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

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Abstract

Interleukin 17 (IL-17) is a proinflammatory cytokine that promotes the expression of different cytokines and chemokines via the induction of gene transcription and the post-transcriptional stabilization of mRNAs. Here we show that IL-17 increases the half-life of the *Zc3h12a* mRNA via interaction of the adaptor protein CIKS with the DEAD box protein DDX3X. Interleukin 17 stimulation promotes the formation of a complex between CIKS and DDX3X, this interaction requires the helicase domain of DDX3X but not its ATPase activity. DDX3X knock-down decreases the IL-17-induced stability of *Zc3h12a* without affecting the stability of other mRNAs. IKK ϵ , TRAF2 and TRAF5 were also required to mediate the IL-17-induced *Zc3h12a* stabilization. DDX3X directly binds the *Zc3h12a* mRNA after IL-17 stimulation. Collectively, our findings define a novel, IL-17-dependent mechanism regulating the stabilization of a selected mRNA.

Introduction

Interleukin-17 (IL-17A) is the signature cytokine produced by CD4⁺ T helper-17 cells, and belongs to a molecular family composed of six members (IL-17A to F), which are structurally unrelated to other cytokines (1, 2). IL-17 is a proinflammatory cytokine that induces transcription and stabilization of different mRNAs encoding for other inflammatory proteins, such as cytokines, chemokines and metalloproteinases, to amplify the inflammatory response. Although IL-17 is required for host defense against bacterial and

fungal infection, it has also been linked to the development of various autoimmune and inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and Systemic Lupus Erythematosus (3–6). IL-17A and the other members of the IL-17 family signal through its binding to heterodimeric receptors composed of members of the IL-17 receptor family (7). Connection to IκB kinase and Stress-activated protein kinase (CIKS) (a.k.a. Traf3ip2 or Act1) is an adaptor protein required for signaling by these receptors(8, 9). After IL-17 receptor triggering, CIKS is recruited to the receptor via a homotypic interaction between its SEFIR domain and the SEFIR domain of the receptor. CIKS, in turn interacts with TRAF6 and this interaction is required for NF-κB and JNK activation, and subsequent transcription of proinflammatory genes (10–12). Other members of the TRAF family (TRAF2 and TRAF5) have been demonstrated to interact with CIKS, but this interaction seems to be dispensable for NF-κB activation, rather it controls the IL-17-induced mRNA stability (13). For this function, IKKε (a.k.a. IKKi) has been rather claimed as essential molecule. After stimulation with IL-17, IKKε forms a complex with CIKS, indeed, mouse embryonic fibroblasts isolated from IKKε KO mice failed to stabilize the IL-17-induced cytokine mRNA(s) (14). Although increased transcription is a requisite for the induced expression of IL-17-target genes, regulation of the half-life of corresponding mRNAs is also critical in determining the magnitude of their expression. Indeed, highly unstable mRNAs require IL-17 to be stabilized during inflammatory responses, and to effectively express the encoded proteins (15). This in turn has fostered the notion that mRNA stabilization is the primary function of IL-17. Stabilization of mRNAs encoding cytokines and chemokines involves regions in the 5'- and 3'-untranslated regions (UTR) of the message, which are specifically recognized by proteins whose function is controlling exonucleolytic degradation of the RNA (16). RNA helicases modulate almost every aspect of RNA metabolism from transcription to translation, and are classified in superfamilies and families based on sequence and structural features (17). DEAD box proteins form the largest helicase family and are characterized by the presence of an Asp-Glu-Ala-Asp (DEAD) motif (18, 19). DDX3X is an ubiquitously expressed member of this family, it consists of 662 amino acids and contains a central core helicase domain. DDX3X, as most of the members of the helicase family, appears to be involved in almost every step of RNA metabolism, and a role for DDX3X in cell cycle control and apoptosis was also proposed (20–24). Recently, DDX3X has also been demonstrated to have a positive role in Interferon (IFN) induction: i) by binding to PolyI:C and to viral RNA in solution; ii) as a component of the IPS-1 and TBK1/IKKε complex; iii) via direct binding to the IFN-β promoter (25– 27).

ZC3H12a (a.k.a. MCP-1-induced protein 1 (MCPIP1), or Regnase-1), is a LPS-inducible gene, and contains a highly conserved Nedd4-BP1, YacP nuclease/deubiquitinase (NYN/DUB) domain with intrinsic RNase and DUB activities at the N terminus, a single CCCH-type ZF domain with RNA-binding potential in the middle region, and a proline-rich (PRR) domain for protein oligomerization at the C-terminus (29, 30). The RNase and DUB activities of ZC3H12a are involved in various biological functions, such as cellular RNA decay and negative regulation of cellular inflammation. The RNase activity of ZC3H12a can directly degrade certain mRNAs of cytokines, such as IL-6 and IL-12p40, via AU-rich element-independent mechanisms (30, 31). The DUB function of ZC3H12A inhibits LPS-, IL-1β-, and TNF-α-mediated NF-κB- and JNK-signaling pathways by removing ubiquitin

moieties of TNFR-associated factors (TRAFs), including TRAF2, TRAF3, and TRAF6 (32). *Zc3h12a*-deficient mice exhibited severe immune syndrome disorders characterized by severe anemia, autoimmune response, and severe inflammation, and most mice died within 12 wk of birth (30).

Although the role of the above mentioned RNA-binding proteins in regulating the half-life of mRNA is well recognized, the high number of proteins involved in such effect, and the signaling pathways downstream of extracellular stimuli (including IL-17) involved are poorly understood. For example, it has been recently demonstrated that the splicing-regulatory factor SF2 (ASF) prolongs the half-life of the chemokine *cxcl1* mRNA, after IL-17 stimulation (13). However, the role of SF2 seems to be restricted to specific RNAs and does not involve all the IL-17-induced mRNAs, further suggesting that multiple, RNA-specific mechanisms exist. Here we demonstrate that the DEAD-box helicase DDX3X is mediating the stabilization of the *Zc3h12a* mRNA. DDX3X interacts with CIKS in an IL-17 dependent manner, and this interaction requires the helicase domain of DDX3, and both the N-terminus and the C-terminus of CIKS. DDX3X knock-down decreases the IL-17-induced stability of *Zc3h12a* without affecting the stability of other mRNAs. This effect is independent on NF- κ B activation, while it depends upon IKK ϵ , TRAF2 and TRAF5. Collectively, our findings unveil a new-, IL-17-dependent mechanism regulating the stabilization of a selected mRNA.

Material and Methods

Reagents, cell lines, and constructs

Recombinant IL-17 and TNF- α 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 antigen. Anti-TRAF6 (sc-7221), anti-TRAF2 (sc-876), anti-TRAF5 (sc-7220), were from Santa Cruz Biotechnology. Anti-IKK ϵ (D61F9) was from Cell Signaling. Anti-CIKS was produced in rabbit using a recombinant peptide spanning the amino acids 382–574 of CIKS as antigen.

HEK293, wild type mouse embryonic fibroblast (MEF), and CIKS^{-/-} MEF (28) were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Sigma-Aldrich), antibiotics (100 μ g/mL penicillin, 100 μ g/mL streptomycin), and 1 mM *L*-glutamine (Invitrogen).

The cDNA encoding CIKS and its mutants were previously described (8, 33). 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 (Invitrogen).

Transfection and immunoprecipitation

Lipofectamine-mediated transfections were performed according to the manufacturer's instructions (Invitrogen). All transfections included supplemental empty vector to ensure that the total amount of transfected DNA was kept constant in each dish culture. For

immunoprecipitation of transfected proteins, HEK293 cells (3×10^6) were transiently transfected 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 additional 15 min on ice, cell extracts were centrifuged for 10 min at $14,000 \times g$ at 4°C , and supernatants were incubated with anti-FLAG antibodies bound to agarose beads (M2, Sigma) for 3 hours, at 4°C . Immunoprecipitates were washed five times with Triton X-100 lysis buffer and subjected to SDS-PAGE.

RNA-interference (RNAi)

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

Mouse DDX3X: 5'-GAUGCUGGCUCGUGAUUUCU-3'

Mouse TRAF2: 5'-CGACAUGAACAU CGCAAGC-3'

Mouse TRAF5: 5'-AAGCCAGUGACCAGAGAUUAGUU-3'

To knock-down mouse IKK ϵ and TRAF6, we used the esiRNA#EMU029351 (Sigma) and esiRNA #EMU046421 (Sigma), respectively. The scrambled control was from Thermo Scientific (siRNA ON-TARGETplus Non-targeting Pool #D-001810-10-05). Forty-eight or seventy-two hours after transfection, cells were collected for RNA or protein extraction.

Lentivirus production and infections

FLAG-CIKS, CIKS E17A and CIKS Ubox mutant cDNAs were subcloned into pWPT lentiviral vector at BamHI/SalI sites. The constructs were sequenced to confirm correct DNA sequence and orientation. Subconfluent 293T lentivirus packaging cells were cotransfected with either pWPT-GFP or pWPT-CIKS 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 at 23000 rpm for 90 min, at 4°C in a Beckman SW28 swinging bucket rotor. For transduction, CIKS $^{-/-}$ MEFs were plated on 12-well plate, and infected with lentiviruses in the presence of 10% foetal bovine serum and polybrene (8 $\mu\text{g/ml}$ final concentration) (Sigma).

Coimmunoprecipitation and mass spectrometry analysis

CIKS $^{-/-}$ MEFs reconstituted with FLAG-CIKS or with empty vector were left untreated or treated with IL-17 at different time points. After stimulation, cells were washed with ice-cold PBS, and then lysed with lysis buffer (20 mM HEPES pH7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM Na₃VO₄) freshly supplemented with protease inhibitors cocktail (Roche). Nuclear and cellular debris were removed by centrifugation at $14,000 \times g$ for 30 min, at 4°C . For identification of the CIKS-interacting proteins, 100 mg of cell extract from CIKS $^{-/-}$ and FLAG-CIKS-reconstituted MEFs were incubated with 500 μl of M2 beads (Sigma), which were preventively washed with 0.1 M glycine pH 3.0, to eliminate non-covalently bound antibody. After 3 hours incubation at 4°C , beads were washed four times with lysis buffer, and twice with high salt (1 M NaCl)

lysis buffer. Proteins were eluted from the beads by using 500 μ l of 3 \times FLAG peptide (Sigma) (200 μ g/ml final concentration). Eluted proteins were separated by SDS-PAGE on a 9–16% gradient gel, and stained with colloidal Coomassie G-250 (34). Gel slices (11 in number) were excised from the lanes corresponding to the bait and mock samples, S-alkylated and *in gel* digested with trypsin (35). After desalting, peptide mixtures were 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 C₁₈ column (100 \times 0.075 mm, 3 μ m) (Proxeon) by using a gradient of acetonitrile in aqueous 0.1% formic acid, at flow rate of 300 nL/min. Spectral acquisition was performed as previously reported (36). Two technical replicates were performed for each gel slice. Proteome Discoverer platform v.1.3 (Thermo) was used to search raw mass data against an updated Uniprot database of mouse sequences (2011/10) with both Sequest (Thermo, USA) and Mascot (Matrix Science, UK) algorithms. nLC-ESI-LIT-MS/MS data were searched by using experimental parameters previously reported (36). 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 then identified by subtracting the components ascertained within the bait with that ones from the corresponding mock.

Cell stimulation, RNA isolation and Real-Time PCR

To measure mRNA stability, MEFs were stimulated with TNF- α (2000U/ml) for 60 minutes, and then after removing the TNF- α , with IL-17 (200 ng/ml) plus actinomycin D (5 μ g/ml) for different time points. Total RNA was extracted by using TRIZOL reagent according to manufacturer's instructions (Invitrogen). Real-Time RT-PCR was carried out with cDNAs reverse-transcribed from total RNA by using GoTaq qPCR Master Mix (Promega) and CFX Manager software (Bio-Rad). Experimental Ct values were normalized to *GAPDH*. Statistical analysis has been performed by using the student's t-test.

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'

IKK ϵ : Fw: 5'-CCACGTCTTTTCCCTACCCC-3' Rev: 5'-GATGGCAATCGTGTGTTGGG-3'

DDX3X: Fw: 5'-GTAGAGGCAGCCTTTGCTCA-3' Rev: 5'-ATAACGCCCTTTGCTTGCTG-3'

CXCL2: Fw: 5'-CCCAGACAGAAGTCATAGCCA -3' Rev: 5'-CAGGTACGATCCAGGCTTCC -3'

CCL2: Fw: 5'-AGCTGTAGTTTTTGTACCAAGC-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'-CGCACCTGTCCCTGTA CTT-3' Rev 5'-AGGCAATGTTTCATCTCGCCA-3'

TRAF6: Fw: 5'-ATCCATAAGGGATGCAGGGC-3 Rev: 5'-GGCACTTTACCGTCAGGGAA-3'

RNA immunoprecipitation

To detect the *Zc3h12a* mRNA in the DDX3X immunoprecipitate, HeLa cells (3×10^6) were transiently transfected with FLAG-DDX3X by using Lipofectamin. Twenty-four hours after transfection, cells were treated with TNF- α (2000U/ml) plus IL-17 (200 ng/ml), and then lysed in Triton X-100 lysis buffer containing RNasin 1U/ μ l. After an additional 15 min on ice, cell extracts were centrifuged for 10 min at $14,000 \times g$ at 4 °C, and supernatants were incubated with anti-FLAG antibodies bound to agarose beads (M2, Sigma) for 3 h at 4°C. Immunoprecipitates were washed five times with Triton X-100 lysis buffer containing RNasin 1U/ μ l. RNA was extracted by using TRIZOL reagent according to manufacturer's instructions (Invitrogen). Immunoprecipitated-RNA was reverse-transcribed by using the GoScript Reverse Transcriptase (Promega). PCR was carried out with cDNAs by Expand High Fidelity PCR System (Roche). To amplify the 5'-region of the human *Zc3h12a* mRNA, we used the following primers:

Fw: 5'-GTCTGAGCTATGAGTGGCCC-3'; Rev: 5'-GGCTGTGGCTGTCCTCAAAT-3'

To amplify the 3'-portion of the human *Zc3h12a* mRNA, we used the following primers:

Fw: 5'-TGAGGCCCAAACCGTCTTTT-3'; Rev: 5'-GCCTCATTGGCGTAGAGGAC-3'

Experimental PCR conditions were: 95°C 30"; 60°C 30"; 68°C 1', 40 cycles. The PCR products were analyzed by 1% agarose gel electrophoresis.

RESULTS

CIKS interacts with DDX3X

To search for CIKS-interacting proteins, we reconstituted CIKS^{-/-} mouse embryonic fibroblasts (MEFs) with FLAG-tagged CIKS, immunoprecipitated FLAG-CIKS, and analysed the products by mass spectrometry. Among the proteins recovered from the reconstituted cells, but not from the CIKS^{-/-} counterpart (control), we identified the DEAD-box RNA helicase DDX3X. We confirmed this interaction by co-immunoprecipitation (Fig. 1A), and we mapped the sites on both CIKS and DDX3X responsible for the interaction. As shown in Fig. 1B, both the N-terminus and the C-terminus of CIKS were required for a proper interaction with DDX3X. Indeed, deletion of the first 100 amino acids, or the last 100 amino acids of CIKS sequence, resulted in a loss of the interaction between CIKS and DDX3X. Internal deletions, such as the one comprising the U-box domain (amino acids 271–334) (CIKS^{Ubox}) did not affect complex formation. Next, a searching for the DDX3X domains responsible for a proper interaction with CIKS was performed, we found that the helicase domain of DDX3X was required for interaction with CIKS (Fig. 1C). Worth noting a DDX3X mutant (K230A) unable to bind ATP was proved to retain the ability to interact with CIKS (data not shown). We also found that the interaction between CIKS and endogenous DDX3X was dependent upon IL-17 stimulation. In fact, as shown in Fig. 1D, treatment of reconstituted CIKS MEF with IL-17 enhanced the interaction of endogenous DDX3X with CIKS.

All together these data demonstrated that CIKS interacted with DDX3X in an IL-17-dependent manner.

DDX3X mediates mRNA stability

Since CIKS is essential for the IL-17-mediated biological responses, we sought to investigate if DDX3X down-regulation was interfering with some of the IL-17-mediated functions, such as gene expression and mRNA stability. To this purpose, we knocked-down DDX3X expression in MEFs by using siRNA (Fig. 2A and B), and in these cells we evaluated: NF- κ B and AP-1 activation after IL-17 stimulation. No differences were observed between parental and DDX3X-interfered cells both in terms of NF- κ B and AP-1 activation (Supplementary figure 1).

Next, we investigated the ability of DDX3X to mediate the IL-17-induced stabilization of some short-lived mRNAs induced by TNF- α . We treated CIKS^{-/-} MEFs, reconstituted MEFs, and DDX3X knocked-down MEFs with TNF- α for 60 minutes to induce transcription of target genes. Then, we treated cells with actinomycin D (to block *de novo* transcription) plus IL-17 to induce mRNA stabilization. After 90 minutes cells were harvested, RNA extracted, and analysed by Real-Time PCR. As shown in Fig. 2C, the level of *Zc3h12a* was significantly decreased in DDX3X knocked-down cells compared to MEFs treated with a control siRNA. The ability of DDX3X to stabilize mRNA was relatively selective for *Zc3h12a*, as the levels other mRNAs known to be stabilized by IL-17, including *Cxcl1*, *Cxcl5* and *Il6*, was not affected in the absence of DDX3X (Fig. 2D–F). Fig. 2G illustrates a time course of mRNA stability for *Cxcl1* and *Zc3h12a*, in cells knocked-

down for DDX3X expression. The half-life of *Zc3h12a* was decreased by almost 50%. These results suggested that DDX3X is involved in the stabilization of selected mRNAs after IL-17 treatment.

IKK ϵ forms a complex with CIKS and DDX3X and is required for mRNA stabilization

In order to gain insights into the mechanism underlying the DDX3X-based stabilization of *Zc3h12a*, we investigated if IKK ϵ , which has already been shown to be involved in mRNA-protecting machineries, was present in a complex containing CIKS and DDX3X. We transfected HEK293 cells with the indicated expression vector (Fig. 3A), immunoprecipitated Myc-tagged IKK ϵ and looked for co-immunoprecipitating DDX3X. As shown in Fig. 3A, IKK ϵ was interacting with DDX3X, suggesting the formation of a complex between CIKS, IKK ϵ and DDX3X. To investigate if the formation of such a complex was dependent upon IL-17 stimulation, we treated CIKS^{-/-} and reconstituted MEF with IL-17, immunoprecipitated FLAG-CIKS and analyzed the presence of IKK ϵ and DDX3X in the immunoprecipitate. As shown in Fig. 3B both IKK ϵ and DDX3X was co-immunoprecipitating with CIKS in an IL-17-dependent manner. It was possible to detect IKK ϵ 15' minutes after stimulation, while DDX3X appeared in the complex at a later time point, thus suggesting that IL-17 treatment induces the formation of a complex containing CIKS, IKK ϵ and DDX3X.

Next, we knocked-down expression of IKK ϵ in MEF (Fig 3 C, D), and we evaluated the stability of the *Zc3h12a* mRNA. Cells were treated with TNF- α for 60 min, and then treated with actinomycin D plus IL-17. As shown in Fig. 3E, the half life of *Zc3h12a* was significantly decreased in IKK ϵ knocked-down cells, compared to MEFs treated with a control siRNA, thus suggesting that the IL-17-induced *Zc3h12a* stabilization requires IKK ϵ .

TRAF2 and TRAF5, but not TRAF6, are involved in *Zc3h12a* stability

TRAF2 and TRAF5 are already known to be involved in the stabilization of mRNAs induced by IL-17. In order to investigate if TRAF2, TRAF5 or both were also involved in the stabilization of *Zc3h12a*, we knocked-down their expression in CIKS-reconstituted MEF (Fig 4A, B, D, E), and we analyzed the stability of the *Zc3h12a* mRNA after IL-17 treatment. As shown in Fig. 4C, in the absence of TRAF2, IL-17 treatment slightly affected *Zc3h12a* stabilization. Similarly, in the absence of TRAF5, *Zc3h12a* stabilization was partially affected after IL-17 stimulation (Fig. 4F). As TRAF2 and TRAF5 are known to have redundant functions (37), we knocked-down expression of both proteins by siRNA. As shown in Fig. 4G, knocking down expression of both molecules resulted in a loss of mRNA accumulation in interfered cells, when compared to the scrambled-treated counterpart. These results suggest that either TRAF2 or TRAF5 (or both) are required for stabilization of *Zc3h12a*.

We also investigated if the ability of DDX3X to stabilize the *Zc3h12a* mRNA was dependent upon the ability of CIKS to bind TRAF6 and to mediate NF- κ B activation. We knocked-down TRAF6 expression in CIKS-reconstituted MEFs, and we analyzed the stability of *Zc3h12a*, after IL-17 treatment. As shown in Fig. 5 A, B, C, TRAF6 knock-down did not affect the stability of the *Zc3h12a*. Similarly, we reconstitute CIKS^{-/-} MEFs with

the point mutant CIKS E17A, unable to bind TRAF6 and to activate NF- κ B (38, D.S. and A.L. unpublished observation). As shown in Fig. 5D, E, CIKS^{-/-} MEFs reconstituted with CIKS or CIKS E17A had the same levels of the *Zc3h12a* mRNA, thus suggesting that TRAF6 and NF- κ B activation were not part of the mechanism regulating mRNA stability.

IL-17 modulates the binding of DDX3X to *Zc3h12a*

To demonstrate whether DDX3X binds the *Zc3h12a* mRNA, we ectopically expressed FLAG-DDX3X in HeLa cells. Cells were treated with TNF- α plus IL-17, lysed, and DDX3X was immunoprecipitated by using anti-FLAG antibody. RNA was extracted from the immunoprecipitate, reverse transcribed, and the presence of *Zc3h12a* was detected by PCR. As shown in fig. 6A it was possible to amplify the *Zc3h12a* mRNA in the DDX3X immunoprecipitate after TNF plus IL-17 stimulation. It was not possible to amplify other mRNAs known to be stabilized by IL-17, such as *il6* and *Cxcl1*, thus demonstrating that DDX3X specifically binds *Zc3h12a*. In order to investigate the functional consequence of the decreased level of *zc3h12a* in the absence of DDX3X, we knocked-down DDX3X expression in MEF, treated the cells with TNF- α plus IL-17 and evaluated the level of *il6*, one of the known target of the RNase activity of ZC3H12a. As shown in fig 6B, the level of *il6* was significantly increased in DDX3X knocked-down MEF, suggesting that the DDX3X-mediated *zc3h12a* stabilization is one of the mechanisms used to down-regulate immune responses.

DISCUSSION

In this manuscript we report that the helicase family member DDX3X contributes to the regulation of 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. CIKS-DDX3X interaction finally stabilizes a selected mRNA. In fact, DDX3X selectively enhances the half life of the *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 involved in different cellular processes linked to RNA metabolism and gene expression (20). Despite its involvement in almost every step of mRNA metabolism, this is the first evidence linking DDX3X to the stabilization of a selected mRNA following IL-17 stimulation. DDX3X has also been demonstrated to be required for translation of selected mRNAs, by directly binding the 5'-end of such RNAs and facilitating the recruitment of eIF4F complex to start translation (39). It is then possible that binding of DDX3X to the *Zc3h12a* mRNA is important to modulate both the stability of the *Zc3h12a* mRNA and its translation. It is not yet clear how DDX3X increases the half life of *Zc3h12a*. One possible 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. ZC3H12a regulates its own mRNA stability by binding to its 3'UTR, thus the physical interaction that here we show between DDX3X and the *Zc3h12a* mRNA might suggest the displacement of ZC3H12a binding as one possible mechanism for its stabilization (40).

DDX3X has also been involved in viral sensing pathway at different levels: by i) enhancing the IPS-1 function; ii) recruiting TBK1 and IKK ϵ ; iii) directly binding to the IFN- β promoter; iv) directly binding to viral RNA (25–27).

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 were not able to detect an interaction between CIKS and DDX3X after stimulation with different substances mimicking viral nucleic acids, such as Poly(I:C) (both low and high molecular weight) and Poly (dA:dT). In addition, we have not detected a significant variation in IRF3/7 phosphorylation in CIKS^{-/-} MEFs treated with Poly(I:C) and Poly(dA:dT), compared to WT MEFs. These data prompt us to envisage a scenario in which DDX3X may function in various signaling pathways by regulating different immune responses: determining mRNA stability in the IL-17 pathway (via interaction with CIKS), and type I interferon production in the innate immune system (via interaction with TBK1/IKK ϵ).

In this manuscript, we also demonstrated that the DDX3X-mediated stabilization of the *Zc3h12a* mRNA requires IKK ϵ , TRAF2 and TRAF5. This finding is not surprising given that TRAF2, TRAF5 and IKK ϵ are required to stabilize other mRNAs, such as *Cxcl1* (13–14). Notably, mRNA stability was completely abrogated only in TRAF2/TRAF5 double deficient cells, suggesting a redundant function of these two molecules in the IL-17 pathway. The functional redundancy of TRAF2 and TRAF5 is not restricted to the IL-17 pathway. For instance, one of the two proteins may compensate the absence of the other one in TNF- α , signaling pathways. In this line, the phenotype of the TRAF2/TRAF5 double KO mice is way more complex than the phenotype of the single KO (37). However, in our experimental system, TRAF2 knock-down was more effective in blocking the IL-17 mediated stabilization of *Zc3h12a*, thus suggesting that TRAF2 is the regulator physiologically relevant. It remains to be determined if after IL-17 stimulation DDX3X forms a complex with TRAF2 and/or TRAF5, and we are currently investigating this hypothesis.

We also present evidence that CIKS, IKK ϵ and DDX3X form a complex after IL-17 stimulation. IKK ϵ and DDX3X are recruited in the complex with a different kinetic. In fact, while it is possible to detect IKK ϵ in the CIKS immunoprecipitate immediately after IL-17 stimulation, DDX3X appeared in the complex at a later time point. It is possible to speculate that after IL-17 stimulation IKK ϵ is first recruited to CIKS and then DDX3X is recruited to the complex to regulate its biological function. To this regard, it has been recently reported that IKK ϵ phosphorylates DDX3X and that this phosphorylation modulated the interaction between DDX3X and IRF3 (41). Therefore, it is likely that the phosphorylation of DDX3X may affect its function also in the IL-17 pathway. We are currently testing if the ability of DDX3X to bind *Zc3h12a* is affected by IKK ϵ . It has been demonstrated that IKK ϵ phosphorylates CIKS and this event is required for the formation of the CIKS-TRAF2-TRAF5 complex that mediates mRNA stability, while the formation of the CIKS-TRAF6 complex, regulating activation of NF- κ B, is not affected (14). Our data confirm and expand this observation, as also the DDX3X-mediated mRNA stabilization relies on a similar mechanism and is independent of NF- κ B activation. In fact, DDX3X-mediated *Zc3h12a*

mRNA stabilization was not affected in CIKS^{-/-} MEFs reconstituted with the CIKS mutant E17A unable to bind TRAF6 and to activate NF-κB. Similarly, the *Zc3h12a* mRNA stability was also retained in CIKS^{-/-} MEFs reconstituted with a CIKS mutant lacking the Ubox domain, unable to activate NF-κB (Suppl. Fig 2). On this basis, it is then possible to speculate that the CIKS-TRAF2-TRAF5 axis may activate different effector mechanisms, each regulating the stability of selected mRNA. The use of different machineries to stabilize different RNAs may reflect the different biological functions exerted by the different RNA products, CXCL1 being pro-inflammatory, and ZC3H12a being anti-inflammatory.

ZC3H12a (a.k.a. Regnase-1 or MCP-1) is a novel RNAase with CCCH-type zinc-finger domain expressed mainly by immune cells. It has been shown that *Zc3h12a* expression is rapidly induced either by LPS or by MCP-1, and it negatively regulates mRNA stability of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (42). It is also able to de-ubiquitinate TRAF2, TRAF3 and TRAF6, and its over-expression can inhibit NF-κB and AP-1 activity (32). However, ZC3H12a^{-/-} macrophages are still able to induce NF-κB and AP-1 dependent genes upon LPS stimulation, thus suggesting that the major role of ZCH12a is to regulate the level of different cytokine genes by affecting their mRNA stability rather than regulate their expression via NF-κB or AP-1 function (43). At present it is still unknown whether ZC3H12a targets mRNA alone or in combination with other protein(s). Our data suggest that DDX3X might be one of these protein partners.

The increased half-life of *Zc3h12a* induced by DDX3X could also play a major role for the stabilization of different cytokine mRNAs induced by IL-17 to finely tune the inflammatory response. It has been reported that ZC3H12a is essential for the inhibition of unwanted T-cell mediated immune response and our data are in line with these observations (43). In fact, the lack of ZC3H12a stabilization in the DDX3X knocked-down MEF results in increased level of the pro-inflammatory cytokine IL6. In this regard it is worth noting that the relative abundance of *il6* mRNA does not change in DDX3X knockdown cells at early time point after IL-17 stimulation (fig. 2F), but it is greatly increased at later time point (8 hours after stimulation, fig 6B). This apparent discrepancy may be simply explained by the fact that the stability of *il6* mRNA is not directly regulated by DDX3X, rather it is regulated by ZC3H12a, that is, in turn, a direct target of the DDX3X-mediated mRNA stabilization activity. Then, the CIKS/DDX3X -mediated stabilization of *Zc3h12a* mRNA upon IL-17 stimulation might be part of a regulatory pathway involved in both innate and adaptive immunity to avoid sustained expression of pro-inflammatory cytokines aimed to suppress inflammatory response.

In brief, we report for the first time the association between CIKS and DDX3X, and we provide evidence that DDX3X mediates the stability of selected mRNA induced by IL-17. The possibility to manipulate this interaction may be an important point to control over activation of the immune response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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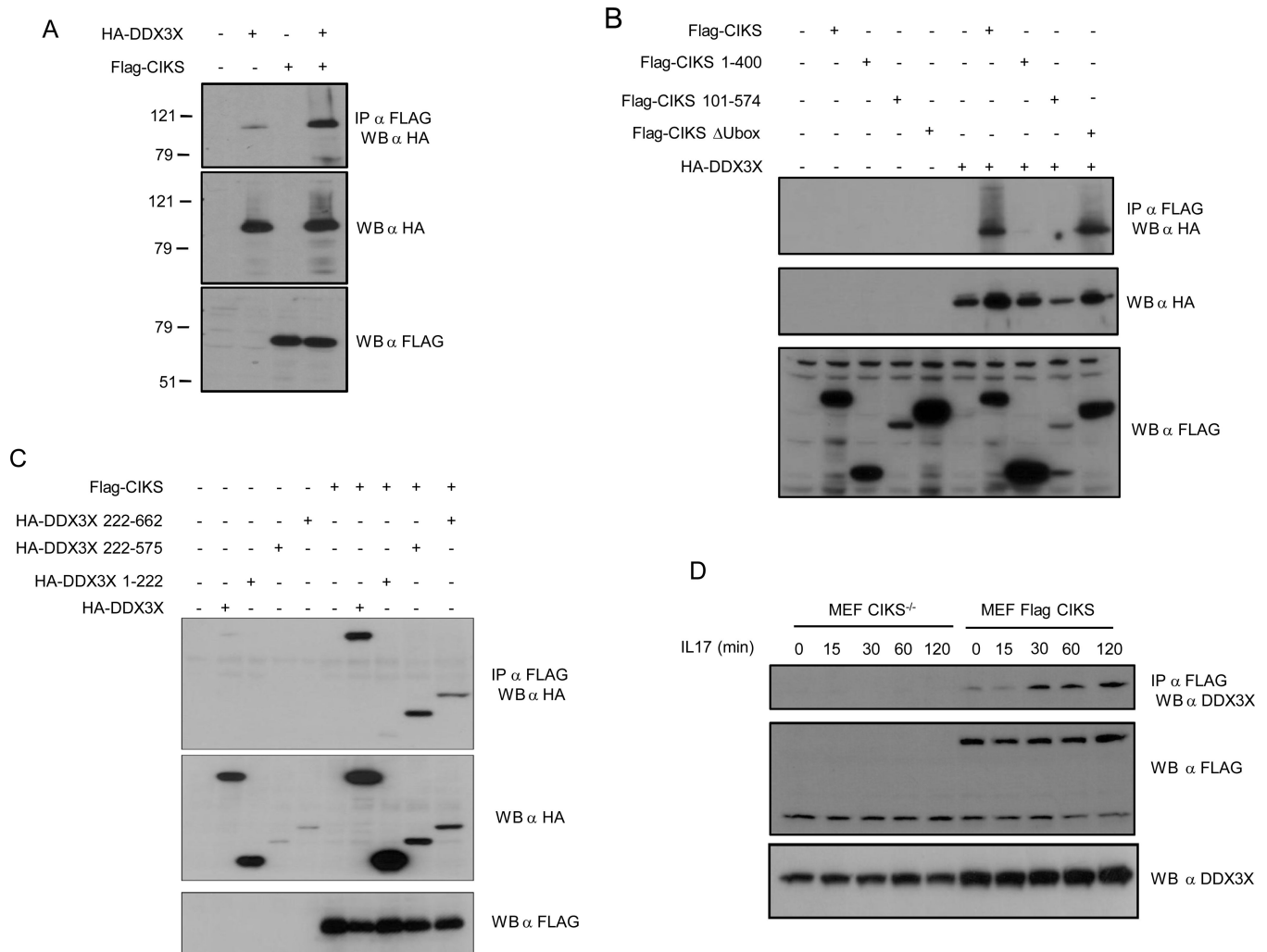


Figure 1. Physical interaction between CIKS and DDX3X

(A) Anti-FLAG immunoprecipitates (IP) of HEK293 cell extracts transfected with FLAG-CIKS and HA-DDX3X were Western-blotted with anti-HA antibodies to detect coimmunoprecipitated DDX3X (*Top panel*). (*Middle and Bottom panels*) Western blots with anti-HA (anti-DDX3X) and anti-FLAG (anti-CIKS) antibodies on whole-cell extracts are shown, respectively. (B) Mapping of the DDX3X-binding domain on CIKS. HEK293 cells were transfected with HA-DDX3X and FLAG-CIKS or different FLAG-CIKS deletion mutants. Cell extracts were immunoprecipitated with anti-FLAG antibodies (CIKS) followed by Western blot (WB) anti-HA (DDX3X). The presence of -HA and -FLAG proteins in the whole cell extract is shown in the middle and bottom panels, respectively. (C) Mapping the CIKS-binding domain on DDX3X. HEK293 cells were transfected with FLAG-CIKS and HA-DDX3X or different HA-DDX3X deletion mutants. Cell extracts were immunoprecipitated with anti-FLAG antibodies (CIKS) followed by Western blot (WB) anti-HA (DDX3X). The presence of -HA and -FLAG proteins in the whole cell extract is shown in the middle and bottom panels, respectively. (D) Interaction between CIKS and endogenous DDX3X. CIKS^{-/-} and reconstituted FLAG-CIKS MEFs, were treated for the

indicated period of time with IL-17 (200ng/ml). Cell extracts were immunoprecipitated with anti-FLAG antibodies and Western blotted (WB) with anti-DDX3X antibodies.

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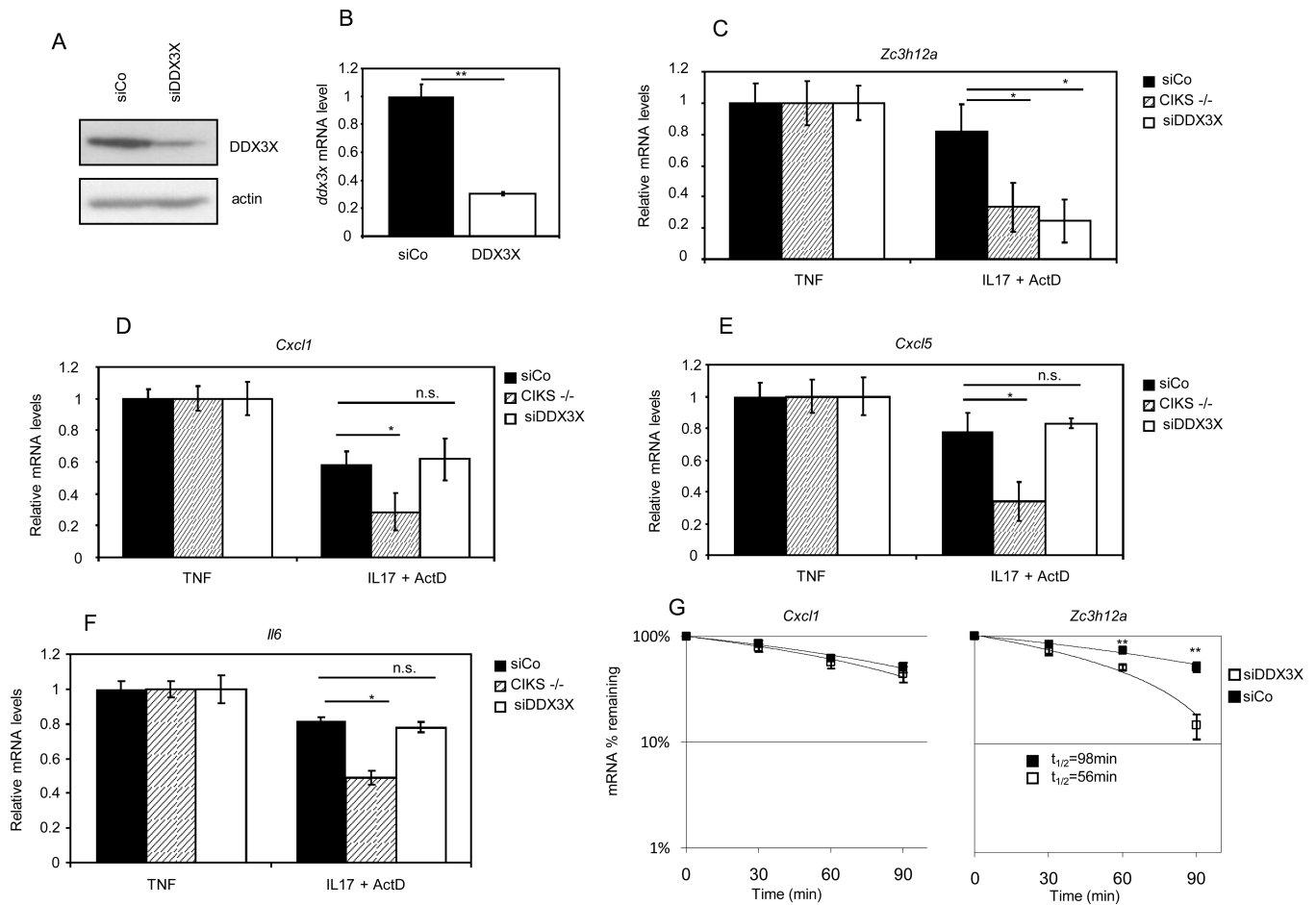


Figure 2. DDX3X controls the half-life of the *Zc3h12a* mRNA

Control MEF (siCo), DDX3X-depleted MEF (siDDX3X) and CIKS^{-/-} MEF were treated with TNF- α (2000U/ml) for 60 minutes, and then with IL-17 (200ng/ml) and actinomycin D (5 μ g/ml). After additional 90 min RNA was extracted and the abundance of the *Zc3h12a* (C), *Cxcl1* (D), *Cxcl5* (E), *Il-6* (F) was measured by Real-Time PCR. (G) Time course analysis of *Cxcl1* and *Zc3h12a* abundance after treatment with TNF- α (60 minutes) followed by the combined addition of IL-17 and actinomycin D. Expression of DDX3X protein (A) and mRNA (B) in control (siCo) and DDX3X interfered (siDDX3X) MEFs is shown. Data are representative of three independent experiments. Statistical analysis by unpaired Student's t-test: * $p < 0.05$, ** $p < 0.01$. N.S. not statistically significant.

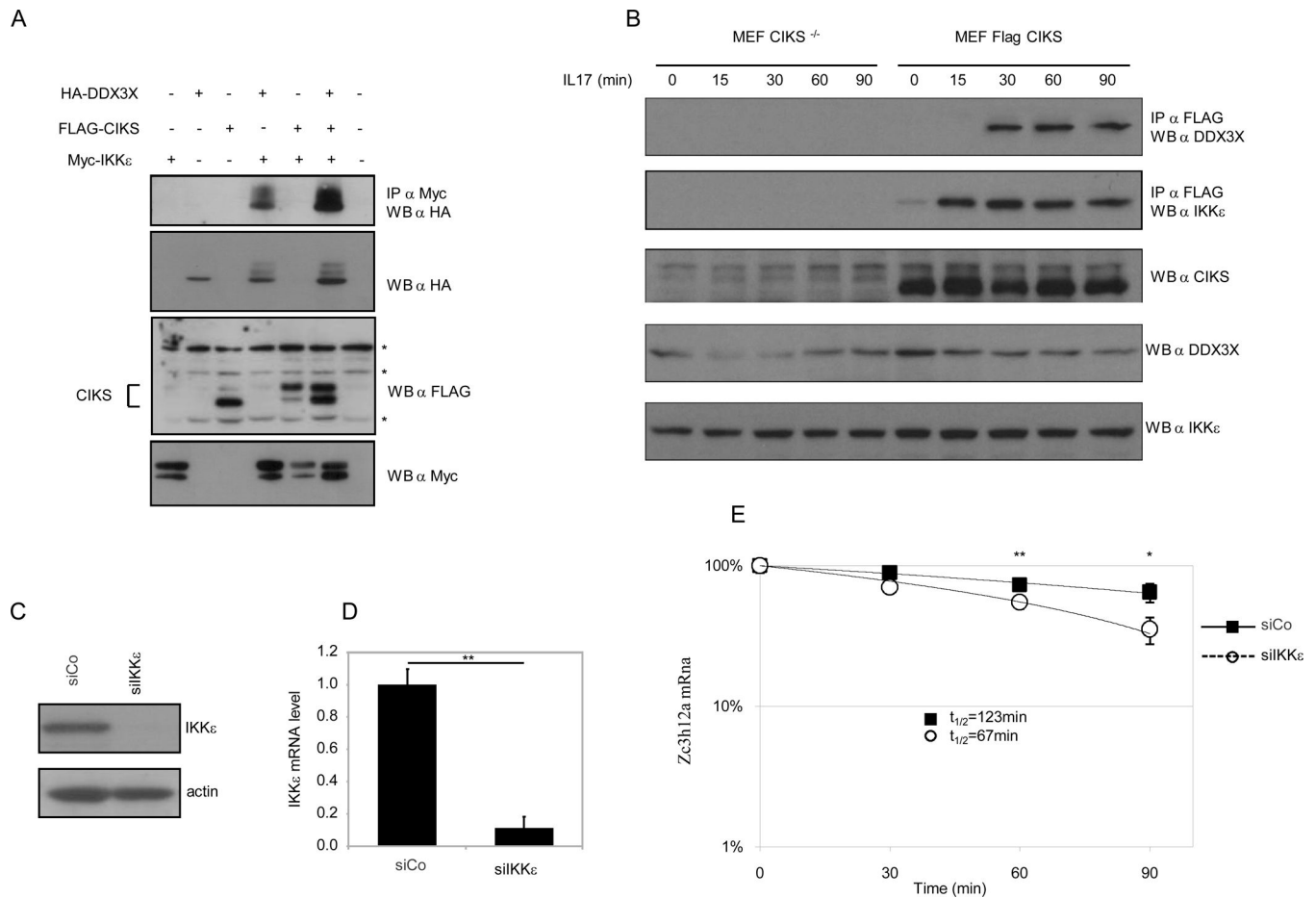


Figure 3. IKK ϵ interacts with DDX3X and controls the *Zc3h12a* mRNA stability

(A) Anti-Myc immunoprecipitates (IP) of HEK293 cell extracts transfected with Myc-IKK ϵ , FLAG-CIKS and HA-DDX3X were Western-blotted with anti-HA antibodies to detect coimmunoprecipitated DDX3X (*Top* panel). Western blots with anti-HA (DDX3X) and anti-FLAG (CIKS) and anti-Myc (IKK ϵ) antibodies on whole-cell extracts are shown. * Indicates non-specific cross-reactive bands.

(B) Interaction between CIKS and endogenous IKK ϵ and DDX3X. CIKS^{-/-} and reconstituted FLAG-CIKS MEFs, were treated for the indicated period of time with IL-17 (200ng/ml). Cell extracts were immunoprecipitated with anti-FLAG antibodies and Western blotted (WB) with anti-IKK ϵ and with anti-DDX3X antibodies. Expression of IKK ϵ protein (C) and mRNA (D) in control (siCo) and IKK ϵ -interfered (siIKK ϵ) MEFs. (E) *Zc3h12a* mRNA level in control (siCo) and IKK ϵ -interfered MEFs (siIKK ϵ), after treatment with TNF- α (60 minutes) followed by combined addition of IL-17 and actinomycin D. Data are representative of three independent experiments. Statistical analysis by unpaired Student's t-test: * p<0.05, ** p<0.01.

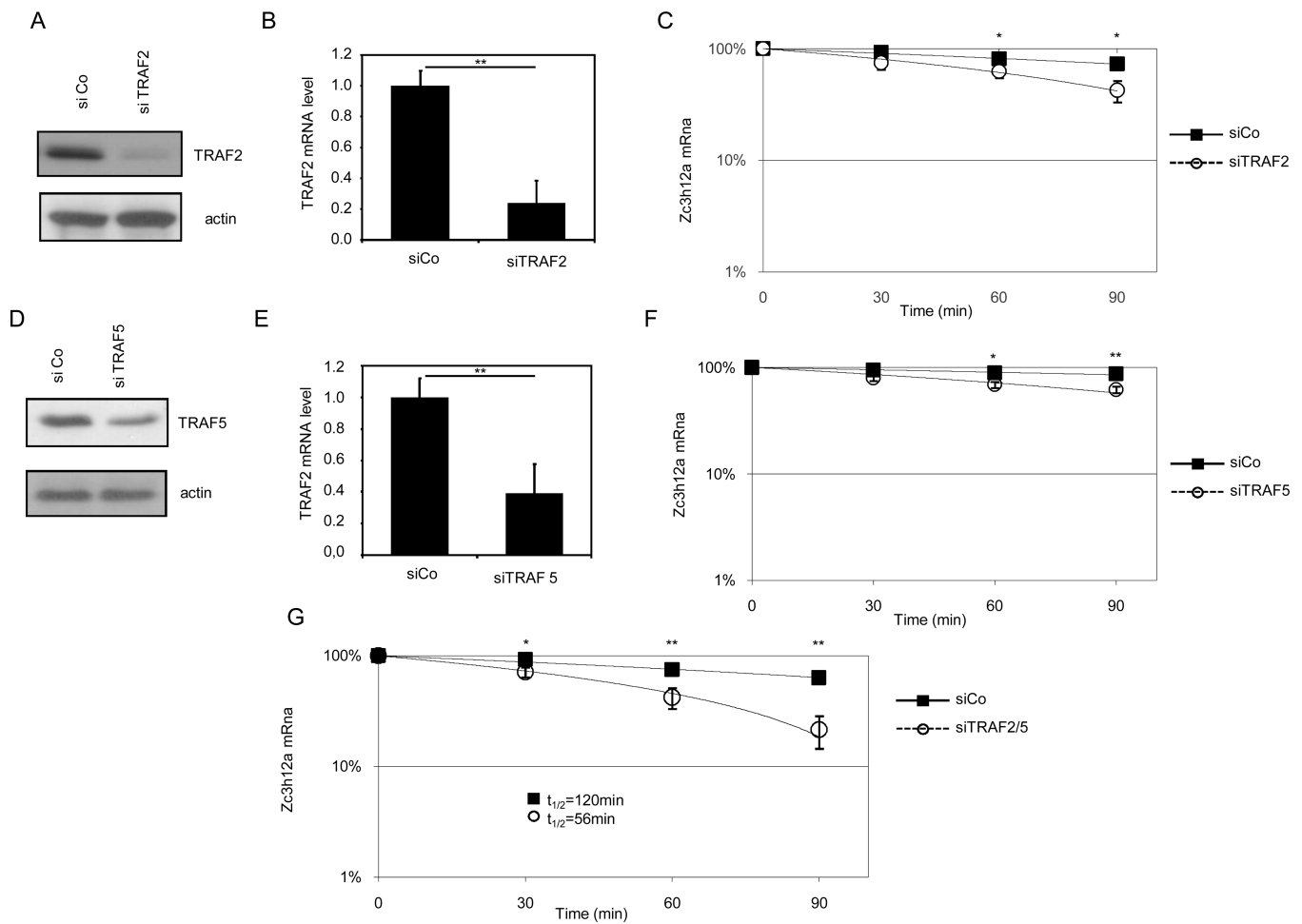


Figure 4. TRAF2 and TRAF5 are required for IL-17-induced stabilization of the *Zc3h12a* mRNA

Expression of TRAF2 (A, B) and TRAF5 (D, E) in control (siCo), TRAF2 (siTRAF2) and TRAF5 (siTRAF5) interfered MEFs. *Zc3h12a* mRNA level in control (siCo), TRAF2 (siTRAF2) (C), TRAF5 (siTRAF5) (F) and double interfered (siTRAF2/5) (G) MEFs, after treatment with TNF- α (60 minutes) followed by combined addition of IL-17 and actinomycin D for the indicated period of time. Data are representative of three independent experiments. Statistical analysis by unpaired Student's t-test: * p<0.05, ** p<0.01.

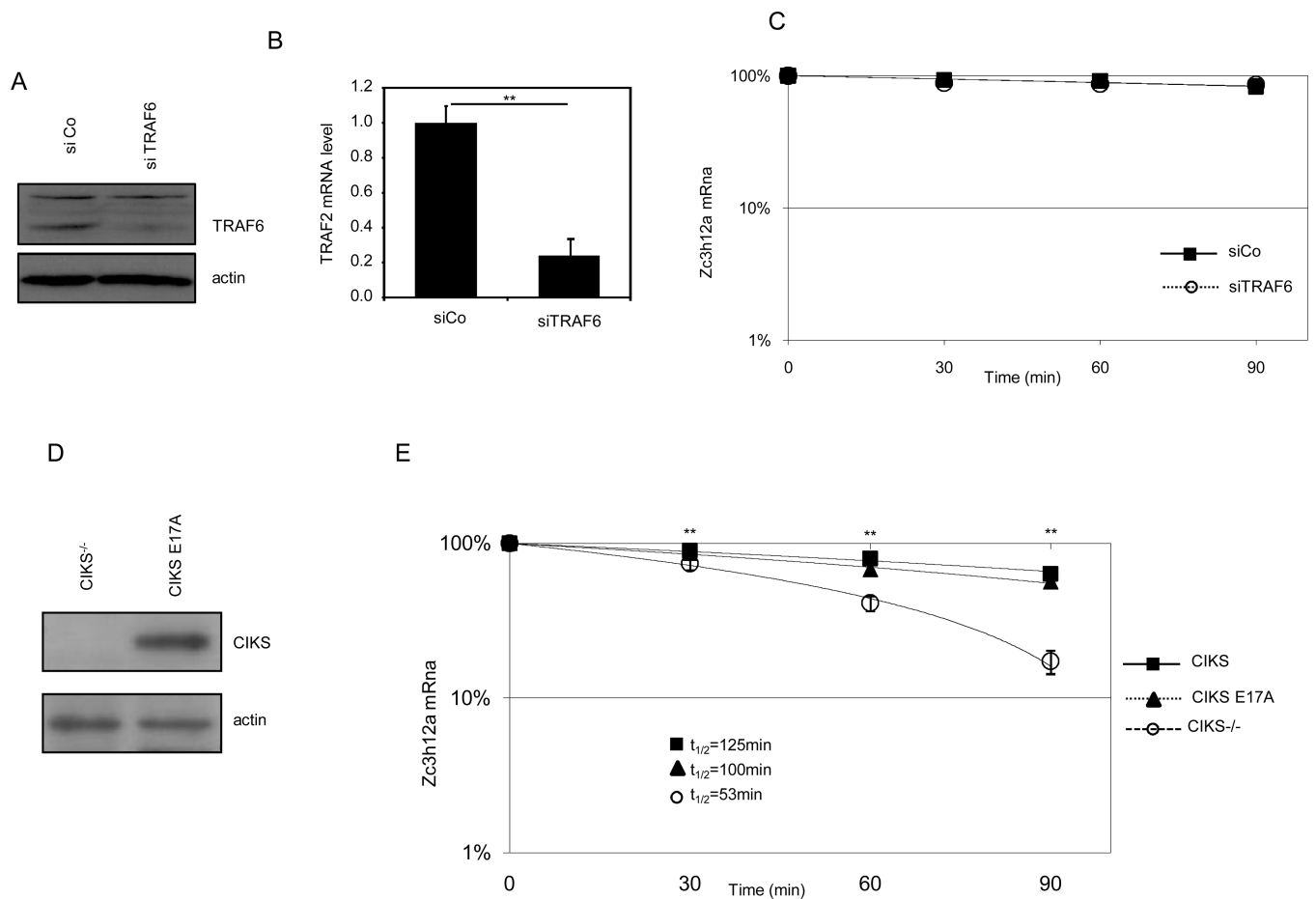
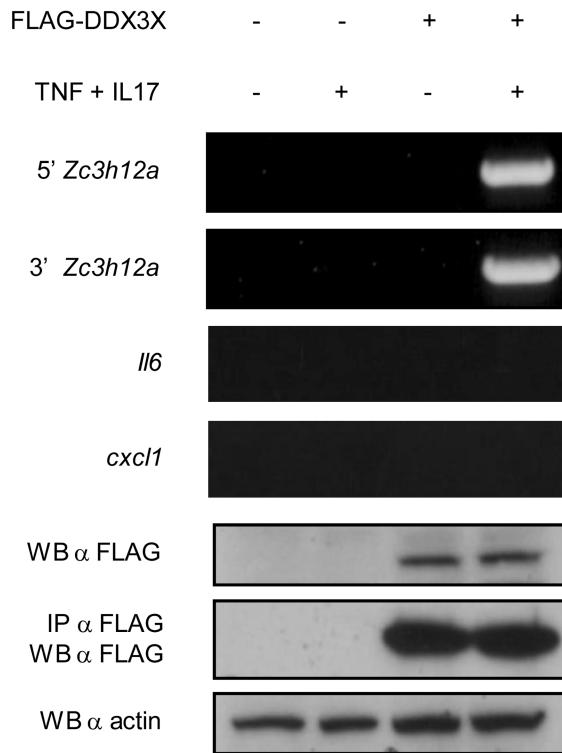


Figure 5. TRAF6 is not required for IL-17-induced stabilization of the *Zc3h12a* mRNA
 Expression of TRAF6 (**A**, **B**) in control (siCo), and TRAF6-interfered (siTRAF6) MEFs. (**C**) *Zc3h12a* mRNA level in control (siCo), and siTRAF6 MEFs, after treatment with TNF- α (60 minutes) followed by combined addition of IL-17 and actinomycin D. (**D**) Expression of CIKS E17A in CIKS^{-/-} MEFs reconstituted with CIKSE17A. (**E**) *Zc3h12a* mRNA level in CIKS^{-/-} MEFs, reconstituted with CIKS or CIKS E17A after treatment with TNF- α (60 minutes) followed by combined addition of IL-17 plus actinomycin D for the indicated period of time. Data are representative of three independent experiments. Statistical analysis by unpaired Student's t-test: ** p<0.01.

A



B

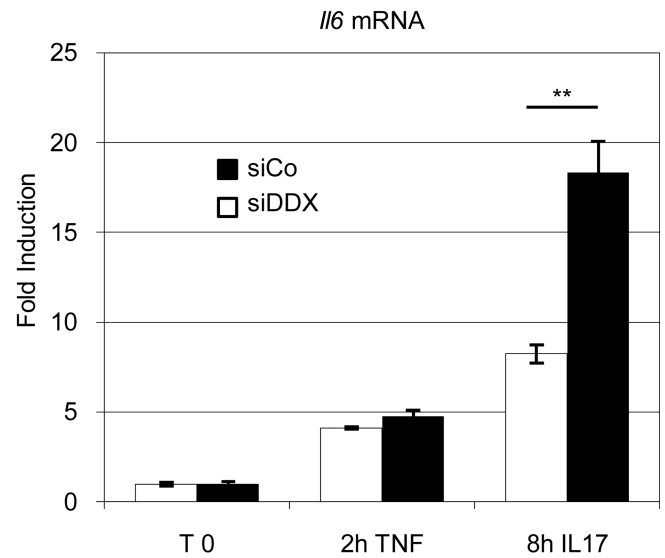


Figure 6. IL-17 modulates the binding of DDX3X to the *Zc3h12a* mRNA

A) HeLa cells were transfected with an expression vector encoding FLAG-DDX3X or with an empty vector. Twenty-four hours after transfection, cells were treated with TNF- α (2000U/ml) plus IL-17 (200 ng/ml) for two hours, and DDX3X was immunoprecipitated by using anti-FLAG antibodies. RNA was extracted from the immunoprecipitates, reverse transcribed, and *Zc3h12a*, *Il6* and *cxcl1* were amplified by PCR. The presence of transfected DDX3X in the whole cell lysate and in the immunoprecipitates is shown. **B)** *Il6* mRNA level in control (siCo) and DDX3X-interfered MEFs (siDDX) after treatment with TNF- α (2 hours) or TNF- α (2 hours) followed by IL-17 for additional 8 hours. Statistical analysis by unpaired Student's t-test ** p<0.01.