011), and amyotrophic lateral sclerosis(Fiala et al., 2010).

The respective contribution of IL-17 produced by these different cell types to disease pathology is still unknown.

Interleukin-17 mediates adverse effects in many autoimmune diseases. Before the discovery of the Th17 it was considered that Th1, Th2 and B cells were the main mediators of pathology in autoimmunity.

Based on these observations, inhibitors — mostly monoclonal antibodies — have been designed, and the results of these clinical trials are now being released.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that may affect many tissues and organs, but principally attacks flexible (synovial) joints. It can be a disabling and painful condition, which can lead to substantial loss of functioning and mobility.

Inflammatory infiltrate of the joint synovium membrane leads to bone and cartilage destruction. The presence of IL-17-positive cells in the affected synovium and the production of functionally active IL-17 by this tissue was first demonstrated in this disorder(Lubberts et al., 2002).

Interleukin-17 induces pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 from cartilage, synoviocytes, macrophages and bone cells(Shen and Gaffen, 2008). Collectively, these pro-inflammatory cytokines contribute to RA increase and also establish a chronic inflammatory state by a positive feedback loop wherein IL-17-induced IL-6 maintains the Th17 T-cell population(Ogura et al., 2008). The IL-17 also stimulates the production of multiple chemokines, including IL-8/CXCL8, CXCL1, CXCL2, CCL20, CCL2 and CCL7(Fossiez et al., 1996; Park et al., 2005; Shen et al., 2005a; Shen and Gaffen, 2008). These serve to recruit neutrophils, macrophages and lymphocytes to the synovium, thereby enhancing inflammation.

Inhibition of IL-17 with antibodies against the ligand IL-17A or its receptor IL-17RA protected against the development and consequences of arthritis(Lubberts et al., 2004). Furthermore, mice lacking IL-17RA develop a very mild form of experimental arthritis.

Psoriasis

Psoriasis is a chronic inflammatory skin disorder characterized by dermal hyperplasia. The key histological features of psoriatic skin are epidermal keratinocyte hyperproliferation, vascular proliferation and infiltration of Dentritic Cells, macrophages, neutrophils and T cells(Nestle et al., 2009).

Psoriasis has been successfully treated using TNFα inhibitors in some but not all patients. Skin biopsy samples taken from patients with psoriasis showed high expression of IL-17 together with high expression of IL-23, IL-22 and IL-6(Zheng et al., 2007). Furthermore, increased numbers of TH1 and TH17 cells were found in blood and skin lesions of patients, showing a positive correlation with disease activity(Lowes et al., 2008). Local production of TH17 cytokines within the plaques appears to contribute to the increased production of chemokine CC motif ligand 20 (CCL20), a key chemokine that is necessary for the migration of TH17 cells(Harper et al., 2009). Mast cells and neutrophils represent additional sources of IL-17 in skin that is affected by psoriasis(Lin et al., 2011).

Multiple sclerosis

Multiple sclerosis is a chronic inflammatory disease that leads to brain inflammation in which the myelin around the axons of the brain and spinal cord are damaged, leading to demyelination leading to a broad spectrum of signs and symptoms

Experimental autoimmune encephalomyelitis (EAE) is a model of multiple sclerosis, a T-cell-mediated autoimmune disease of the central nervous system. It is elicited by immunization of neuroantigens, such as myelin basic protein and proteolipid protein. As with many other autoimmune conditions, Th1 cells were long thought to be responsible for EAE pathology, despite the

fact that IFN-/-, IFNcR-/- and IL-12p35-/- mice were susceptible(Ferber et al., 1996; Becher et al., 2002; Zhang et al., 2003). Landmark studies comparing the IL-12p35-/- and IL-23p19-/- mice showed clearly that the Th17 path-way was responsible for pathology(Langrish et al., 2005).

Further evidence for the role of Th17 cells in driving EAE was shown in STAT6-/- / T-bet-/- doubly deficient mice, lacking Th1 and Th2 cells(Das et al., 2009). IL-17 drive the tight junctions distruption that form the blood–brain barrier, enabling Th17 cells to migrate into the central nervous system and cause neuronal damage(Kebir et al., 2007). Furthermore, EAE in the mice could be ameliorated by treatment with anti-IL-17 antibodies.

Asthma

Asthma is a common airway disorder that is characterized by chronic airway inflammation, mucus production, and airway hyperresponsiveness with airway remodeling.

Mouse models of asthma have yielded significant new insights recently. In particular, the cytokine IL-17E (IL-25) has been shown to have a role(Tamachi et al., 2006).

IL-17E was proposed to initiate and maintain Th2 responses. Furthermore, by directly and/or indirectly inducing the expression of, in particular, IL-13 and IL-5, IL-17E may be responsible for the observed mucus production and lung infiltration of eosinophils during allergic reactions(Claudio et al., 2009).

In asthmatic patients, IL-17 expression has been shown to increase in sputum, lung cells, bronchoalveolar lavage fluids, and peripheral blood(Barczyk et al., 2003).

Interleukin 17 Family

The IL-17 gene and IL-17 protein were first discovered as a product of T cells in rodents, but it was not immediately recognized as a cytokine, owing to its unusual amino acid sequence(Rouvier et al., 1993).

It is well established that IL-17 activity contributes to various aspects of acute inflammation. The IL-17-mediated release of IL-6 and IL-8 from mesenchymal cells leads to fever, an acute phase response (caused by IL-6) and the accumulation of neutrophils in blood and tissue (caused by IL-8)(Fossiez et al., 1996). IL-17 activity also contributes to chronic inflammation(Miossec, 2003), which is often — but not always — associated with matrix destruction.

The IL-17 molecule is composed of two monomers that are linked by intramolecular disulphide bonds on cysteine residues to form a homo or eterodimer. IL-17 is now formally referred to as IL-17A in the literature and is the founding member of the IL-17 family, which is composed of six members — from IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F(Aggarwal and Gurney, 2002).

IL-17A and IL-17F are the best characterized cytokines of the IL-17 cytokine family. Both are covalent homodimers, and recent findings show that they also form IL-17A–IL-17F heterodimers(Chang and Dong, 2007).

IL-17A, IL-17F and the IL-17A–IL-17F heterodimer signal through the same receptor subunits: IL-17RA and IL-17RC(Kuestner et al., 2007; Wright et al., 2008). Results from mouse models, comparing IL-17A-/- and IL-17F-/- mice, indicate that some of the pro-inflammatory functions are not identical: IL-17A has a more important role in driving autoimmunity than IL-17F(Yang et al., 2008).

This effect is probably due to more activity of IL-17A in inducing inflammation: IL-17F-induced responses are 10–30 fold weaker in terms of downstream gene activation than those of IL-17A, with IL-17A–IL-17F heterodimers acting at an intermediate level(McAllister et al., 2005).

In contrast to the other members, IL-17E (IL-25) induces allergic responses and activation of the TH2 pathway and it is produced by mucosal epithelial cells and many immune cell types(Wang et al., 2007). Moreover, IL-17E inhibits Th17 cell development by inducing the expression of IL-13 by dendritic cells (DCs) or by inhibiting IL-23 production(Kleinschek et al., 2007; Dong, 2008a).

IL-17B, IL-17C and IL-17D functions are poorly defined .

IL-17 Receptors

The first receptor to be identified for IL-17 was initially referred as IL-17 receptor (IL-17R) but is now known as IL-17RA(Yao et al., 1995). Afterwards additional receptor components, required to form a functional receptor complex for IL-17 signaling, were identify(Yao et al., 1997).

Four additional receptors have been identified in the IL-17R family, based on sequence homology to IL-17RA: IL-17RB, IL-17RC, IL-17RD and IL-17RE, thus IL-17RA appears to be a common receptor chain for the IL-17 family of ligands.

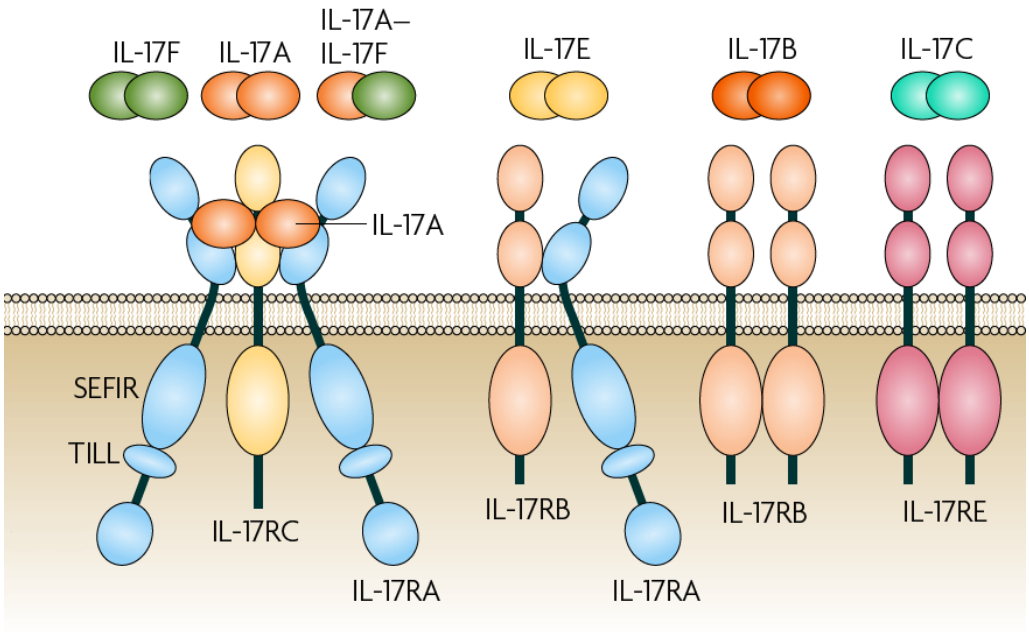

Figure 2: ***IL-17R family ligand–receptor relationships and main structural features.*** *Nat Rev Immunol.* 2009 Aug;**9**(8):556-67.

All receptor subunits are single transmembrane domain-containing proteins, these receptor subunits contain certain conserved structural motifs, including an extracellular fibronectin III-like domain and a cytoplasmic domain named SEFIR, a member of STIR-domain superfamily.

STIR-domain superfamily comprising TIR (Toll/Il-1 Receptor) domain, and SEFIR (SEF/IL-17R) domain.

TIR domain has three 'boxes' of conserved residues and a important binding loop named BB-loop, SEFIR domain keep the same structure of TIR domain, but lack the TIR box 3 subdomain and the BB-loop(Novatchkova et al., 2003). However, a region at the carboxy-terminal side of the SEFIR domain in IL-17RA has marked sequence homology to BB-loops. Deletion or point mutations in this region render IL-17RA non-functional, so this motif is referred to as a TIR-like loop (TILL)(Maitra et al., 2007; Shen et al., 2009).

Surprisingly, although all IL-17Rs have a SEFIR motif, the TILL domain seems to be unique to IL-17RA, perhaps explaining why IL-17RA functions as a common subunit shared by several receptors in the family.

As mentioned IL-17RA is necessary for signal transduction mediated by IL-17A, IL-17A–IL-17F and IL-17F(Hymowitz et al., 2001). Indeed, IL-17RA pairs with IL-17RC to induce responses to IL-17A and IL-17F(Toy et al., 2006). Similarly, IL-17RA–/– mice are refractory to the effects of IL-17E, suggesting that IL-17RA is also a component of this receptor complex(Rickel et al., 2008).

IL-17RA is expressed ubiquitously, with particularly high levels in haematopoietic tissues(Yao et al., 1995; Ishigame et al., 2009), but only a limited number of IL-17A-induced genes has been documented in lymphocytes, and these genes are distinct from those induced by IL-17A in other cell types(Hsu et al., 2008; Ishigame et al., 2009), like endothelial cells and fibroblasts.

Another function of IL-17RA seems to limit signalling by receptor-mediated internalization of the ligand. Indeed, surface expression of IL-17RA rapidly decreases after IL-17A binding(Lindemann et al., 2008).

The precise stoichiometry of the IL-17A-binding receptor complex has not been determined, but some data suggest the existence of a trimeric complex containing two IL-17RA subunits and one IL-17RC subunit(You et al., 2006).

In contrast to IL-17RA, IL-17RC expression is low in haematopoietic tissues and high in non-immune cells of the prostate, liver, kidney, thyroid and joints(Haudenschild et al., 2002; Kuestner et al., 2007). This differential expression of IL-17R subunits is coerent with a mechanism for tissue-specific signalling by IL-17A and/or IL-17F.

IL-17RB instead binds both IL-17B and IL-17E. This subunit is expressed by various endocrine tissues as well as kidney, liver and Th2 cells(Lee et al., 2001). Recent evidence indicates that IL-17RB pairs with IL-17RA to form a functional receptor complex for IL-17E(Rickel et al., 2008). IL-17RB is the one IL-17R that has a TRAF6-binding motif in its cytoplasmic tail(Maezawa et al., 2006).

IL-17RD and IL-17RE has no known ligand and their function is

unknown.

IL-17: molecual pathway

IL-17 stimulation lead to two major biological responses: 1) de novo transcription and, 2) stabilization of mRNA. Both biological responses are medieted by CIKS(Sun et al., 2011) (also known as ACT1 or TRAF3IP2) that is recruited to the receptor after IL-17 stimulation. Insteed genetic ablation of CIKS gene in mice, leads to unresponsiveness to IL-17 stimulation(Claudio et al., 2009).

CIKS contains a SEFIR domain, an E3 ubiquitin ligase domain(Liu et al., 2009), and a binding site to TRAF6(Sønder et al., 2012). The binding between CIKS and IL-17RA is mediated by a SEFIR-SEFIR domains interaction. In particular CIKS binds to the CC′ loop region of the cytoplasmic tail of IL-17RA.

After receptor binding CIKS recruits and ubiquitinates TRAF6 through K63 in a U-box – dependent manner(Liu et al., 2009).

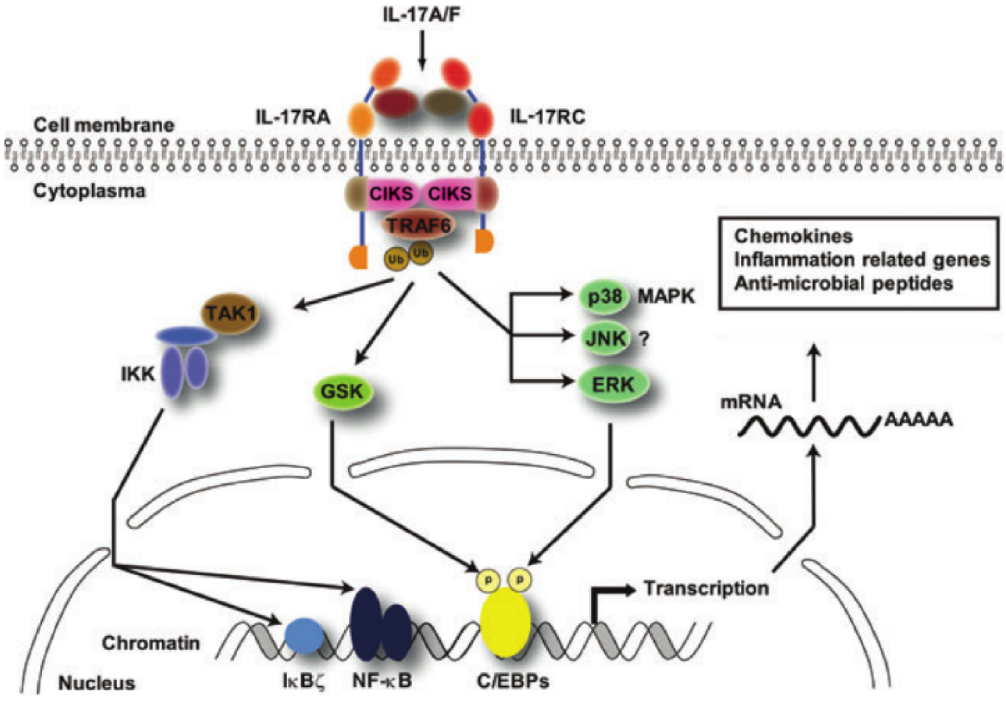

Figure 3: ***IL-17 signaling pathway****. Modified by Ann N Y Acad Sci.* 2011 Jan;1217:60-76

This signal drive TRAF6 to the activation of NF-κB via TAK-IKK complex(Deng et al., 2000). Like the activation of NF-κB by TLR, IKK complex induces the phosphorylation, degradation of IκBα and subsequent activation of NF-κB(Schwandner et al., 2000; Liu et al., 2009). Some data demonstrate that NF-κB p65 and p50 subunits quickly associate with NF-κB response element upon IL-17 stimulation(Ruddy et al., 2004; Shen et al., 2006). There is no evidence of processing of other noncanonical NF-κB subunits. Interestingly, IL-17 also induces expression of IκBζ(Shen et al., 2005a), a positive regulator of NF-κB activation(Yamamoto et al., 2004). Recently, additional evidence suggests that IκBζ plays an important role in IL-17–induced expression of human β-defensin 2 and neutrophil gelatinase-associated lipocalin(Karlsen et al., 2010).

IL-17 signaling also activates C/EBPs. Among all C/EBP family members, only C/EBP- β and C/EBP-δ are transcriptionally up-regulated by IL-17(Shen et al., 2005b). The cooperation of NF-κB and C/EBPs can only partially explain the synergistic effect of IL-17 and TNFα.

IL-17A also activates the MAPK pathway. Within 30 minutes of IL- 17A stimulation, ERK is rapidly phosphorylated and then regulates various downstream signaling components, including NF-κB, C/EBP, and AP1(Maitra et al., 2007).

mRNA stability IL-17 dependent

Once mRNAs are transcribed they are subjected to post-transcriptional events that regulate mRNA metabolism including stability and translation. These two processes normally dictate the protein levels encoded by mRNA. RNA-binding proteins that bind mature mRNA sequences normally have an important regulatory effect on the mRNA.

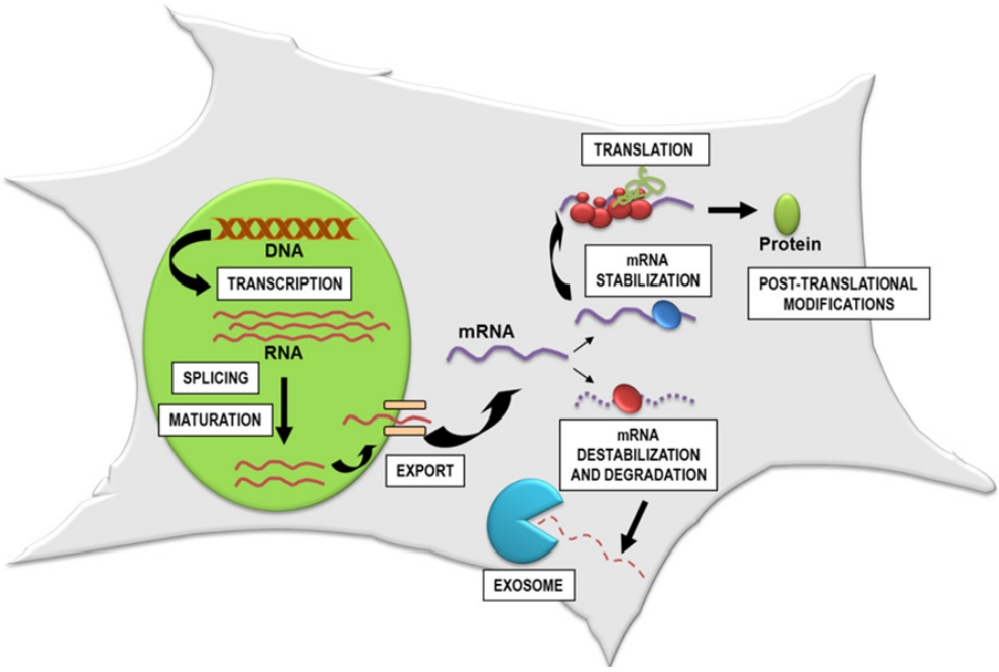

Figure 4: ***mRNA metabolism.*** *From Abdelmohsen, K.in Binding Protein (Abdelmohsen, K.) (InTech, 2012)*

One major effect of IL-17 is an increase in mRNA stability, this effect is evident when IL-17 is used in combination with other cytokines like TNFα. The IL-17 mediated mRNA stability has been demonstrated for different genes including G-CSF and CXCL1(Henness et al., 2004; Hartupee et al., 2007).

This increas half-life of chemokine mRNA is mediated by an CIKS-dependent but TRAF6-independent pathway(Hartupee et al., 2007). This observations indicate that IL-17-mediated stabilization of CXCL1 mRNA involves a distinct signal-transduction pathway(Hamilton et al., 2010).

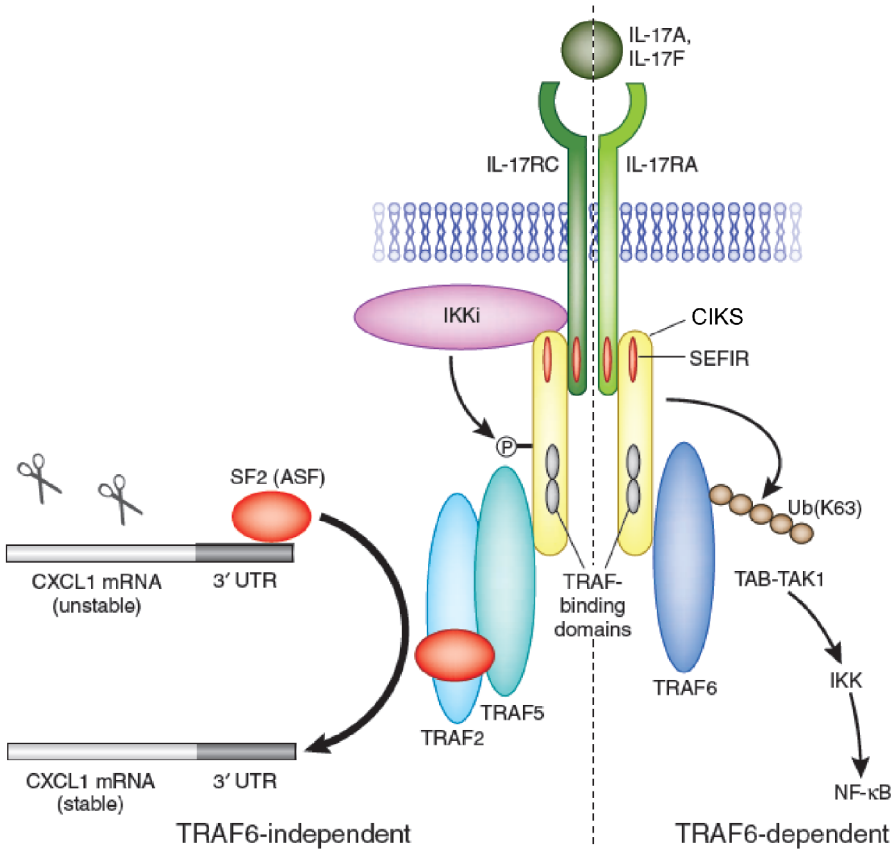

Figure 5: ***TRAF6-dependent and TRAF6-independent IL-17R signaling pathways****. Nat Immunol.* 2011 Aug 18;**12**(9):813-5

Receptor triggering also promoted interaction between CIKS and TRAF2 or TRAF5, and IKKε (also named IKKi). These proteins are involved for mRNA stabilization of Cxcl1(Bulek et al., 2011; Sun et al., 2011).

IKKε is able to phosphorylate CIKS on Ser 311, located adjacent to a putative TRAF-interacting consensus motif(Liu et al., 2009). Computational modeling analysis suggest that phosphorylation of CIKS Ser 311 modulated its affinity for TRAF5/TRAF2.

IKKε deficiency and substitution of CIKS Ser 311 indeed abolished IL-17-induced formation of the CIKS-TRAF2/TRAF5 complex and stabilization of chemokine mRNA, whereas the CIKS–TRAF6–NF-κB axis was retained(Bulek et al., 2011).

The complex formed by CIKS, IKKε and TRAFs induce also the

association of SF2 (also named ASF), a multifunctional RNA-splicing regulatory factor. SF2 selectively enhanced the decay of target mRNA by binding to sites in 3′ UTRs. This interaction is disrupted by IL-17, perhaps via TRAF5-dependent sequestration of limiting amounts of cytoplasmic SF2 (ASF).(Sun et al., 2011)

Notably, mRNA-decay mechanisms can be regulated via MAPK-dependent signaling, including signaling by p38, Erk and Jnk(Rowlett et al., 2008).

Target genes IL-17 dependent

IL-17 downstream targets can be categorized into five major groups:

1) chemokines;
2) proinflammatory cytokines and inflammatory mediator related genes;
3) antimicrobial peptides;
4) tissue remodeling genes;
5) signaling components such as C/EBPβ , C/EBPδ , and IκBζ

CXCL1, CXCL2, and CXCL5 are the major mediators of the IL-17–induced neutrophil response in host defense and various inflammatory disease models. IL-17F alone or in combination with IFNγ can stimulate CXCL10 production in bronchial epithelial cells(Kawaguchi et al., 2007). Other CXC chemokines such as CXCL6, CXCL8, and CXCL12 are also regulated by IL-17. CCL2 and CCL20 are among the few CC chemokines up-regulated by IL-17(Kao et al., 2005).

IL-17A induces production of other proinflammatory cytokines in various cell types. For example, IL-6 is one of the earliest target genes reported in IL-17A–stimulated fibroblasts(Yao et al., 1995). G-CSF is another important cytokine that is induced by the IL-17 pathway.

IL-17 (like IL-1 and TLR) also induce a anti-inflammatory RNase: Zc3h12a (also know as MCPIP or MCPIP1) that selectively degrades specific target mRNA, like IL-1, IL-6 or IL-12, to modulates the immune response and inflammation(Huang et al., 2012). Zc3h12a recognizes the 3'-untranslated region (UTR) of the mRNA, it is able to cleaved the mRNA and it is required for normal decay of mRNA. It triggers apoptosis and promotes angiogenesis in response to the binding of CCL2 to CCR2(Matsushita et al., 2009). Zc3h12 is

also a Interferon Stimulated Genes (IGSs) induced by interferon-γ, and it has been hypothesized to suppress the antiviral state(Abe et al., 2011).

Antimicrobial peptides are important mediators of innate immunity; and IL-17A is a potent inducer of various antimicrobial peptides. Lipocalin 2 (24p3) plays an important role in host defense against E. coli and Klebsiella pneumoniae by limiting bacterial siderophores to bind free iron(Goetz et al., 2002). In fibroblast cells, IL-17A alone or with TNFα results in quick induction of 24p3 protein(Shen et al., 2006). As another example, human β-defensin 2 (hBD2) is an antimicrobial peptide and a chemo-attractant to CCR6+ cells. hBD2 is markedly up-regulated by IL-17A in human airway epithelium and during virus infection(Kao et al., 2008).

IL-17A also induces expression of MMPs, including MMP1, MMP2, MMP9, and MMP13, which are the major players in extracellular matrix degradation and bone resorption(Sylvester et al., 2004). Mice lacking IL-17RA fail to express MMPs in streptococcal cell wall induced-arthritis and show amelioration of cartilage destruction(Koenders et al., 2005).

DEAD-box protein 3 - DDX3X

DEAD box proteins form the largest helicase family, with 37 members in humans(Fairman et al., 2004), and are characterized by the presence of an Asp-Glu-Ala-Asp (DEAD) motif. DEAD-box protein are involved in all aspects of RNA metabolism, from transcription and translation to mRNA decay(Jankowsky, 2011).

Although all DEAD box proteins contain a structurally highly conserved core with conserved ATP-binding and RNA-binding sites, different members of the family have been associated with diverse and seemingly unrelated functions, including the disassembly of RNPs, chaperoning during RNA folding and even stabilization of protein complexes on RNA(Cordin et al., 2006; Jarmoskaite and Russell, 2011).

Members of the DEAD box family contain a highly conserved helicase core that harbours the binding sites for ATP and RNA(Caruthers and McKay, 2002; Fairman et al., 2004; Singleton et al., 2007). The core is surrounded by variable auxiliary domains, which are thought to be critical for the diverse functions of these enzymes.

Within this helicase core, at least 12 characteristic sequence motifs are located at conserved positions. Some of these motifs are conserved across the entire SF2 family, whereas others are found only in the DEAD box family(Fairman et al., 2004). Motif II, which contains the Asp-Glu-Ala-Asp motif, inspired the name DEAD box for this family(Linder et al., 1989).

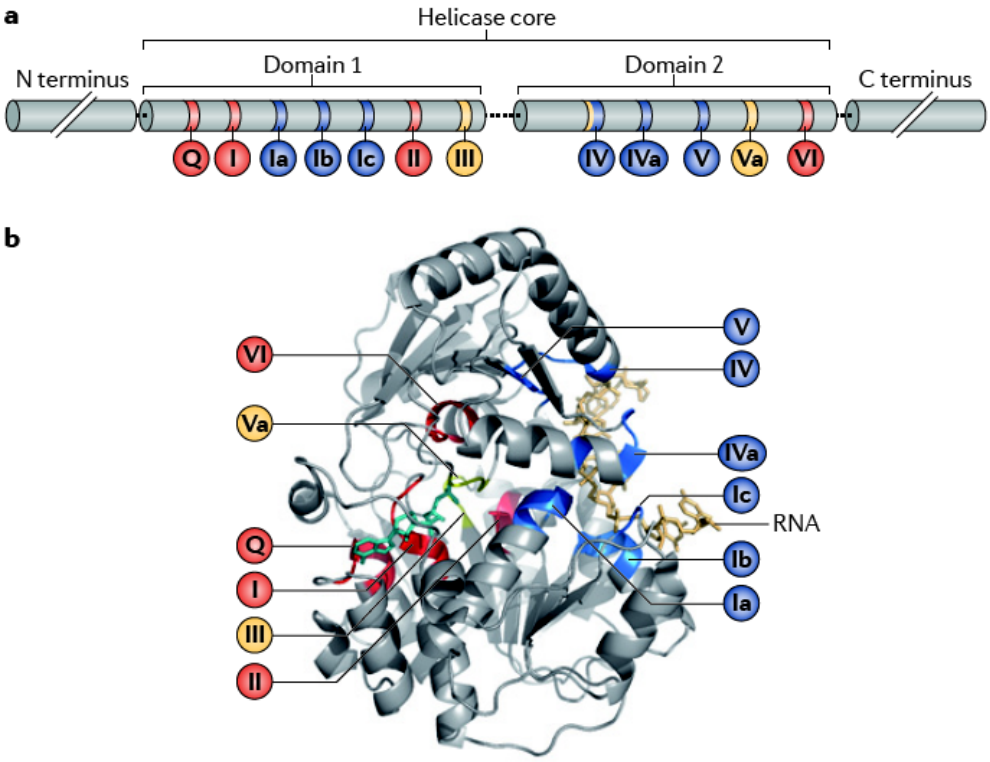

Figure 6: ***Structure of the DEAD box helicase core.*** *Nat Rev Mol Cell Biol.* 2011 Jul 22;**12**(8):505-16

The two helicase domains form a cleft that harbours the ATP-binding site, which is located between the two domains(Hilbert et al., 2009). Despite the high structural conservation of the helicase core, DEAD box proteins have been associated with an intriguing array of cellular functions, from disassembling RNPs to serving as immobile RNA clamps(Tanner and Linder, 2001; Jankowsky, 2011). Similarly to most proteins involved in RNA metabolism, DEAD box proteins usually function within complexes containing dozens or even hundreds of components, such as the spliceosome or the nascent ribosome. In addition to ATP-dependent RNA unwinding and clamping, DEAD box proteins can remove proteins from RNA in an ATP-driven reaction(Kistler and Guthrie, 2001; Fairman et al., 2004).

DDX3X (also know as DBX or DDX3) was first identified in 1997(Park et al., 1998). DDX3X was found to be part of cytoplasmic complexes containing eIF2, eIF3, eIF4A, eIF4E, and mRNA translation was affected by DDX3X overexpression or DDX3X knock down(Geissler et al., 2012). Furthermore DDX3X is able to bind 5' free extremity of the specific mRNA, and this is essential to starting the translation(Soto-Rifo et al., 2012).

DDX3X interact with the HBV (hepatitis B virus) protein pol (polymerase). DDX3X was incorporated into nucleocapsids together with HBV pol and inhibited the initial step of reverse transcription in a manner that was dependent on the ATPase activity of DDX3(Wang et al., 2009).

This suggested an antiviral role of DDX3 in HBV infection; however, it remained unclear how DDX3X interfered with viral replication. Interestingly, it has been shown that DDX3X levels are decreased in a fraction of HBV-induced hepatocellular carcinoma cases(Chang et al., 2006). It is possible that the virus down-regulates DDX3X levels to relieve the inhibitory effect of DDX3 on its own replication(Schröder, 2011). Some studies demonstrated that HBV pol disrupts the interaction between IKKε and DDX3X(Wang and Ryu, 2010). DDX3X is also inibithed by VACV (vaccinia virus) protein K7(Schröder, 2011).

AIM OF THE STUDY

IL-17 plays a critical role in host defense against different pathogens in mucosal epithelium. IL-17 induces and sustains tissue inflammation and drives the immune responses, mainly by two different mechanisms. The first is transcription dependent and involves transcription of genes controlling inflammation, the second is dependent upon the stabilization of different mRNA induced by other inflammatory cytokines, such as TNFα.

In the last few years some of the mechanisms regulating these two functions have been elucidated. However, much remains to be understood about this molecular pathway. Aim of this study is the identification of new proteins involved in the IL-17 signaling, in order to unravel the molecular mechanism regulating the IL-17 dependent inflammatory responses, and to identify new potential target for therapeutic intervention.

MATERIAL AND METHODS

Reagents, cell lines, and constructs.

Recombinant IL-17 and TNF were from Peprotech, and were used at (200ng/ml) and 2000U/ml, respectively. Anti-M2 (Flag) was from Sigma-Aldrich. Anti DDX3X was produced in rabbit using a recombinant fragment spanning the amino acids 1-222 of mouse DDX3X as antigene. Anti-TRAF6, anti-TRAF2, anti-TRAF5, anti-IKKe were from Santa Cruz Biotechnology.

HEK293, wild type mouse embryonic fibroblast (MEF), CIKS$^{-/-}$ MEF, HeLa were maintained in Dulbecco modified Eagle medium (DMED) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin), and 1 mM *L*-glutamine (Invitrogen). WT and CIKS$^{-/-}$ MEF were a gift of U. Siebenlist (NIAID, NIH).

The cDNA encoding CIKS and its mutants were previously described (PNAS, BBRC). The cDNA encoding DDX3X was a gift of Dr Tilmann Bürckstümmer (Haplogen GmbH , Wien, Austria). DDX3X mutants were generated by PCR and cloned in pCDNA3.1-HA or FLAG (Invitrogene).

RNA-interference (RNAi) and transfection.

Cells were transfected with small interfering RNA oligonucleotides (20nM final concentration) and Interferin (PolyPlus) according to manufacturer's instruction. The siRNA sequence used are listed below:

Mouse DDX3X 5′-GAUGCUGGCUCGUGAUUUCU-3′

Mouse TRAF2 5′-CGACAUGAACAUCGCAAGC-3′

Mouse TRAF5 5'-AAGCCAGUGACCAGAGAUUAGUU-3'

To knock-down mouse IKKε we used the esiRNA#EMU029351 (Sigma)

The scrambled control was from Thermo Scientific (siRNA ON-TARGETplus Non-targeting Pool #D-001810-10-05).

Seventytwo hours after transfection cells were collected for protein or RNA extraction.

Lentivirus production and infections.

FLAG-CIKS cDNA was subcloned into pWPT lentiviral vector at BamHI/SalI sites. The construct was sequenced to confirm correct DNA sequence and orientation. Subconfluent 293T lentivirus packaging cells were cotransfected with either pWPT-GFP or Wip1-FLAG-pWPT and pMD2G and pCMV-R8.91 by calcium phosphate precipitation. After 24h medium was changed, and supernatant was harvested after 48 and 72 hours. Lentiviral supernatant, cleared of cell debris, was concentrated by centrifugation for 90 min at 23000 rpm at 4°C. For transduction, $CIKS^{-/-}$ MEFs were plated on 12-well plate, infected with lentiviruses in the presence of 10% foetal bovine serum and 8μg/ml polybrene (Hexadimethrine bromide; Sigma-Aldrich, Milano, ITALY).

Coimmunoprecipitation and immunoblot analysis.

$CIKS^{-/-}$ MEF reconstituted with FLAG-CIKS or with empty vector were left untreated or treated with IL-17 at different time points, were harvested by washing with ice-cold PBS, and then lysed with Lysis buffer (20 mM HEPES pH7.5, 150 mM NaCl, 1% Triton X-100, 10% Glycerol, 10mM NaF, 1mM Na_3VO_4) freshly supplemented with protease inhibitors cocktail (Roche). Nuclear and cellular debris were removed by centrifugation at 14,000 x *g* for 30 min at 4°C. Cell extracts were normalized for protein content, and incubated with 20 ml of M2 beads for 3 hours at 4°C. Beads were washed five times with Lysis buffer, and boiled in SDS sample buffer, and the resulting supernatants were subjected to 10% SDS/PAGE.

For identification of the CIKS-interacting proteins, 100mg of cell extract from $CIKS^{-/-}$ and reconstituted MEF were incubated with 500 μl of M2 beads (Sigma) pre-washed with Glycine pH3.0 to eliminate not covalently bound antibody. After 3 hours incubation at 4°C, beads were washed four times with Lysis buffer, 2 times with High Salt (1M NaCl) Lysis buffer. Proteins were eluted from the beads by using 500μl of 3xFLAG peptide (Sigma) 200mg/ml. Eluted proteins were separated by SDS/PAGE on a 9-16% T gradient gel, which was then stained with colloidal Coomassie G-250 (Candiano). Gel image was acquired by using an Image Scanner III densitometer (GE Healthcare Life Sciences). Slices (15 in number) were excised from the lanes corresponding to the bait and mock samples present in the resolving gel, triturated, *in-gel* reduced, S-alkylated and digested with trypsin as previously reported (D'Ambrosio).

Peptide mixtures were desalted on home-made desalting tips (Empore C_{18} extraction disk, 3M USA) and analyzed by nLC-ESI-LIT-MS/MS; a LTQ XL mass spectrometer (Thermo,San Jose, CA, USA) equipped with a Proxeon nanospray source connected to an Easy-nanoLC (Proxeon, Odense, Denmark) was used to this purpose. Peptide mixtures were separated on an Easy C_{18} column (100 x 0.075 mm, 3 μm) (Proxeon) by using a linear gradient of acetonitrile containing 0.1% formic acid in aqueous 0.1% formic acid; acetonitrile was ramped from 5% to 40%, over 40 min, and from 40% to 80% in 10 min, at flow rate of 300 nL/min. Spectra were acquired in the range *m/z* 400–2000. Acquisition was controlled by a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 1 min).

The mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively. Two technical replicates were performed for each sample. Proteome Discoverer platform (Thermo, San Jose, CA, USA) was employed to search raw mass data against an updated Uniprot database of human sequences (2011/6) using both Sequest (Thermo, USA) and Mascot (Matrix Science, UK) algorithms. nLC-ESI-LIT-MS/MS data were searched by using a mass tolerance value of 2 Da for precursor ions and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, a missed-cleavage maximum value of 2, and Cys carbamidomethylation and Met oxidation as fixed and variable modifications, respectively. Candidate proteins with more than 2 assigned peptides with an individual MASCOT score >25, or filtered by Sequest Xcorr (>1.8 for +1, >2.0 for +2, >2.2 for +3 and higher charges), and with a significant threshold $p < 0.05$, were further considered for protein identification. CIKS-interacting proteins were identified by subtracting the components ascertained within the bait with that ones from the corresponding mock.

Gel filtration of cellular extract.

Reconstituted CIKS$^{-/-}$ MEF were lysed in Triton X-100 lysis buffer. Lysates were incubated for 15 min on ice and clarified by centrifugation at 14,000 rpm for 15 min, 4 °C. Supernatants were collected and recentrifuged for 1 h at 100,000xg at 4 °C. One milligram of the S-100 extracts (0.5 ml) was loaded onto a Superdex S200 HR (Amersham–Pharmacia Biotech). Proteins were eluted from the column at the flow rate of 0.3 ml/min. Fractions (0.5 ml) were precipitated with 10% trichloroacetic acid, resuspended in Laemmli's buffer, and analyzed by SDS–PAGE followed by Western blotting using appropriate antibodies.

RNA isolation and Real-Time PCR.

Total RNA was extracted by using TRIZOL reagent according to manufacturer's instruction (Invitrogene). Real-time RT-PCR was carried out with cDNAs reverse-transcribed from total RNA by using GoTaq qPCR Master Mix (Promega, Madison, WI, USA) and Bio-Rad CFX Manager software (Bio-Rad, Segrate, MI, ITALY). The primers used were:

GAPDH: FW: 5'-ATGGTGAAGGTCGGTGTGAAC-3' REV: 5'-CCATGTAGTTGAGGTCAATGAAG-3'

Zc3h12a: FW: 5'-AACTGGTTTCTGGAGCGAGG-3' REV: 5'-CGAAGGATGTGCTGGTCTGT-3'

Cxcl1: FW: 5'-AGCCACCCGCTCGCTTCTCT-3' REV: 5'-GTCCCGAGCGAGACGAGACCA-3'

IKKε: FW: 5'-CCACGTCTTTTCCCTACCCC-3' REV: 5'-GATGGCAATCGTGTTGTGGG-3'

DDX3X: FW: 5'-GTAGAGGCAGCCTTTGCTCA-3' REV: 5'-ATAACGCCCTTTGCTTGCTG-3'

CXCL2: FW: 5'-CCCAGACAGAAGTCATAGCCA -3' REV: 5'-CAGGTACGATCCAGGCTTCC -3'

CCL2: FW: 5'-AGCTGTAGTTTTTGTCACCAAGC-3' REV: 5'-GTGCTGAAGACCTTAGGGCA-3'

CXCL5: FW: 5'-AGAAGGAGGTCTGTCTGGATCCA-3' REV: 5'-CGAGTGCATTCCGCTTAGCTTTC-3'

IL6: FW: 5'-AAAGCCAGAGTCCTTCAGAGAGA-3' REV: 5'-GGTCCTTAGCCACTCCTTCTGTG-3'

LCN2: FW: 5'-ACAGAGCTACAATGTGCAAGTG-3' REV: 5'-CAGCTCCTTGGTTCTTCCATACA-3'

TRAF2: FW: 5'-GCCTTTCCAGATAACGCTGC-3' REV: 5'-TCGTGGCAGCTCTCGTATTC-3'

TRAF5: FW: 5'-CGCACCTGTCCCTGTACTTT-3' REV 5'-AGGCAATGTTCATCTCGCCA-3'

RESULTS

Identification of DDX3X as a specific CIKS-interacting protein.

In order to clarify the molecular mechanism regulating IL-17 signaling we decided to isolate new CIKS interactors.

We set up an experimental system constituted by MEF (Mouse embryonic fibroblast) isolated from CIKS$^{-/-}$ mice, and the same cells reconstituted by lentiviral infection, with FLAG-CIKS.

The level of expression of FLAG-CIKS in the reconstituted cells, is comparable to the endogenous protein. In fact, no transcription of IL-17 dependent genes is detected in unstimulated cells, while a strong transcriptional response is observed after IL-17 stimulation, comparable to that detected in wt MEF (Fig 7). In addition, when the cells were stimulated with IL-17 plus TNFα, there was a synergistic effect on mRNA levels. This effect was due to mRNA neo-synthesis through TNFα signaling, and to mRNA stabilization induced by IL-17. As shown in figure 7 treatment of wt and reconstituted MEF with IL-17 results in upregulation of different IL-17 target genes: *cxcl1* (B), *ccl2* (C), *cxcl5* (D), *IL6* (E), *Lcn2* (F). In the absence of CIKS both the IL-17 dependent transcription and the IL-17 dependent mRNA stabilization were absent.

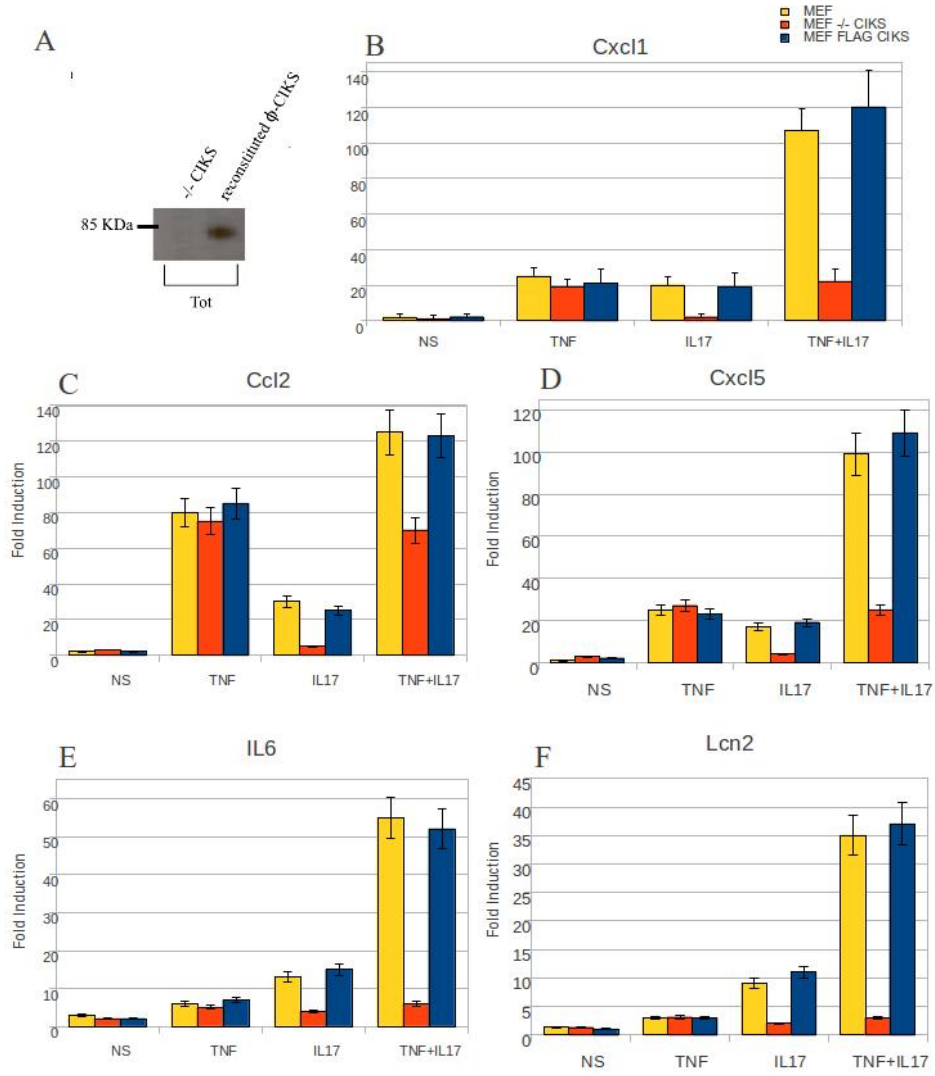

Figure 7: Functional caracterization of WT, CIKS$^{-/-}$, and reconstituted MEF. Cells were stimulated with TNFα, IL-17 or both cytokines for 6 hours, RNA extracted and the level of the indicated RNAs were assessed by Real Time PCR.

To search for CIKS-interacting proteins, cell extracts from CIKS$^{-/-}$ and reconstituted MEF were immunoprecipated and analysed by mass

spectrometry. I found about 30 interactors immunoprecipitated from the reconstituted cells but not in the CIKS$^{-/-}$ cells, some of them are already known as CIKS interactors, such as IKKε and SF2.

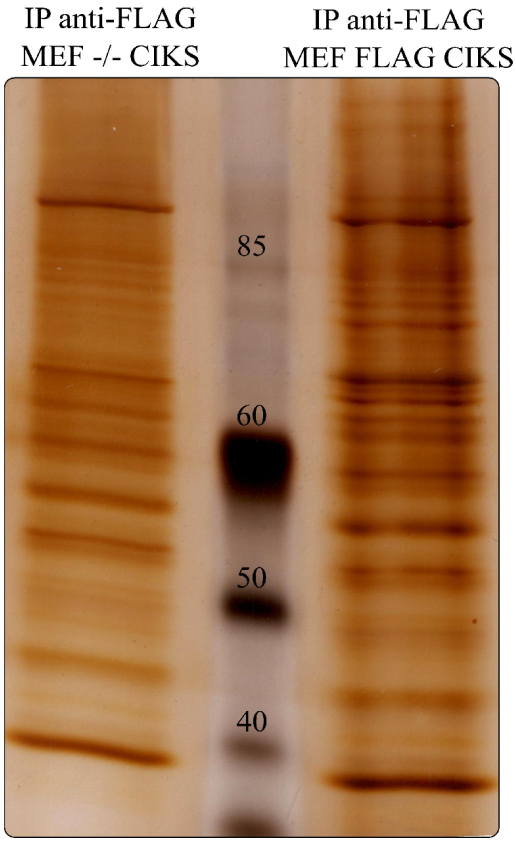

Figure 8: Immunoprecipitation of FLAG-CIKS followed by mass spectrometry analysis. Cell lysate were immunoprecipitated with anti-FLAG Ab and eluted wit 3xFLAG peptide. The eluate is used to SDS-PAGE followed by silver stain. The gel was sliced and sended fot mass spectrometry analysis.

We decided to investigate one of this interactor: DDX3X to confirm this interaction. DDX3X was cloned in pcDNA3.1 and transiently trasfected in HEK-293 cells. As shown in figure 9 DDX3X was co-immunoprecipitating with FLAG-CIKS.

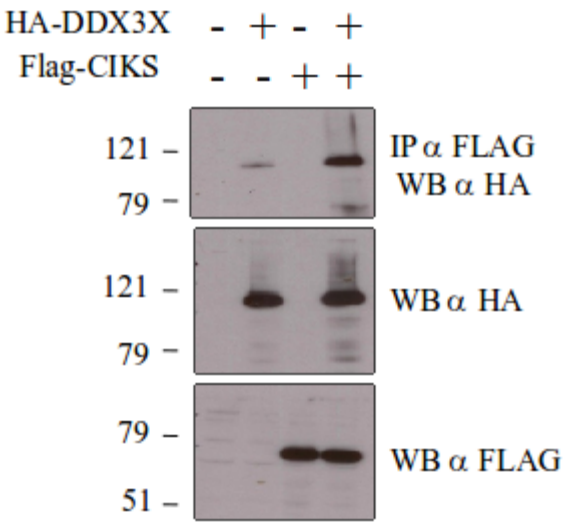

Figure 9: CIKS interact with DDX3X. HEK 293 were trasfected with expression plasmids for HA-tagged DDX3X and FLAG-tagged CIKS . The immunoprecipitates were analysed by Western blot with anti-HA (upper panel). Expression of CIKS and DDX3X was confirmed by western blot with anti-HA (middle panel) and anti-FLAG (lower panel) Abs, respectively.

Domain mapping of the interaction between CIKS and DDX3X.

To map the region of CIKS necessary to interact with DDX3X some deletion mutants of CIKS were used. We used CIKS full length, CIKS 1-400 lacking the SEFIR domain, CIKS 101-574 lacking the first 100 aminoacids containing the TRAF6 binding site, and CIKS ΔUb that lose the Ubox domain. A schematic representation of these constructs is illustrated in figure 10B. We trasfected these constructs in HEK-293 together with DDX3X, immunoprecipitated CIKS and looked for DDX3X. As shown in figure 10A both the N- and the C-terminal of CIKS are essential for the interaction with DDX3X because by immunoprecipitating FLAG-CIKS, the HA-DDX3X bands was revealed only when both N- and C-term were present.

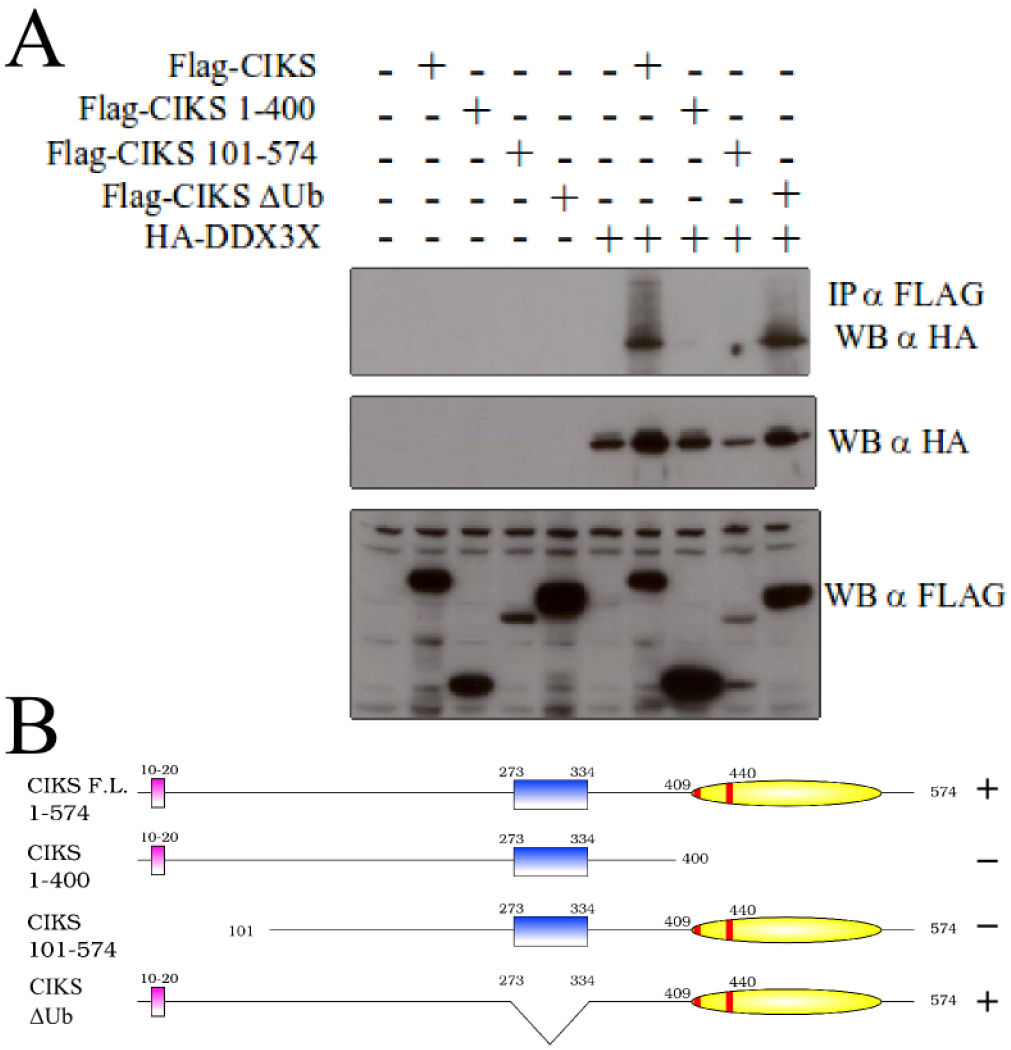

Figure 10: Mapping of CIKS interaction with DDX3X. HEK 293 were trasfected with expression plasmids for HA-tagged DDX3X and FLAG-tagged CIKS . The immunoprecipitates were analysed by Western blot with anti-HA (upper panel). Expression of CIKS and DDX3X was confirmed by western blot with anti-HA (middle panel) and anti-FLAG (lower panel) Abs, respectively. B, graphical rappresentation of DDX3X mutants.

Next, we investigated the region of DDX3X involved in the binding to CIKS. The deletion mutants of DDX3X are illustrated in figure 11B. Figure 11A shown a co-immunoprecipitation assay between FLAG-CIKS and HA-tagged DDX3X full length or bearing different mutations. Deletion of the Gly/Ser-rich domain at the C-terminus of DDX3X or deletion of the N-terminus of DDX3X did not affect the binding of DDX3X with CIKS. Deletion of the helicase domain disrupted the binding, thus suggesting that helicase core

domain is involved in CIKS interaction.

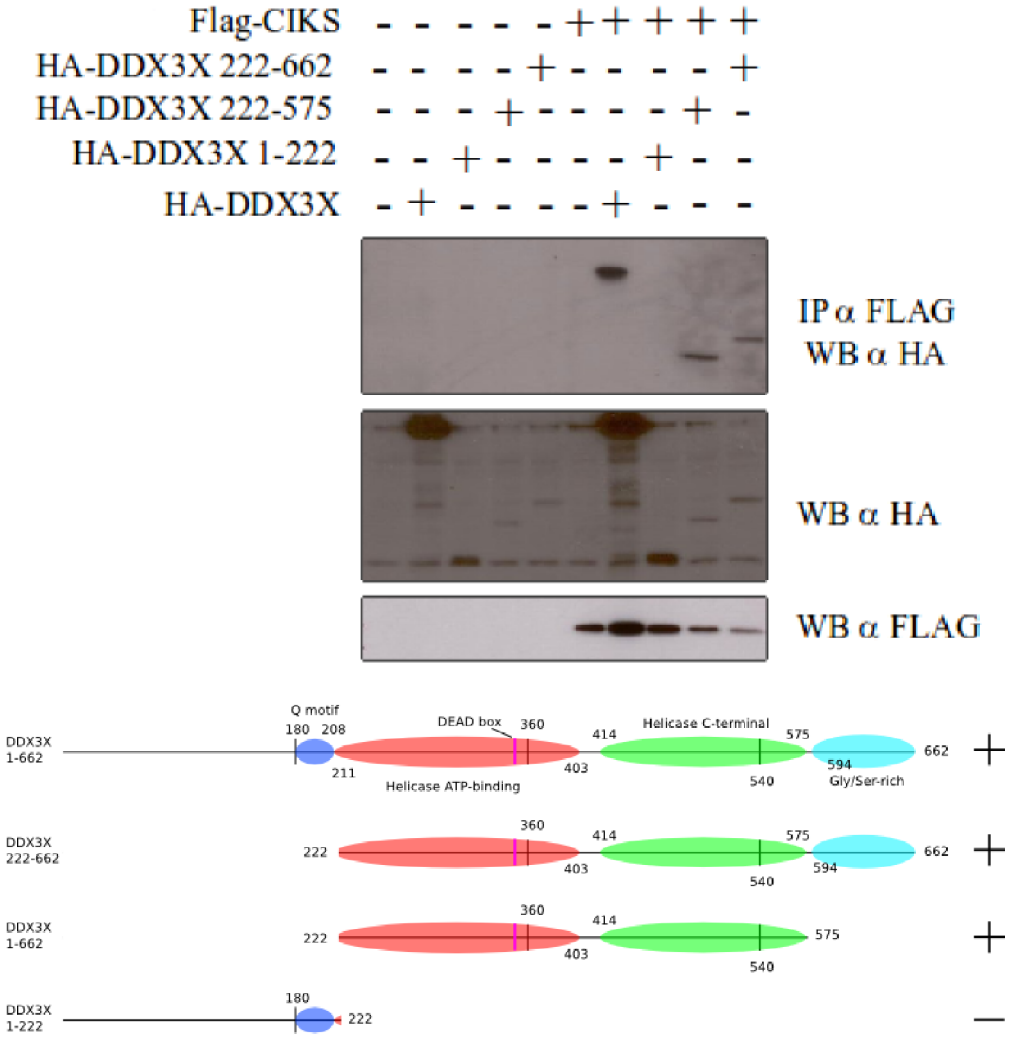

Figure 11: Mapping of DDX3X interaction with CIKS. HEK 293 were transfected with some expression plasmids for HA-tagged DDX3X (wt or DDX3X 222-662 that lose the N-terminal portion, or DDX3X 1-222 that lose the helicase core domain and the Gly/Ser rich domain at C-terminal domain or DDX3X 222-575 that lose the N-terminal portion and Gly/Ser rich domain.) and FLAG-tagged CIKS. The immunoprecipitates were analyzed by Western blot with anti-HA Ab (upper panel). Expression of CIKS and DDX3X was confirmed by western blot with anti-HA (middle panel) and anti-FLAG (lower panel) Abs, respectively. B, graphical representation of DDX3X mutants.

CIKS/DDX3X interaction is modulated by IL-17.

In order to detect the interaction between CIKS and endogenous DDX3X, we generated an antibody. We cloned the portion 1-222 of DDX3X in a bacterial expression vector, purified the protein by Nickel affinity chromatography and used it as antigen (Fig. 12A e B). Specifity of the antibody is illustrated in figure 12. We then investigated if the interaction between CIKS and DDX3X was dependent upon IL-17 stimulation. As shown in figure 13 treatment of reconstituted MEF with IL-17 for different time enhanced the interaction with endogenous DDX3X.

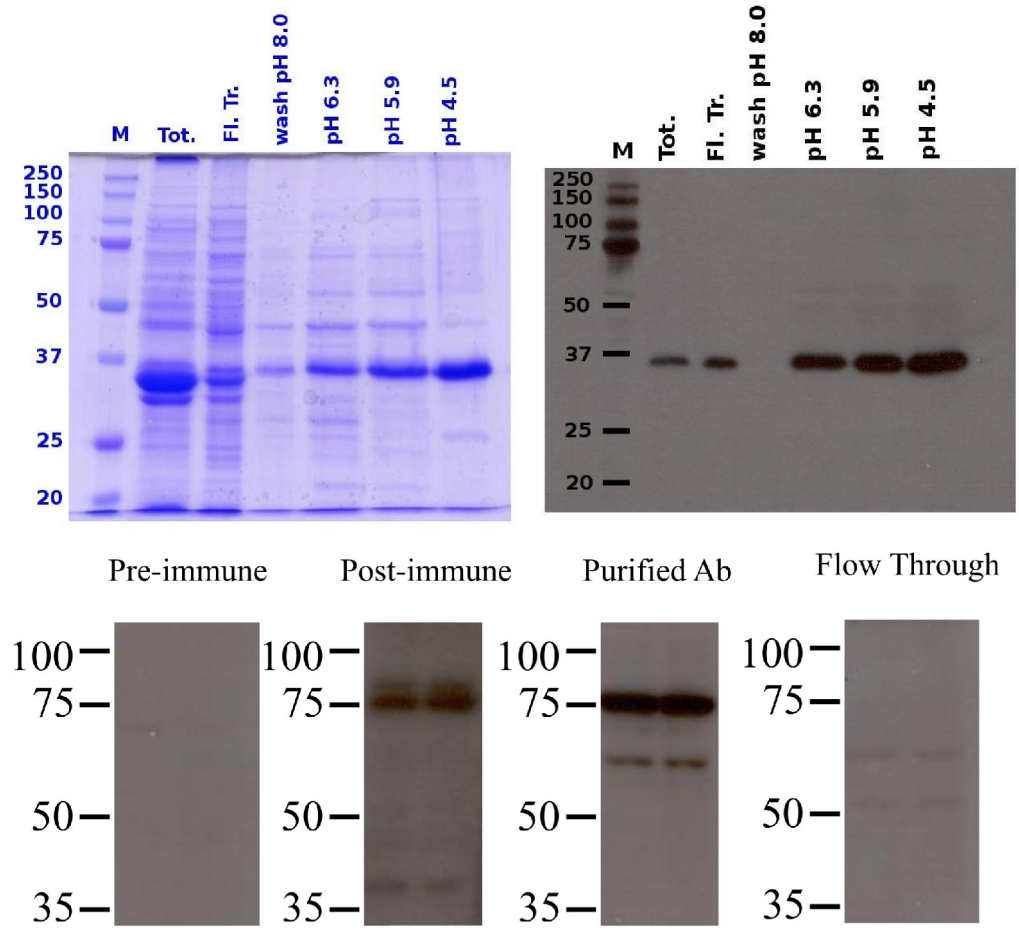

Figure 12: anti-DDX3X Ab production. A, SDS-PAGE with Blue Comassie stain loaded with molecular marker (M), total extract (Tot), and the seguent fraction by nikel resin purification: flow through (Fl. Tr.), wash pH8, elution pH 6,3, elution pH 5,9, elution pH 4,5. B, SDS-PAGE with western blot anti-His. C, D, western blot with pre-immune serum and immune serum from rabbit inoculated with recombinant DDX3X respectively. E,F, purified Ab after affinity cromatography with His-DDX3X and the flow through.

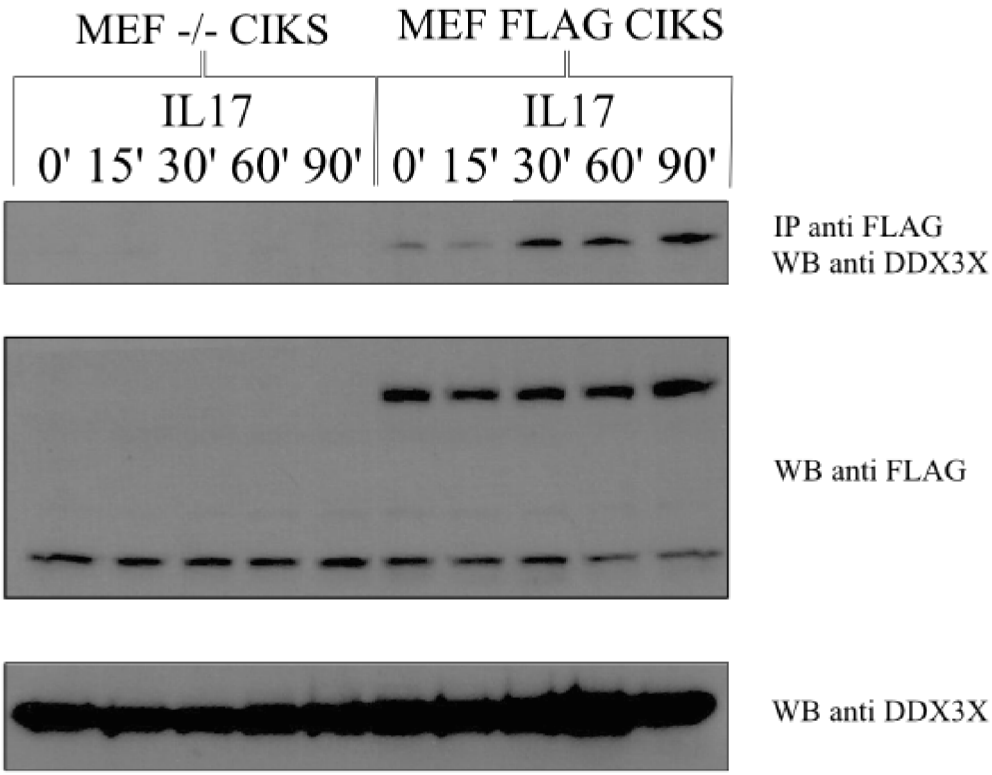

Figure 13: IL-17 stimulation increase CIKS-DDX3X binding. CIKS$^{-/-}$ MEF and MEF FLAG CIKS were stimulated with IL-17 (200 ng/ml) for the indicated time. The immunoprecipitates were analyzed by Western blot with anti-DDX3X (upper panel). Expression of FLAG-CIKS and DDX3X was confirmed by western blot with anti-HA (middle panel) and anti-FLAG (lower panel) Abs, respectively.

To further confirm the DDX3X was involved in IL-17 signaling we treated reconstituted MEF with IL-17, separated the cell extract by gel filtration chromatography and analysed the fractions by Western blot. The figure 14 shows that following IL-17 stimulation, endogenous DDX3X was eluted from the column in the same fraction containing CIKS, further suggesting the formation of complex between the two proteins after IL-17 stimulation.

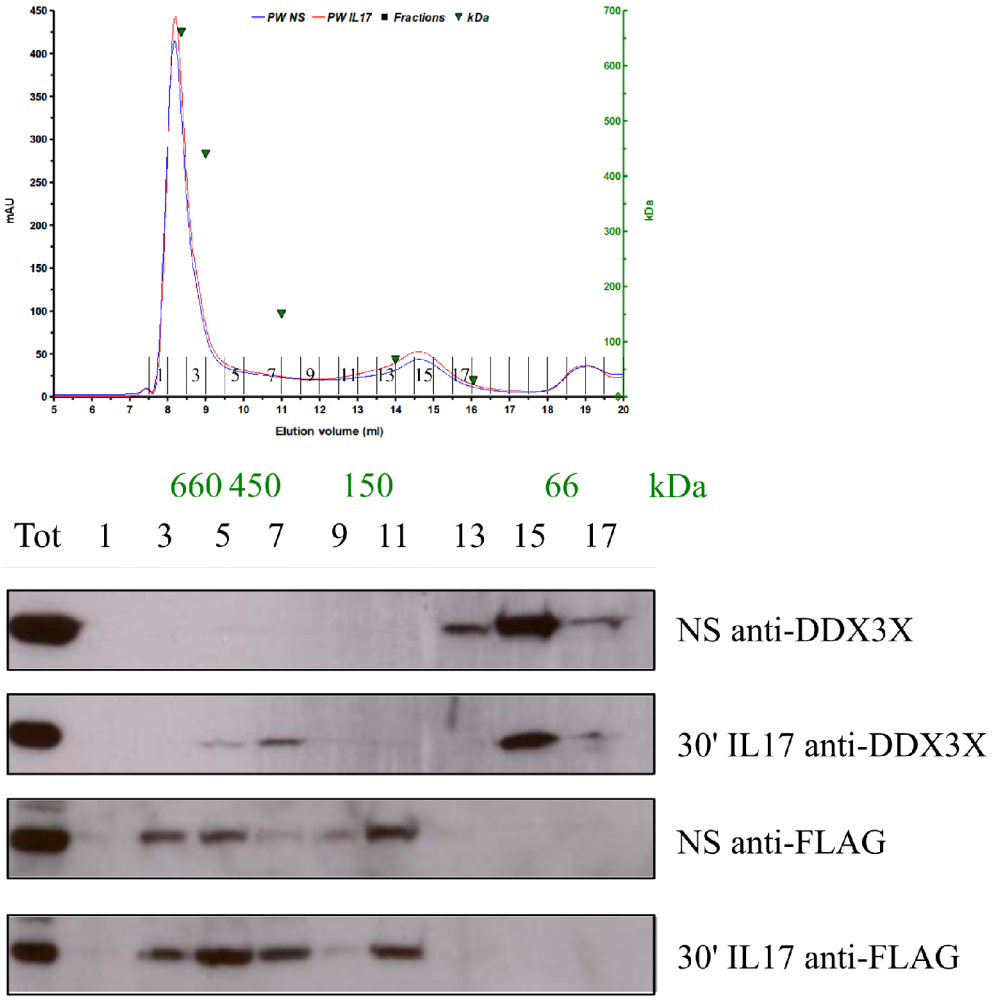

Figure 14: Follow IL-17 stimulation DDX3X moves to high molecular weight complex. MEF FLAG CIKS were not stimulated (NS) or stimulated for 30 minutes with IL-17, and cell lysate was separate by Gel Filtration. A, chromatogram of protein separation by chromatography of MEF FLAG CIKS NS and stimulated with IL-17, with molecular weight. B, The fractions results by chromatography were loaded on SDS-PAGE and analyzed with anti-DDX3X antibody (first and second panel) or anti-FLAG (third and four panel).

DDX3X control Zc3h12a mRNA half-life.

Because CIKS is essential for IL-17 mediated biological responses we sought to investigate if DDX3X was involved with some of the IL-17 mediated functions, such as gene expression and mRNA stability.

No difference were observed by DDX3X over-expression in terms of NF-κB activation (Fig.15)

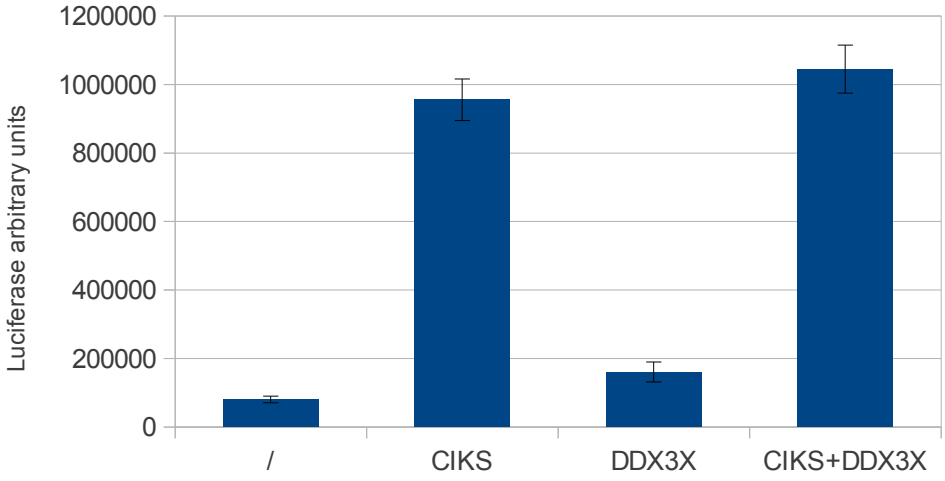

Figure 15: DDX3X is unable to activate NF-κB. Relative reporter activity was evaluated in HEK-293 cells co-transfected with the Ig-κB-luciferase plasmid and the indicated expression vectors.

We knocked down DDX3X expression in MEF by using siRNA (fig. 16) and in these cells we evaluated the expression of different IL-17-regulated genes.

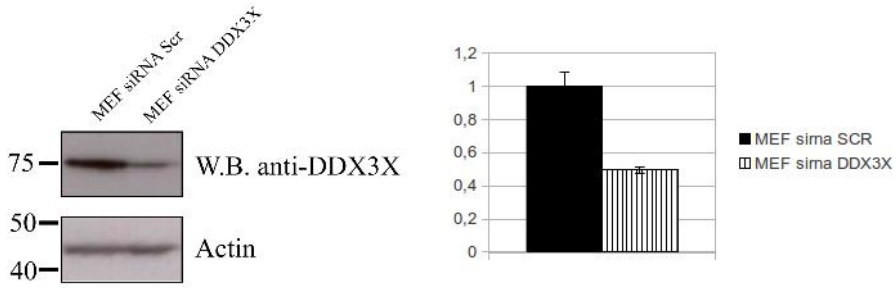

Figure 16: sirna against DDX3X. A, western blot with MEF FLAG CIKS treated with siRNA scramble and siRNA DDX3X specific. B, real time analysis to evaluate the mRNA levels of MEF FLAG CIKS treated with siRNA scramble and siRNA DDX3X specific.

Next, we investigated the ability of DDX3X to mediate the IL-17-induced stabilization of the some short-lived mRNA induced by TNFα. We treated CIKS$^{-/-}$, reconstituted, and DDX3X KD MEF with TNFα for 60 minutes to induce transcription of target genes. Then we treated cells with Actinomycin D (to block de novo transcription) and IL-17 to induce mRNA stabilization. After 90 minutes cells were harvested, RNA extracted, and analyzed by Real Time PCR. As shown in figure 17 the half-life of z*c3h12a* was significantly decreased in DDX3X KD cells, compared to MEF treated with a scrambled siRNA. The ability of DDX3X to stabilize mRNA was selective for *zc3h12a*, as the half life of other mRNA known to be stabilized by IL-17, including *cxcl1*, *cxcl5*, *LCN2* and *IL6* was not affected in the absence of DDX3X.

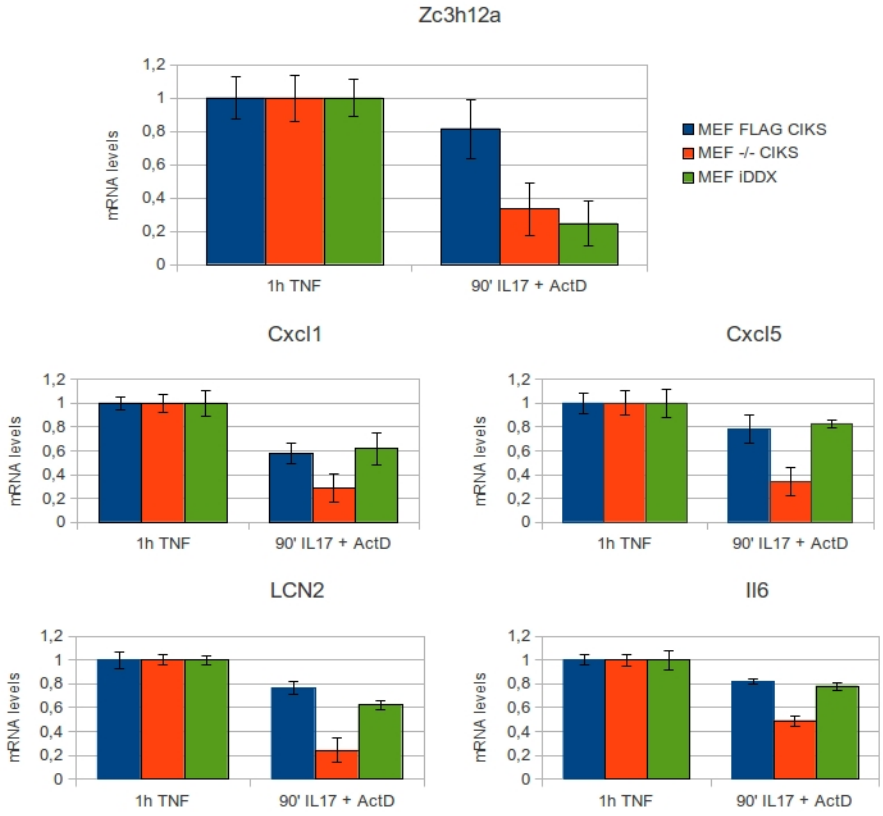

Figure 17: DDX3X control zc3h12a mRNA half-life. zc3h12a, cxcl1, cxcl5, lcn2 and il6 mRNA levels in MEF FLAG CIKS, CIKS$^{-/-}$ MEF and MEF sirna DDX treated with TNFα for 1 hour and the same cells after IL-17 and actinomycin D to block mRNA neo-syntesis.

To further demonstrate that the half-life of *zc3h12a* mRNA depended on DDX3X I performed a time-course with IL-17/ActD treatment, and the difference between *zc3h12a* mRNA levels in MEF FLAG CIKS and MEF siRNA DDX was already significant after 60 minutes of treatment (Fig. 18). Cxcl1 was used as negative control (Fig. 18). In this experiment I used cxcl1 as negative control, because its mRNA level is CIKS-dependent, but not DDX3X-dependent.

These results suggest that DDX3X is involved in the stabilization of selected mRNA after IL-17 treatment.

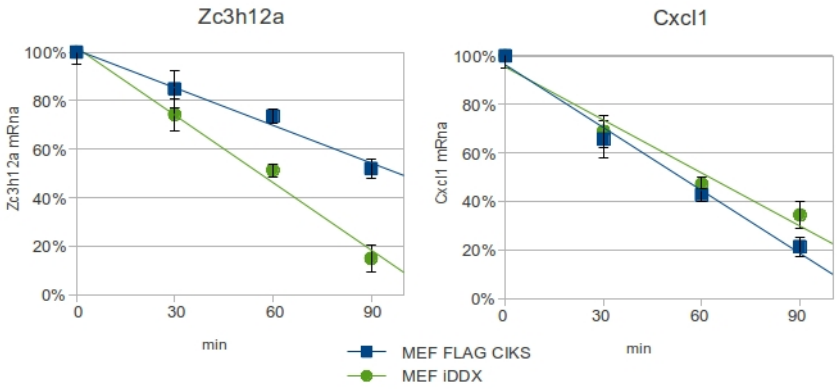

Figure 18: Zc3h12a mRNA is stabilized just then 60 minutes. Time corse of MEF FLAG CIKS and MEF sirna DDX3X treated with TNFα and IL-17/actinomycin D to induce mRNA stabilization and block the trascription. Cxcl1 was used such as control.

IKKε interacts with DDX3X and is required for mRNA stabilization

In order to gain insight into the mechanism used by DDX3X to stabilize *zc3h12a*, we investigated if IKKε, that has already been shown to be involved in this function was interacting with DDX3X. We transected HEK293 with the indicated expression vector (Fig 19), immunoprecipitated Myc-tagged IKKε and looked for co-immunoprecipitating DDX3X. As shown in figure 19 IKKε was interacting with DDX3X, and this interaction did not depend upon the presence of CIKS. Of note, in the presence of IKKε different form of DDX3X with apparent decreased relative mobility, appeared. The relative mobility of these forms of DDX3X were increased by treatment with phosphatase (data not shown), suggesting that DDX3X is phosphorylated by IKKε.

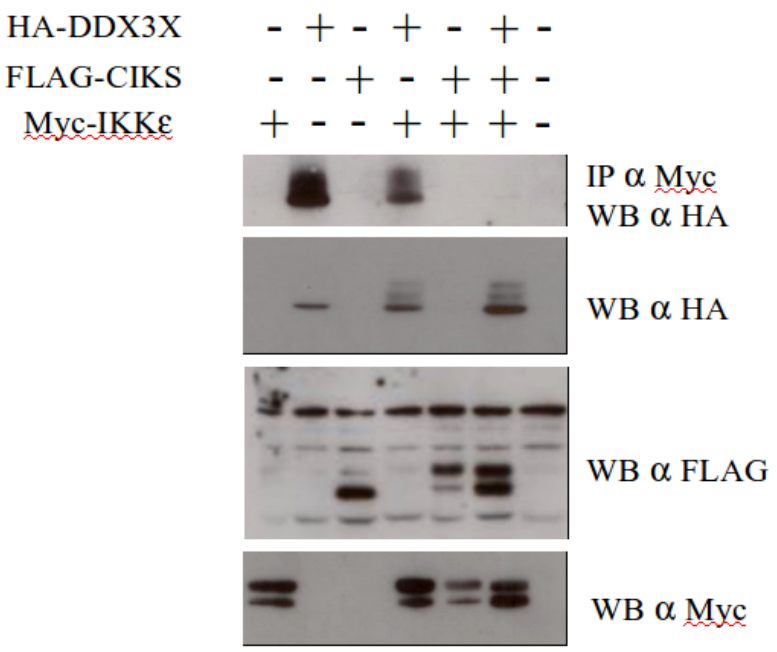

Figure 19:IKKε interact with DDX3X. HEK 293 were transfected with some expression plasmids for HA-tagged DDX3X, FLAG-tagged CIKS and MYC-tagged IKKε. The immunoprecipitates with anti-IKKε Ab were analyzed by Western blot with anti-HA Ab (first panel). Expression of DDX3X, CIKS and IKKε was confirmed by western blot with anti-HA (second panel), anti-FLAG (third panel) and anti-Myc (fourth panel) Abs, respectively.

Next, we knocked down expression of IKKε in MEF (Fig. 20) and evaluated the stability of *zc3h12a* mRNA. Cells were treated with TNFα for 60 minutes, and then treated with Actinomycin D plus IL-17. As shown in figure 20 the half life of *zc3h12a* was significantly decreased in IKKε knock-down cells, compared to MEFs treated with a scrambled siRNA, thus suggesting that the IL-17-induced *zc3h12a* stabilization required IKKε.

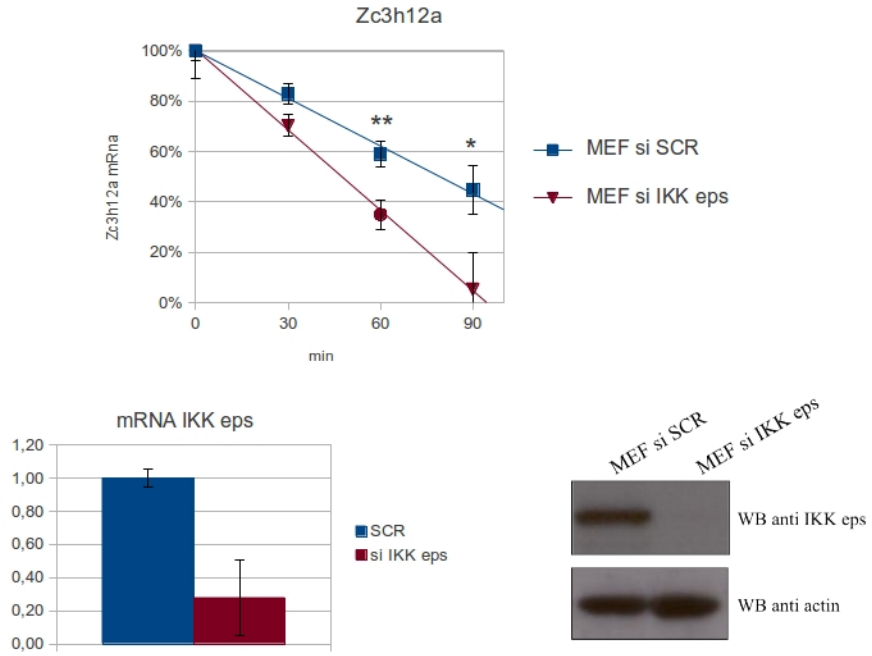

Figure 20: IKKε is necessary to zc3h12a mRNA stabilization. A, abundance of zc3h12a mRNA by Real Time PCR Analysis (normalized to that of GAPDH mRNA), presented as decay over time, of MEF FLAG CIKS, MEF si IKKε treated with TNFα/IL-17/ActD at different time. B, C, IKKε levels in MEF FLAG CIKS treated with siRNA screamble and sirna specific to IKKε by Real Time PCR analysis and western blot analysis respectively.

DDX3X is in TRAF6-independent pathway.

Next we investigated what domain of CIKS was necessary to stabilize *zc3h12a.* We reconstituted CIKS$^{-/-}$ with E17A, unable to bind TRAF6. The results (Fig. 21) indicated that TRAF6 is not involved in this process since MEF CIKS E17A and MEF FLAG CIKS had the same *zc3h12a* mRNA level.

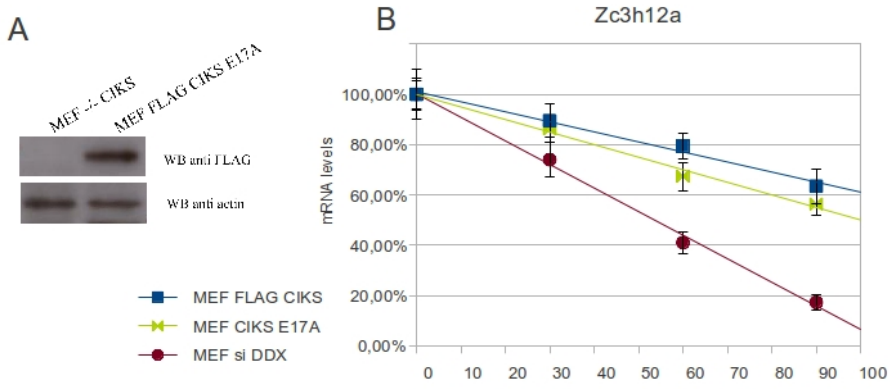

Figure 21: Zc3h12a mRNA stabilization is TRAF6-indipendent. A, Western blot with cell lysate from CIKS$^{-/-}$ MEF and MEF reconstituted with FLAG-CIKS E17A with anti-FLAG Ab (upper panel) and anti-actin (lower panel). B, abundance of zc3h12a mRNA by Real Time PCR Analysis (normalized to that of GAPDH mRNA), presented as decay over time, of MEF FLAG CIKS, MEF CIKS E17A and MEF si DDX3X treated with TNFα/IL-17/ActD at different time.

We investigated whether CIKS U-box domain was required for *zc3h12a* mRNA stabilization. We reconstituted CIKS$^{-/-}$ MEF with CIKS ΔUbox, a deletion mutant lacking the U-box domain. In figure 22 is shown that the U-box was not required for *zc3h12a* mRNA stabilization.

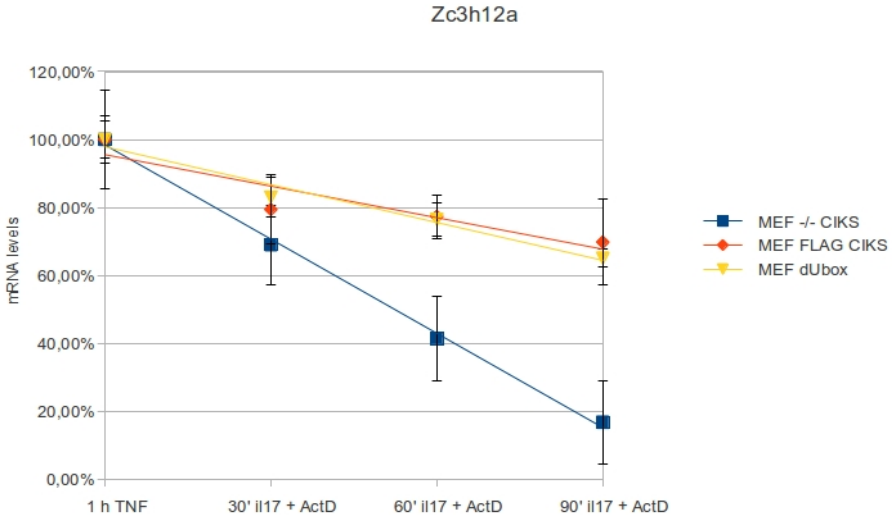

Figure 22: CIKS U-box domain is dispensable for zc3h12a mRNA stabilization. Abundance of zc3h12a mRNA by Real Time PCR Analysis (normalized to that of GAPDH mRNA), presented as decay over time, of MEF FLAG CIKS, MEF CIKS dUbox and CIKS$^{-/-}$ MEF treated with TNFα/IL-17/ActD at different time.

TRAF2 and TRAF5 are required for Zc3h12a mRNA stabilization

TRAF2 and TRAF5 are two proteins involved in IL-17-dependent mRNA stabilization. To study the involvement of these two proteins in mediating the stabilization of *zc3h12a* induced by DDX3X, we down-regulated TRAF2 and TRAF5 by siRNA transfection, and evaluated the levels of *zc3h12a* mRNA levels. Figure 23 shows that both TRAF2 and TRAF5 was involved in *zc3h12a* stabilization. In fact knocking down TRAF2 or TRAF5 partially affected *zc3h12a* levels, while knocking down both proteins the *zc3h12a* stabilization was impaired (Fig. 23).

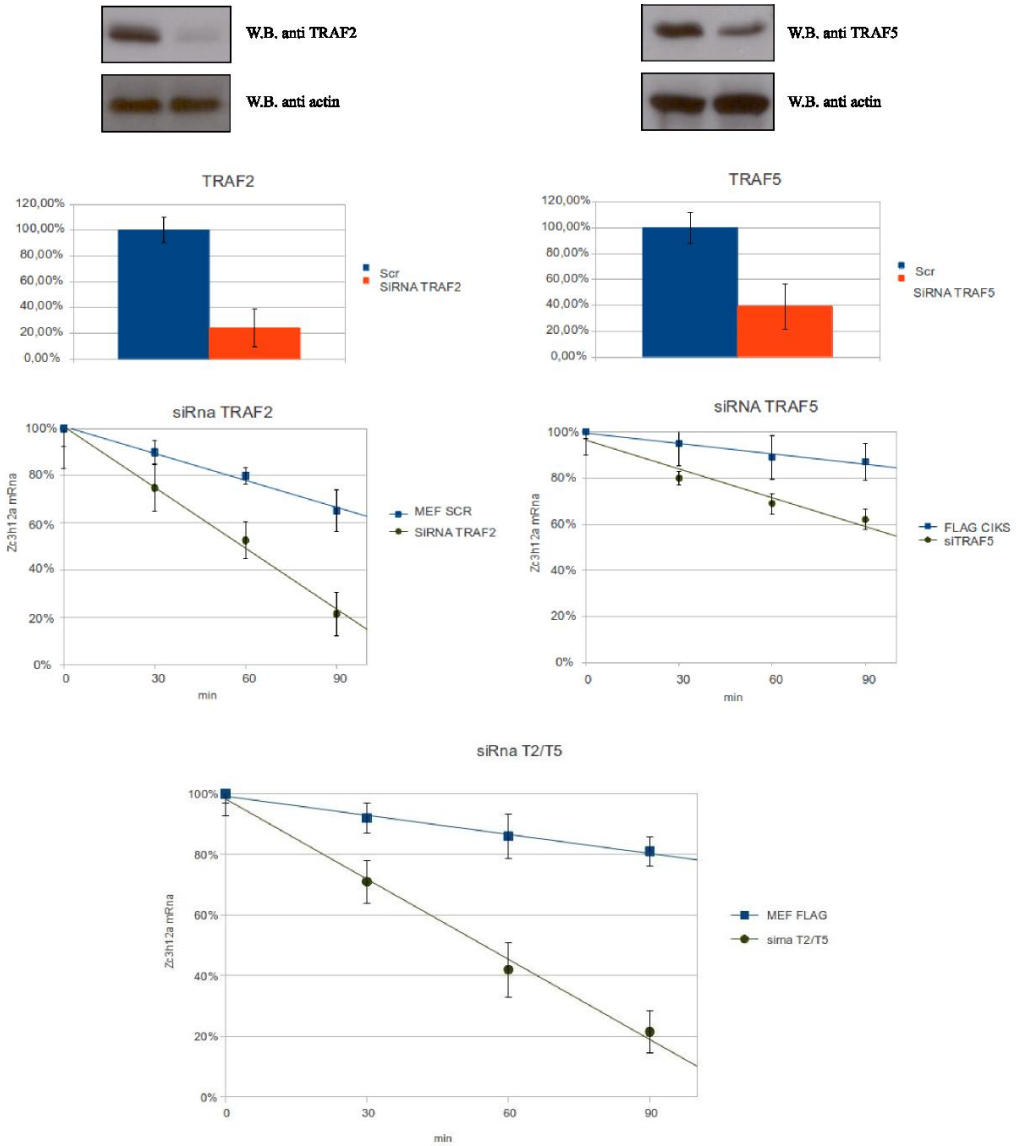

Figure 23: TRAF2/5 are necessary for zc3h12a mRNA. A-D TRAF2 and TRAF5 levels in MEF FLAG CIKS treated with siRNA screamble and sirna specific to TRAF2/5 by Real Time PCR analysis and western blot analysis. E-G, abundance of zc3h12a mRNA by Real Time PCR Analysis (normalized to that of GAPDH mRNA), presented as decay over time, of MEF FLAG CIKS, MEF siRNA TRAF2 alone, sirna TRAF5 alone or both, treated with TNFα/IL-17/ActD at different time.

DDX3X bind directly Zc3h12a mRNA

To demonstrate whether DDX3X binds directly *zc3h12a* mRNA, we ectopically expressed DDX3X in HeLa cells. Cells were treated with TNF plus IL17, cells were lysed, DDX3X was immunoprecipitated by using anti-FLAG antibody, and the presence of the *zc3h12a* mRNA in the immunoprecipitate was detected by PCR. As shown in figure 24 it was possible to amplify the *zc3h12a* mRNA in the DDX3X immunoprecipitate after IL17 stimulation. thus proving that DDX3X is directly binding *zc3h12a* after IL-17 stimulation.

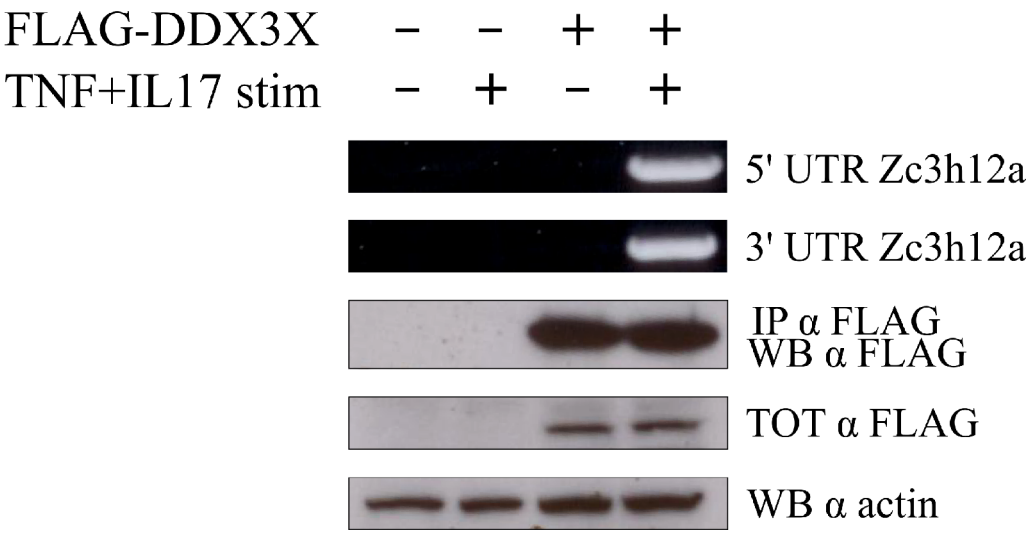

Figure 24: DDX3X binds directly zc3h12a mRNA follow IL-17 stimulation. HeLa cells were transfected with FLAG-DDX3X and stimulated with TNFα and IL-17. After immunoprecipitation anti-FLAG we extract the RNA DDX3X-binding and underwent to PCR analysis to reveal zc3h12a mRNA. In first and second panel there are the results of PCR to 5' and 3' UTRs of zc3h12a. In third there is a western blot analysis

DISCUSSION

In the present manuscript we present evidence that the helicase family member DDX3X is part of the mechanism regulating mRNA stability induced by IL-17. We demonstrate that DDX3X is interacting with CIKS, an essential component of the IL-17 signaling pathway, and that this interaction is dependent upon IL-17 stimulation. The functional consequence of this interaction is the stabilization of selected mRNA. In fact, DDX3X selectively enhance the half life of *zc3h12a* mRNA by directly binding the mRNA in an IL-17-dependent manner, possibly protecting it from degradation. The DEAD-box RNA helicase DDX3X is a multifunctional protein, which has been implicated in different cellular processes linked to RNA metabolism and gene expression. Despite its involvement in almost every step of mRNA metabolism, this is the first evidence linking DDX3X to the stabilization of selected mRNA following IL-17 stimulation. DDX3X has also been demonstrated to be required for translation of selected mRNA, by directly binding the 5' end of such RNAs and facilitating the recruitment of eIF4F complex to start translation. It is then possible that binding of DDX3X to the *zc3h12a* is important to modulate both the stability of the *zc3h12a* mRNA and its translation. How DDX3X is increasing the half life of *zc3h12a* is not currently clear. The simplest explanation is that after receptor triggering DDX3X binds the mRNA displacing other factors controlling the mRNA decay, and recruits the translational machinery to start translation.

DDX3X has also been involved in viral sensing pathway at different levels: by enhancing the IPS-1 function; by recruiting TBK1 and IKKε; by directly binding the IFN-β promoter, and by directly binding viral RNA. These results suggest that DDX3X may be an important component of the innate immunity, and that it may use the adaptor protein CIKS to signal the interferon response. However, at least in our experimental system, we have not been able to detect an interaction between CIKS and DDX3X after stimulation with substances mimicking viral nucleic acids such as poly(A:T), poly(C:G), poly(I:C). In addition, we have not detected a statistically significant variation in IFN-β production (measured by ELISA), and IRF3/7 phosphorylation in CIKS -/- MEF treated with poly(A:T), poly(C:G), poly(I:C). It is possible that DDX3X may function in various signaling pathways regulating different immune responses, such as mRNA stability in the IL-17 pathway, and type I interferon production in the innate immune system.

We demonstrated that the DDX3X-mediated stabilization of *zc3h12a* mRNA requires IKKε, TRAF2 and TRAF5. This finding was not unexpected given that the stabilization of other mRNA, such as *CXCL1*, has also been demonstrated to be mediated by TRAF2, TRAF5 and IKKε. Notably, TRAF2 and TRAF5 seems to provide redundant functions in the IL-17 pathway, as

mRNA stability was completely abrogated only in double deficient cells. The functional redundancy of TRAF2 and TRAF5 is not restricted to the IL-17 pathway, as also in other signaling pathways, such as TNF, the two proteins may compensate for the absence of the other. Indeed, the phenotype of the TRAF2/TRAF5 double KO mice is way more complex that the phenotype of the single KO. However, in our experimental system, TRAF2 knock-down was more effective in blocking the IL-17 mediated stabilization of *zc3h12a*, which suggests that TRAF2 is the more physiological regulator. It remains to be determined if after IL-17 stimulation DDX3X forms a complex with TRAF2 and TRAF5. This hypothesis is currently under investigation.

We also present evidence that a complex composed of CIKS, IKKε and DDX3X is formed after receptor triggering. Notably, in the presence of IKKε, DDX3X seems to be phosphorylated by IKKε, suggesting that this effect may be important to regulate DDX3X function. It has been recently reported that IKKε phosphorylates DDX3X and that this phosphorylation modulated the interaction between DDX3X and IRF3. It is possible that also in the IL-17 pathway the phosphorylation of DDX3X affects DDX3X function, or its ability to interact with CIKS. However, we did not detect difference in the amount of CIKS immunoprecipitating with DDX3X in the presence or in the absence of IKKe. We are currently investigating if the ability of DDX3X to bind the *zc3h12a* is affected by IKKε. It has been demonstrated that IKKε phosphorylates CIKS and that this event is required for the formation of the CIKS-TRAF2-TRAF5 complex mediating mRNA stability, while the formation of the CIKS-TRAF6 complex, regulating activation of NF-κB, is not affected. Our data confirm and expand this observation, as also the DDX3X mediated mRNA stabilization, relies on a similar mechanism, independent on NF-κB activation. In fact, in $CIKS^{-/-}$ MEF reconstituted with the CIKS mutant E17A unable to bind TRAF6 and to activate NF-κB, the DDX3X mediated mRNA stabilization is not affected. Similarly, also in $CIKS^{-/-}$ MEF reconstituted with a CIKS mutant lacking the Ubox domain (unable to activate NF-κB), the mRNA stability is retained. It is then possible to speculate that the CIKS-TRAF2-TRAF5 axis activates different effector mechanisms, each regulating the stability of selected mRNA.

In summary, we have identified a previously not reported association between CIKS and DDX3X, and we provide evidence that DDX3X mediates the stability of selected mRNA induced by IL-17. .

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Appendix: Original Papers

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NGAL Controls the Metastatic Potential of Anaplastic Thyroid Carcinoma Cells

Vincenzo Volpe, Zelinda Raia, Luca Sanguigno, Domenico Somma, Paola Mastrovito, Fortunato Moscato, Stefano Mellone, Antonio Leonardi, and Francesco Pacifico

Dipartimento di Biologia e Patologia Cellulare e Molecolare (V.V., Z.R., L.S., D.S., P.M., A.L.), "Federico II" University of Naples, and Istituto di Endocrinologia ed Oncologia Sperimentale (F.M., S.M., F.P.), Consiglio Nazionale delle Ricerche, 80131 Naples, Italy

Context: We have previously identified neutrophil gelatinase-associated lipocalin (NGAL) as one of the genes mediating the oncogenic activity of nuclear factor-κB in human anaplastic thyroid carcinomas (ATCs).

Objectives: To further investigate the role of NGAL in thyroid cancer, we established NGAL knocked-down and NGAL overexpressing ATC cell lines.

Results: We found that the ability of NGAL knocked-down cells to degrade Matrigel in a transwell invasion assay and to form lung metastasis in nude mice was decreased. Because NGAL binds matrix metalloproteinase-9 (MMP-9), to form a macromolecular complex involved in the regulation of metastatic spread of cancer cells and given the strong expression of both genes in tissue specimens from human ATCs, we analyzed the MMP-9 enzymatic activity in NGAL-null ATC cells. Enzymatic immunoassays show that MMP-9 activity is reduced in NGAL-null ATC cells, even if its expression is not affected by NGAL inhibition. Ectopic expression of NGAL in an ATC cell line not expressing NGAL determines an increase of its metastatic property. The use of a mutated form of NGAL, unable to bind MMP-9, has no positive effect on the invasive potential of ATC cells and does not improve the MMP-9 enzymatic activity.

Conclusions: Our results indicate NGAL as a novel target of nuclear factor-κB prometastatic activity in thyroid cancer through enhancement of MMP-9 enzymatic activity. **(*J Clin Endocrinol Metab* 98: 0000–0000, 2013)**

One of the most problematic aspects of cancer is represented by metastatic disease that is largely incurable because of its systemic nature and the increased resistance of metastatic cells to chemo- or radiotherapy. In the vast majority of cases, the mortality from cancer is due to metastases, not to the primary tumor (1, 2). Therefore, a great effort has been attempting to understand the molecular mechanisms underlying the metastatic process to identify novel targets for more effective therapeutic intervention. Metastasis development is a very complex process which requires a number of coordinated steps to successfully promote the onset of secondary neoplastic lesions at organs distant from primary tumor. The initial event of metastatic cascade is represented by local invasion of primary tumor cells to adjacent tissues. This process is achieved by cancer cells via degradation of basement membrane, a specialized extracellular matrix that plays vital roles in organizing epithelial tissues. The degradation of basement membrane occurs by proteolysis of extracellular matrix components mediated by the activity of a family of zinc and calcium dependent endopeptidases collectively termed matrix metalloproteinases (MMPs) (3). One of the most important metalloproteinases involved in the invasion process is MMP-9, a 92-kDa protease able to

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Abbreviations: ATC, Anaplastic thyroid carcinoma; HA, hemagglutinin; HEK, human embryonic kidney; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase-associated lipocalin; NF-κB, nuclear factor-κB; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; TBST, buffer of Tris-HCl, Tween 20, and NaCl.

degrade a broad range of substrates including collagens, fibronectins, and laminins (4). MMP-9 is highly expressed in many human tumors, especially of more aggressive phenotype, and its levels generally correlate with a poor prognosis (5–8). Its enzymatic activity is, at least partially, regulated by neutrophil gelatinase-associated lipocalin (NGAL), via the formation of a macromolecular complex that allows MMP-9 to preserve its proteolytic activity (9).

We have previously demonstrated that nuclear factor-κB (NF-κB) is strongly activated in human thyroid cancer in which it plays an important role, given that the inhibition of its activity in anaplastic thyroid carcinoma (ATC) cells blocks their oncogenic potential (10). In the effort to identify target genes of NF-κB activity in thyroid cancer, we found that NGAL is able to recapitulate some of protumorigenic functions of NF-κB in these tumors, such as the ability to protect ATC cells from growth factors deprivation-induced apoptosis (11). NGAL, also known as lipocalin-2, is a member of the large family of lipocalins, a group of small extracellular proteins with great functional diversity (12). It is overexpressed in a number of human tumors, including breast (13), lung (14), colon (15), ovary (16), and pancreas (17) carcinomas. Several studies have demonstrated that NGAL has protumorigenic activity that occurs through the enhancement of neoplastic cell survival and proliferation, very likely due to its iron-carrier function. In addition, NGAL contributes to cancer by promoting the metastatic potential of tumor cells because of its ability to protect MMP-9 from autodegradation (9, 18).

We found a strong coexpression of both NGAL and MMP-9 in tissue specimens from primary human ATCs so that we decided to investigate the role of NGAL in the ATC metastatic process by modulating its expression in two ATC cell lines, BHT101 and Act1.

Materials and Methods

Cell culture and biological reagents

BHT101 and Act1 cells were kindly provided by Professor M. Santoro, "Federico II" University of Naples. To knock down NGAL expression, BHT101 cells were infected with pLL 3.7 lentiviral vector containing double-stranded oligonucleotides sequences derived from the human NGAL in forward and reverse orientation, separated by a 7-bp spacer region (caagaga) to allow the formation of the hairpin structure in the expressed small interfering RNAs (siRNAs): NGAL sc-siRNA, sense strand, 5′-CCATCTATGAGCTGAAAGAcaagagaTCTTTCAGCTCATAGATGG-3′, antisense strand, 5′-CCATCTATGAGCTGAAAGAtctcttgTCTTTCAGCTCATAGATGG-3′; NGAL siRNA1, sense strand, 5′-GGGAATGCAATTCTCAGAGTTcaagagaAACTCTGAGAATTGCATTCCC-3′, antisense strand, 5′-GGGAATGCAATTCTCAGAGTTctcttgAACTCTGAGAATTGCATTCCC-3′; NGAL siRNA2, sense strand, 5′-GGACTTTTGTTCCAGGTTGTTcaagagaAACAACCTGGAACAAAAGTCC-3′, antisense strand, 5′-GGACTTTTGTTCCAGGTTGTTctcttgAACAACCTGGAACAAAAGTCC-3′. Act1 cells were infected with pLENTI CMV GFP-2A-PURO vector (ABM, Richmond, British Columbia, Canada) alone or containing the cDNA coding for human wild-type or mutated NGAL fused in frame with the hemagglutinin (HA) tag. All cell lines were grown in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma). Anti-HA (sc-805 and sc-7392) and antiactin (sc-8432) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-FLAG (F7425 and F3165) was from Sigma, and anti-NGAL (AF1757) was from R&D Systems (Minneapolis, MN).

Patients

The study has been approved by the local ethics committees. Informed consent of the patients had been obtained before their inclusion in the study. Five cases of surgically resected ATCs and two specimens of the normal controlateral thyroid lobe from surgically resected benign adenomas were analyzed for their ability to express NGAL and MMP-9 mRNAs. The clinicopathological characteristics of the ATC selected cases are shown in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

mRNA quantification by real-time RT-PCR

Real-time RT-PCR was carried out with cDNAs reverse transcribed from total RNA by using GoTaq qPCR master mix (Promega, Madison, WI) and Bio-Rad CFX Manager software (Bio-Rad Laboratories, Segrate, Milan, Italy), according to manufacturers' procedure. The primers used were: glyceraldehyde-3-phosphate dehydrogenase, forward, 5′-AAACAGAAGGCAGCTTTACGATG-3′, reverse, 5′-AAATGTTCTGATCCAGTAGCG-3′; NGAL, forward, 5′-GAAGACAAAGACCCGCAAAAG-3′, reverse, 5′-CTGGCAACCTGGAACAAAAG-3′; and MMP-9, forward, 5′-CGAACTTTGACAGCGACAAG-3′, reverse, 5′-CACTGAGGAA TGATCTAAGCCC-3′.

Immunoprecipitation and Western blots

For immunoprecipitation of transfected proteins, human embryonic kidney (HEK) 293 cells (3×10^6) were transiently transfected by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and 24 h after transfection, cells were lysed in Triton X-100 lysis buffer (20 mM HEPES, pH 7.4; 150 mM NaCl; 10% glycerol; 1% Triton X-100; and Complete protease inhibitor mixture). After an additional 15 min on ice, cell extracts were centrifuged for 10 min at 14,000 × *g* at 4 C, and supernatants were incubated for 4 h at 4 C with anti-HA antibodies bound to agarose beads (Sigma). The immunoprecipitates were washed five times with Triton X-100 lysis buffer and subjected to SDS-PAGE.

For Western blots, 20 μg of total proteins from cell lysates or supernatants was analyzed by 10% SDS-PAGE and blotted onto nitrocellulose membrane (Schleicher & Schuell, Whatman GmbH, Dassel, Germany). Filters were blocked for 1 h 30 min at room temperature with 5% nonfat dry milk in TBST buffer [10 mM Tris-HCl (pH 8), 0.1% Tween 20, 150 mM NaCl] and incubated with 1:2000 dilution of anti-HA, anti-FLAG, anti-NGAL, or antiactin antibodies for 1 h 30 min. After TBST washing, blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (NXA931; Amersham Biosciences, Buckinghamshire, UK) diluted 1:5000 in

TBST buffer and then revealed by enhanced chemiluminescence (Amersham Biosciences).

Matrigel invasion assays

Cell invasion was examined using a reconstituted extracellular matrix (Matrigel; BD Biosciences, Bedford, MA). Polycarbonate membranes (8 mm pore size) on the bottoms of the upper compartment of the transwells (6.5 mm; Costar, Cambridge, MA) were coated with 1.2 mg/ml Matrigel. The 1×10^5 cells in 100 μl of serum-free Opti-MEM I (Invitrogen), alone or added with recombinant NGAL (11), were placed on the Matrigel-coated polycarbonate membrane in the upper compartment, whereas 600 μl of Opti-MEM I supplemented with 10% fetal bovine serum was added to the lower compartment. The plates were incubated at 37 C in a 5% CO_2 atmosphere saturated with H_2O for 24 h. At the end of incubation, the cells and Matrigel at the upper side of the polycarbonate filter were mechanically removed. Cells that had invaded the Matrigel and migrated to the lower side of the filter were fixed with 11% glutheraldeyde for 15 min at room temperature, washed three times with PBS, and stained with 0.1% crystal violet-20% methanol for 20 min at room temperature. After three PBS washes and complete drying at room temperature, the crystal violet was solubilized by immerging the filters in 300 μl of 10% acetic acid. The concentration of the solubilized crystal violet was evaluated as absorbance at 590 nm. Experiments were performed at least in triplicate. Results are ±SD of at least three separate experiments.

In vivo experiments

To analyze *in vivo* the metastatic activity of neoplastic cells, 1×10^7 cells were resuspended in 200 μl of PBS and injected into the tail vein of 6-wk-old athymic mice divided into four groups of six animals each one. Groups were injected as follows: group 1, PBS alone; group 2, BHT cells; group 3, BHT pLL sc-siRNA cells; and group 4, BHT pLL siRNA2. Four weeks after injection, mice were killed and lung metastasis was analyzed by standard hematoxylin and eosin staining.

Analysis of MMP-9 activity

The quantitative determination of human active MMP-9 in conditioned media from NGAL-proficient and -deficient BHT101 and Act1 cell lines was performed by Fluorokine E immunoassay, according to the manufacturer's instructions (R&D Systems). The relative fluorescence units were determined with the PerkinElmer LS50B luminescence spectrometer (New York, NY) using an excitation wavelength of 320 nm and an emission wavelength of 405 nm. Experiments were performed at least in triplicate. Results are ±SD of at least three separate experiments.

Statistics

Data were analyzed with ANOVA and a Student's *t* test analysis. Data are presented as the means ± SD. $P < 0.05$ was considered significant.

Results

MMP-9 and NGAL are overexpressed in human ATCs

We have previously shown (11) that NGAL is strongly expressed in human thyroid carcinomas, especially that of the anaplastic type. Because it is known that in many tumors NGAL expression parallels that of MMP-9 (19–21), we verified whether both genes were overexpressed in human thyroid tumors from the same tissue specimen. As shown in Fig. 1A, MMP-9 expression is clearly more pronounced in human ATCs than in normal thyroid tissues, as assessed by quantitative RT-PCR (qRT-PCR) analysis, and, importantly, it overlaps that of NGAL in the same tissues (Fig. 1B), indicating that both genes are highly expressed in the most aggressive and metastatic types of human thyroid carcinomas.

Establishment of knocked-down and overexpressing NGAL anaplastic thyroid carcinoma cell lines

To study the role of NGAL in the metastatic activity of ATC cells, we modulated its expression in two ATC cell lines, BHT101 and Act1, that show different levels of NGAL expression. BHT101 cells express high levels of NGAL and secrete the protein in the culture medium, whereas Act1 cells do not express NGAL that is not detectable either in the whole cell extract or in the culture medium (Fig. 2, A and B). Therefore, we knocked down NGAL in BHT101 cells by infecting cells with two different lentiviruses containing siRNA sequences that bind two different regions of NGAL mRNA (pLL siRNA1 and -2). In parallel, we ectopically expressed NGAL in Act1 cells by infecting cells with a lentivirus carrying the cDNA en-

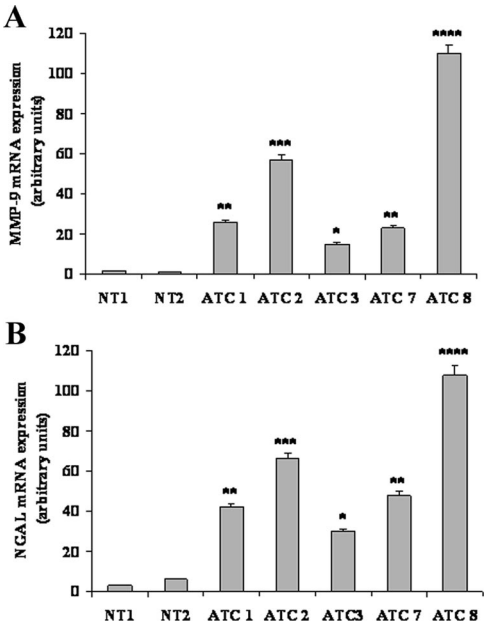

FIG. 1. MMP-9 and NGAL expression in primary human ATC specimens. qRT-PCR of MMP-9 (A) and NGAL (B) in normal thyroid (NT1 and NT2) and ATCs (ATC1, ATC2, ATC3, ATC7, and ATC8) from human specimens. The relative amount of MMP-9 and NGAL mRNA was normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA. *, $P < 0.001$; **, $P < 0.0001$; ***, $P < 0.00001$; ****, $P < 0.000001$.

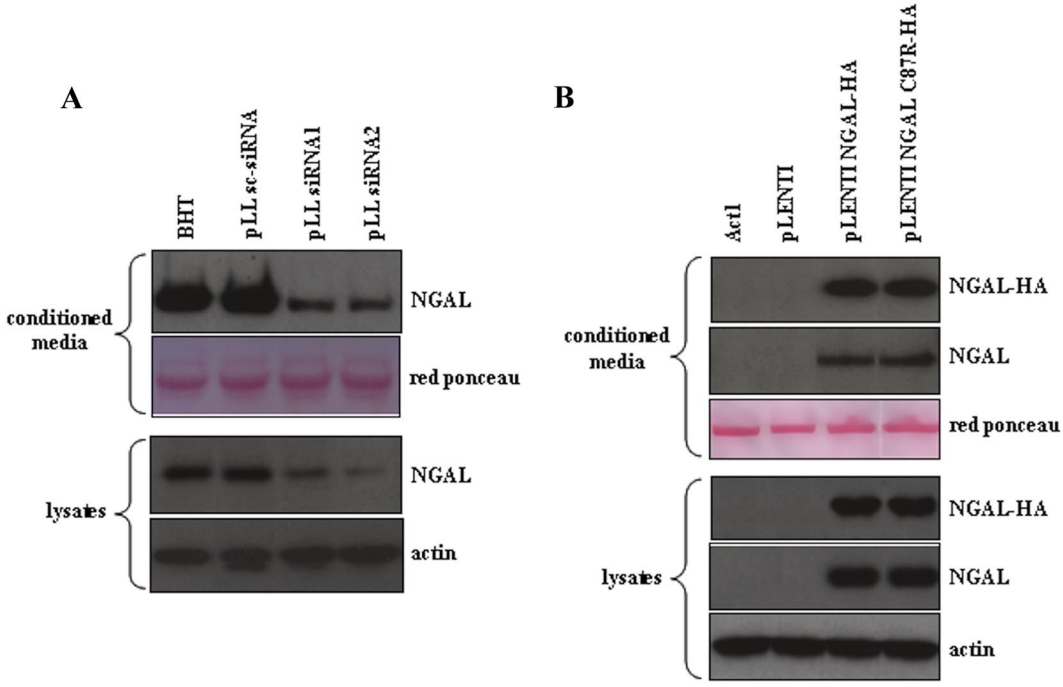

FIG. 2. Modulation of NGAL expression in ATC cell lines. Western blot of NGAL on conditioned media and lysates from BHT101 cells uninfected and infected with pLL 3.7 NGAL siRNA1, siRNA2, or scrambled (sc) siRNA (A) and from Act1 cells uninfected and infected with pLENTI alone, pLENTI NGAL-HA, or pLENTI NGAL C87R-HA (B). The expression levels of NGAL protein from lysates were normalized on actin content (A and B, *bottom panels*), whereas those of conditioned media were normalized on total protein content determined by a Bradford assay (Bio-Rad Laboratories).

coding for human NGAL. BHT101 cells infected with pLL siRNA1 and pLL siRNA2 showed a strong decrease of NGAL expression compared with uninfected cells and to control scrambled siRNA (Fig. 2A). The ectopic expression of NGAL in Act1 cells was successfully achieved both in lysates and extracellular media, as shown in Fig. 2B.

In addition, we also infected Act1 cells with a mutated form of NGAL (C87R), carrying a substitution of cysteine residue in position 87 with an arginine residue (C87R) (Fig. 2B). This mutation impairs the ability of NGAL to form the disulfide bridge with MMP-9, thereby interfering with NGAL-mediated MMP-9 protection from auto-degradation and preventing association of tissue inhibitor of metalloproteinase-1 with MMP-9 N-terminal domain (22). To test the effectiveness of this substitution, we transiently cotransfected HEK293 cells with FLAG-MMP-9 and NGAL-HA or with FLAG-MMP-9 and NGAL C87R-HA, respectively, and we coimmunoprecipitated them with anti-HA antibodies. Western blot analysis of immunoprecipitated proteins with anti-FLAG antibodies revealed that only wild-type NGAL-HA was able to bind FLAG-MMP-9, whereas the mutated NGAL C87R-HA was completely unable to interact with the metalloproteinase (Fig. 3).

NGAL enhances the invasiveness of BHT101 and Act1 cells

Both NGAL knocked-down and NGAL overexpressing cell lines were tested for their ability to degrade matrigel in a transwell invasion assays. As shown in Fig. 4, all BHT cell lines migrated through the transwell at similar extent, independently of the presence of NGAL in the culture medium (Fig. 4, A–C, *left panels*). However, they not degraded matrigel with the same efficiency (Fig. 4, A–C, *right panels*). In particular, knocking down NGAL expression determined the decrease of BHT cells invasiveness compared with that of parental and sc-siRNA BHT cells, as

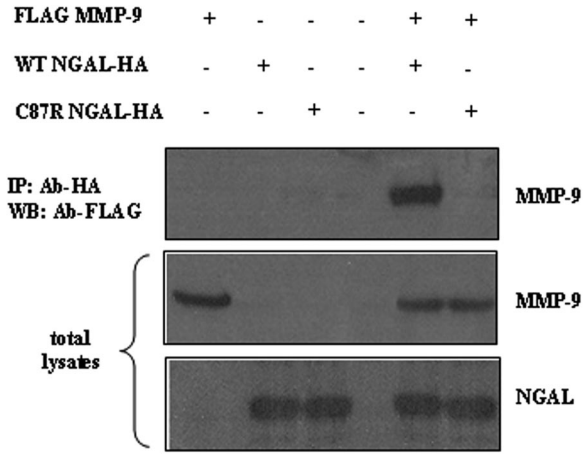

FIG. 3. The substitution of cysteine residue in position 87 with arginine (C87R) impairs NGAL binding to MMP-9. HEK293 cells were transfected with constructs encoding MMP-9, wild-type (WT), and mutated (C87R) NGAL. Cell lysates were immunoprecipitated (IP) with anti-HA antibodies (NGAL) and Western blotted (WB) with anti-FLAG to reveal the coprecipitation of MMP-9 and NGAL. The presence of HA and FLAG proteins in total lysates is shown in *lower panels*.

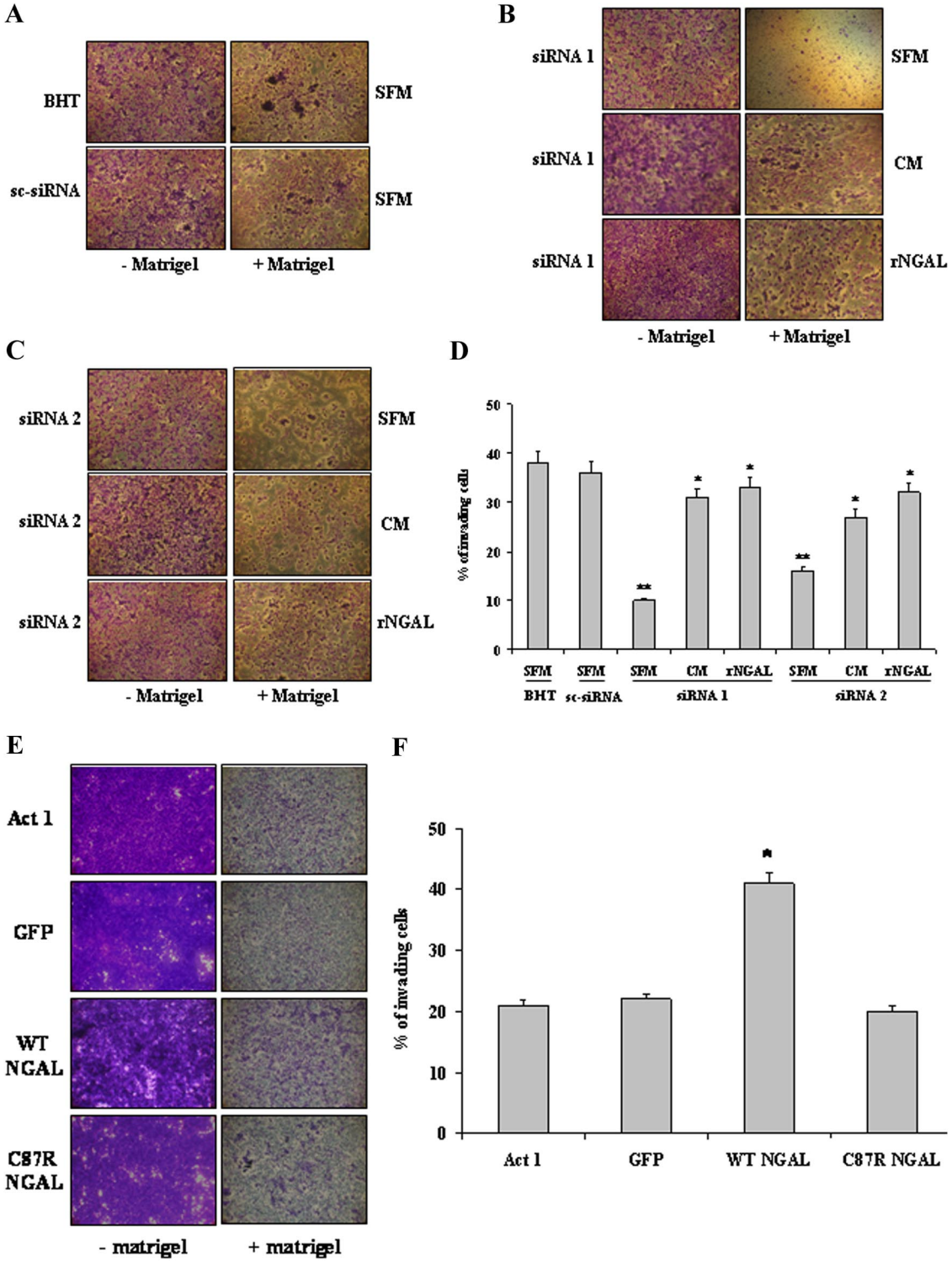

FIG. 4. *In vitro* invasive properties of NGAL-proficient and -deficient ATC cell lines. Parental and infected BHT101 (A–C) and Act1 (E) cell lines were used to analyze their ability to degrade Matrigel (BD Biosciences) in transwell assays. The extent of invasive potential was measured as the number of Matrigel degrading cells on total migrating cells (D, *, $P < 0.01$, **, $P < 0.001$; and F, *, $P < 0.001$). SFM, Serum-free medium; CM, conditioned medium; rNGAL, recombinant NGAL.

assessed by the reduced number of both BHT pLL siRNA1 and siRNA2 cells that degraded matrigel (Fig. 4D). The addition of NGAL-containing conditioned medium or recombinant NGAL to the upper chamber of transwells restored the invading activity of BHT pLL siRNA1 and siRNA2 cells (Fig. 4, B–D). On the other hand, the ectopic expression of NGAL in Act1 cells induced an increase of their invasiveness, as assessed by a more pronounced ability of NGAL-expressing Act1 cells to degrade matrigel compared with the parental and control vector cells (Fig.

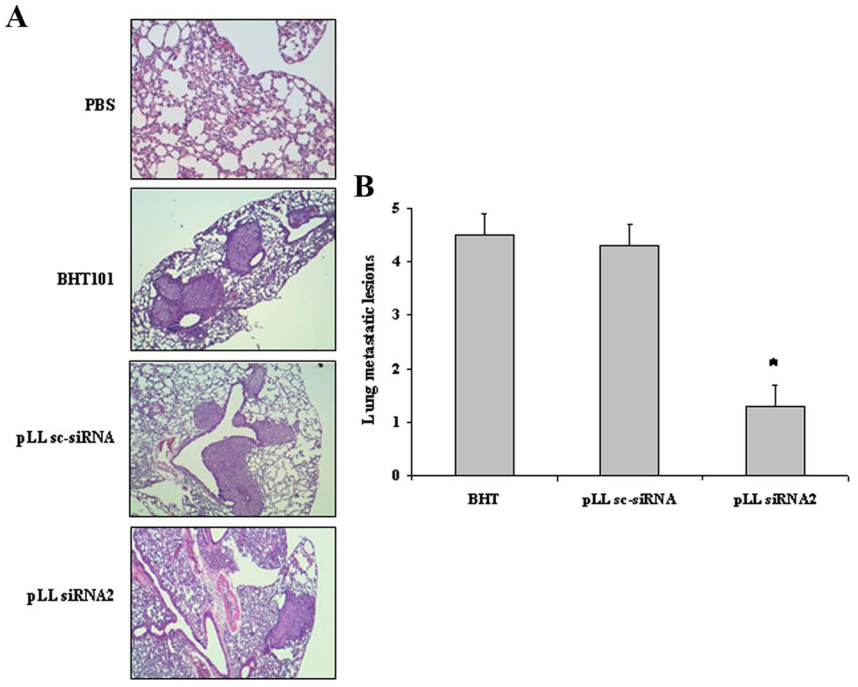

FIG. 5. *In vivo* analysis of metastatic activity of NGAL-proficient and -deficient ATC cells. Parental and infected BHT101 cells were injected in athymic mice tail vein to analyze their ability to form lung metastasis (A). The number of metastatic lesions in the lung was determined for each group of injected mice (B). *, $P < 0.0001$.

To confirm these results *in vivo*, we injected parental and NGAL knocked-down BHT101 cells in the tail vein of athymic mice, which represents a preferential way of access for tumor cells migration to future metastatic sites, *i.e.* lung and liver. Figure 5 shows the hematoxylin-and-eosin staining of lung metastases from mice injected with uninfected BHT101, BHT pLL sc-siRNA, and BHT pLL siRNA2 cells. All three cell lines gave rise to lung metastases but at different extent. In particular, the number of lung metastases from BHT101 NGAL-null cells injected mice were lower than those originated from parental and scrambled BHT101 cells (Fig. 5, A and B).

These results suggest that the presence of functional NGAL increased the metastatic potential of ATC cells, *in vitro* and *in vivo*, very likely through the improvement of MMP-9 activity.

4, E and F). Importantly, Act1 cells expressing the C87R NGAL mutant reversed the proinvading effects of wild-type NGAL, suggesting that interfering with the formation of NGAL/MMP9 complex is important in regulating the prometastatic activity of MMP-9.

MMP-9 enzymatic activity is enhanced by the presence of NGAL

To test our hypothesis, we evaluated the MMP-9 activity in the parental and infected BHT101 and Act1 cell lines by an immunoenzymatic assay. To this purpose, cells

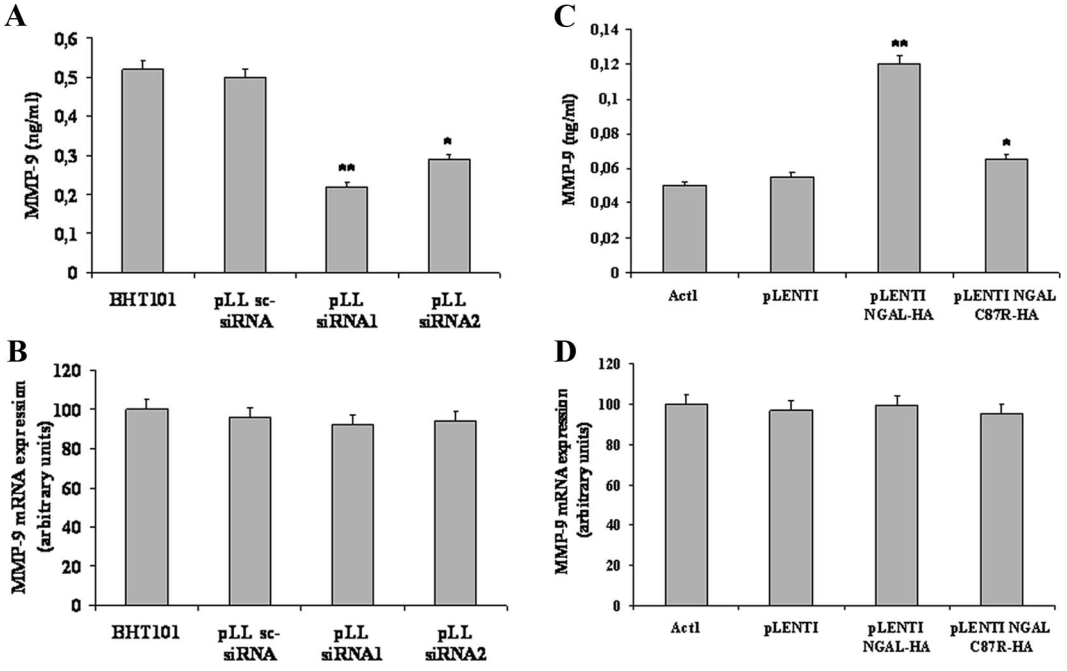

FIG. 6. Determination of MMP-9 activity in conditioned media from ATC cell lines. Conditioned media from parental and infected BHT101 (A, *, $P < 0.001$, **, $P < 0.0001$) and Act1 (C, *, $P < 0.001$, **, $P < 0.0001$) cell lines were collected to determine the concentration of active MMP-9. qRT-PCR analysis showed that no difference of MMP-9 expression was detected among cell lines (B and D).

were grown in serum-free medium, and after 48 h their conditioned media were collected to measure the levels of endogenous active MMP-9. As shown in Fig. 6A, the absence of NGAL in BHT101 cells determined a strong decrease of active MMP-9 content compared with that measured in the extracellular medium of parental and scrambled BHT101 cells. On the contrary, the concentration of active MMP-9 from the conditioned medium of NGAL-overexpressing BHT101 cells was higher than that from conditioned media of control cells (Fig. 6C). Importantly, the presence of the mutated form of NGAL in BHT101 extracellular medium had no effect on MMP-9 activity. The up- or down-regulation of enzymatic activity in the different experimental systems was not dependent on MMP-9 expression because RT-PCR analysis showed that no differences of MMP-9 mRNA levels were detectable in the various cell lines (Fig. 6, B and D).

Discussion

In the present paper, we analyzed the role of NGAL in promoting ATC tissue invasion through enhancement of MMP-9 activity. The inhibition of NGAL expression, which is strongly elevated in the same primary human ATC showing high MMP-9 expression, decreases the *in vitro* and *in vivo* metastatic potential of BHT101 ATC cell line. This effect is the consequence of the reduced enzymatic activity of MMP-9 in the absence of NGAL. However, knocking down NGAL expression in BHT101 cells does not completely abrogate the ability of these cells to invade matrigel and to form lung metastasis (Figs. 4, A and D, and 5, A and B). This is probably due to the presence of a residual NGAL expression in BHT siRNA cells, as assessed by Western blot (Fig. 2A), that still preserves MMP-9 from autodegradation, as proven by the partial but not absolute decrease of MMP-9 enzymatic activity in the extracellular medium of BHT NGAL-null cells (Fig. 6A).

The importance of NGAL as regulator of ATC cells metastatic potential is further supported by increased MMP-9 enzymatic activity in the Act1 after ectopic expression of NGAL. In this case, the secretion of extracellular NGAL improves the MMP-9 enzymatic activity of Act1 cells causing the increased invasion through matrigel in the transwell assay. This effect is clearly NGAL mediated because Act1 cells overexpressing the C87R mutant of NGAL show a MMP-9 activity and an invasive behavior similar to those of control cells. The evidence of a basal MMP-9 activity independent of NGAL expression in Act1 cells (Figs. 4, D and E, and 6C) points out that MMP-9 can function in the absence of NGAL; even so, the presence of NGAL significantly improves its enzymatic activity.

In the light of these findings, the strong coexpression of NGAL and MMP-9 in primary human ATC suggests a pro-metastatic role of NGAL/MMP-9 complex in aggressive thyroid carcinomas. Anaplastic thyroid carcinomas constitute the most undifferentiated type of thyroid cancer with marked invasive properties so that the presence of an active NGAL/MMP-9 complex could explain in part the metastatic potential of ATC cells. The biological importance of NGAL/MMP-9 complex in cancer has been extensively studied in other human tumors, such as breast cancer (18, 23), esophageal (20) and oral (24, 25) squamous cell carcinomas, cholangiocarcinomas (21), bladder cancer (26), gastric cancer (19), and rectal cancer (27). In these reports the activity of the complex generally correlates with the more aggressive behavior of neoplastic cells and, in the majority of cases, with a poor prognosis. Thus, the role of NGAL/MMP-9 complex in metastatic thyroid cancer, up to now not yet elucidated, fits with that exerted in other types of human tumors.

The identification of NGAL as a regulator of ATC-induced metastasis adds a novel piece to the puzzle of NF-κB functions in thyroid cancer (10, 11, 28–31). In fact, our previous results and the findings reported here indicate that NF-κB activity could influence the invasive potential of ATC cells through the up-regulation of NGAL other than of MMP-9, whose mRNA transcription, as it is known, is in part under NF-κB control (32). This could also explain why the expression of both genes is so elevated in ATCs in which NF-κB is strongly activated. In this way, NGAL could be viewed as a novel target gene of NF-κB-mediated metastatic activity of ATC cells and its inhibition as a potential tool for therapeutical intervention in advanced ATC therapy without altering NF-κB physiological functions.

Acknowledgments

Address all correspondence and requests for reprints to: Francesco Pacifico, Istituto di Endocrinologia ed Oncologia Sperimentale, Consiglio Nazionale delle Ricerche, Via Sergio Pansini 5, 80131 Naples, Italy. E-mail: f.pacifico@ieos.cnr.it; or Antonio Leonardi, Dipartimento di Biologia e Patologia Cellulare e Molecolare, "Federico II" University of Naples, Via Sergio Pansini 5, 80131 Naples, Italy. E-mail: leonardi@unina.it.

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