UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXII CYCLE

Dr. Michele Cillo

Adaptor protein CIKS is involved in STING-mediated antiviral innate immunity
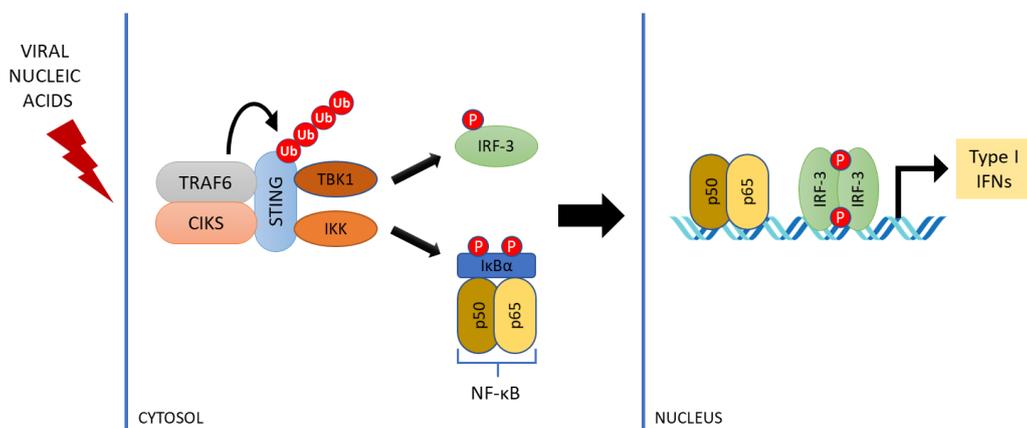
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Adaptor protein CIKS is involved in STING-mediated antiviral innate immunity

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS USED

CARD	Caspase activation and recruitment domain
cGAMP	2'-5'cyclic di-GMP/AMP
CIKS:	Connection to IKK and SAPK/JNK
IFN	Interferon
IP	Immunoprecipitation
IRF3	Interferon regulatory factor 3
MEFs	Mouse Embryonic Fibroblasts
NF-κB	Nuclear Factor kappa-B
NLR	NOD-like receptor
pA:T	poly-deoxyadenylic:thymidylic acid
PAMPS	pathogen-associated molecular patterns
pI:C	poly-inosinic:cytidylic acid
PRR	pattern recognition receptor
RLR	RIG-like receptor
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
TIR	Toll/interleukin-1 receptor
TLR	Toll-like Receptor
TRAF6	TNF receptor associated factor 6
TUBEs	Tandem Ubiquitin Binding Entities

ABSTRACT

Innate immunity plays a critical role in controlling the early stage of a viral infection and its spread into the organism. The efficacy of innate immunity relies on a set of germ-line encoded receptors, belonging to the family of pattern recognition receptors (PRRs), that can bind conserved features of pathogens, collectively called pathogen-associated molecular patterns (PAMPs). In the case of viruses, PAMPs are mainly represented by their nucleic acids. Their recognition by numerous PRRs activates NF-κB and IRF3 leading to the production and secretion of type I interferons (IFNs). By using as experimental model constituted by MEFs knock-out for CIKS, we show that the IL-17R adaptor protein CIKS is involved in this process.

CIKS$^{-/-}$ cells produce and secrete lower amount of IFNβ when challenged by nucleic acids that mimic viral DNA or RNA (pA:T and pI:C respectively). This phenotype is reverted when FLAG-CIKS expression is restored in CIKS$^{-/-}$ cells by lentiviral transduction. IFNβ reduction is not due to alterations in its mRNA stability, rather to a differential phosphorylation of IRF3 between wt and CIKS$^{-/-}$ cells. We also found that wt and ΔUbox CIKS, but not E17A mutant, interacts with Stimulator of interferon genes (STING) and influences its ubiquitination via TRAF6. Moreover, this interaction is a consequence of viral stimulation, especially after DNA treatment.

Here, we describe the role of CIKS in the STING-mediated antiviral signaling, its molecular interactors and the dynamics of this pathway.

INTRODUCTION

Viruses: structure and infection routes

Viruses represent the most abundant and the smallest microorganisms in the biosphere (Edwards and Rohwer, 2005; Kristensen *et al.*, 2010). In 1892, the Russian botanist Dmitri Iosifovich Ivanovsky isolated the first virus: Tobacco mosaic virus. Since then, more than 5500 different species of viruses have been classified [International Committee on Taxonomy of Viruses, ICTV 2018 report], and they can be found in virtually every ecosystem. All viruses are obligate intracellular parasites: they replicate only inside living cells and they can infect every known living organism, including other viruses (La Scola *et al.*, 2008).

Virus structure is relatively simple (Fig. 1). From inside to outside it is possible to find:

- The nucleic acid that contains all the genetic information needed to the production of all viral proteins. It can be either single or double stranded DNA or RNA. The Baltimore classification of viruses is based on their genome and identify seven classes (originally six) (Baltimore, 1971).
- A proteic shell called capsid. It is made up of one or few proteic subunits (capsomer) that multimerize conferring at the capsid a precise symmetry which allows a further characterization of viruses based on their symmetry: helical, icosahedral or complex (Lodish *et al.*, 2000). The gene(s) for the capsomers are encoded in the viral genome. The assembled capsid is directly or strictly connected to the genome of the virus. For this reason, the capsid is often called nucleocapsid. It has three main functions: I) to protect the genome from possible enzyme digestion (DNA/RNAases); II) to facilitates the interaction between the virion and the host cell; III) in certain class of viruses (Bacteriophages) the capsid forms an head-tail structure. The head contains the nucleic acid while the tail can attach the host cell and physically inject the viral genome inside the target (Taylor, van Raaij and Leiman, 2018).
- Some viruses (e.g. HIV, Influenza Virus etc.) have another layer called envelope (or pericapsid). It is a phospholipid bilayer that originates from the plasma membrane of the host cell during the process of viral budding. In the envelope there are present different proteins that may facilitate the process of infection. These proteins protrude from the membrane bilayer representing the most exterior part of an enveloped virus (Lucas, 2010).
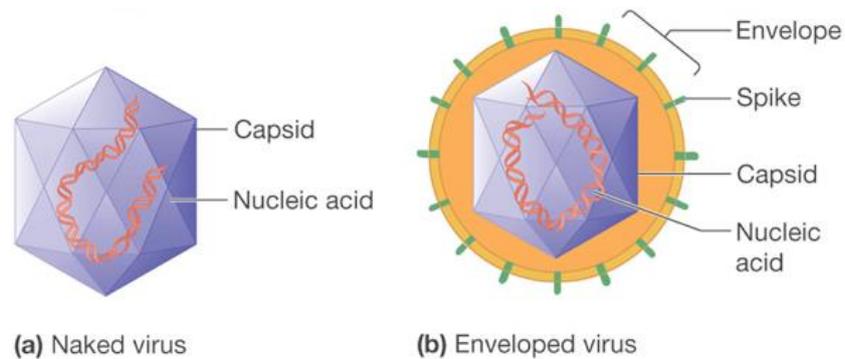
Figure 1: schematic representation of virus structure. (a) Naked (or non-enveloped) viruses are composed of a proteic "shell" which contains the viral genome; (b) Enveloped viruses have an external phospholipidic bilayer membrane with spike proteins (Willey, 2011).

Since all viruses are intracellular obligate parasites, they must enter inside a living cell to replicate. Depending on the nature of the viruses, they can infect cells via two routes: while non-enveloped viruses can infect only through receptor mediated endocytosis, enveloped viruses can infect cells also through direct fusion of membranes (Fig. 2) (Plemper, 2011).
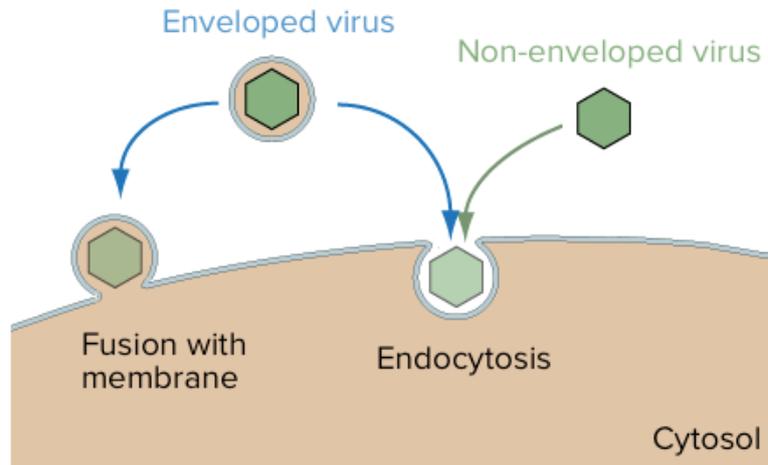
Figure 2: simplified depiction of the two viral routes of infection. Naked or non-enveloped viruses can infect cells only through the endosomal compartment. Enveloped viruses can also directly enter in the cytoplasm through membrane fusion.

Innate immunity and viral infections

The establishment of a viral infection is often a race between the virus and the immune system: the first one need to replicate itself as much as possible to spread the infection; the latter must identify the virus as a pathogen and eliminate it before it can harm the organism. For this reason, million years of evolution have selected several and redundant recognition systems, called

patterns recognition receptors (PRRs), for conserved motifs or molecules located upon and/or inside microorganisms. These are collectively named pathogen associated molecular patterns (PAMPs). A convenient classification of PRRs is based on their localization: cytoplasmic or membrane bound.

The first discovered and most famous category of PRRs is represented by the Toll-like Receptors (TLRs) (Fig. 3) (Medzhitov, Preston-Hurlburt and Janeway, 1997; Rock *et al.*, 1998; O'Neill, Golenbock and Bowie, 2013). To date, 10 different TLR proteins have been characterized in humans (13 in mouse) and all are transmembrane receptors located both on the plasma membrane and the endosomal compartment (Gay *et al.*, 2014). Structurally, the TLRs have a ligand binding domain made up of ~20 leucine-rich repeats (LRR), that folds in a characteristic solenoid-like structure. This, together with the multiple N-glycosylation, is responsible for the TLRs ligand biding specificity (Botos, Segal and Davies, 2011). The intracellular domain is called Toll/interleukin-1 receptor (TIR) homology domain and, by binding other TIR containing protein such as MyD88 and TRIF they start the signal cascade that culminate with the production of inflammatory cytokines (IL-1, IL-6, TNFα etc.) and/or interferons (Kawai and Akira, 2006). At resting state all TLRs are
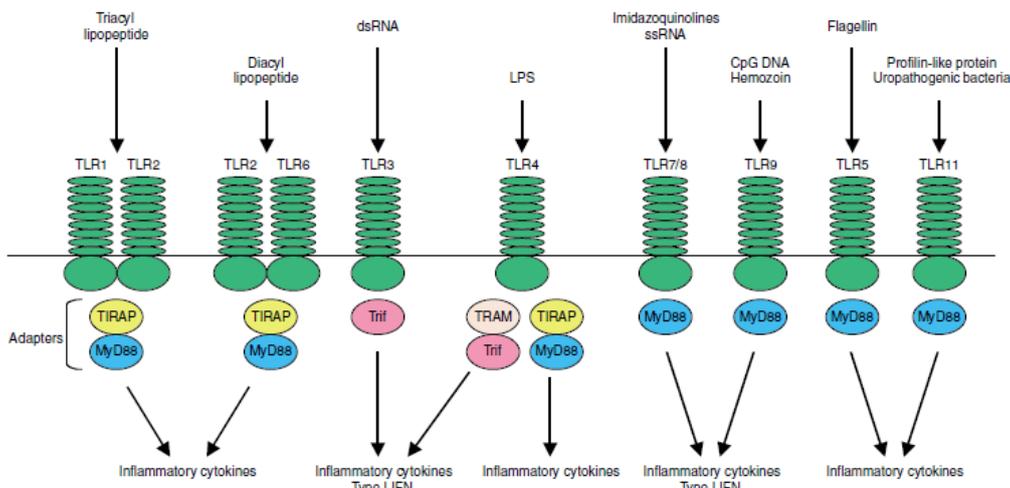
Figure 3: Toll-like receptors (TLRs) with their main ligands and adaptor molecules needed for signal transduction. TLRs are composed of several LLRs domain that forms the ligand biding region, a transmembrane domain, and the TIR domain always in the cytoplasm. All TLRs makes use of the adaptor molecule MyD88 to transduce signal, except for TLR3 that is exclusively TRIF dependent (Kawai and Akira, 2006).

monomeric proteins. After their engagement they homo- or hetero-dimerize enhancing their affinity for the ligand and, most important, generating a fully activated TIR domain. In fact, each monomeric TLR has only half of the complete TIR domain and, in this state, it is unable to transduce any signal. Differently, it has been demonstrated that endosomal located TLR8 and TLR9 are pre-assembled as inactive homodimer. Once engaged by ssRNA and CpG

rich DNA respectively, they undergo a conformational change that allows the activation of the signal transduction (Latz *et al.*, 2007; Tanji *et al.*, 2013). As for the signal transduction, all TLRs trigger a MyD88 dependent pathway, except for the TLR3 which is exclusively TRIF dependent (Yamamoto *et al.*, 2003).

TLRs sense a wide plethora of stimuli derived both from bacteria (LPS, flagellin, lipoproteins etc.) and viruses (viral nucleic acids). The distribution of TLRs reflects their function related to their ligands: TLR-1, -2, -4, -5, -6 and -10 recognize bacterial components and have their binding domain protruding in the extracellular space, therefore they are located on the plasma membrane; TLR-3, -7, -8 and -9 recognize nucleic acids (both viral and bacterial) and they are located in the endosomal compartment to protect cells from pathogens that take advantage of the endosomal entry route (Gay *et al.*, 2014). Interesting exceptions of this are TLR2, TLR4: although they are extracellular protruding, they can also recognize capsomer and spike proteins and efficiently activate the innate response (Oliveira-Nascimento, Massari and Wetzler, 2012; Olejnik, Hume and Mühlberger, 2018).

However, in the case of viruses PAMPs are mainly represented by their nucleic acids, but viral genome is exposed (hence detectable) only after the entry of the virus inside the cell, when the capsid is degraded. Even if the whole TLRs system is able to detect a broad spectrum of pathogens and activates an efficient innate immune response, none of them can detect free PAMPs inside the cytoplasm. To fill this potential and extremely dangerous gap in the innate immunity, evolution has selected a second class of PRRs that is composed of all the cytoplasmic receptors that bind non-self, as well as self-molecules, that signal for cellular damage (DAMPS) or incorrectly located molecules as nucleic acids which are physiologically not present inside the cytoplasm (dsRNA/DNA or 5'ppp RNAs) (Fig. 4) (Sui *et al.*, 2017). These receptors are further classified in sub-families:

1) Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) comprising RIG-I, MDA5 and LGP2. These proteins share 3 helicase domains, responsible for the ability of these receptors to bind RNAs, a C-Terminal domain (CTD) that act as a repressor domain to prevent autoactivation and, except for LGP2, two Caspase activation and recruitment domains (CARDs). In steady state, the CTD and CARD domains are kept in a "closed" conformation. Upon RNA binding to the CTD the CARD domains are no longer kept inactive and became exposed. Several RLRs can multimerize on a single RNA molecule, facilitating the downstream signaling mediated by the mitochondrial antiviral-signaling protein (MAVS) (Goubau, Deddouche and Reis e Sousa, 2013). In addition, numerous DExH/D-Box helicases have been proposed to be PRRs since they directly bind to dsRNA, but the

mechanism by which they are able to trigger the antiviral response is still not known (Tanner and Linder, 2001)

2) Nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs) are PRRs that recognize bacterial cell wall components or DAMPs. The structure of the ligand binding domain resembles the one of the TLRs composed of multiple LLR. The differences between TLRs and NLRs resides in the presence of a NACHT domain, responsible for the multimerization of NLRs, and in the transactivating domain that can be a CARD, PYR or BIR (Proell *et al.*, 2008; Shaw *et al.*, 2008). In the
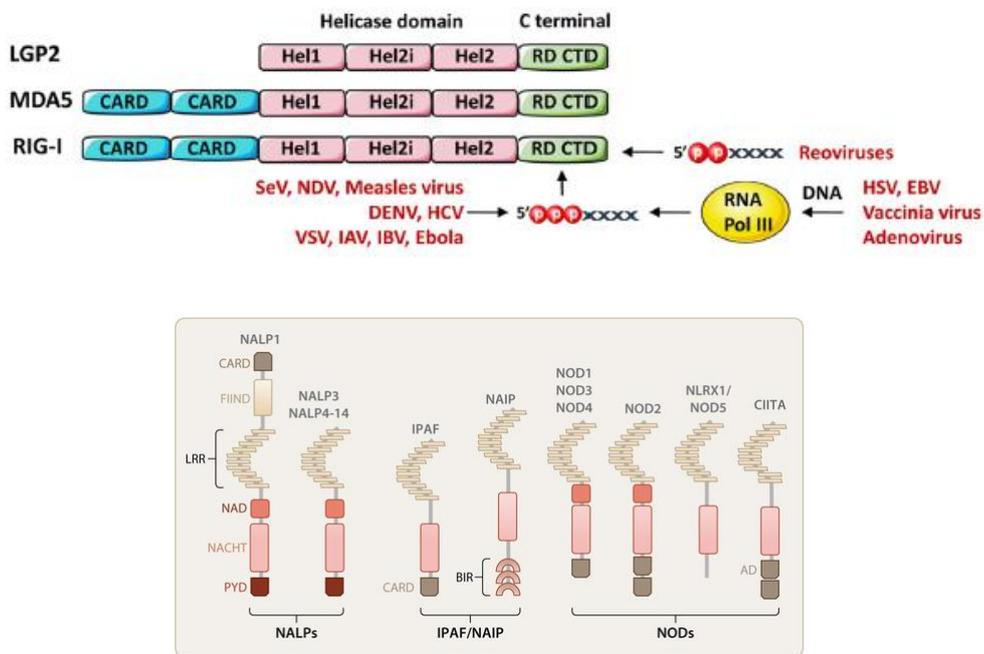
Figure 4: schematic illustration of RLRs (top panel) and NLRs (bottom panel) and their functional domains (Martinon, Mayor and Tschopp, 2009; Liu, Olagnier and Lin, 2017).

last decade, the role that certain NLR proteins like Nod2, NLRP1, NLRP3 play in the antiviral innate response has become clearer. Nod2 can bind viral RNA and induce Type I IFNs via its CARD domain. Other NLRs known to be involved in the innate antiviral response are NLRP1, NLRP3 and NLRC4 which form the so-called inflammasome. When activated it rapidly produce active IL-1β from pro-IL-1β via Caspase-1 (Kanneganti, 2010).

The cGAS-STING axis
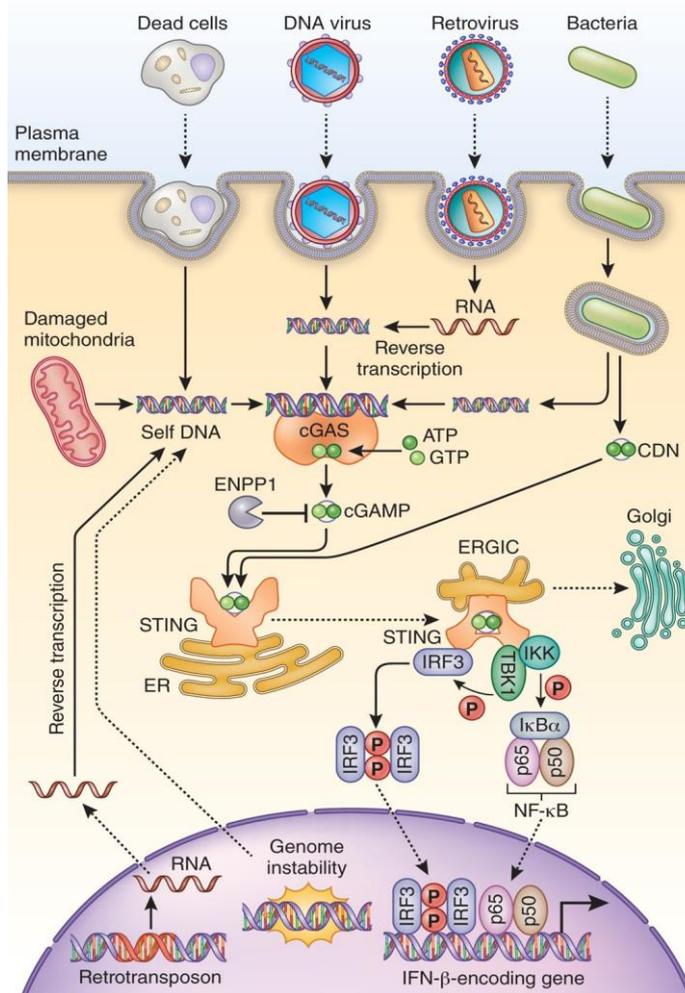
Figure 5: the cGAS-STING patway. Both cGAS and STING are PRRs triggered by dsDNA and CDN respectively. Activation of this pathway leads to the transcription of Type I IFNs driven by the activation of IRF3 and NK-κB transcription factors (Chen, Sun and Chen, 2016).

Among the PRRs that sense nucleic acids in the cytoplasm it is noteworthy the cGAS-STING axis (Fig. 5). This pathway is peculiar because the two proteins involved are both PRRs. cGAS (cyclic GMP-AMP synthase) senses dsDNA and produces the second messenger 2'-5'cyclic di-GMP/AMP (cGAMP), which is in turn one of the ligands for STING (Stimulator of interferon genes)(Ablasser et al., 2013; Xin Li et al., 2013; L. Sun et al., 2013). The latter is considered a PRR because it also binds several cyclic di-nucleotides (c-di-GMP/AMP or 3'-3'cGAMP) that can be produced directly by some pathogens, especially bacteria. Interestingly, it has been demonstrated that cGAMP is a transmembrane diffusible second messenger that can signal to adjacent bystander cells the danger signal derived from infected cells (Ablasser, Schmid-Burgk, *et al.*, 2013).

Under steady state conditions STING is located on the endoplasmic reticulum. Upon activation, it translocates to ER-Golgi intermediate compartment (ERGIC) and Golgi complex (Sun *et al.*, 2009). Here, the phosphorylation in Ser366 is essential for the binding of IRF3 and its subsequentially phosphorylation by TBK1 (Liu et al. 2015). Along with IRF3, another essential transcription factor that drives the induction of Type I IFNs is NF-κB. IKK is

recruited by STING upon CDNs binding and the canonical pathway of NF-B is triggered (Abe and Barber, 2014).

A critical aspect of this signal pathway is the tight regulation of STING by ubiquitination (Fig. 6). It is widely reported in literature that STING undergoes heavy ubiquitination. In general, K63-linked polyubiquitination by TRIM56 and TRIM32 are needed to activate STING trafficking and activation (Tsuchida *et al.*, 2010; Zhang *et al.*, 2012). K27 poly-ubiquitination by AMFR and INSIG1 are essential to allow the recruitment of TBK1 (Wang *et al.*, 2014). On the contrary, K48-linked polyubiquitination mediated by TRIM30α and RNF5 disrupt STING dimerization and attenuates the antiviral response addressing STING to proteasomal degradation (Zhong *et al.*, 2009; Wang *et al.*, 2015). Although the evidence that STING is substrate for different E3 ubiquitin ligases, the full "ubiquitin code" underlying STING fine and deep regulation has still to be decoded.

Figure 6: regulation of STING activity. STING undergoes several post translational modifications aimed both to enhance (green arrows) and suppress (red arrows) its activity (Sokolowska and Nowis, 2018).

Type I Interferons as the first-line molecules against viral infection spreading

IFNs were first described in 1957 as molecules secreted by cells that can "interfere" with viral replication, hence the name interferon (Isaacs and Lindemann, 1957). Since then, the importance of IFNs has become clear.

All IFNs are generally classified in 3 major groups according to the receptor they engage. Here, a brief description of these groups:

- **Type I IFNs**: In mammals several Type I IFN genes are present: IFN-α, IFN-β, IFN-κ, IFN-δ, IFN-ε, IFN-τ, IFN-ω, and IFN-ζ.

  In humans 17 members are known: 13 different IFN-α and IFN-β/ε/κ/ω. These IFNs bind to the heterodimeric receptor composed of IFNAR1 and IFNAR2 which are almost ubiquitous. All the genes are clustered-on chromosome 9 in a region of ~400 kb. The only exception is *IFNK* which is on the same chromosome but ~7Mb distant.

  Type I IFNs, especially IFN-α and IFN-β are the main antiviral IFNs;

- **Type II IFNs:** This class comprises only one member that is IFN-γ, which gene is located on chromosome 12. It is mainly produced by lymphocyte and it is a strong activator of macrophages. It binds to the receptor composed of IFN-γR1 and IFN-γR2 subunits;

- **Type III IFNs:** The recently described, IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ 3 (IL-28B) and IFN-λ4. They bind the type III IFN-specific receptor composed by the heterodimerization of IL-28RA and IL-10RB. The genes coding for lambda IFNs are located on chromosome 19 and differently from Type I IFNs they have introns. Their activity, as well as the activated pathway, seems to be similar respect to the Type I IFNs but their receptor is expressed mainly on epithelial-origin cells. (Hemann 2017; Kotenko 2011).

The critical role of IFNs, especially Type I IFNs, is confirmed by evolutionary studies. Nowadays, it is well established that the genes encoding for IFNs appeared early in the evolution. To date, no eukaryote lacks the gene encoding for IFN-β and at least two for IFN-α (Stetson and Medzhitov, 2006).
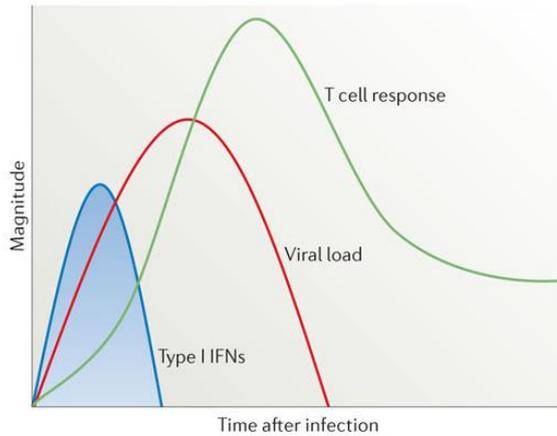
Figure 7: kinetics of antiviral response. At early stages of viral infections, the early response of the innate immune system is the production of Type I IFNs. This gives time to the adaptive immunity to efficiently mount a pathogen-specific response (Crouse, Kalinke and Oxenius, 2015).

All Type I IFNs are intronless genes. This is probably due to the production kinetics of these class of molecules (Fig. 7). They must be produced very quickly by the infected cells to limit as long as possible the diffusion of the virus, while giving time to the adaptative immunity to mount a pathogen-specific response which is slower compared to the innate response, especially in the case of the first exposition to the pathogen. In this context, it must be noted that Type I IFN are not directly cytotoxic towards the pathogens. On the contrary, their function is to signal in an autocrine and paracrine manner that a virus managed to breach the first line defenses of the organism.
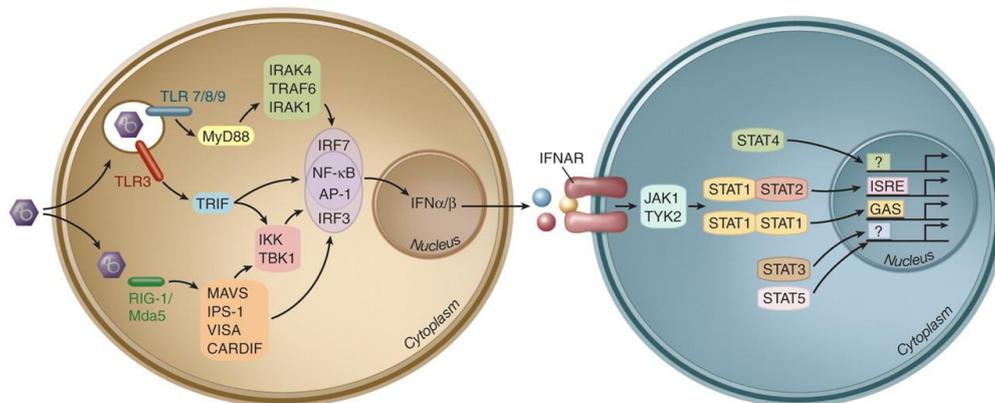
Figure 8: production and effects of Type I IFNs. Infected cells can detect viral nucleic acids both in the cytoplasm and in the endosomal compartment through the PRRs system. The pathways activated converge in the phosphorylation of several transcription factors such as IRF3/7, NF-κB and AP-1, which in turn drive the transcription of Type I IFNs. Once secreted, these proteins bind IFNAR, eventually leading to the transcriptional activation of hundreds of genes to induce the "antiviral-state" (García-Sastre and Biron, 2006).

When a virus managed to infect a cell, exposed viral nucleic acids are sensed by TLR or cytosolic sensors (previously described), and the antiviral signaling is triggered either by IKK, TRIF, IRAK1/4 and TRAF6. This, in turn, leads to the phosphorylation of several transcription factors, in particular one Type I IFNs specific inducer which belongs to the interferon-regulatory factor (IRF)

family: IRF3 (Honda, Takaoka and Taniguchi, 2006). After their production, Type I IFNs are secreted in the extracellular space where they bind IFNAR located upon neighborhood uninfected cells. This triggers the JAK-STAT signal pathway and transcriptionally activates hundreds of interferon-stimulated genes (ISGs) that eventually induce the so-called "antiviral state" (Fig. 8) (García-Sastre and Biron, 2006) . This represent the condition in which a healthy cell degrades mRNAs through the activation of RNAse L, and inhibits their translation by phosphorylating the eukaryotic Initiation Factor 2α (eIF-2α) mediated by PKR, to prevent, or at least limit, the spread of the viral infection (Fig. 9).
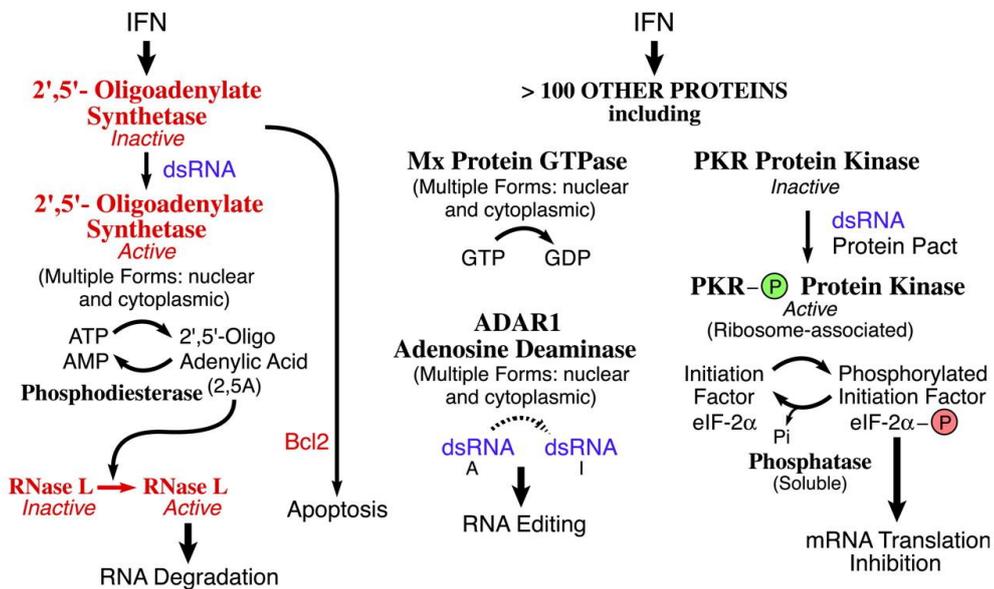
Figure 9: the antiviral state. Type I IFNs activates the transcription of several genes involved in the limitation of viral spreading. Among these: 2'-5' Oligoadenylatate synthetase which eventually leads to the activation of RNAse L and consequently the mRNA degradation; protein kinase R (PKR) which, by phosphorylating the eucariotic Initiator Factor 2α (eIF-2α), blocks mRNA translation (Samuel, 2002).

CIKS: Connection to IKK and SAPK/JNK

The adaptor protein CIKS, also known as ACT1 or TRAF3IP2, was first identified as a NEMO/IKKγ interactor (Leonardi *et al.*, 2000; Li *et al.*, 2000). It is a 574-aa protein composed of different domains (Fig. 10):

- two TRAF-binding regions (aa 35-42 and 333-337);
- a putative helix-loop-helix domain (aa 135-190);
- the central Ubox domain (aa 274-338), that is responsible for the only known catalytic activity of CIKS, which can act as an E3-ubiquitin ligase (Liu *et al.*, 2009);
- the SEFIR domain (Similar Expression to Fibroblast growth factor genes and IL-17R) (aa 409-550), located at the C-terminus;
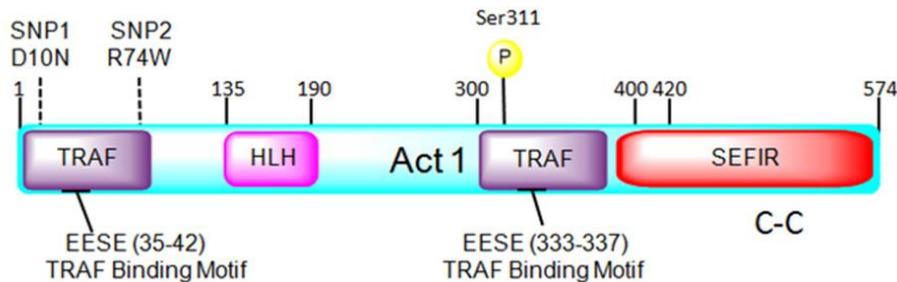
Figure 10: CIKS structure and domains organization (Doyle et al., 2012).

The SEFIR domain is particularly relevant because it mediates homotypic interactions among same family members, which comprises IL-17 receptor chains (Novatchkova *et al.*, 2003). In fact, CIKS is the fundamental adaptor in the IL-17 signaling (Seon, Park and Dong, 2006). Upon stimulation, CIKS is recruited to the cytoplasmic tail of IL-17 through the SEFIR-SEFIR dimerization and it can trigger a TRAF6-dependent or independent pathway (Fig. 11).

The first one involves CIKS catalytic activity: TRAF6 is ubiquitinated in a non-degradative way by CIKS (Liu *et al.*, 2009). This lead, in turn, to the recruitment of TAB-TAK1, the activation of the IKK complex and then the canonical pathway of NF-kB promoting the transcriptional activation of several cytokines and chemokines (Kanamori *et al.*, 2002).

The second one is a pathway of mRNA stabilization that begins with the phosphorylation of CIKS in Ser311 mediated by IKKi and followed by the recruitment of TRAF2 and TRAF5 (Hartupee *et al.*, 2007, 2009). Together, they can enhance the stability of several mRNAs, among which different pro-inflammatory cytokines as for example CXCL1(Bulek *et al.*, 2011; Somma *et al.*, 2015). This also occurs via the recruitment of the splicing factor 2 (SF2/ASF), mediated by TRAF2 and TRAF5, which binds to the 3' UTR of several mRNA enhancing their stability (Sun *et al.*, 2011).
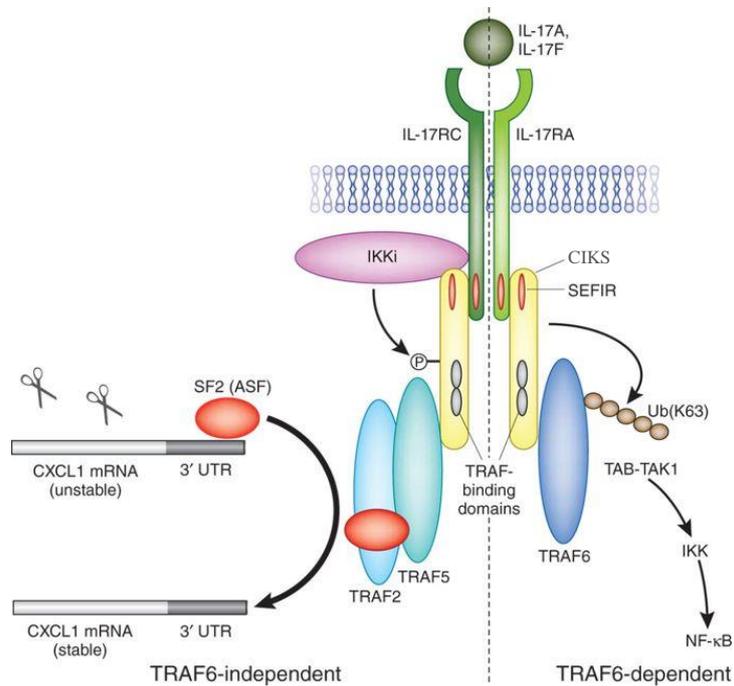
Figure 11: IL-17 signaling. IL-17 receptor engagement can trigger two distinct signals. The TRAF6- dependent (or transcriptional) that activate canonical NF-kB, and the TRAF6-independent signal which is mainly an mRNA stabilization mechanism (May, 2011).

Recently, a possible involvement of CIKS in antiviral response has been proposed. Human fibroblasts silenced for CIKS are less responsive to viral RNA stimuli. CIKS binds IRF3 while deletion mutants of CIKS reduce this interaction. In addition, silenced fibroblasts show a reduced phosphorylation of IRF3 after stimulation with viral RNA mock (Ryzhakov *et al.*, 2012).
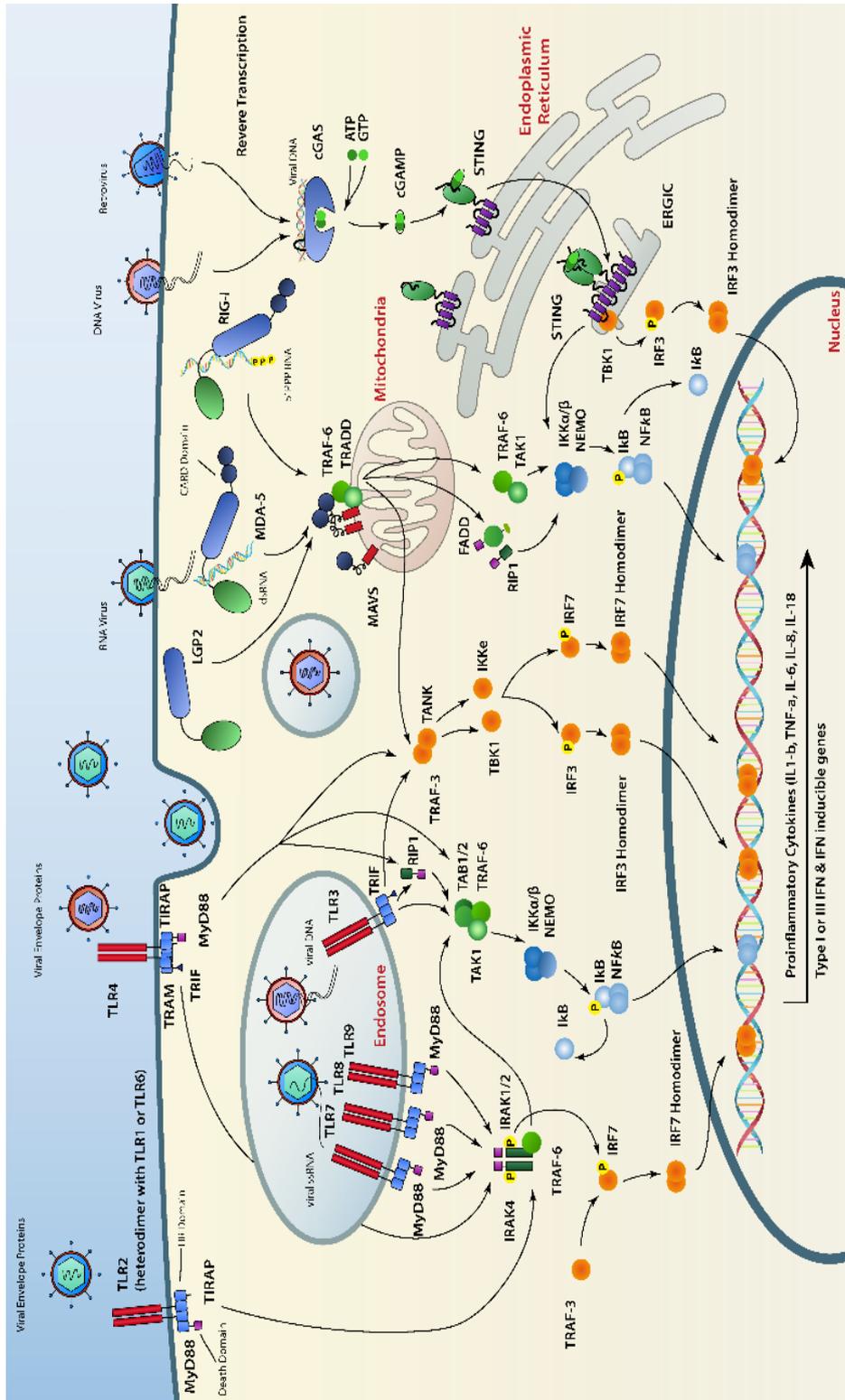
Figure 12: *overview of the viral routes of infection, the complex and redundant PRRs system dedicated to viral sensing and the responses triggered by their engagement. (Majzoub, Wrensch and Baumert, 2019)*

AIM

To date, very little is known about the involvement of CIKS in antiviral response. Previous literature (Ryzhakov 2012) has shown that human fibroblasts in which the expression of CIKS is reduced by RNAi, produce less IFNβ mRNAs when virally challenged by oligonucleotides that mimic an RNA viral infection (pI:C).

To evaluate a possible involvement of CIKS in the innate antiviral response, we used as experimental model composed by Mouse Embryonic Fibroblasts (MEFs) knock-out for CIKS and CIKS$^{-/-}$ cells reconstituted with FLAG-CIKS.

The aim of this study is to investigate the role that CIKS plays in the innate antiviral response along with the molecular mechanism and the dynamics of this pathway.

MATERIAL AND METHODS

Reagents and cells

Recombinant IL-17 and TNF-α were from PeproTech and were used at 200 ng/ml and 2000 U/ml, respectively. ANTI-FLAG ® M2 Affinity Gel, anti-FLAG and anti-Actin antibodies were from Sigma-Aldrich. Anti-HA was from SantaCruz. Anti-MycTag, anti-IRF3 and anti pIRF3 antibodies were from CellSignaling Technologies. Synthetic nucleic acids (pA:T and pI:C) were from Invivogen.

Anti-CIKS monoclonal antibody was produced in mouse using a recombinant peptide spanning the amino acids 382–574 of human CIKS.

Mouse embryonic fibroblasts (MEFs) and CIKS$^{-/-}$ MEFs were obtained as previously described (Claudio *et al.*, 2009). All cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich), 100 U/ml penicillin/streptomycin (Sigma) and 1 mM l-glutamine (Sigma), at 37°C and 5% CO_2 in fully humidified incubators.

Plasmids

Human CIKS, E17A point mutant, ΔUbox deletion mutant and human STING were cloned into pCDNA3.1 FLAG/HA-Tag vector as indicated in the figures. Human TBK1 was cloned in pCDNA3.1 Myc-Tag.

To produce fluorescent tagged proteins, cDNA encoding for mRUBY3 and mCLOVER3 were amplified from pKK-BI16-ORF1-3C-mRuby3_ORF2-TEV-mClover3 construct (Addgene #105802) and ligated in frame at the C-terminus of CIKS and STING respectively. Then, the construct obtained was subcloned in the transfer vector pLV-EF1a-IRES-Puro (Addgene #85132) used to generate lentiviral vectors. All constructs were Sanger sequenced to check both sequence and frame.

RNA extraction, cDNA synthesis, and quantitative PCR

Total RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription reactions were performed on at least 500 ng of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosistems). qRT-PCR was carried out using cDNAs reverse transcribed from total RNA by using SensiFAST SYBR® No-ROX Master Mix (Bioline), in LightCycler480 system (Roche). Experimental ΔΔCt values were normalized to Rplp0 housekeeping gene. Statistical analysis was performed using Student t test.

qRT-PCR primers used were:

CXCL1	FW	5'- AAC CGA AGT CAT AGC CAC AC - 3'
	REV	5'- TTG GGG ACA CCT TTT AGC ATC - 3'
IL-6	FW	5'- TCC TCT CTG CAA GAG ACT TCC - 3'
	REV	5'- TGA AGT CTC CTC TCC GGA CTT - 3'

IFNβ	FW	5'- ATG AGT GGT GGT TGC AGG C - 3'
	REV	5'-TGA CCT TTC AAA TGC AGT AGA TTC A - 3'
ISG15	FW	5'- AGC AAT GGC CTG GGA CCT AAA - 3'
	REV	5'- TCG CTG CAG TTC TGT ACC AC - 3'
RPLP0	FW	5'- GCT TTC TGG AGG GTG TCC G - 3'
	REV	5'- ACG CGC TTG TAC CCA TTG AT - 3'

Lentiviral vectors production and cells transduction
Lentiviral vectors were produced and concentrated as described by Kutner et al. (2009), with minor modifications. Briefly, a total of 7×10^6 HEK293T cells were seeded in 150mm-dishes 24 hours prior to transfection in 20 ml complete medium. A total of 42 μg of plasmid DNA was transfected in each dish: 7 μg of the envelope plasmid pMD2G, 14 μg of packaging plasmid pR8.91, and 21 μg of transfer vector plasmid. The medium was harvested after 24, 48 and 72 hours after the transfection, pooled and filtered using 0.45 μm PES filtration units. Viral particles were then subjected to PEG concentration.

Transfections, Western blot and Immunoprecipitation (IP)
All transfections were performed using Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. Cells were lysed in Triton X-100 lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA] supplemented with Complete Protease Inhibitor (Roche) and Phosphatase-Inhibitor-Mix II solution (Serva). IP were performed on equal amounts of proteins using 10 µl/sample of ANTI-FLAG ® M2 Affinity Gel (Sigma). The binding reaction was performed for at least 3 hours at 4°C with gentle rotation. IP where then washed 5 times with TBS-T 1x and eluted with SDS sample buffer [50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02 % bromophenol blue].

Tandem Ubiquitin Binding Entities (TUBEs) production
The pGEX5 vector encoding the GST-TUBEs construct was kindly provided by Dr. Domenico Somma. TUBEs were produced in BL-21 bacterial strain. After transformation, a single colony was growth in standard Luria-Bertani broth at $OD_{600} \sim 0.5$. Then expression of the construct was induced with IPTG (Sigma) for 2 hours. Cells were harvested and subjected to 3 cycles of freeze-thaw-sonication in lysis buffer [50mM Tris/HCl pH 8.5, 150 mM NaCl, 10 mM DTT] supplemented with complete protease inhibitors (Roche). Lysates were clarified by centrifugation at 7000g for 20 minutes and GST-TUBEs were purified using Glutathione-Agarose beads (Sigma) according to manufacturer's instructions. Purified TUBEs were then dialysed against the lysis buffer used for Western Blot experiments.

Ubiquitination assays

For ubiquitination analysis performed by double IP experiments, lysates were first precipitated under described conditions. The captured proteins were eluted with 100µl of 1% SDS and boiled at 95 °C for 5 minutes. Concentration of SDS was then reduced to 0.1 % by adding 900µl lysis buffer and all samples were subjected to a second round of IP.

For TUBEs-based ubiquitination analysis cells were lysed in the usual buffed supplemented with 200 µg/ml TUBEs. Equal amounts of proteins were subjected to Glutathione-Agarose beads pulldown overnight at 4°C. Pulldowns where then washed 5 times with TBS-T 1x, eluted with SDS sample buffer and analysed by SDS-PAGE.

Luciferase assays

For luciferase assays, cells were transfected with the Interferon Stimulated Response Element construct (ISRE-Luc) and the control vector coding for Renilla Luciferase (pRL-Luc), which allow a normalization based on DNA uptake. Cells were then treated as indicated and lysed in Luciferase Passive Buffer (Promega). Luminescence was detected using GloMax® system (Promega).

IFNβ quantization by ELISA assay

For IFNβ quantization in cell culture supernatants $5x10^4$ cells/well were seeded in 6-well plates. 24 hours after seeding cells were stimulated by transfection with pA:T or pI:C at 1000 ng/ml. 16 hour after stimulation cell culture supernatants were collected and analysed using Mouse IFNβ ELISA Kit (Elabscience).

Confocal microscopy

To obtain confocal images, fluorescent-tagged CIKS and STING expressing cells were seeded in 8-well chamber slides (Nunc). After 24 hours, cells were stimulated by transfection with pIC or pAT for the indicated time. Cells were then fixed with cold 4% paraformaldehyde and incubated for 5 minutes at room temperature. After 3 washes with PBS, fixed cells were counterstained with DAPI (0.125 µg/ml) for 5 minutes at room temperature. 50% Glycerol in PBS was used as mounting medium. All the procedures were performed in the dark. Images were acquired with the same settings using Zeiss LSM-710 confocal microscope and analysed with FIJI software.

Statistical analysis

Data are presented as means of at least three independent experiments with Standard Deviation (SD), or representative data are shown. Statistical analysis was performed using two-tailed paired Student t test.

RESULTS

Antiviral response is impaired in CIKS$^{-/-}$ fibroblasts

To date, very little is known about the involvement of CIKS in antiviral response. Previous literature has shown that cells in which the expression of CIKS is reduced by RNAi, produce less IFNβ and ISG15 mRNAs if challenged with poly-inosinic:cytidylic acid (pI:C), an oligonucleotide that mimic a viral dsRNA (Ryzhakov *et al.*, 2012). To evaluate a possible involvement of CIKS in the innate antiviral response, we used as experimental model Mouse Embryonic Fibroblasts (MEFs) knock-out for CIKS (CIKS$^{-/-}$) and CIKS$^{-/-}$ cells in which the expression of FLAG-CIKS is reconstituted by lentiviral transduction.
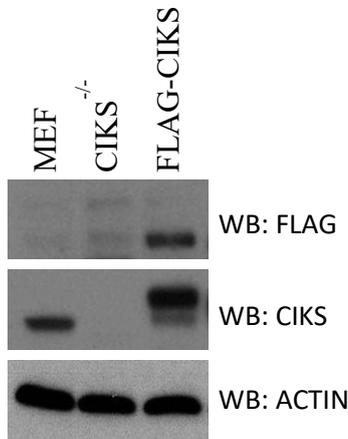
Figure 13: validation of the experimental system. Lysates from MEF, CIKS-/- and reconstituted cells were analysed by SDS-PAGE.

Firstly, to validate our model, we confirmed CIKS ablation and restitution by Western Blot analysis, both by using anti-CIKS and anti-FLAG antibodies (Fig. 13).

We also functionally tested the ability of reconstituted cells to respond to IL-17 and TNFα, alone or in combination (Fig. 14). MEFs physiologically respond to IL-17 by producing several cytokines and chemokines such as CXCL1 and IL-6. Due to the lack of CIKS, their production in CIKS$^{-/-}$ cells is impaired, while they efficiently respond to TNFα stimuli. In wt and FLAG-CIKS reconstituted cells, the response to IL-17 is comparable.
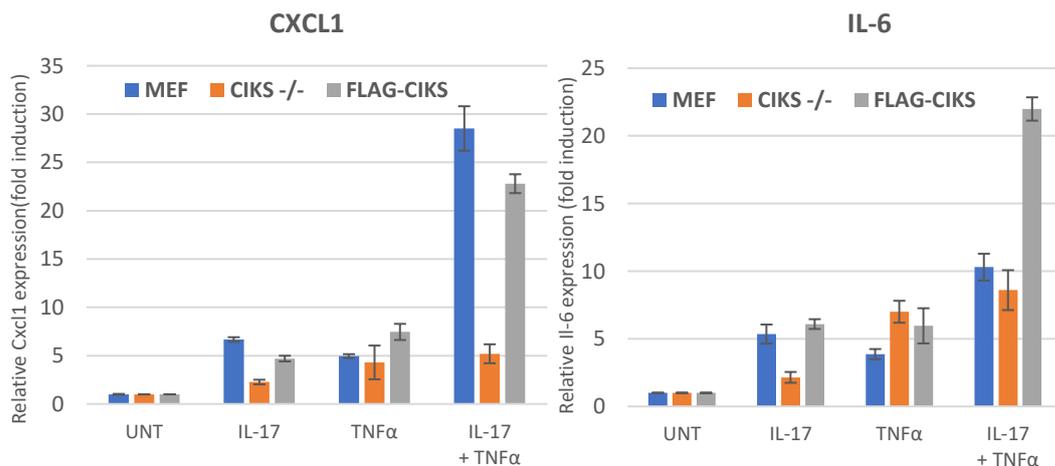
Figure 14: functional characterization of the experimental system. MEF, CIKS -/-and FLAG-CIKS reconstituted cells were stimulated with IL-17(200ng/ml) and TNFα (2000U/ml) alone or in combination for 6 hours. RNA was isolated and Cxcl1/Il-6 gene expression was assessed by qRT-PCR.

Then, we proceeded by challenging our system with pI:C to assess the capacity of these cells to react to RNA-based viral infections. We found, as reported in literature, that CIKS^{-/-} cells produce significantly less Ifnβ and Isg15 (a ubiquitin-like protein induced as a consequence of Type I IFNs stimulation (Perng and Lenschow, 2018)) mRNAs compared to MEFs. Interestingly, we
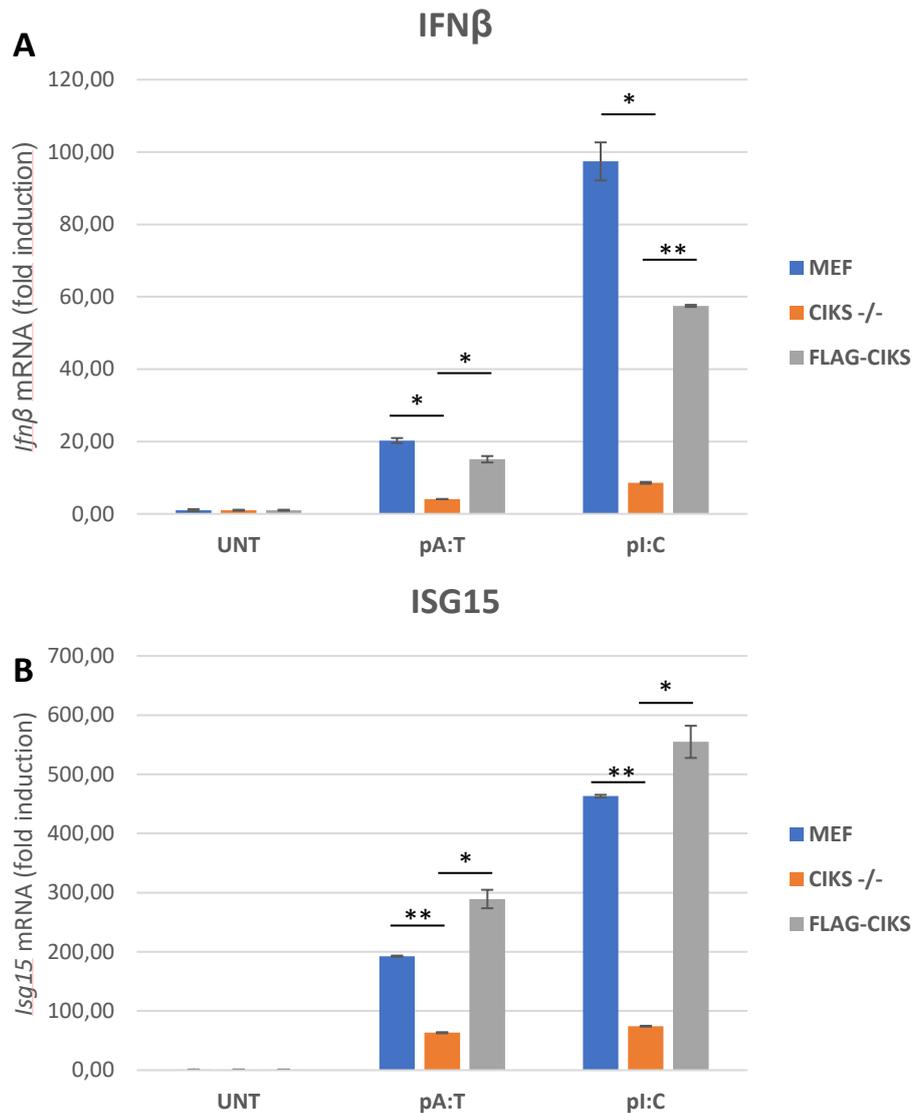
Figure 15: MEF, CIKS^{-/-} and reconstituted cells were stimulated either with pA:T or pI:C (1000ng/ml) for 4 hours.(A) *Ifnβ* and (B) *Isg15* relative expression were assessed by qRT-PCR. * $p<0.05$, **$p<0.001$

obtained analogous results by stimulating cells with poly-deoxyadenylic:thymidylic acid (pA:T), a synthetic analogue of B-DNA, that mimics a viral DNA genome (Fig. 15).

Since IFNβ is a secreted protein we also measured, by ELISA assay, this protein level in the media where cells were cultured. MEFs and CIKS^{-/-} were

stimulated overnight either with pA:T or pI:C, and media were used to quantify the amount of IFNβ secreted in the extracellular space. We found that the reduction of *Ifnβ* mRNA also reflects a reduction in IFNβ protein secretion in CIKS^{-/-} cells respect to wt cells, both after stimulation with pI:C and pA:T (Fig. 16).
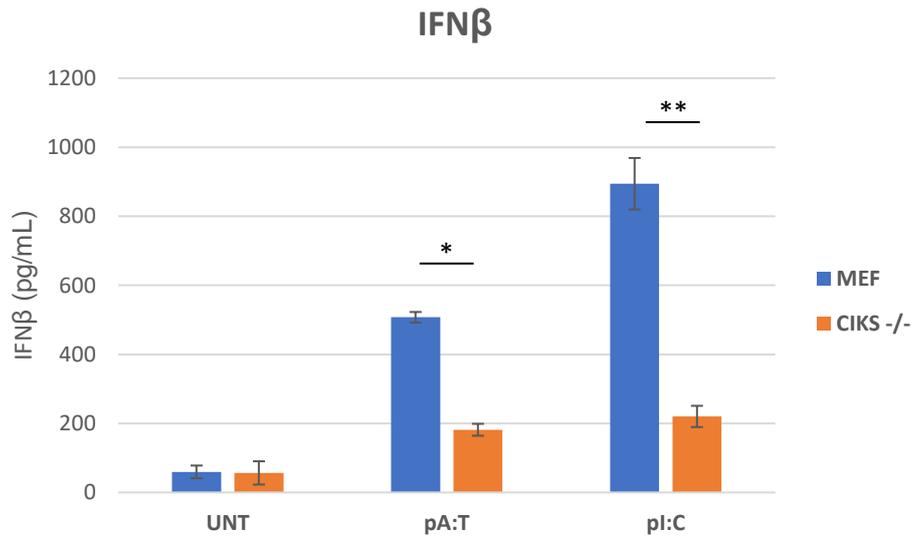
Figure 16: MEF and CIKS -/- cells were stimulated overnight with pA:T or pI:C (1000ng/ml). *Ifnb* levels were measured by ELISA essay. * p<0.05, ** p<0.001

IFN-β reduction is not due to altered mRNA stability
Given that CIKS may regulate both de novo transcription and messenger stability, we questioned if the reduction in both *Ifnβ* mRNA and protein was due to mRNA altered stability. On this basis, we performed mRNA decay assays, in which cells were challenged either with pI:C or pA:T for 4 hours. Then transcription was blocked by inhibiting RNA polymerase II with Actinomycin D (ActD) up to 120 minutes, and the level of IFN mRNA was measured by real time PCR at different time points. We found no significant differences between the decay rate of *Ifnβ* mRNA in MEF and CIKS^{-/-} cells, both by using pA:T or pI:C, suggesting that the reduction observed by qRT-PCR and ELISA assays is not due to its mRNA altered stability (Fig. 17). Therefore, since IFNβ mRNA decay rate was comparable between MEF and CIKS^{-/-} we speculated that the TRAF6-independent pathway (the one

controlling mRNA stability) is not involved in CIKS-mediated antiviral signaling.

A

B
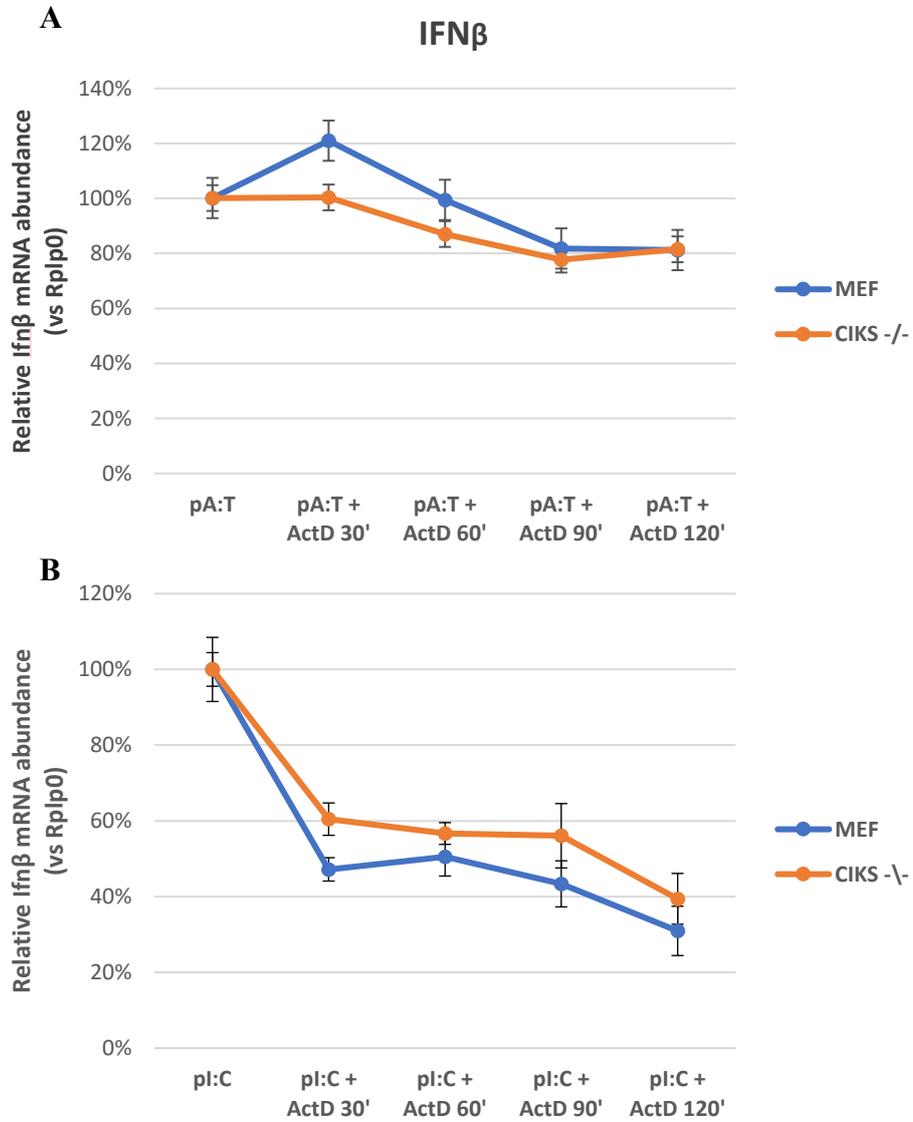
Figure 17: mRNA decay assays. Cells were stimulated with **(A)** pA:T or **(B)** pI:C at 1000 ng/ml. After 4 hours, de novo transcription was blocked with 5 µg/ml Actinomicin D (ActD) for 30, 60, 90 and 120 minutes. At the end of each time point total RNA was isolated and Ifnβ abundance was assessed by pRT-PCR.

CIKS is implicated in de novo transcription of IFN-β via IRF3 activation

IFN-β gene expression is controlled by transcription factors of the IRF family. In viral-infected cells, IRF3 is phosphorylated on Ser 386 at the C-terminus, allowing its dimerization and translocation into the nucleus where it can activate IFN-β transcription. To assess if CIKS could have a role in de novo transcription of type I IFNs we performed luciferase assay using an IFN-specific reporter (ISRE-Luc). We observed a significant increase of the reporter activity in MEFs cells when they were challenged with pA:T or pI:C, that was partially reduced in CIKS$^{-/-}$ cells (Fig. 18).
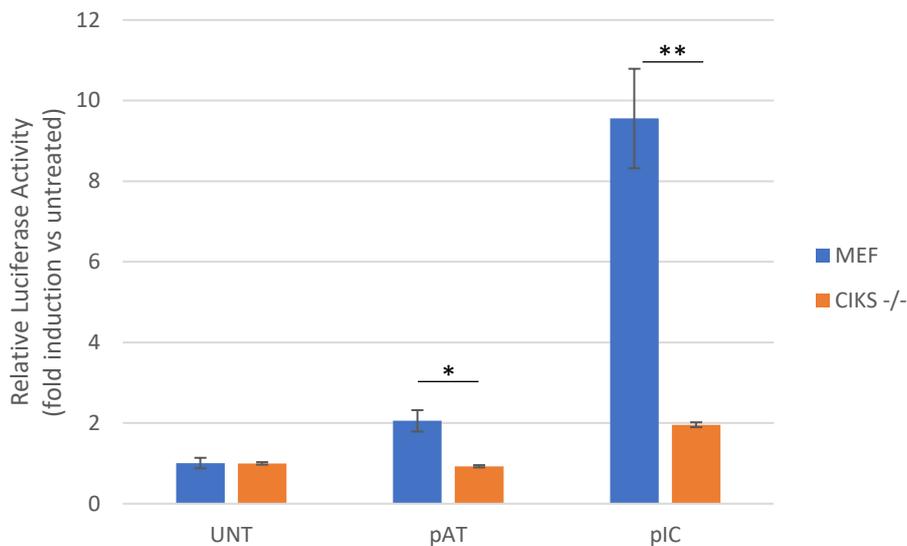
Figure 18: Luciferase assay. MEF and CIKS$^{-/-}$ cells were transfected with luciferase interferon reporter (ISRE-Luc) and control construct (pRL-Luc). 24 hours after transfection cells were stimulated with pA:T or pI:C, or left untreated. After 8 hours, cells were lysed, and luciferase activity was measured. All data are normalized on reporter constructs uptake. * p<0.05, **p<0.001

The triggering of IRF3 was also confirmed by time course Western blot analysis, which revealed a decreased and delayed phosphorylation of IRF3 in CIKS$^{-/-}$ cells compared to MEFs regardless of the stimulus was DNA or RNA (Fig. 19).
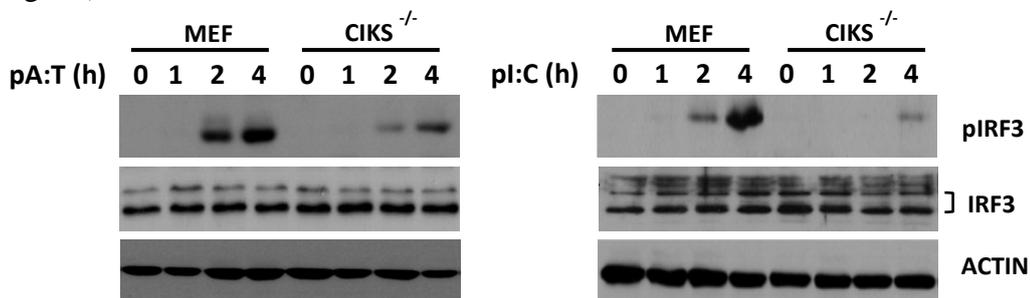
Figure 19: MEF and CIKS -/- cells were stimulated with pA:T or pI:C (1000 ng/ml) for 1, 2 and 4 hours. At the end of each time point, cells were lysed, and extracts were analysed by SDS-PAGE.

CIKS interacts with STING

One of the main pathways known to activate IRF3, allowing the production of IFN-β, is the cGAS-STING axis. STING binds atypical cyclic dinucleotides (CDNs) as 2'-3' cGAMP directly produced by pathogens or produced as second messengers by other PRRs, such as cGAS, following nucleic acid recognition.

By performing co-IP experiments in HEK293T cells, we found the protein STING as a strong interactor of CIKS, together with TBK1, the kinase that directly phosphorylate IRF3 enabling the transcription of Type I IFNs. We confirmed the physical interaction either by immunoprecipitating CIKS or STING (Fig. 20).

Considering this result, we decided to investigate the molecular aspect of this interaction by using two different CIKS mutants: the E17A point mutant which ablates one of the two TRAF6 binding domains and the deletion mutant ΔUbox (Δ274-338) which lacks the E3 ubiquitin ligase domain. Interestingly, CIKS ΔUbox mutant still strongly interacted with STING, suggesting that the E3 ubiquitin ligase domain was not involved, at least in the interaction between CIKS and STING. Strikingly, the overexpression of CIKS E17A mutant almost completely abolished the interaction, implying a possible involvement of TRAF6 in the process of interaction (Fig. 21).

Figure 20: CIKS interacts both with STING and TBK1. HEK293T cells were transfected as indicated and lysates were subjected to anti-FLAG IP. Upper panel: FLAG-STING IP; Lower panel: FLAG-CIKS IP.

Figure 21: CIKS-STING interation. HEK293T cells were transfected as indicated and lysates were subjected to IP using anti-FLAG conjugated agarose beads.

CIKS co-localize with STING following Poly I:C and Poly dA:dT stimulation

To better clarify the dynamics of CIKS-STING interaction we performed time course IP experiments in CIKS$^{-/-}$ and FLAG-CIKS reconstituted cells stimulated with pA:T or pI:C for the indicated time (Fig. 22).
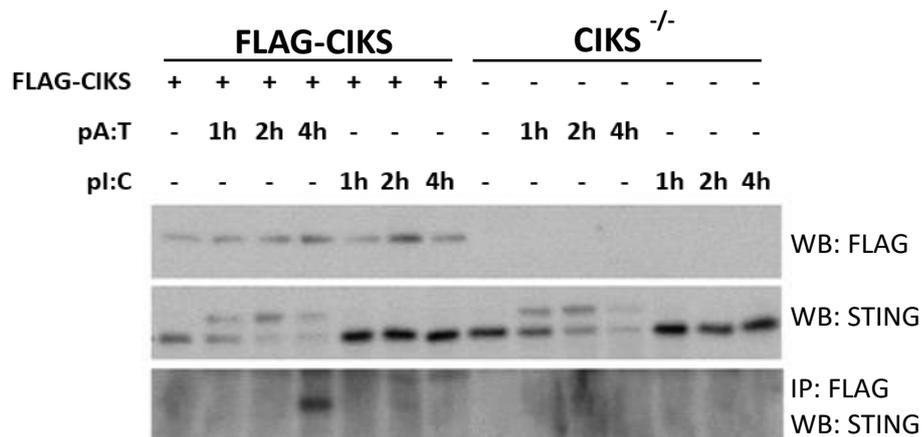
Figure 22: CIKS-STING interaction in stimulated cells. CIKS$^{-/-}$ and reconstituted cells were stimulated with pA:T or pI:C(1000ng/ml) up to 4 hours. Lysates were immunoprecipitated with anti-FLAG resin. Total inputs and IP were then analyzed by SDS-PAGE.

pA:T stimulation leaded to a rapid phosphorylation of STING in both CIKS$^{-/-}$ and reconstituted cells. The same phenomenon was not observable in pI:C

25

treated cells, independently from CIKS. In addition, by immunoprecipitating FLAG-CIKS we were able to detect an interaction between CIKS and STING in pA:T treated cells after 4 hours, suggesting that this was a consequence of pA:T treatment. Interestingly, this interaction was absent in pI:C treater cells.

To further investigate the dynamics of this interaction we also cloned CIKS and STING in frame with the fluorescent protein mRuby3 and mClover3 respectively. We then used pLV-EF1α-IRES-Puro as transfer vector to produce lentiviral constructs and we stably transduced CIKS$^{-/-}$ cells. The double fluorescent cells obtained were stimulated either with pAT or pIC for 4 hours and cells were analyzed by confocal microscopy (Fig. 23). As a result, untreated cells show a diffuse and mostly not localized signal both of CIKS and STING. After stimulation with synthetic oligonucleotides the STING and CIKS fluorescent signal accumulated in vesicle-like structures where they co-localize. This is particularly evident in pA:T treated cells.
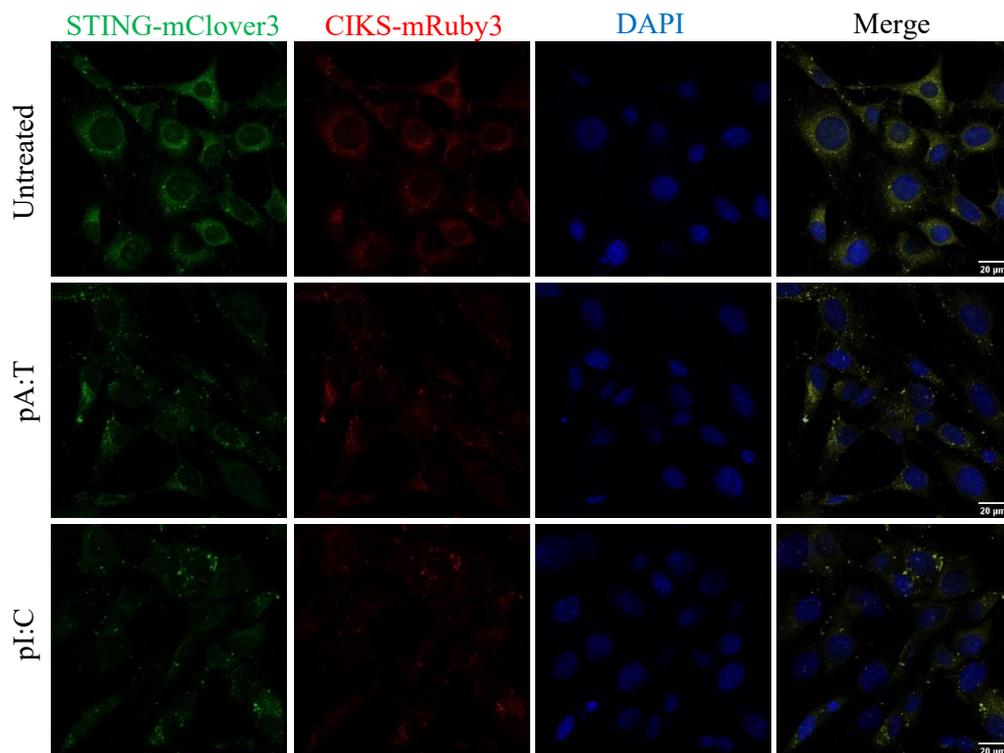
Figure 23: CIKS-STING co-localization by confocal microscopy. CIKS$^{-/-}$ cells were transduced with lentiviral vectors encoding for STING-mClover3 and CIKS-mRuby3. Cells were stimulated with pA:T or pI:C (1000ng/ml) for 4 hours, or left untreated, then fixed with 4% PFA and counterstained with DAPI. Scalebar is 20μm.

CIKS influences STING ubiquitination via TRAF6 activity

It is well known that STING undergoes heavy ubiquitination in order to regulate its function (Zhong *et al.*, 2009; Tsuchida *et al.*, 2010; Wang *et al.*, 2014, 2015; Ni, Konno and Barber, 2017). For this reason, we studied if CIKS could somehow modulate STING ubiquitination, also considering that the ectopic expression of both STING and wt/ΔUbox CIKS, but not E17A CIKS, leads to an increase of STING dimerization visible by Western Blot (see Fig. 28). In this context it should be considered that STING is active only in dimeric form (Sun *et al.*, 2009).

Double IP experiments allowed us to assess the ubiquitination status of STING without the interference of possible ubiquitination of other interacting proteins. As a result, when wt CIKS is exogenously expressed along with STING, the latter ubiquitination significantly increases. ΔUbox mutant behaves like the wt protein suggesting that the ubiquitin ligase domain is not directly responsible for STING ubiquitination. It is also possible to detect a CIKS-STING interaction even in the stringent condition of a double IP (Fig 24 left panel). On the contrary, the E17A mutant clearly decreases STING ubiquitination (Fig. 24 right panel). Overall, these results prompted us to investigate deeper the involvement of TRAF6.
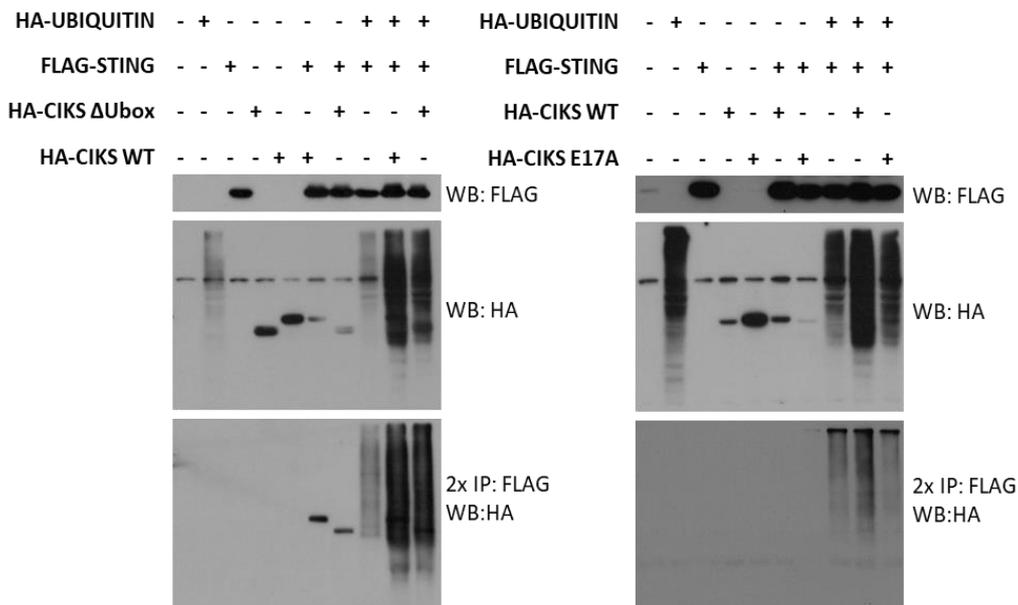
Figure 24: Ubiquitination analysis of STING in HEK293T cells using wt CIKS and ΔUbox (left panel) or E17A (right panel) mutants. Cells were transfected with the indicated constructs and lysates were subjected to two rounds of IP. Immunoprecipitated proteins were analysed by SDS-PAGE.

Together with CIKS, TRAF6 is an E3 ubiquitin ligase itself. Moreover, it is known in literature that CIKS and TRAF6 are interacting proteins. On these assumptions, we verified whether TRAF6 could enhance STING ubiquitination by performing the same double IP experiment using wt TRAF6 and TRAF6-ΔN, which display lower ubiquitination activity, due to the lack of the RING-finger domain. We found that the overexpression of TRAF6 dramatically increased STING ubiquitination. By contrast the presence of TRAF6-ΔN had not the same effect, with only a slight enhance of STING ubiquitination respect to the basal level (Fig. 25).
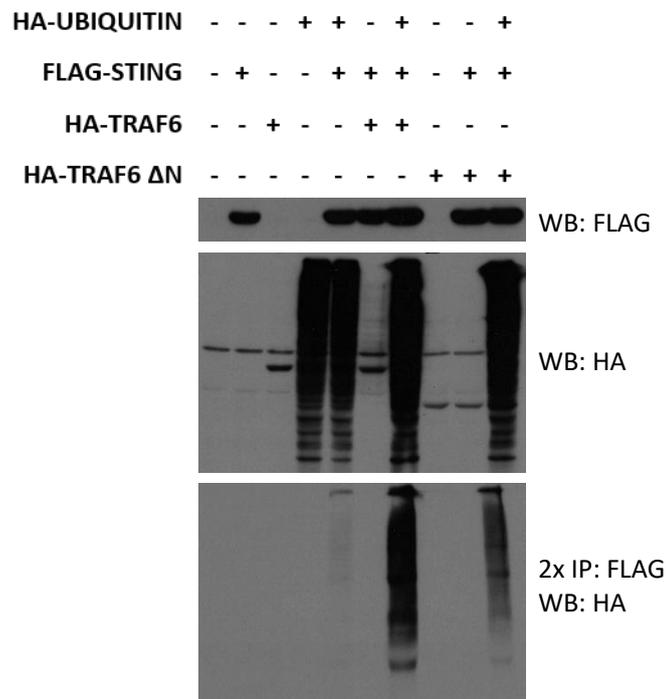
Figure 25: TRAF6 influences STING ubiquitination. HEK293T were transfected with the indicated constructs and lysates were subjected to two rounds of IP. Captured proteins were analysed by SDS-PAGE.

In addition to these results, we moved our attention on MEF and CIKS^{-/-} system. Here, the ubiquitination of STING in cells lacking CIKS, was almost completely absent, further strengthening the deep involvement of CIKS in the STING-mediated antiviral signaling (Fig. 26).
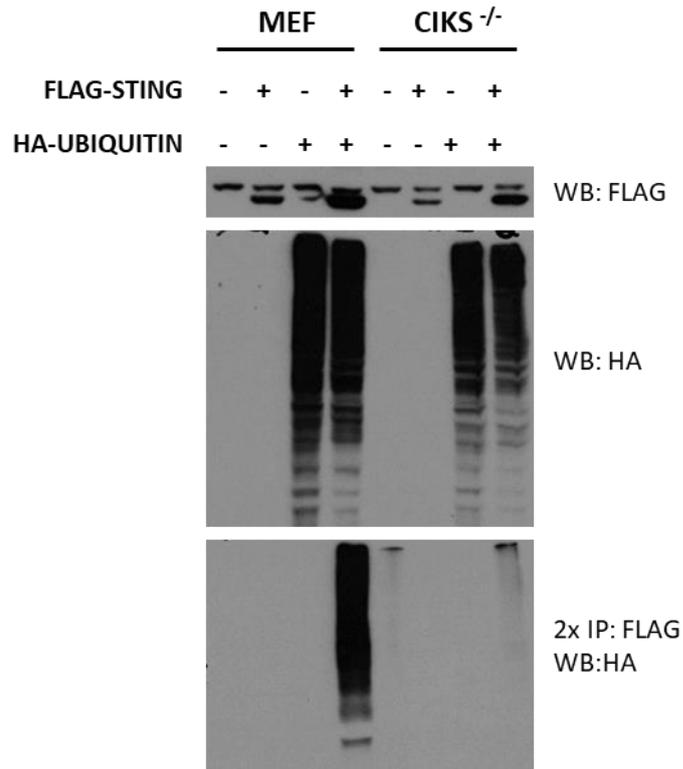
Figure 26: Analysis of STING ubiquitination in MEF and CIKS^{-/-} cells. Cells were transfected with the indicated plasmids and lysates were subjected to two rounds of IP. Captured proteins were analysed by SDS-PAGE.
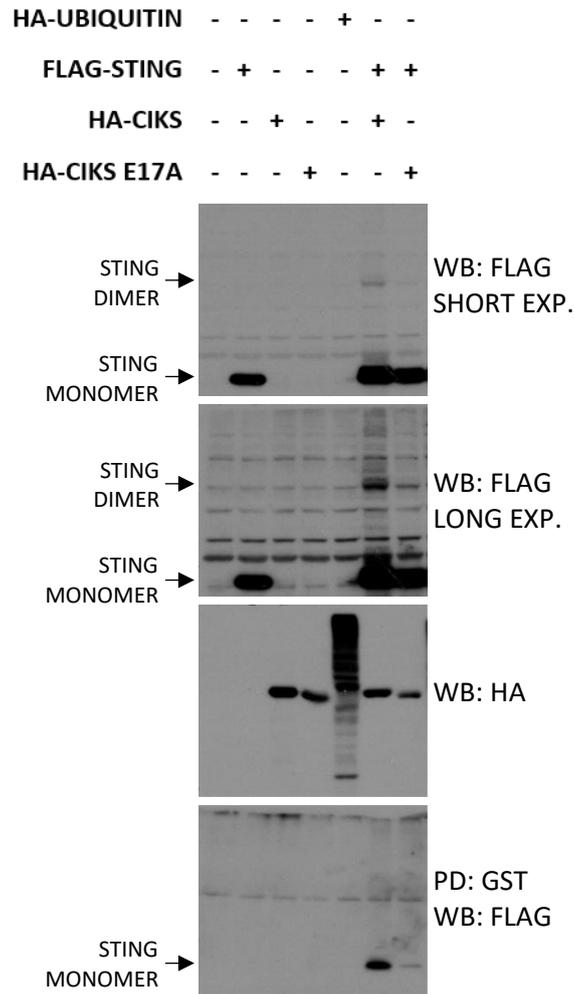
Figure 27: TUBEs-based analysis of STING ubiquitination. HEK293T were transfected as indicated. Cells were lysed in TUBEs containing buffer (200μg/ml) and all ubiquitinated proteins were precipitated using Glutathione-Agarose beads. Ubiquitination status of STING was assessed by SDS-PAGE analysis.

To further validate these findings, we also studied STING ubiquitination through an alternative approach based on the Tandem Ubiquitin Binding Entities (TUBEs), which binds ubiquitin with high affinity, and have a GST-Tag that allows the pull-down (PD) of all ubiquitinated proteins. We focused on wt CIKS and E17A mutant since previous results showed no differences between wt and ΔUbox mutant behavior. This technique confirmed the results obtained from double IP experiments, at least in overexpression conditions: the ubiquitination of STING was strongly enhanced when both CIKS and STING were ectopically expressed, while the E17A point mutant does not show the same effect (Fig. 27).

DISCUSSION

In this work, we presented data suggesting the involvement of the adaptor protein CIKS in STING-mediated antiviral Type I IFNs response, through the activity of the ubiquitin ligase TRAF6. Some authors have associated CIKS to the antiviral signaling. It has been demonstrated that RNAi for CIKS in human fibroblasts leads to a strong reduction in the antiviral response triggered by pI:C (Ryzhakov *et al.*, 2012). We confirmed this finding in MEF and CIKS^{-/-} fibroblasts. We also expanded to the existing knowledge by stimulating cells with pA:T, the analogue of a viral DNA genome. In the absence of CIKS, MEFs are less sensitive to both DNA and RNA viral challenges, with a significant decrease of *Ifnβ* mRNA compared to wt population. The same trend is observable also for other interferon stimulated genes, such as *Isg15*. In addition, the decrease of IFNβ is also detectable at protein level: the amount of IFNβ secreted by stimulated cells is significantly lower in CIKS^{-/-} respect to wt cells. This phenotype is almost completely reverted when CIKS^{-/-} cells are stably reconstituted by lentiviral transduction with FLAG-CIKS, confirming that it is somehow involved in the induction of Type I IFNs. Anyway, even though the expression level of CIKS in wt and reconstituted cells seems to be comparable, the lentiviral transduction is not finely tunable. The variability observable between wt cells and FLAG-CIKS reconstituted cells may be due to a differential expression of CIKS between the two cell lines.

One of the functions of CIKS, following IL-17 stimulation, is to trigger a non-transcriptional pathway (TRAF-6 independent) which leads to several mRNA stabilization and consequently a longer lasting protein expression (May, 2011). Our mRNA decay assay experiments shows that the decay rate of IFNβ mRNA induced after pA:T or pI:C challenge is completely overlapping between MEFs and CIKS^{-/-} cells, thus excluding that the CIKS mRNA stabilization pathway is involved. On the contrary, luciferase reporter experiments showed that a de novo transcription of IFNβ gene is involved, rather than alteration in its mRNA stability. This is observable especially in pI:C treated cells: dsRNA, or its structural analogue, is an extremely strong activator of the innate antiviral response. The reason is to be found in the fact that dsRNAs are inside the cytoplasm only in case of a viral infection, and so its recognition must efficiently and rapidly induce the antiviral response. On the contrary, dsDNAs in the cytosol are more tolerated since they can have a self-origin (DAMPS, mitochondrial or nuclear DNA leakage etc.). In addition to these data, time course experiments aimed to assess the phosphorylation status of IRF3, the key inducer of Type I IFNs, confirmed a weaker and delayed activation of IRF3 in CIKS^{-/-} respect to wt cells, confirming a defect in signal transduction in the absence of CIKS.

One of the main pathways that induce the production of Type I IFNs is the cGAS-STING axis(Motwani, Pesiridis and Fitzgerald, 2019). We questioned if

CIKS could participate somehow in the signal transduction of this axis. CIKS physically interacts both with STING and TBK1 when overexpressed in HEK293T cells suggesting that CIKS cooperate with STING in antiviral signaling. This interaction is not affected by using CIKS ΔUBox mutant, suggesting that the ubiquitin ligase domain of CIKS is not involved in this function. Strikingly, the interaction is dramatically reduced when the point mutant E17A is overexpressed. Since this mutation abolish one of the TRAF6 binding domain of CIKS we hypothesized that TRAF6 could be a facilitator of CIKS-STING interaction, also considering that the interaction between CIKS and TRAF6 is already described, and that TRAF6 is a key E3 ubiquitin ligase involved not only in the activation of NF-κB, but also in the regulation of other key proteins involved in the innate antiviral response, such as cGAS and MAVS (Lee *et al.*, 2018; Chen and Chen, 2019). Interestingly, it is possible to observe that the overexpression of both STING and CIKS, but not the E17A mutant, is sufficient to induce STING dimerization. We were not able to observe in vitro interaction between purified CIKS and STING, suggesting that their interaction is indirect or requires post-translational modifications (e.g. ubiquitination) not occurring in the in vitro translation system (or in E. Coli).

STING undergoes heavy K11, K27, K63 and K48 poly-ubiquitination upon activation. Some of these (K63-K27) are necessary to its dimerization and translocation to the ERGIC and Golgi compartments. K48 is needed for the proteasomal degradation to switch off the signal. Both CIKS and TRAF6 are E3 ubiquitin ligases and for this reason we performed experiments to find if CIKS could influence STING ubiquitination, directly or indirectly. The overexpression of CIKS and STING in HEK293T resulted in a strong enhancement of STING ubiquitination. Again, in this context, the presence of the E17A mutant strongly reduced STING ubiquitination, while the ΔUbox mutant behaves like wt CIKS. Moreover, the ectopic expression of TRAF6 and STING resulted in a clear increase of STING ubiquitination, whereas the TRAF6-ΔN produced only a slight increase. The fact that TRAF6-ΔN still enhance somehow STING ubiquitination may be explained with a residual ubiquitination activity of this TRAF6 mutant. In addition, it must be noted that there is the possibility of a redundant E3 ubiquitin ligase (e.g TRAF3 or TRAF5, similarly to TNF signaling) which can independently ubiquitinate STING. Another possibility is the existence of a ubiquitin ligase that act on STING through TRAF6 even though its ubiquitination domain is functionally impaired.

Although double IP is a validated tool to study protein-protein interaction as well as protein ubiquitination, we also performed experiments to confirm the data obtained using an alternative technique that allows to study ubiquitination: TUBEs pulldown. The preliminary data obtained with this tool is confirming what we found in double IP experiments, that is a massive increase in STING ubiquitination when CIKS and STING are co-expressed. On the contrary, when CIKS is replaced with its point mutant E17A, the ubiquitination is not efficient.

The results obtained in overexpression system are completely transferable to MEF and CIKS$^{-/-}$ cells. The absence of CIKS in knock-out cells almost completely abrogate STING ubiquitination. Although the involvement of a third ubiquitin ligase cannot be excluded from our system (at least until ubiquitination assays on purified proteins), these results suggest that CIKS is not directly ubiquitinating STING and strengthen the involvement of TRAF6 in this antiviral pathway.

The evidence that, in overexpression, CIKS and STING are interacting proteins prompted us to investigate the dynamics of this interaction. It is known that STING responds to CDNs directly produced by pathogens or synthetized by other PRRs that recognize dsDNA, such as cGAS (Motwani, Pesiridis and Fitzgerald, 2019). Our experiments confirm this evidence: the stimulation with pA:T leads to a rapid phosphorylation of STING, that occur within 1 hour both in CIKS$^{-/-}$ and reconstituted cells. In the pI:C treated cells this phenomenon does not occur, suggesting that STING-mediated signaling is not activated by dsRNA. Also, the co-IP of FLAG-CIKS in reconstituted cells resulted in a detection of STING-CIKS interaction only in 4 hours pA:T treated cells. By confocal microscopy instead, using fluorescent-tagged CIKS and STING, it is possible to observe a co-localization between STING and CIKS also in untreated cells, even though the signal is predominantly diffuse. After treatment with synthetic oligonucleotides, the co-localization signal becomes shaped in vesicle-like structures with apparently no precise localization, since they can be found broadly distributed in the cytoplasm. From our preliminary data in confocal microscopy, the extent of this interaction seems to be lower in pI:C respect to pA:T treated cells. This might in part explain why we did not find any interaction by Western Blot analysis in pI:C treated cells. It is possible that the stimulation with DNA leads to a higher stability of the CIKS-STING complex respect to RNA stimulation. This could result in a loss of interaction when performing IP experiments. Moreover, two issues should be taken into account: 1) it must be considered that the sensitivity of confocal microscopy is higher than the IP experiments; 2) even using a "more physiological" promoter to drive the lentiviral transduction of both fluorescent STING and CIKS, such as the EF1α, we are *de facto* still working under overexpression conditions.

Overall, these data highlight the role played by CIKS in the innate antiviral immunity. This opens to novel questions:1) which is the receptor that senses viral infection and starts the signal cascade? CIKS is known to interact with some helicases, such as DDX3X (Somma *et al.*, 2015), which role in viral sensing is emerging during the last years (Fullam and Schröder, 2013). In addition, CIKS present an HLH domain that can potentially bind dsDNA (Leonardi *et al.*, 2000; Li *et al.*, 2000), allowing the possibility to be a PRR in itself; 2) is TRAF6 the only CIKS-interacting ubiquitin ligase that can regulate STING ubiquitination? The existence of numerous E3 ubiquitin ligases that act on STING, both to activate and suppress its activity, prospects the possibility of redundant activation/regulation routes, in which CIKS may be involved.

This consideration is strengthened by the evidence that several viruses deploys evasion systems for STING-mediated, as well as other antiviral pathways. Among these,   (Chan and Gack, 2016); 3) what signal goes through CIKS? Even if we described the general mechanism for this model, much is still to be known to unveil the detailed molecular pathway. 4) Does CIKS undergo any post-transcriptional/translational regulation related to its antiviral involvement? It is known that CIKS expression is induced in B lymphocytes stimulated with BAFF, CD40L, and LPS (Qian *et al.*, 2004), or in fibroblasts challenged with pI:C (Ryzhakov *et al.*, 2012). Moreover, except for the phosphorylation on Ser 311, no additional post-translational modification, such as ubiquitination, have been studied.

In conclusion, we propose a novel model for STING-mediated activation of Type I IFNs anti-viral response, mediated by the adaptor protein CIKS, through the activity of the E3 ubiquitin ligase TRAF6.

LIST OF PUBLICATIONS

The results obtained by Dr Michele Cillo have been included in the following publications:

• Pacifico F, Pisa L, Mellone S, **Cillo M**, Lepore A, Leonardi A. NGAL promotes recruitment of tumor infiltrating leukocytes. Oncotarget. 2018;9(56):30761–30772.

• Merlo R, Caprioglio D, **Cillo M**, Valenti A, Mattossovich R, Morrone C, Massarotti A, Rossi F, Miggiano R, Leonardi A, Minassi A, Perugino G. Expanding the SNAP-tag technology: an innovative chemo-enzymatic approach by using a universal azide substrate. In preparation.

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