RIP1 and FADD's Role in Innate Immunity

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UNIVERSITY OF MIAMI

RIP1 AND FADD’S ROLE IN INNATE IMMUNITY

By

Jinhee Hyun

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of the University of Miami
in partial fulfillment of the requirements for
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the requirements for the degree of
Doctor of Philosophy

RIP1 AND FADD’S ROLE IN INNATE IMMUNITY

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Rapid production of type I Interferon is pivotal to initiate cellular antiviral host defense and adaptive immunity. In order to facilitate innate immune processes, a cell harbors pattern recognition receptors (PRRs) which sense distinctive forms of pathogen associated molecular patterns (PAMPs). For example, Toll like receptors (TLRs) and RIG-I like receptors (RLRs) were discovered as PRRs for pathogen derived molecules and the production of type I Interferon (IFN). To induce type I IFN, several transcription factors such as nuclear factor-kappaB (NF-kB), interferon regulatory factor 3 (IRF3), interferon regulatory factor 7 (IRF7), and activating protein-1 (AP-1) need to be stimulated through the specific signaling adaptors. Among them, our lab is interesting in the death domain (DD) containing proteins Receptor interacting kinase1 (RIP1) and Fas-associated death domain protein (FADD), which we showed were important for innate signaling processes.

RIP1 and FADD were initially identified as Fas and TNFR interacting proteins which were involved in death receptor mediated apoptosis. Aside from apoptotic function, recent publications indicate that RIP1 and FADD mediate cell survival, proliferation, and cytokine production through NF-kB activation.
Here, we show that RIP1 and FADD are essential for efficient TLR-independent signaling. We report that RIP1 and FADD lacking MEF cells are sensitive to viral cytolysis and also exhibit impaired IFN production against dsRNA virus infection.

RIP1 acts as a scaffolding protein for death receptor mediated apoptosis and NF-κB activation, necrosis, and innate immunity. As mentioned, we demonstrate that cells lacking RIP1 are sensitive to RNA virus infection. To understand the detailed mechanisms of RIP1 function in innate signaling, we first tested whether RIP1 is involved in RIG-I signaling. We found that RIP1 forms a complex with RIG-I in the presence of dsRNA. Additionally, we showed that RIP1 is required for optimal RIG-I and melanoma differentiation-associated protein 5 (MDA-5) activity. We also find that FADD, a RIP1 interaction protein, is implicated in innate immunity. To study the precise mechanisms of FADD in type I IFN signaling, we generated FADD variants and used luciferase reporter assays to indicate that the FADD death effector domain (DED) is crucial for IFN-β signaling.

In order to identify interacting partners of FADD, yeast two hybrid assays were performed and indicated that FADD binds to protein inhibitor of activated STAT (PIAS1), part of the SUMO machinery. SUMOylation is a reversible post-translational modification of a protein by SUMO, a 100 amino acid protein. The consequence of SUMOylation alters specific proteins’ function by affecting activity, localization, stability or influencing molecular interactions by interfering with or linking to a target protein. To confirm FADD-PIAS interactions, we conducted \textit{in-vitro} SUMOylation assays by using Ubc9 conjugated FADD and found possible FADD SUMOylation sites. We also discovered that FADD and SUMO are co-localized in the nucleus. This result
reveals that FADD undergoes SUMOylations and its modification might regulate FADD’s function, including role in innate signaling.

Furthermore, we report here that HTLV-1 Tax protein interacts with RIP1 and inhibits IFN-β inducing signaling by abrogating RIP1 and IRF7 interaction. This implies that RIP1 is involved in the regulation of IRF7 and is essential for IFN-β production.

Collectively, our data demonstrate the significance of RIP1 and FADD in dsRNA recognition pathways in mammalian cells that are essential for the optimal induction of type I IFNs and other innate genes important for host defense.
DEDICATION

Jesus Christ in my heart

The grass withers and the flowers falls,
but the word of our God stands forever.
- Isaiah 40:8
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CHAPTER 1: Introduction

Innate immunity

Overview

Innate immunity is a first line defense mechanism against pathogens. It is a fast acting response usually within minutes of infection. In order to immediately respond to foreign pathogens, a cell harbors pattern recognition receptors (PRRs) which recognize specific microbial proteins called pathogen associated molecular patterns (PAMPs). The interaction between PRRs and PAMPs results in the induction of inflammatory cytokines, and chemokines and the expression of co-stimulatory molecules that facilitate further adaptive immune response.

Currently, several PRRs such as the Toll like receptors (TLRs), RIG-I like receptors (RLRs), Nod like receptors (NLRs), and DNA receptors have been discovered which play an essential role in host innate immunity.

Toll like receptors, RIG-I like receptors, and DNA sensors.

TLRs are type I glycoproteins which were initially isolated in Drosophila. TLR’s consist of extracellular leucine repeats (LRRs) responsible for pathogen recognition and a cytosolic toll/interleukin-1 domain (TIR) for downstream signaling adaptor functions. In humans, 10 TLRs have been identified and each member recognizes distinctive PAMPs (pathogen associated molecular pattern). In particular, TLR2, TLR4, TLR5, and TLR6 recognize peptidoglycan, lipopolysaccharide, flagellin and diacyl lipopolypeptide in
Figure 1-1 Toll like receptors (TLRs).

Membrane bound TLRs locate in plasma membrane or ER. Each TLR recognizes different PAMPs. TLR3, 7 and 9 lie on ER and move to endosome upon microbial invasion and senses the PAMPs (Pathogen Associated Molecular Patterns).
cell membranes and TLR3, TLR7 and TLR9 are important in sensing dsRNA, ssRNA, and dsDNA in the endosomes, respectively (Fig 1-1). All TLRs recruit TIR containing adaptors such as myeloid differentiation primary response gene 88 (MyD88) for the downstream signaling except for TLR3. In the MyD88 pathway, MyD88 binds to DD containing serine threonine kinase interleukin-1 receptor-associated kinase 1 (IRAK-1) and interleukin-1 receptor-associated kinase 4 (IRAK-4), and transmits the signal through TNF receptor-associated factor 3 (TRAF3), and TNF receptor-associated factor 6 (TRAF6) for mitogen-activated protein kinase (MAPK) family and nuclear factor-kappaB (NF-κB) activation. This results in proinflammatory cytokines or type I interferon (IFN) induction. In the TLR3 and TLR4 pathway, TIR and RHIM containing protein toll-like receptor adaptor molecule 1 (TRIF) associates with TLR3 or TLR4 following dsRNA or LPS ligand stimulation respectively and recruits TRAF3, receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIP1), MAPK, and TANK-binding kinase 1 (TBK1) for activating protein-1 (AP-1), NF-κB and interferon regulatory factor 3 (IRF3) in order to induce type I IFN production.

RLRs are dsRNA sensors, and include retinoic acid-inducible gene I protein (RIG-I), melanoma differentiation-associated protein 5 (MDA-5) and laboratory of genetics and physiology 2 (LGP-2) (Fig 1-2). Except for LGP-2, RLRs contain a CARD domain for signal transmission. All three contain helicase domains for sensing viral genomes and transcripts, and a C-terminal repression domain. It is reported that different subsets of viruses are sensed by these receptors. RIG-I recognizes triphospho groups 5’-end group and negative stranded viruses and short lengths of Poly (I:C) 7. On the other hand,
Cytosolic dsRNA receptors, RIG-I and MDA-5 detect a short form and a long form of dsRNA through helicase domain respectively. Interaction with dsRNA and RLRs results in exposure of CARD of RLRs for downstream signaling transmission.
MDA-5 senses long RNA, such as Poly (I:C) and positive stranded virus such as picornoviruses. The consequence of RLRs interaction with dsRNA triggers conformational changes in these receptors. This alteration exposes the N-terminal CARD domain, resulting in recruitment of another CARD domain containing protein, referred to as interferon beta promoter stimulator protein 1 (IPS1) also MAVS, VISA, and Cardif. IPS1 resides on the mitochondria membrane and triggers the downstream signaling by forming an ‘innateosome’ through TRAF3, Fas (TNFRSF6)-associated via DD (FADD), RIP1 and TNFRSF1A-associated via DD (TRADD) from both receptors (Fig 1-4). These adaptors culminate in the activation of IRF3 and NF-κB through TBK1/IKKe and a IκB kinase (IKK) complexes. Additionally, ER localized adaptor STimulator of INterferon Genes (STING) is also reported to be important in RIG-I mediated dsRNA signaling as well as DNA signaling and links translocon complexes with TBK1. TBK1 is a pivotal kinase for IRF3 and IRF7 phosphorylation. Phosphorylated IRF3 and IRF7 dimerize causing nuclear translocation and formation of enhancesome with NF-κB as well as AP-1 for IFN-β induction.

Although TLR9 recognizes CpG DNA and induces type IFN, TLR9 knock-out mice are still able to induce immune response to DNA, indicating the existence of unknown DNA sensors. So far, four DNA sensors for IFN production are reported in the literature. DAI is reported as DNA dependent activator of IRFs (also ZBP) and reported as an intracellular DNA sensor. However, DAI may be specific for L929 cells since DAI knock-out mice showed normal IFN production in response to DNA. HMGB (high mobility group box) is a conformational modulator of nucleosomes. HMGB binds to B form DNA and is required for Poly (I:C) or DNA mediated IFN production. It is
suggested as a crucial co-receptor for innate immune cytosolic receptors and is involved in RLR, TLR, AIM2, and STING signaling pathways. Interestingly, RIG-I is also involved in DNA sensing. It is illustrated that cytosolic DNA transcribed by Polymerase III and genomic RNA is sensed by RIG-I to induce type I interferon. Recently, a PYHIN protein IFI16 has been reported as being a cytosolic DNA sensor which interacts with STING and is required for DNA mediated innate gene induction. All these DNA receptors are reported to recruit STING and TBK for IFN induction but the underlying mechanism and adaptors still needs to be clarified\(^1\)\(^8\)\(^-\)\(^2\)\(^1\).

**Type I Interferon**

Interferon is a cytokine that inhibits viral replication. It was discovered in the 1950s and comprises two types I and II. Although both types inhibit viral replication, only Type I interferon is inducible by virus infection. Type I interferon interacts with a heterodimer form of the IFNRI and IFNRII receptors. It is produced from most cells and has several classes such as IFN-\(\alpha\) and IFN-\(\beta\). The type I Interferon signaling pathway is divided into two stages; IFN inducing signaling and IFN response signaling (Fig 1-3). At the inducing stage, recognition of viral genomes recruits several adaptors in order to activate essential transcription factors such as IRF3, IRF7, NF-\(\kappa\)B, and AP-1. These factors interact with four positive regulatory domains (PRDs) in the IFN-\(\beta\) promoter region and form an enhancesome for IFN-\(\beta\) gene induction. Accordingly, interferon is secreted and binds to IFN receptors, triggering IFN responsive genes. In this stage, ligand interaction to IFNRI induces Janus kinase (JAK) and signal transducers and activators of transcription (STAT) phosphorylation. Subsequently, phosphorylated JAK/STATs, as well as IRF9, form
Upon microbial infection, receptors recognize pathogen associated molecular patterns (PAMPs) and recruit several adaptors for activation of four transcription factors – NF-κB, IRF3/IRF7 and AP-1. These transcription factors form an enhancesome and initiate IFN-β mRNA induction. Induced IFN-β is secreted out of the cell and binds to IFN receptors. This interaction result in JAK/STAT phosphorylation and ISGF3 formation with IRF9 for induction of interferon stimulated gene expression.
dsRNA genomes or transcripts of virus are sensed by RLRs or TLR3 and trigger IFN signaling through IPS1 or TRIF in order to activate NF-κB, IRF3, IRF7 and AP-1. Those transcription factors form an enhancesome and transcribe $IFN-\beta$ mRNA. Yellow box indicates ‘innateosome’ which consists of RIP1 and FADD for IFN production. an interferon stimulated gene factor 3 (ISGF3) complex. This complex binds to the interferon-sensitive responsive element (ISRE) promoter present in hundreds of genes, which are up regulated and induce an anti-viral state$^{22, 23}$. 

**Figure 1-4 dsRNA mediated IFN-β induced pathway and ‘innatesome’**

PRD IV  PRD III-I  PRD II

IFN-β Promoter

Mitochondria

Endoplasmic Reticulum

Nucleus

Endosome
RIP1 (Receptor interacting Kinase 1)

Gene, mRNA, and protein structure

RIP1 is a receptor interacting protein kinase encoded on the short arm of chromosome 6\textsuperscript{24}. Its mRNA is ubiquitously expressed in most tissues at basal levels and is inducible by TNF, Type I IFN, or T cell activation signals. RIP1 is composed of 671 amino acids and bears a kinase domain (KD) as well as intermediate domain (ID), and death domain (DD) (Fig 1-5). Each domain of RIP1 is conserved among vertebrates and plays a different role in various signaling pathways such as those involving cell survival, proliferation, cell death and innate immunity. It is reported that lysine residue 45 of RIP1 is essential for its kinase activity and its autophosphorylation and necrosome formation. The ID of RIP1 bears a polyubiquitination site on lysine at 377 and a RHIM (RIP homophilic interaction motif). These domains are required for RIP1 mediated NF-κB and AP-1 activation because mutation on lysine at 377 suppresses the signaling pathway. A major function of the DD of RIP1 is to induce death receptor (DR) 3-5 mediated apoptosis and anoikis through homophilic association with other death receptors and different adaptors like FADD and TRADD. Therefore, deletion of the DD impairs signaling interactions and leads to suppression of DD associated signaling\textsuperscript{25,26}.

Function of RIP1

RIP1 was isolated as a FAS or TRADD interacting protein by yeast two hybrid analysis as an apoptosis inducer and a NF-κB signal activator\textsuperscript{24}. Currently, RIP1 is considered a pleiotropic protein which controls diverse signaling pathways as described
Figure 1-5 Schematic of RIP1.

75KDa of RIP1 protein contains four domains - kinase domain, intermediate domain, RHIM (RIP1 homophilic interacting motif) and death domain. Polyubiquination site is marked as a red asterisk. Amino acid position is marked by numbers.
Among the well characterized functions of RIP1 include a role in TNF-α signaling. In detail, RIP1 is recruited to the TNFR-I receptor after TNF-α ligand stimulation and forms a complex I with TRADD, TRAF2 and the cellular inhibitor of apoptosis (cIAPs) near the plasma membrane. Subsequently, RIP1 is ubiquitinated by the cIAPs through K-63 linkage and assembles an IKK complex for NF-κB activation and anti-apoptotic gene induction. If an anti-apoptotic gene is insufficiently induced, or RIP1 is deubiquitinated by A20 or cylindromatosis (CYLD), RIP1 preferentially associates with TRADD, FADD, apoptosis-related cysteine protease (Caspase8), and Caspase10 resulting in apoptosis. Under this condition, if Caspase activity is inhibited, RIP1/FADD associates and leads to necrotic cell death via a RIP3 dependent manner.

RIP1 also functions as a scaffold protein upon genotoxic stress. Upon genotoxic stress, RIP1 interacts with ataxia telangiectasia mutated (ATM) for NF-κB activation and stimulates phosphoinositide-3-kinase/ v-akt murine thymoma viral oncogene homolog (PI3K/AKT) pathways, respectively. Furthermore, RIP1 knock-out mice, which exhibit postnatal lethality, display considerable apoptosis in the lymphoid and adipose cells, indicating that RIP1 is required for cell survival and proliferation.

**RIP1 role in innate immunity**

Similar to TNF-α signaling, RIP1 is responsible for NF-κB activation in TRIF dependent TLR3 and TLR4 pathways. RIP1 binds to TRIF via a RHIM domain and is ubiquitinated possibly by TRAF6. In the TLR independent pathway, RIP1 forms an ‘innateosome’ with FADD through DD interactions following VSV infection. RIP1 is necessary for NF-κB and IRF3 signaling axis, similar to FADD. (Fig 1-4) We reported
that RIP1-deficient MEFs are susceptible to VSV cytolysis and exhibit decreased mRNA and protein levels of Type I IFN production following VSV infection\textsuperscript{12,36}. Additionally, it is reported that RIP1’s ubiquitination enhances IRF7 activity by recruiting TRAF6 in the presence of the Epstein-Barr virus (EBV) oncoprotein latent membrane protein 1 (LMP1)\textsuperscript{37}. Therefore, RIP1 is essential in innate antiviral signaling but the mechanisms of RIP1-mediated Type I IFN signaling remain to be fully understood.

**FADD (Fas associating DD containing protein)**

*Gene, mRNA, and protein structure*

FADD was originally identified as a Fas interacting protein through DD interactions. Its gene consists of two exons on chromosome 11q13.3 and its mRNA is ubiquitously expressed in adult and embryonic tissue\textsuperscript{38,39,40}. FADD is composed of six antiparallel $\alpha$-helix bundles with two functional domains which are highly conserved death effector domains (DED) and death domains (DD). Protein domain analysis reveals that FADD possesses a nuclear localization signal (NLS) and a nuclear export signal (NES) in the DED part of FADD and undergoes post-translational modifications such as phosphorylation\textsuperscript{41-43} (Fig 1-6).

*Function of FADD*

FADD functions as a regulator for death receptor mediated apoptosis, cell survival, inflammation and innate immunity\textsuperscript{44}. A very well known function of FADD is Fas death
**Figure 1-6 Schematic of FADD.**

FADD consists of DED and DD domain. Red asterisk and numbers indicate phosphorylation site and amino acid position, respectively.
Recent data show that FADD also associates with other DRs such as tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and tumor necrosis factor (TNFR) and governs apoptosis. In order to execute apoptosis, FADD forms a complex called a DISC (death-inducing signaling complex) with Caspase8 or Caspase10 following Fas ligand stimulation. In DISC, FADD interacts with Caspase8 through its DED and Fas with its DD and forms a trimer in a perpendicular arrangement. Formation of a DISC subsequently stimulates auto-cleavage of Caspase8 or Caspase10 for its activation and promotes downstream effector Caspase activation such as Caspase3 and Caspase6, resulting in apoptosis. Moreover, FADD also forms a second complex with Caspase8 and cFLIP, but not Fas in B cells. In TRAIL-R1 (DR-4) and R2 (DR-5) signaling, FADD forms a TRAIL associated DISC. In this formation, which is different from Fas signaling, the DED of FADD participates in TRAIL interaction. Since FADD is involved in TNF RI signaling in complex II, FADD also plays a role in TNFR induced apoptosis and necrosis. It is considered that the DD part of FADD might engage in necrosis by interacting with RIP1 and then the DED part may participate in apoptosis by binding to Caspase8.

Although FADD functions as an initiator of cell death, FADD is also required for DR mediated NF-κB activation and cell survival. FADD defective Jurkat T cells fail to stimulate NF-κB upon DRs signaling. In addition, homozygous FADD null mice die at E11.5 and show signs of cardiac failure and abnormal hemorrhage, suggesting a role for FADD in embryonic development and cell survival. FADD appears to regulate the cell cycle and proliferation following phosphorylation. It is reported that the phosphorylated form of FADD is mainly observed in G2/M stages and the unphosphorylated form is
dominant in the G1 stage of the cell cycle. Additionally, mutation of serine194 to aspartic acid in FADD and FADD-DN (c-terminal FADD) impairs proliferation of mature T cells when these vectors are reconstituted in FADD-deficient cells mice. Although the exact stimulus leading to FADD’s phosphorylation is unknown, it is selectively phosphorylated on a serine 194 at its C-terminus by casein kinase1 α (CK1α), Fas-interacting serine/threonine-protein kinase (FIST), and protein kinase C (PKC). However, the phosphorylation of FADD seems to be independent of death receptor signaling because a mutant incapable of being phosphorylated could elicit apoptosis and NF-κB activation by Fas ligand.

For Fas interaction, FADD is thought to be localized in the cytoplasmic membrane. However, it turns out that FADD is also in the nucleus. FADD contains a NLS and a NES and mutation of these sequences alters FADD localization to cytoplasm. Supporting this data, FADD interacts with Exportin-5 which mediates protein transport between the nuclear and cytoplasmic compartments.

**FADD role in innate immunity**

FADD is known to play different roles in innate immune signaling. For example, in the TLR pathway, it is reported that FADD is required for B cell proliferation in response to TLR3 and TLR4 and works as a negative regulator by abrogating the MyD88-IRAK-1 interaction through DD interactions.

In the TLR independent pathway, FADD functions as a positive signal transmitter for dsRNA-mediated IFN-β signaling. But the questions are which signaling axis is regulated by FADD and what is downstream of FADD? Three papers show that FADD is involved
in NF-κB as well as IRF3 and IRF7 activation. We reported that FADD deficient MEFs are sensitive to VSV infection and exhibit decreased mRNA and protein levels of IFN-α and IFN-β after Poly (I:C) stimulation. Furthermore, IRF3 nuclear localization is defective in FADD knock-out MEFs in respond to Poly (I:C). This process seems to be independent of Caspase8 since MEFs lacking Caspase8 do not display VSV cytolysis nor decreased levels of IFN-α and IFN-β production similar to FADD knock-out cells. Moreover, we reported that FADD is required for optimal activity of RIG-I, MDA-5, and IPS1 by activation of NF-κB and induction of the IRF7 gene. Akira et al showed that FADD is only essential for NF-κB activation through Caspase8 and Caspase10. It was also reported that FADD interacts with IPS1 via RIP1 and TRADD through the DD interaction. In contrast with a positive role, recently a paper suggested that FADD was a negative regulator for IRF7 degradation by binding to tripartite motif-containing 21 (TRIM21).

Collectively, multiple FADD roles have been discovered in type I IFN signaling pathways. However, how FADD performs these functions still needs further investigation.

Our data shows that the regulation of FADD and RIP1 may involve SUMOylation – a post translational event that can control protein function. We therefore describe the function of this process as follows.
SUMOylation

SUMOylation is a reversible post-translational modification of proteins; a process where a small protein, SUMO, a 101 amino acid protein is attached to a lysine residue of the substrate protein similar to the conjugation of ubiquitin\textsuperscript{70}. Like ubiquitination, SUMOylation requires four main components such as an E1 activating enzyme, E2 conjugating enzyme, E3 ligase, and SUMO protease and works by a comparable mode of action. However, SUMOylation differs from ubiquitination in the process due to the E2 ligase. Compared with ubiquitin E2 ligase, the SUMO system uses a single E2 ubiquitin-conjugating enzyme E2I (Ubc9) which has the ability to recognize a target without the E3 ligase\textsuperscript{71}. Therefore, substrates do not need to be conjugated with E3 ligases, and E3 ligases function as the SUMOylation enhancer\textsuperscript{72}. The process of SUMOylation is briefly displayed in Figure 1-7.

Components of SUMOylation.

SUMO is a 10 KDa protein with a 3D structure similar to ubiquitin with 20% identity in amino acid sequence\textsuperscript{73}. In humans, four SUMO families have been identified and are expressed in most tissues except for the preferential expression of SUMO-4 in lymph node, spleen, and kidney. Sequence homology analysis indicates that SUMO-1 shares 50% sequence identity with SUMO-2 and SUMO-3 and SUMO-2 and SUMO-3 are 97% identical\textsuperscript{72,74,75,76}.
As previously mentioned, Ubc9 is an E2 conjugation enzyme that confers substrate specificity and recognizes a consensus SUMO motif ($\psi$KxE/D; $\psi$ is a bulky aliphatic residue and x is any amino acid) on a target protein, if it is located in an extended loop or non-structural area. Recently, two extended consensus SUMO motifs have been found. A common feature of both extended motifs is a negatively charged group next to the SUMO motif. The first is PDSM (phosphorylation dependent SUMOylation motif) which is a consensus SUMO motif followed by a phospho serine or proline residue ($\psi$KxE/DxxpSP). Heat shock factor-1 (HSF-1) is known to contain a PDSM. The second extended motif is NDSM (negatively charged amino-acid dependent SUMOylation motif) which found in myocyte enhancer factor 2A (MEF2A). However, a number of studies have reported that SUMO can be attached to a non-canonical lysine such as TKET in proliferating cell nuclear antigen (PCNA) and VKYC in sterile alpha motif domain containing 4 (Samd 4).

Since Ubc9 is a unique E2 ligase, deletion of Ubc9 completely abrogates the SUMOylation process, leading to destructive effects on cells. Ubc9 knock-out blastocysts die of apoptosis after a second day of culture and exhibit severe nuclear structure
SUMO is processed by SENP for maturation and activated by E1 complex, a heterodimer SAE1 and SAE2 in an ATP dependent manner. Activated SUMO is transferred to Ubc9 which catalyzes the conjugation of SUMO to a target protein. E3 ligases such as PIAS family members facilitate this conjugation process to a target.
disruption and chromosome abnormality, thus emphasizing the importance of SUMOylation in the cell. The SUMO E3 ligase might serve 4 functions; it may (1) promote the affinity of Ubc9 for target proteins, (2) stabilize Ubc9 and target interaction, (3) help to properly orient the lysine residue, or (4) contribute to SUMO conjugation. Three types of SUMO E3 ligases have been discovered so far. The most well-known ligase is the SP-Ring motif containing protein inhibitor of activated STAT (PIAS) family. It was originally identified as SAP and Miz1 domain (SIZ) protein in Yeast and later it is found as the PIAS family in mammals. Five members of PIAS (PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy) have been isolated. The PIAS family contains the SP-Ring domain and SIM domain (SUMO interacting motif: IDIEV) in the ligase. The SP-Ring domain directly interacts with Ubc9 and the target, while the SIM/SBM domain non-covalently binds to SUMO, thereby forming a platform for favorable transfer. Currently, other members of SP-Ring E3 ligase such as mitochondrial E3 ubiquitin protein ligase 1 (MUL1) and TOPOROS were described in the literature. A second type of SUMO E3 ligase is RanBP2. RanBP2 is a nuclear pore complex component and has no homology to E3 ubiquitin ligases. It binds to Ubc9 and SUMO, and seems to promote the catalysis of the SUMO-Ubc9 thioester bond for optimal attack of subjective lysines in the substrate. Although the \textit{in-vivo} target of RanBP2 is not known, \textit{in-vitro} data suggest that RanBP2 facilitates SUMOylation of histone deacetylase 4 (HDAC4) and Sp-100. The last class of SUMO E3 ligase is a polycomb group member (Pc2). The mechanism of action is not clear, but it may function as a scaffolding protein. HDAC4, Ras homolog enriched in
striatum (Rhes), and TRAF7 are considered new members of the Pc2 E3 ligase family\textsuperscript{90-92}. Lastly, SENP is a serine specific protease; the SUMO-specific isopepdiase which requires nascent SUMO maturation, and reversible reaction of SUMOylated substrates. Among seven SENP members in mammals, based on preference to SUMO paralogs and enzymatic features, they are divided into three groups. SENP1 and SENP2 have processing and deconjugating activity with a broad range of specificity. The second group of SENP3 and SENP5 is preferential to SUMO-2 and SUMO-3 paralogs. The last group also favors SUMO-2 and SUMO-3 but it seems to select a polymer form for editing\textsuperscript{93,94}.

\textit{Consequence of SUMOylation.}

The consequence of SUMOylation has been extensively studied for a decade. SUMOylation alters specific proteins’ function by affecting activity, localization, and stability of a protein or influencing molecular interactions by interfering with or linking to a target protein, thereby regulating essential processes such as the cell cycle, DNA repair/replication, nuclear transport/localization, RNA stability, ubiquitination or acetylation\textsuperscript{72,95}.

\textit{SUMOylation, Innate Immunity, and Virus Infection.}

Recently, the interplay between SUMOylation and innate immunity is emerging as new research area. Several innate immune signaling components as well as viral products are reported to undergo SUMOylation and these modifications negatively or positively
affect IFN signaling or host immunity. For example, SUMOylation of the IRFs is known to down regulate type I IFN inducible or responsive signaling. IRF1 is a positive transcription factor for the induction of interferon responsive genes while IRF2 antagonizes IRF1. Yet, both SUMOylation of IRF1 and IRF2 lead to the repression of IRF1 transcriptional ability\textsuperscript{96, 97}. Regarding IFN-β induced signaling, it has been reported that IRF3 and IRF7 are modified by SUMO after VSV infection. Lysine residues K406 in IRF7 and K152 in IRF3 were identified as SUMOylation sites and mutation of these sites increased endogenous IFN-β mRNA production in response to VSV\textsuperscript{98}. However, this event is independent of phosphorylation and JAK/STAT signaling. Another example of negative regulation mediated by SUMOylation is TRAF family member-associated NF-κB activator (TANK) in TLR7 signaling. Following TLR7 stimulation, SUMO-I is attached to TANK resulting in an impaired interaction with IKKe, leading to restoration of IKK activity after phosphorylation\textsuperscript{99}.

In contrast, SUMOylation can positively regulate type I IFN signaling as well. dsRNA cytosolic sensors such as RIG-I and MDA-5 are reported to be SUMOylated which increases IFN-β inducing signaling\textsuperscript{100, 101}. The underlying mechanisms of MDA-5 SUMOylation are not clear, but SUMOylation on RIG-I augments its interaction with IPS1.

Since fine tuning of innate immunity is required for intact anti-viral responses, viruses also deregulate the SUMOylation machinery by altering overall SUMOylation processes, by inducing the SUMOylation of innate signaling proteins or by utilizing the SUMOylation system to their advantage. For instance, avian adenovirus Gam1 binds to E1 heterodimer and decreases the overall SUMOylation process. Moreover, viral VP35
protein in Ebola Zaire virus inhibits interferon responses by inducing IRF7 SUMOylation\textsuperscript{102}. For viral advantage, it is reported that immediate–early protein transcription activator (RTA) of The Kaposi's sarcoma associated herpesvirus (KSHV) is SUMOylated, resulting in stimulation of transactivation for viral lytic cycle and HTLV Tax protein SUMOylation is required for nuclear localization so as to facilitate Tax mediated transactivation\textsuperscript{103, 104}. 

A number of viruses have been shown to inhibit innate immune signaling. Through these studies, we observed that HTLV Tax inhibits FADD and RIP1 function in IFN production. We therefore provide information on this virus as follows.

**HTLV-1 (Human T cell Leukemia Virus-1)**

**ATL (Adult T cell Leukemia)**

ATL is an aggressive non-Hodgkin T cell cancer with infiltrated T cells in various tissues especially skin\textsuperscript{105}. Lobulated nuclei termed ‘flower-like T cells’ are usually seen in patients and these T cells express CD3, CD4, CD7, CD8 and CD25 on the cell surface\textsuperscript{106}. Depending on the clinical features, ATL is classified into four types: acute, lymphoma, chronic, and smoldering ATL. The acute and lymphoma type are an aggressive form of ATL in which combination chemotherapy, allogenic stem cell transplant, and monoclonal anti-CCR4 or anti-CD25 antibody are option for treatment. In chronic and smoldering types, a combination of Zidovudine (ZDV) and interferon-\(\alpha\) is used for ATL treatment\textsuperscript{107}. 
Figure 1-8 Schematic of HTLV-1 genome and Tax protein.

A. All viral protein produced by alternative splicing through pX regions. Except HBZ, viral products are transcribed from 5’LTR.

B. Tax contains NLS (nuclear localization signal), Zn (zinc finger), LZR (leucine zipper like region), NES (nuclear export signal), G (Golgi localization motif), S (secretion motif), and PDZ (PDZ binding domain).
**HTLV-1 as an etiological agent for ATL**

HTLV-1 is the first human cancer causing retrovirus$^{108, 109}$. At present, HTLV-1 infects around 20 million individuals especially in endemic area such as Japan, Africa, Caribbean islands, and central South America. Among them, a small percentage (6% of males and 2.1% of females) of infected individuals develop ATL after a 10-40yrs latent period. It is unclear why only a few percentages of infected people develop human malignancy, but genetic background and an individual’s immune system may contribute to the disease$^{110}$. The role of HTLV-1 as the etiological agent of ATL was reported during the 1980s. It was found that a type C retrovirus was isolated in Cutaneous T cell Lymphoma which had DNA polymerase activity. Later in Japan, ATLV (Adult T cell Leukemia Virus) was detected in ATL patients’ samples by using southern blot analysis and DNA polymerase activity assays. Based on the fact that both share identical genomic sequence, it was designated HTLV$^{111-113}$.

**HTLV-1 retrovirus**

HTLV-1 is a human retrovirus which belongs to the delta retrovirus family$^{112}$. There are 4 types of HTLV including recently identified HTLV-3 and HTLV-4$^{114, 115}$. However, only HTLV-1 is known as a causative agent for human malignancy. HTLV-1 contains a 9 Kbp diploid plus strand RNA virus which has gag, env, pol structural genes and a pX region flanked by 5’ and 3’ long term repeats (LTRs). This pX region is unique in that there is no homology to any human oncogene. Alternative splicing of pX region generates Tax, Rex, p21, p12, p13 and p30 from the 5’LTR region and HTLV bZIP protein (HBZ) from the 3’LTR. Accessory proteins such as p12, p30, Rex, and p13 are
reported to contribute viral infectivity and proviral load\(^{116}\). However, the most essential and extensive regulatory proteins for ATL are Tax and recently identified HBZ\(^{117,118-121}\).

\textit{Oncoprotein Trans-Activating factor of pX region (Tax)}

At first Tax was discovered as a transcriptional activator for the viral LTR. Later studies have reported that viral Tax has oncogenic properties in rat cells and primary T-cells as well as in Tax expressing transgenic mice\(^{117-119}\). The oncogenic property of Tax may originate from Tax’s multiple interacting domains, several localization signals, post-translational modifications like phosphorylation, ubiquitination, and SUMOylation\(^{122}\). These allow Tax to interact with wide ranges of cellular proteins and deregulate several signaling pathways such as cellular survival and proliferation pathways, DNA damage and DNA repair system, genomic stability, cell cycle, apoptosis and tumor suppressor.

1. Activation of cellular survival and proliferation pathways.

Abnormal NF-\(\kappa\)B and AKT activation is a common feature of cancer, and prerequisite for tumor progression. Accordingly, the activation of these factors by Tax is crucial for leukemogenesis. In NF-\(\kappa\)B signaling, Tax binds to NF-kappa-B essential modulator (NEMO) and IKK-\(\alpha\) and causes consistent stimulation in the both classic and alternative NF-\(\kappa\)B pathways\(^{123}\). Additionally, Tax interacts with TAXBP1 and then inhibits negative feedback regulation\(^{124}\). In the AKT pathway, Tax associates with PI3 kinase and enhances AKT phosphorylation at Ser 473 and 308 and subsequently leads to activation of survival genes\(^{125}\). It is reported that NF-\(\kappa\)B defective Tax mutants exhibit the inability to form tumors in transgenic mice, and treatment with a PI3 kinase inhibitor induces cell death in Tax expressing cells\(^{126-128}\). Aside from these factors, Tax also targets other
transcription factors such as cAMP responsive element binding protein (CRE) or serum response factor (SRF) in order to open the nucleosome and increase Fos binding capacity to DNA\textsuperscript{122}.

2. Induction of DNA damage and attenuation of DNA repair system.

Although Tax is primarily a positive regulator for gene activation, it also works as a repressor for certain genes. In particular, Tax suppresses the induction of basic excision repair, nuclear excision repair, and DNA mismatch repair genes\textsuperscript{129-131}. Attenuation of Tax in DNA repair signaling is also reported. Tax inactivates ATM/CHK2 axis by direct interaction\textsuperscript{132, 133}. Moreover, it is reported that Tax induces reactive oxygen species (ROS) formation\textsuperscript{134}. Collectively, Tax promotes DNA damage, and contributes to overall tumorigenesis.

3. Genomic instability.

Aberrant chromosomal structures are common phenotypes of human cancer. Tax causes aneuploidy in ATL cells. Tax also triggers multipolar mitosis by targeting TAX1BP2, RAN binding protein (RANBP) and mitotic arrest deficient-like protein 1 (MAD1)\textsuperscript{135}. TAX1BP2 and RANBP1 are chromosome replication controllers. Association of Tax with these protein provokes abnormal chromosome replication and multipolar mitosis\textsuperscript{136}. MAD1 and MAD2 are members of the spindle assembly checkpoint (SAC) family which ensure appropriate movement of chromosome segregation during mitosis\textsuperscript{137}. Tax binds to MAD1 and deregulates SAC function. These events lead to aneuploidy and together they also contribute to cellular transformation.

4. Deregulation of cell cycle.
One phenotype of Tax expressing cells is stimulation of G1 to S phase transition\textsuperscript{138}. Tax increases levels of cyclin E and cyclin D expression, and directly binds cyclin-dependent kinase 4/6 (CDK4/6) holoenzyme complexes with cyclin D\textsuperscript{139-141}. This interaction enhances CDK4/6 kinase activity for retinoblastoma (Rb). Subsequently, Rb becomes hyperphosphorylated and its modification leads to deactivation of Rb’s tumor suppressor function. Tax also triggers degradation of Rb. Another function of Tax in the cell cycle involves repressing the cell cycle inhibitors by downregulating the transcription levels of p27, p18 and p19. In addition, Tax hinders interactions between cyclin-dependent kinase 4 and cyclin-dependent kinase inhibitors\textsuperscript{142}.

5. Inhibition of apoptosis and tumor suppressor.

It is reported that HTLV infected T cells exhibit resistance to apoptosis. The underlying mechanisms include the induction of anti-apoptotic genes such as B-cell lymphoma 2 (BCL-2) and the repression of the Bax gene\textsuperscript{143}. Moreover, Tax targets tumor suppressors like Rb and p53. As described above, Tax enhances Rb phosphorylation and subsequently leads to proteasomal degradation. Additionally, Tax inhibits p53 but the mechanism needs further delineation\textsuperscript{144-148}.

\textit{HBZ (HTLV bZIP protein)}

Although Tax is crucial for malignancy, freshly isolated ATL cells do not express Tax protein\textsuperscript{149-151}. Also, 60% of the provirus genome can contain a 5’ LTR region deletion, or a genetically silenced Tax gene. On the other hand, the 3’ LTR region of the HTLV genome can remain in an unmethylated state without any deletion\textsuperscript{121}. HBZ is the only protein which uses the 3’UTR for its expression\textsuperscript{120}. HBZ expression is observed in all
ATL patients, and correlates with proviral load\textsuperscript{152}. Originally, HBZ was identified as an inhibitor of Tax mediated viral transcription and decreased c-Jun and Jun B activity with abrogation of the canonical NF-κB pathway\textsuperscript{153}. However, HBZ activates JunD for induction of human telomerase, reverse transcriptase, and preserves the non canonical NF-κB pathway, which is central for certain types of cancer\textsuperscript{154}. Additionally, HBZ RNA possesses growth enhancing capacity\textsuperscript{155}. Therefore, HBZ is considered a key factor for maintaining ATL malignancy\textsuperscript{156}. However, the molecular mechanisms of HBZ in tumorigenesis still need to be further elucidated.

Here I will describe my results which attempt to further unravel the mechanisms of innate immune signaling, including the importance of RIP1 and FADD in these processes. We also present data about a possible FADD regulatory mechanism in SUMOylation. Furthermore, we provide data indicating that HTLV Tax may inhibit these processes to avoid host defense countermeasures.

**SPECIFIC AIMS**

The innate immune system is a first line defense mechanism against invading microorganisms such as viruses. To eradicate virus infection, the host possesses specific cellular receptors that activate antiviral host responses by producing select cytokines such as type I Interferon \textsuperscript{1,2}. Following viral infection, cellular cytosolic receptors RIG-I or MDA-5 recognize viral dsRNA and trigger IFN-β induction through adaptors such as IPS1 and DD containing proteins RIP1 and FADD\textsuperscript{2}. These adaptors form a complex called an ‘innateosome’ and activate transcription factors required for IFN-β production.
(Fig 1-8). Subsequently, IFN binds to IFN receptor on cell surface and activates JAK/STAT signaling in order to induce hundreds of genes important for inducing an antiviral state\(^4\).

Our lab has focused on the DD containing protein RIP1 and FADD in regulating RLR-mediated IFN production. Our data indicates that RIP1 and FADD play pleiotropic functions as adaptors in several pathways such as apoptosis, cell survival, cell proliferation, and innate immunity\(^{12, 44}\). However, their roles in antiviral signaling and their mechanism of action have not been clarified. Therefore, in this study we investigated how RIP1 and FADD are involved in the RLR pathway. Understanding how RIP1 and FADD function in the RLR pathway will provide extended knowledge for IFN-\(\beta\) signaling. Recently SUMOylation is emerging as an essential modification which modulates protein activity and localization\(^{72}\). In previous studies, we found that FADD interacts with the SUMO machinery and contains SUMO subjective consensus lysines. Accordingly, we postulate that FADD may be a target of SUMOylation. This study will enlighten the relevance of FADD’s function in the context of SUMOylation.

Through these studies we also noted that HTLV1 Tax was able to inhibit RLR-signaling. Our lab is interested in the HTLV-1 Tax protein which is a viral protein implicated in ATL (Adult T cell Leukemia). Comprehending the anti-viral mechanism of HTLV-1 Tax will contribute to our understanding of innate immunity.

Given this the following issues will be discussed in each chapter.

Chapter 1: Introduction
Chapter 2: will address the role of RIP1 and FADD in RLR signaling. How do they perform their functions in RLR signaling?

Chapter 3: we will address whether FADD is a target of SUMOylation. What is the role of FADD SUMOylation? We show evidence for FADD SUMOylation, and additionally, we identified FADD’s SUMOylation site.

Chapter 4: we will address whether HTLV-1 Tax inhibits anti-viral signaling.

To illustrate the anti-viral mechanism of the HTLV-1 Tax protein, we examined IFN signaling and identified interacting adaptors which lead to abrogation of IFN-β induction.

Our Hypothesis

1. RIP1 and FADD play an important role in innate signaling. We hypothesize that RIP1 and FADD regulate RLR signaling.

2. We found that FADD may be SUMOylated. We hypothesized that SUMOylation regulates FADD function.

3. Our data indicates that Tax inhibits innate signaling. We hypothesize that Tax does this by inhibiting RIP1 and FADD function.
CHAPTER 2: RIP1 and FADD’s role in innate immunity

RESULTS

RIP1 role in RLH Signaling

Previously, it was shown that RIP1 is involved in IFN-β induction signaling. RIP1 knock-out MEFs display increased VSV-induced cytolysis and a lower level of type I IFN mRNA compared to wild type MEFs\textsuperscript{12}. However, the underlying mechanisms describing how RIP1 is related to the RIG-I pathway is not yet clear. As a start to these studies, we examined which innate immune genes could bind dsRNA. After treating with IFN-β, cell lysates were pulled down with Poly (I:C) (synthetic analog of dsRNA) beads and we performed immuno blot analysis with anti-RIG-I, MDA-5, RIP1, FADD and PKR antibodies. Following IFN-β treatment, RIG-I, MDA-5, RIP1, and PKR protein levels were increased. Interestingly, although RIP1 does not contain a RNA binding motif, RIP1 strongly associated with Poly (I:C) beads. However, FADD is undetectable in a complex with Poly (I:C) (Fig 2-1, A). This observation makes us wonder if there is a possible interaction between RIP1 and RIG-I-like helicase receptors. To evaluate RIP1 and RIG-I binding, we performed a yeast two hybrid assay and co-immunoprecipitation analysis in 293T cells after transiently overexpressing RIP1 and variants of RIG-I and MDA-5. The bait RIP1 and prey vectors FADD, RIG-I, and MDA5 were co-transfected into yeast and interactions were examined. We found that RIP1 binds to the CARD domain of RIG-I or domain of MDA-5 in the yeast system, but the interaction was not as strong as with full-length FADD (Fig 2-1, B). This was also observed in 293T cells when we transiently
Figure 2-1 RIP1 associates with RIG-I in presence of dsRNA.

A. Poly (I:C) beads pull down with endogenous RIG-I, MDA-5, PKR and RIP1 but not FADD after treating with IFN-β (18hr) and Poly (I:C) (3hr) in Hela Cells.

B. GAL4 binding domain (BD) conjugated with full length RIP1, BD-RIP1 interacts with full length FADD, 1<sup>st</sup> CARD of RIG-I, 2<sup>nd</sup> CARD of MDA-5 variants fused to the GAL4 activation domain (AD-FADD, RIG-I (a:1-200aa, b:1-100aa, c:101-200aa)), and MDA-5 (a:1-200aa, b:1-100aa, c:101-200aa) in yeast-two hybrid system.

C. 293T cells were co-transfected with HA-RIP1, FLAG-RIG-I variants or MDA5 variants and lysates were immunoprecipitated (IP) and performed WB analysis with anti HA antibody.
introduced RIG-I or MDA-5 with RIP1 and co-immunoprecipitated the lysates. RIG-I and MDA-5 associated with RIP1 and the interactions were enhanced especially with CARD domain of the receptors. But, no interactions were observed with the C-terminal end of RIG-I or MDA-5 with RIP1 (Fig 2-1, C). In order to further detect which part of RIP1 mediates the interaction with ∆RIG-I (CARD domain of RIG-I), we generated variants of RIP1, as described in the figure legend (Fig 2-2, A). Accordingly, RIP1 variants with ∆RIG-I were transfected into 293T cells and the cells were used for co-immunoprecipitation analysis. The data indicated that ∆RIG-I binds to full length RIP1 or a mutant form lacking the KD (∆Kinase), indicating that kinase activity is not necessary for the interaction (Fig 2-2, B). Moreover, the binding capacity was not detected when we introduced only the KD or DD of RIP1. However, the interaction was observed in the presence of the ID of RIP1 (Fig 2-2, B). This illustrated that the RIP1 ID is responsible for RIG-I interactions. To validate the functional outcome of the interaction, we carried out luciferase reporter gene assays under control of the IFN-β promoter. We introduced an empty vector or ∆RIG-I along with full length RIP1, the KD, ID or DD of RIP1 in 293T cells and tested luciferase activity from the lysates. Compared to empty vector, RIG-I activates the IFN-β promoter 200 fold. RIP1 and RIP1 variants induce low IFN-β promoter activity (data not shown); however, co-expression of RIP1 together with ∆RIG-I, augments IFN-β induction 2-3 times that of ∆RIG-I activity. This result indicates that RIP1 can regulate RIG-I activity (Fig 2-2, C). Since IFN-β promoter region consists of three transcription factors such as NF-κB, IRF3/IRF7 and AP-1 for its induction, we questioned which signaling axis RIP1 regulates. Activated IRF3 and IRF7 bind to the
Figure 2-2 ID of RIP1 interacts with RIG-I and facilitates RIG-I activity.

A. Schematic of RIP1 and RIP1 variants.
B. 293T cells were transfected with an HA-ΔRIG-I (CARDs of RIG-I, 1-200aa) and RIP1 variants and the immunoprecipitates and cell extracts were analyzed by WB with anti HA antibody.
C. 293T cells were transfected with an IFN-β-Luc plasmid and RIP1 variants in presence of empty vector or ΔRIG-I.
D. 293T cells transfected as in C with PRD-III-I-Luc.
PRD III-I promoter region. Accordingly, a luciferase reporter vector encoding the PRD III-I promoter region was transfected into 293T cells with ΔRIG-I and luciferase activity was evaluated. Similar to the results from IFN-β promoter, we observed that the ID of RIP1 increased RIG-I activity up to 4-fold. Collectively, these data indicate that the RIP1 ID associates with ΔRIG-I and these interactions enhance IRF3 and IRF7 activation (Fig 2-2, D).

**FADD DED function in RIG-I pathway**

Previously, we reported that FADD is required for optimal RIG-I and MDA-5 signaling by mediating NF-κB activation and IRF7 induction. To further elucidate the functions of FADD, we generated a mutant FADD including only the DED or DD. Each of the domain recruits different adaptors (Fig 2-3, D). The DED of FADD recruits Caspase8 or cFLIP through DED homophilic interactions. On the other hand, the DD of FADD engages death receptors such as Fas and DD containing adaptors like RIP1 and TRADD. Overexpression of DED or DD is reported to initiate RIP1 mediated necrosis or Caspase8 mediated apoptosis, respectively. To test how those mutants change the modulation of IFN-β signaling, we performed a luciferase reporter assay using the IFN-β promoter construct and observed how FADD deletion changes IFN-β signaling in 293T cells. Our data indicate that similar to RIP1, FADD augments RIG-I or IPS1 activity up to 3-5 fold (Fig 2-3, A, B). The enhanced activity was mainly caused by DED. Next, to determine which signaling axis is regulated by FADD in RLR signaling, we utilized luciferase reporter assays within the PRD III-I promoter region. In accordance with IFN-
Figure 2-3 DED of FADD facilitates ΔRIG-I activity.

A. 293T cells were transfected with an IFN-β-Luc plasmid and Full length FADD, DED of FADD, DD of FADD in presence of empty vector or ΔRIG-I (CARDs of RIG-I, 1-200aa).

B. 293T cells were transfected with an IFN-β-Luc plasmid and Full length FADD, DED of FADD, DD of FADD in presence of empty vector or IPS1.

C. 293T cells were transfected with an PRD III-I-Luc plasmid and DED of FADD, DD of FADD in presence of empty vector or ΔRIG-I.

D. Schematic of FADD and FADD variants.
β promoter regions, DED of FADD manifested enhanced RIG-I activity for IRF3 and IRF7 activation. (Fig 2-3, C). Overall, these results suggest that FADD facilitates RIG-I signaling through the death effector domain of FADD for optimal IRF3 / IRF7 activity. Thus, RIP1 and FADD facilitate RIG-I mediated innate signaling by possibly forming a complex.

DISCUSSION

RIP1 and FADD Role in Innate Immunity

Following viral infection, the genome or transcripts of a virus is recognized by RLR or TLR and triggers a signaling cascade for IFN-β induction. In the RIG-I Like helicase pathway, two representative receptors RIG-I and MDA-5 harbor similar structural domains with 75% homology and share the same adaptor protein termed IPS1. IPS1 interacts with the exposed CARD domain of RIG-I or MDA-5 after changing conformation following infection with viral nucleotides16.

In this study we observed RIP1 but not FADD forms a complex with Poly (I:C) and may associate with the CARD domain of RIG-I and MDA-5. The interaction between RIP1 and RLRs is through the ID of RIP1 resulting in amplification of RLR signaling. The DD of RIP1 also slightly enhances RIG-I activity. Although it is not clear whether FADD associates with RIG-I or MDA-5, we also observed that overexpression of FADD synergizes with RIG-I activity primarily through its DED. Previously, we have reported that FADD and RIP1 form a complex which we termed an ‘innateosome’ to regulate TBK1 activation of IRF3 and IRF712. Our data reveals that RIP1 and FADD may play a
role as scaffold proteins in ‘innateosome’ formation. In this complex, RIP1 functions as a signaling apex by gathering DD proteins and other adaptor proteins to dsRNA complexes and FADD facilitates this process.

In detail, the DD of FADD and RIP1 associate through DD electrostatic interaction\textsuperscript{157}. After RIP1 is associated with the RLR complex, RIP1 links FADD to RLRs and IPS1 complexes. The ID of RIP1 contains a K63 linked ubiquitination and a RHIM domain. Several proteins such as NEMO, TRAF3, TRAF6, IPS1, IRF7, TRIF and RLRs are reported to interact with the ID of RIP1.\textsuperscript{26} Since this region interacts with RLRs, it is possible that RIP1 modulates RIG-I function by increasing the association between IPS1 and the RLRs or by linking downstream signaling.

Similar to the DD, the DED domain connects to other DED proteins and promotes protein complex formation\textsuperscript{158}. Downstream of FADD, Caspase8 seems to be involved only in NF-κB signaling since Caspase8 deficient MEF did not show the same phenotype as FADD knock-out cells\textsuperscript{12}. Therefore, the DED of FADD might recruit other signaling molecules for IRF3 and IRF7 activation. For example, the DED of FADD may engage the same signaling molecules as the ID of RIP1 or might recruit other DED containing proteins.

Similar to the ‘innateosome’, a TRADDsome has been reported in the literature. In this case TRADD, RIP1, and FADD form a complex as a signaling apex through DD interactions and TRADD recruits the RIP1 and FADD to IPS1 in Mitochondria\textsuperscript{36}.

Forming a proper multi signaling complex is required for optimal activation of signal transduction. In the process, scaffolding proteins are emerging as key components by intensifying signaling although they do not contain intrinsic enzyme activity\textsuperscript{159}. Based on
our data we think that RIP1 and FADD act as scaffold proteins which form a signaling platform in the ‘innateosome’.(Fig 2-4).
**Figure 2-4 ‘innateosome’ formation in RLRs pathway**

RIP1 and FADD form a signaling apex for ‘innateosome’ complex through death domain interaction and facilitate RLRs innate signaling.
CHAPTER 3 – A possible regulation of FADD: SUMOylation

RESULTS

FADD is a substrate of SUMOylation.

Previously, to find FADD-associating proteins and FADD regulation mechanisms, a yeast two hybrid assay was performed in a Epstein-Barr Virus (EBV) transformed human B-cell cDNA library. Consequently, we discovered that FADD associates with SUMO machinery. SUMOylation is the reversible post-translational modification which is mediated by SUMO and can influence protein stability, localization and activity. SUMO targets lysine at the consensus \( \psi KXD/E \) motif where \( \psi \) hydrophobic, \( X \) any amino acids, \( K \) lysine and \( D/E \) aspartate or glutamine\(^{72} \). Based on SUMO plot analysis, FADD contains two potential SUMO consensus motifs in the K35 and K125 positions (Fig 3-1, A, B). Based on these experimental results, we hypothesized that FADD may be a target of SUMOylation and SUMOylation of FADD may modulate FADD’s function. To test this possibility, we performed \textit{in-vitro} SUMOylation assay using the UFDS (Ubc9 fusion directed SUMOylation) system (Fig 3-2). We utilized this system due to low steady state level SUMOylated protein\(^{160} \). Subsequently, we made a translational fusion protein with FADD and Ubc9 and carried out \textit{in-vitro} SUMOylation assays. In the absence of SUMO-1, Ubc9 and FADD-Ubc9 construct were expressed as a single protein (~20KDa and ~50KDa respectively) in the \textit{in-vitro} translation system. In the presence of SUMO-1, 70% of FADD-Ubc9 was detected as a SUMO modification form with three major shifted bands (~70KDa, ~83KDa, and ~100KDa) while Ubc9 did not shift (Fig 3-3, A)
A

1 MDPFLVLLHS VSSSLSSSEL TELKFLCLGR VGKRKLERVQ SGLDLFSMML
1 MDPFLVLLHS LSGSLSGNDL MELKFLCRER VSKRKLERVQ SGLDLFTVLL
61 EQNDLEPGHT ELLRELLASL RRHDLRLRRVD DFEAGAAAGA APGEEDLCAA
61 EQNDLERGHT GLLRELLASL RRHDLRQRDL DFEAGTATAA PPGEADLQVA
101 FNVICDNVGK DWRRRLARQLK VSDTKIDSIE DRYPRNLTER VRESLRIWKN
101 FDIVCDNVGR DWKRLARELK VSEAKMDGIE EKYPRSLSER VRESLKVWKN
161 TEKENATVAH LVGALRSCQM NLVADLVQEV QQARDLQNRS GAMSPMSWNS
161 AEKKNASVAG LVKALRTCRL NLVADLVEEA QESVSKSENAM SPVLRDSTVS
201 DASTSEAS human FADD
201 SSETP mouse FADD

B

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<td>None</td>
<td>SVSKSEN</td>
</tr>
</tbody>
</table>

Figure 3-1 Putative SUMOylation site in hFADD and mFADD.

A. Red colors indicate lysine residue in human and mouse FADD sequence. Human
and mouse FADD have 8 lysine residues and 13 lysine residues respectively.
B. Consensus motif for SUMOylation.
Figure 3-2 Schematic view Ubc9-fusion-directed SUMOylation system and FADD-Ubc9 fusion protein.

A. In general SUMOylation way, SUMO protein attached to Ubc9 transfers to a substrate with SUMO E3 liagse.
B. UFDS (The Ubc9 fusion dependent SUMOylation) system.
C. The feature of FADD-Ubc9 protein. Human Ubc9 construct (pCU) which harbors BamHI and EcoR I sites and Stop codon deleted FADD gene was added to the pCU after polymerase chain reaction with corresponding cloning enzyme sites.
This result suggested that FADD might be a possible target of SUMOylation. Additionally, three different migratory patterns of FADD-Ubc9 suggested the possibility that FADD-Ubc9 could be SUMOylated at multiple sites. To affirm SUMOylation of FADD, we tested whether these modifications were an isopeptide link that is present between SUMO and a substrate by conducting an \textit{in-vitro} deSUMOylation assay. As seen in Figure 3-3, shifted bands were not present during SENP1 treatment. This result confirmed that the delayed migratory patterns of FADD-Ubc9 resulted from SUMOylation.

To validate FADD SUMOylation \textit{in-vivo}, total lysates were collected from 293T cells transfected with FADD, FADD-Ubc9, or empty vector control were immunoprecipitated with either anti-FADD or anti-SUMO antibody. Then the signal was detected by immunoblotting using anti-FADD or anti-SUMO-1 antibody. In accordance with the result from the \textit{in-vitro} SUMOylation assay, the three major SUMO-1 modified bands (~70KDa, ~83KDa, and ~100KDa) were detected in both blots (Fig 3-4, A, B). This illustrates FADD SUMOylation \textit{in-vivo}.

SUMO-1 is reported to predominantly reside in the nucleus and regulate the nuclear localization of other proteins. FADD is also known to localize either in the nucleus or cytoplasm\textsuperscript{41}. Accordingly, if SUMO-1 can modify FADD, it is likely to localize within the same compartment of the cells. To detect a possible interaction with endogenous FADD and SUMO-1, hTert (human telomerase fibroblast) cells were stained with anti-SUMO and anti-FADD antibody. SUMO-1 resides in the nucleus as a spot. FADD also forms a cluster in nucleus region and is hardly detectable in cytoplasm in the cells. Merging image of FADD and SUMO indicates that FADD and SUMO-1 were
**Figure 3-3 in-vitro FADD-Ubc9 SUMOylation.**

A. Ubc9 and FADD-Ubc9 were incubated in TNT T7 *in-vitro* system with S35-Methionine in presence or absence of SUMO-1 and reaction mixtures were analyzed with SDS-PAGE and autorgraphy.

B. After SUMOylated reaction mixtures were incubated with SENP1 and were analyzed with SDS-PAGE and autorgraphy.
A. 293T cells were co-transfected with empty vector, Ubc9, FADD, and FADD-Ubc9 in presence of SUMO-1 and lysates were immunoprecipitated (IP) with anti FADD antibody and performed WB analysis with anti SUMO-1 antibody.

B. 293T cells were co-transfected with empty vector, Ubc9, FADD, and FADD-Ubc9 in presence of SUMO-1 and lysates were immunoprecipitated (IP) with anti SUMO-1 antibody and performed WB analysis with anti FADD antibody.
Figure 3-5 Immunofluorescence analysis of FADD and SUMO-1 in hTert.

Human telomerase fibroblasts were stained with an anti-FADD (green) and anti-SUMO-1 (red) antibody.
co-localized in nucleus as spot-like clusters (Fig 3-5). This indicates that endogenous FADD might be SUMOylated \textit{in-vivo} and SUMOylated FADD may be located in nucleus.

\textit{Identification of SUMOylation sites in FADD-Ubc9.}

FADD contains a SUMOylation consensus motif at K35 and K125. To determine if these lysine residues are responsible for SUMOylation of FADD, we mutated the lysines to arginines and performed in-vitro SUMOylation assay. Compared to wild type, single or double mutations of those lysines did not shift the mobility on SDS-PAGE (data not shown, Figure 3-6). Since these lysines are not responsible for SUMOylation, non-consensus lysines of FADD were then mutated and subjected to in-vitro SUMOylation assay. Among eight lysines in FADD, replacement of lysine at 110 partially abolished the two shifted bands (~83KDa and ~100KDa) (Fig 3-6). We next mutated all the lysines of FADD. However, we still observed additional SUMOylated sites in FADD-Ubc9 (~70KDa). This may indicate Ubc9 SUMOylation although we were unable to detect it. According to the literature, it has been reported that SUMO is attached to Ubc9 in-vitro. A recent paper also showed that K14 in Ubc9 is a SUMOylation site and that this modification regulates target specificity by enhancing or reducing SUMO conjugation. Additionally, Rhes (Ras homologue enriched in stratum) was discovered as an enhancer for Ubc9 SUMOylation. Accordingly, it is possible that SUMOylated Ubc9 is a prerequisite for FADD SUMOylation or FADD is necessary for Ubc9 SUMOylation.

In order to evaluate first whether Ubc9 SUMOylation is specific within the FADD fusion, we decided to use p53 as a control. p53 is a tumor suppressor and SUMOylation
of p53 at lysine 386 is reported to be required for transcriptional activity. Subsequently, p53 was cloned into Ubc9 and a mutation was made in SUMO lysine 386. The outcome of in-vitro SUMOylation assay reveals that the p53-Ubc9 fusion protein was translated into a ~70KDa product and approximately 30% of p53-Ubc9 became SUMOylated (~100KDa) in the presence of SUMO-1. On the other hand, we detected only a ~70KDa of K386R p53-Ubc9 and were unable to detect additional sites for Ubc9 SUMOylation in the presence of SUMO-1 (Fig 3-8, A). This indicates that p53-Ubc9 fusion protein may not need Ubc9 SUMOylation. This also signifies that FADD may be required specifically for Ubc9 SUMOylation.

To confirm Ubc9 SUMOylation in our FADD-Ubc9 fusion protein, we did a SUMO plot analysis. Ubc9 contains a consensus motif at K65 and K76 (Fig 3-7). We selected these two sites along with other known Ubc9 SUMOylation sites K14 and K153. Among four putative SUMO sites, we only obtained the K58R and K153R FADD-Ubc9 construct and performed in-vitro SUMOylation assay. Compared with wild type FADD-Ubc9, SUMOylated Ubc9 bands (~70KDa) remain unchanged. The results suggested that neither K65 nor K153 were responsible for Ubc9 SUMOylation (Fig 3-8, B).

Taken together, our data indicate that K110 is one of the major SUMOylation sites of FADD. Moreover, Ubc9 is potentially SUMOylated in the presence of FADD. It suggests that FADD might be required for Ubc9 SUMOylation or Ubc9 SUMOylation is a prerequisite for FADD SUMO attachment. Thus, FADD may be a part of the SUMO machinery.
Figure 3-6 Detection of FADD-Ubc9 SUMOylation site.

Indicated lysine residues in FADD-Ubc9 were mutated to arginine by using XL-Blue site-directed mutagenesis kits. Then, plasmids were used for *in-vitro* SUMOylation assay as described previously.
A

1 MSGIALSRLA QERKAWRKDH PFGFVAVPTK NPDGTMLMN WECAPGKKG
61 TPWEGGLFKL RMLFKDDYPS SPPKCKFEPP LFHPNVYPSG TVCLSILEED
101 KDWRPAITIK QILLGIQELL NEPNIQDPAQ AEAYTIYCN RVEYEKRVRA
161 QAKKFAPS human Ubc9

B

<table>
<thead>
<tr>
<th>Pos</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>MLFKDDY</td>
</tr>
<tr>
<td>76</td>
<td>PKCKFEP</td>
</tr>
</tbody>
</table>

Figure 3-7 Putative Ubc9 SUMOylation site.

Human Ubc9 have 14 Lysine residues and red color letters indicate lysine residues consensus motif for SUMOylation.
Figure 3-8 Ubc9 SUMOylation.

A. p53 was fused with pCU by using polymerase chain reaction cloning method with Hind III and EcoRI enzyme site integrated primer. After cloning, mutagenesis of Lysine 136 residues were carried out, then *in-vitro* SUMOylation was conducted.

B. By using site-directed mutagenesis, lysine residues at 65 and 153 in Ubc9 were mutated in arginine and then *in-vitro* SUMOylation was carried out.
DISCUSSION

**FADD’s SUMOylation.**

Our study illustrates that FADD is modified by SUMOylation at Lysine 110 in a polySUMOylated pattern and may occur in the nucleus of cells. Additionally, we observed specific Ubc9 SUMOylation by FADD indicating that FADD may require Ubc9 SUMOylation or Ubc9 SUMOylation might require FADD.

Since FADD was initially isolated as a Fas adaptor protein, localization of FADD is thought to be in the plasma membrane. However, it is revealed that FADD shuttles between the nuclear and cytoplasmic compartment\(^4\). Our data found that FADD is located in the nucleus and interacts with SUMO-1 like speckled structures under normal condition. This implies that FADD SUMOylation might be related to FADD localization. SUMO’s role in nuclear localization have been reported in several papers\(^{162, 163}\). Caspase8 and Caspase7 are reported to be associated with SUMO and the modification leads to nuclear localization although SUMOylation of Caspase8 does not alter functional Caspase activity\(^{162, 163}\). But, it is possible that SUMOylated forms of Caspase8, and Caspase7 or FADD may protect cells against apoptosis in normal condition’s by compartmentalizing FADD in the nucleus\(^{162, 163}\). Supporting this notion, it is reported that overexpression of SUMO-1 in BJAB or rheumatoid arthritis synovial fibroblasts displays resistance to apoptosis upon Fas stimulation\(^{164}\). Fas itself undergoes SUMOylation in the DD which mediates FADD apoptotic activity\(^{165}\).

To identify targets of SUMOylation in FADD we mutated SUMO consensus motif (K35, K125) and nonconsensus lysines in FADD as a FADD-Ubc9 fusion protein and
performed in-vitro SUMOylation assay. Our results reveal that K110 is the single polySUMOylation site for FADD’s SUMOylation. K110 of FADD along with R113, R114, R117 and R140 are the essential positively charged groups for homophilic DD mediated electrostatic interactions. As we reported in the previous section, DD or DED-mediated homophilic interactions are required for FADD’s scaffolding function. Accordingly, further studies are required to determine whether SUMOylation of FADD may abrogate DD mediated interaction and function as a general FADD inhibitory mechanism.

While mutating all the lysines in FADD, we noticed SUMO attachment on Ubc9. Additionally, it was specific to FADD fusion, since p53-Ubc9 did not display Ubc9 SUMOylation. These data have led us to speculate whether Ubc9 SUMOylation is required for FADD or vice versa. It has been reported that Ubc9 SUMOylation preferentially determines substrate SUMOylation. In particular, SUMOylated Ubc9 increases sp100 SUMOylation through a SIM domain but does not alter the SUMOylation status of HDAC4, E2-25K, and PML. Accordingly, Ubc9 SUMOylation may be necessary for FADD SUMOylation. Another possibility is FADD’s role in Ubc9 SUMOylation. Currently, few molecules have been discovered as SUMO related accessory proteins. RWD domain containing 3 (RSUME) is reported to increase general SUMOylation by increasing E1 transfer to Ubc9. Rhes enhances Ubc9 SUMOylation on K14, 49, and 153 and is thought to increase thioester or isopeptide linkages. SF2/ASF, which is a regulator of RNA processing, has been reported as a general SUMOylation enhancer upon heat shock stress. The relationship between FADD and Ubc9, however needs further investigation.
Collectively, SUMOylation is a reversible process where ubiquitin-like SUMO proteins are tagged onto a protein at a lysine residue, resulting in a change of cellular localization or biological function of a target protein. We found here that FADD is a substrate for SUMO-1 and reported that FADD SUMOylation might regulate FADD localization. Additionally, FADD may be involved in Ubc9 SUMOylation but further studies are necessary for this issue to be clarified.
CHAPTER 4 - HTLV-1 Tax abrogates RLRs mediated antiviral signaling

RESULTS

*HTLV oncprotein Tax inhibits antiviral signaling.*

To investigate the regulation of IFN-β signaling, we performed a luciferase assay using the IFN-β promoter and screened for anti-viral proteins involved in suppression of the RLH signaling pathway. We found that the HTLV-1 Tax protein potently inhibits IFN-β-induced signaling. To confirm this result, we performed a luciferase assay with the IFN-β promoter in 293T cells after treating with Poly (I:C) in the presence or absence of Tax. Poly (I:C) stimulated the IFN-β promoter up to 14 fold and this induction was decreased in the presence of Tax in a dose dependent manner (Fig 4-1, A). Similar inhibitory effects of Tax with regard to IFN-β signaling were also observed in full length RIG-I or MDA-5 expressing cells during Poly (I:C) treatment in ΔRIG-I or ΔMDA-5 expressing cells (Fig 4-1, B, C). To examine if IFN-β protein level were decreased in the presence of Tax due to abrogation of signaling, we conducted an IFN-β ELISA while co-expressing ΔRIG-I and Tax in 293T cells. Our data illustrated that the amount of IFN-β in ΔRIG-I expressing cell is suppressed by Tax and this indicated that Tax abrogates signaling prior to IFN-β production (Fig 4-1, D).

To further elucidate the mechanism of suppression, MEFs expressing Tax were stimulated with Poly (I:C) (3 hr, 6 hr, and 9 hr) and IFN-β mRNA levels were measured in the cells by RT-PCR analysis. IFN-β mRNA levels began to increase after 6 hr Poly
Figure 4-1 Tax inhibits type I IFN induction mediated by RIG-I and MDA-5.

A. 293T cells were transfected with an IFN-β-Luc plasmid and with Tax in absence and presence of Poly (I:C) stimulation.
B. 293T cells were transfected with an IFN-β-Luc plasmid and full length RIG-I and MDA-5 along with Tax.
C. 293T cells were transfected with a PRD III-I-Luc plasmid and ΔRIG-I and ΔMDA-5 (CARDs of MDA-5, 1-200aa) as in B.
A. Primary C57Bl/6 MEFs were transfected with either an empty control vector, or with a Tax expressing vector for 24hr and treated with Poly (I:C) for the indicated periods and \( \text{ifn}_\beta \) transcription measured by real-time PCR.

B. Tax inducible Jurkat T cells were stained using anti Tax (red) antibody after treating with doxycycline (1ug/ml) for 24 hr.

C. Tax inducible Jurkat T cells were infected with the indicated m.o.i.s with VSV\( \Delta M \) for 6hr and \( \text{ifn}_\beta \) transcription measured by real-time PCR in presence or absence of Tax.

D. Primary C57Bl/6 MEFs were transfected with either empty vector or Tax for 24 hr and cells were infected with the indicated m.o.i.s of VSV-GFP. Photomicrographs were taken 24 hr post infection.

E. VSV progeny yield was determined in culture supernatants from cells treated as in D by standard plaque assay.

Figure 4-2 Tax abrogates antiviral signaling.
(I:C) stimulation. After 9 hours of stimulation, the Poly (I:C) induced IFN-β mRNA level were increased 24-fold although Tax decreased the mRNA production by 60% (Fig 4-2, A). Since HTLV-1 provirus is commonly detected in T cells, we performed similar experiments in Jurkat cells expressing Tax in a Tet On system. After inducing Tax by treating cells with doxycycline, Tax expression was confirmed by immunofluorescence analysis (Fig 4-2, C). We then infected the cells with VSV ΔM at different MOIs and IFN-β mRNA levels were tested in the cells by RT-PCR analysis. In accordance with our results in MEFs, the inhibition of IFN-β mRNA was also observed in Jurkat T cells expressing Tax (Fig 4-2, C).

If IFN-β mediated anti-viral signaling is abrogated by Tax, Tax expressing cells should be more susceptible to VSV replication. Accordingly, after transfecting with empty vector or Tax in MEFs, we infected the cells with VSV-GFP at MOIs of 0, 0.1, and 1 PFU/cell and viral replication was monitored by fluorescence microscopy. VSV replication was significantly increased in the presence of Tax, measured both by levels of GFP-expressing cells and by plaque assay (Fig 4-2, D, E).

Taken together, our data illustrate that Tax suppresses the induction of IFN-β protein production in IFN-β driven luciferase reporter assays. Moreover, endogenous IFN-β mRNA inducibility upon viral infection is abrogated in 293T cells, MEF and Jurkat T cells in the presence of Tax. Overall, the data indicate that HTLV-1 Tax protein blunts IFN-β induced signaling by inhibiting IFN-β production.
Figure 4-3 Tax inhibition point in antiviral signaling.

A. 293T cells were transfected with an IFN-β-Luc plasmid and IPS-1, TRIF, TBK1, IRF3 (SuperActive), IRF7 (SuperActive), in presence of increasing amounts of Tax.

B. 293T cells were transfected with a PRD-III-I-Luc plasmid and empty vector, ΔRIG-I, ΔMDA-5, TBK1 in presence of increasing amounts of Tax.

C. 293T cells were transfected with a PRD-II-Luc plasmid and empty vector, ΔRIG-I, ΔMDA-5, TBK1 in presence of increasing amounts of Tax.
**Tax Inhibition point in antiviral signaling.**

To further investigate the mechanism by which Tax suppresses RLH signaling, we conducted an epistatic analysis by performing IFN-β driven luciferase reporter assay in 293T cells with several adaptor proteins such as IPS1, TRIF, TBK1, IRF3 (SA: SuperActive form S396D), and IRF7 (SA: Super Active form inhibitory domain deletion Δ238-410) in the presence and absence of Tax. IPS1 or TRIF are essential adaptors that interact directly with RLRs or TLR3/4 and robustly switch on type I IFN responses. The overexpression of the RLH signal adaptor, IPS1, or the TLR3 and TLR4 adaptor, TRIF, augment IFN-β promoter activity; however, Tax blocks those adaptors from inducing IFN-β promoter activity. This result implies that Tax not only inhibits the RIG-I pathway, but also inhibits IPS1, TLR3, and TLR4 mediated antiviral signaling. Further downstream molecules were tested using the same reporter assay. TBK1 is an essential kinase which phosphorylates IRF3 and IRF7. Phosphorylation of IRF3 and IRF7 induces a conformational change and allows them to associate with the IFN-β promoter. Consequently, we tested the inhibitory effect of Tax on TBK1, or the superactive forms of IRF3 (S396D) and IRF7 (inhibitory domain deletion 238-410). The overexpression of TBK1, IRF3 (SA) and IRF7 (SA) overcome Tax mediated inhibition of IFN signaling. This suggests that Tax may blunt the signaling upstream of TBK1 but downstream of IPS1 or TRIF (Fig 4-3, A).

The IFN-β promoter region contains four positive regulatory domains that are responsible for distinctive transcription factors. In particular, NF-κB binds to PRD II promoter regions while the homo or hetero form of IRF3 and IRF7 interact with PRD III-I regions. According to previously published results, Tax stimulates NF-κB activity.
Therefore, we assume that Tax may inhibit IRF3 and IRF7 activation. ∆RIG-I, ∆MDA-5, TRIF, and TBK1 were transfected into 293T cells with PRD II or PRD III-I luciferase reporters and the activity was measured in lysates. Consistent with luciferase reporter assays encoding IFN-β promoter regions, Tax inhibits luciferase activity induced by ∆RIG-I, ∆MDA-5, TRIF but not TBK1 (Fig 4-3, B). On the other hand, luciferase induction of PRD II regions is persistently activated by Tax indicating that Tax likely inhibits IRF3 and IRF7 activation (Fig 4-3, C). Thus, Tax inhibits RLRs as well as TLR signaling.

Interaction of RIP1 with Tax.

Several molecules are essentials for IRF3 and IRF7 activation in RLH signaling. To further explore how Tax impedes signaling, we searched for Tax interacting partners by performing yeast two hybrid assays and co-immunoprecipitation studies. We expressed ∆RIG-I, ∆MDA-5, IPS1, FADD, RIP1 together with Tax as bait in yeast and plated on His-Leu-Trp-Ade- deficient media. We detected colonies in RIP1-expressing yeast, indicating Tax potentially interacts with RIP1 (Fig 4-4, A). This interaction was further confirmed in 293T by conducting co-immunoprecipitation assays. In this assay, we included the RIP1 variants in order to identify the domains of RIP1 that interact with Tax. The results indicate that Tax interacts with the full length and the ID of RIP1 but not the KD nor DD. This indicates that Tax interacts with RIP1 through the ID that possesses the RHIM (RIP1 homophilic interaction motif) (Fig 4-4, B). We confirmed the association in primary ATL cells by confocal microscopy. Primary ATL cells were grown for 3 days \textit{in-vitro} culture until Tax was expressed and we stained with anti-Tax and anti-RIP1
antibody. In agreement with previous results, we observed that Tax co-localizes with RIP1 in the perinuclear regions, affirming the association of RIP1 and Tax (Fig 4-4, D). Since TRIF contains a RHIM domain and Tax abrogates TRIF responses on the IFN-β promoter, we tested whether Tax also binds to TRIF by conducting co-immunoprecipitation in 293T cells. FLAG-TRIF and RIP1 with or without Tax were introduced to 293T cells and the lysates were pulled down with FLAG and immuno blotted with anti-Tax antibody. Indeed, we found that Tax associates with TRIF as well as RIP1 (Fig 4-4, C).

Given that RIP1 enhances RIG-I activity and interacts with Tax, we tested whether Tax blunts RIP1 effects with on RIG-I activity by performing IFN-β driven luciferase assays. The result indicates that Tax decreases RIG-I mediated IFN induction up to 80%, revealing that Tax inhibition of RLR signaling possibly results from RIP1 interactions. We also observed that Tax inhibits FADD synergy on RIG-I activity (Fig 4-4, E, F). Thus, Tax binds RIP1 and TRIF and blocks RLR and TLR signaling.

**Tax abrogates the association of RIP1 and IRF7.**

Since RIP1 functions as an adaptor for several signaling pathway and interacts with Tax, we hypothesized that the inhibitory effect of Tax may be due to defects in intramolecular interactions between adaptors such as RIG-I, TRAF3, and IRF7 and RIP1. First, we tested RIG-I and RIP1 interaction in the presence of Tax because Tax and RIG-I associate through the ID in RIP1. However, Tax was unable to inhibit RIG-I and RIP1 interactions (Fig 4-5, A). Subsequently, we chose common adaptors such as TRAF3 which are involved in IRF3 or IRF7 activation and which interact with RIP1 in RLR and
A

Leu-Trp-  His-Leu-Trp-Ade-  + Tax

B

Full length  Kinase  RHIM  Death

Kinase Dead (Kd)

Death Domain Deletion (DDD)

Kinase domain (KD)

Intermediate Domain (ID)

Kinase Domain Deletion (KDD)

Death Domain (DD)

C

HA-ΔRIG-I

1. Vector

2. Full

3. Kd

4. DDD

5. KD

6. ID

7. KDD

8. DD

IP: FLAG

WB: Tax

Input

WB: Tax

IP: FLAG

WB: FLAG

D

Vec  FLAG-TRIF  FLAG-RIPI (314-671)

IP:FLAG

WB:Tax

Input 5%

WB:Tax

IP:FLAG

WB:FLAG

FLAG-TRIF

FLAG-RIPI variants
Figure 4-4 Tax associates with RIP1 and TRIF, resulting in inhibition of RIP1 synergetic effect on ΔRIG-I activity.

A. GAL4 binding domain (BD) conjugated with Tax, BD-RIP1 interacts with full length RIP1 but not with RIG-I variants MDA-5 variants, empty vector, IPS1, FADD, fused to the GAL4 activation domain (AD- RIP1, FADD, IPS1, empty vector, RIG-I, ΔRIG-I, MDA-5, and ΔMDA-5) in yeast-two hybrid system.
B. Schematic of RIP1 and RIP1 mutants
C. 293T cells were co-transfected with Tax, FLAG-RIG-I variants and lysates were immunoprecipitated (IP) and performed WB analysis with anti-Tax antibody.
D. 293T cells were co-transfected with empty vector, FLAG-TRIF, FLAG-RIP1 variants along with Tax and lysates were immunoprecipitated (IP) and performed WB analysis with anti Tax antibody.
E. Primary ATL cells were stained with an anti-RIP1 (green) and Tax (red) antibody after 3 days in-vitro culture.
F. 293T cells were transfected with an IFN-β-Luc plasmid and empty vector, ΔRIG-I, ΔRIG-I along with RIP1 in presence of increasing amounts of Tax.
G. 293T cells were transfected with an IFN-β-Luc plasmid and empty vector, ΔRIG-I, ΔRIG-I along with FADD in presence of increasing amounts of Tax.
TLR pathways. co-immunoprecipitation assays showed that the association of TRAF3 with RIP1 was not affected by Tax (Fig 4-5, B). Next, we selected IRF7 since RIP1 associates with IRF7 in the presence of the EBV LMP1 oncoprotein\(^\text{37}\). IRF7 is a master transcription factor for IFN-α/β induction in the MyD88 independent TLR and RLR pathways.\(^\text{169}\) Therefore, it is possible that Tax may abrogate the interaction of IRF7 and RIP1 and consequently lead to the impairment on IRF7 activation for type I IFN induction. To test this hypothesis, we expressed Myc-IRF7 and FLAG-RIP1 in 293T cells with wild type Tax and Tax mutants M22 and M47. Tax M22 is defective in NF-κB activation and unable to inhibit IFN induction. On the other hand, Tax M47 is incapable of activating viral LTR and blocking IFN response. Subsequently, lysates were immunoprecipitated with anti-FLAG antibody and immunoblot analysis carried out with anti-Myc antibody. The association of RIP1 and IRF7 was detected but the interaction disappeared in presence of Tax, Tax mutant M22 (Fig 4-5, C). The result indicates that Tax blocks RIP1 and IRF7 interactions and this may lead to a defect in IRF7 activation.

**Tax inhibitory effects on IRF7**

IRF3 and IRF7 are activated by TBK1-mediated phosphorylation and this leads to their homodimerization, and migration to the nucleus. Following activation, IRF3 forms heterodimers with IRF7, enhancing IFN-β production and the induction of various IFN-α subtypes that fully drive the antiviral state. Accordingly, we validated IRF7 phosphorylation status by using IRF7 stable 293T cells. After infection with VSVΔM for 6hr, cells were subjected to SDS-PAGE and we performed immunoblot analysis with anti-IRF7 antibody. A shifted IRF7 band was observed upon VSV infection and the
**Figure 4-5 Tax abrogates RIP1 and IRF7 interaction.**

A. 293T cells were co-transfected with HA-ΔRIG-I and FLAG-RIP1 in presence and absence of Tax and lysates were immunoprecipitated (IP) and performed WB analysis with the indicated antibodies.

B. 293T cells were co-transfected with HA-TRAF3 and FLAG-RIP1 in presence and absence of Tax and lysates were immunoprecipitated (IP) and performed WB analysis with the indicated antibodies.

C. 293T cells were co-transfected with HA-ΔRIG-I and Tax mutants and co-immunoprecipitation assay were performed with Myc-IRF7 and FLAG-RIP1 in presence and absence of Tax variants and lysates were immunoprecipitated (IP) and performed WB analysis with the indicated antibodies.
**Figure 4-6 Tax abrogates IRF7 activity.**

A. 293T expressing IRF7 stable cells were transfected with Tax and infected with VSVΔM for 6hr and cell extracts were analyzed by WB.

B. 293T cells were co-transfected with HA-ΔRIG-I and FLAG-RIP1 in presence and absence of Tax and lysates were immunoprecipitated (IP) and performed WB analysis with the indicated antibodies based on EZ-ChIP protocol.
Figure 4-7 Tax inhibition is independent of IRF3.

A. 293T cells were co-transfected with HA-ΔRIG-I or stimulated with Poly (I:C) for 4hr in presence or absence of Tax and lysates were analyzed by native gel and SDS-PAGE.

B. 293 T cells were stained with anti IRF3 (green) and Tax (red) antibody after co-transfecting empty vector, ΔRIG-I and ΔRIG-I with Tax.

C. 293T cells were co-transfected with HA-ΔRIG-I in presence or absence of Tax and nuclear lysates were analyzed by TransAM IRF3 ELISA kit for IRF3 binding capacity.
modification was not observed in presence of Tax (Fig 4-6, A). This implies that Tax may block IRF7 phosphorylation. If IRF7 phosphorylation was inhibited by Tax, IRF7 should be unable to bind the IFN-β promoter region. To further affirm whether Tax blocks the association of IRF7 on IFN-β promoter region, we performed ChIP analysis (Fig 4-6, B). RIG-I was transfected into 293T cells with or without Tax for 24 hrs and IRF7 was immunoprecipitated after UV cross-linking. The lysates were washed with ChIP buffer and PCR analysis performed by using IFN-β primers after purifying the DNA from the lysates. The amplified IFN-β region was detected in ΔRIG-I expressing cells but not in ΔRIG-I and Tax expressing cells. These data manifest a clear defect in IRF7 DNA binding in the presence of Tax.

It is reported that RIP1 strongly interacts with IRF7. Therefore, we assumed that Tax may not have an effect on IRF3. To test this hypothesis, we tested the dimerization status of IRF3 which is an indication of IRF3 phosphorylation. 293T cells were transfected with Tax and stimulated with Poly (I:C) for 4 hr or were co-transfected with Tax and ΔRIG-I. The dimer formation was observed by native gel analysis. Compared with mock, we detected endogenous IRF3 dimerization upon Poly (I:C) stimulation or ΔRIG-I expression. More of the dimerized form was detected in ΔRIG-I expressing cells than Poly (I:C)-stimulated cells. Thus, Tax does not disrupt the dimerization of IRF3 (Fig 4-6, A). We also tested IRF3 translocation status in Tax expressing cells. Expression vectors encoding ΔRIG-I, with or without Tax, were transfected into 293T cells and then endogenous IRF3 was stained with anti-IRF3 antibody. IRF3 is localized to the nucleus in the presence of ΔRIG-I. Nuclear location of IRF3 is not changed regardless of Tax expression. This indicates that Tax does not block IRF3 nuclear translocation nor
sequester IRF3 in cytoplasm (Fig 4-7, B). To further confirm whether the inhibitory mechanism of Tax is independent of IRF3, we checked whether IRF3 could associate with the IRF3 binding site by using the ELISA-based TRANS AM kit. After overexpressing ΔRIG-I in 293T cells, with or without Tax, nuclear fractions were prepared from the lysate and used for ELISA. Optical density corresponded to IRF3 binding capacity. Compared to mock, ΔRIG-I expressing cells exhibited a higher optical density. But, the level of optical density from ΔRIG-I expressing cell was not decreased with Tax. The data illustrates that IRF3 associates with the IFN-β promoter region despite Tax expression (Fig 4-7, C). Taken together, these results imply that Tax inhibits IRF7 activation but not IRF3. Thus, Tax may subvert RLR and TLR signaling by inhibiting IRF7 activation through inhibition of RIP1 and TRIF activity.

**DISCUSSION**

*Tax protein abrogates RIG-I mediated anti-viral signaling.*

HTLV-1 was discovered as the first tumor causing retrovirus in humans for Adult T cell leukemia (ATL) and Tropical spastic paraparesis/HTLV-1 associated myelopathy (TSP/HAM)\(^{156}\). During the process of pathogenesis, the viral transcription factor Tax is a pivotal factor for oncogenesis by affecting several signaling processes and performing pleiotropic function due to its broad range of interactions\(^{122}\). Here, we observed that Tax inhibits RIG-I and MDA-5 mediated antiviral signaling. Tax interacts with RIP1 and abrogates the interaction between RIP1 and IRF7, resulting in the inactivation of IRF7 then leading to a defect in IFN-β induction.
Due to the biological life cycle of retroviruses, three forms of the HTLV genome including ssRNA, DNA, and dsRNA are generated during its life cycle\textsuperscript{170}. When HTLV-1 infects cells, two identical copies of single stranded RNA are reverse transcribed to viral DNA. Then, viral DNA is integrated into the host genome referred to as a provirus. Later, integrated viral DNA transcribes into genomic RNA to produce proteins. Although HTLV-1 can infect most cells, in vitro the virus mainly replicates and forms a provirus in CD4\textsuperscript{+}T cells. Since HTLV-1 contains three forms of nucleic acid, each form could elicit type I IFN via different receptors.

Similar to HIV, the ssRNA of HTLV-1 may be recognized by TLR7 and induce a type I IFN response in pDCs\textsuperscript{171-173}. However, it is not clear whether HTLV-1 genomic RNA may be sensed by RIG-I or MDA-5 or whether viral DNA is possibly recognized by an unknown DNA sensor. Involvement of the RLR pathway in retrovirus infection was reported recently\textsuperscript{174}. It is noted that HIV genomic RNA can be recognized by RIG-I, but not MDA-5, to induce IFN-\beta. Then, activated RIG-I signaling by HIV genomic RNA is antagonized by the HIV protease by relocating RIG-I in the lysosome\textsuperscript{174}. Accordingly, it is possible that HTLV genomic RNA could trigger anti-viral signaling through RLRs, but Tax counteracts the IFN response. Another possibility of RIG-I signaling by HTLV-1 is though Polymerase III\textsuperscript{175}. Here, microbial DNAs are converted into 5 tri-phosphate dsRNA which can be sensed by RIG-I. DNA sensors for retroviruses have not been discovered yet, but a retro-transcribed DNA form of HTLV may be sensed by RIG-I through Polymerase III\textsuperscript{175}. Recently, it has been reported that the HIV genome can induce type I interferon in T cells or macrophages in the absence of the cytosolic nuclease Trex1, through the STING pathway\textsuperscript{176}. Our preliminary data indicates that Tax interacts with
STING and inhibits STING mediated IFN response, indicating that DNA of HTLV-1 may be recognized by a Sting related DNA sensor (data not shown). Additionally, Trex1 also may inhibit HTLV-1 virus induced DNA mediated immune responses\textsuperscript{176}.

Although our data is the first to show Tax inhibition of the RLRs pathway, a number of papers also illustrate negative regulation of type I signaling by HTLV-1. In IFN responsive pathways, HTLV-1 Gag and Pr inhibit STAT1 and STAT2 phosphorylation\textsuperscript{177}. Moreover, HCV replication and IFN-\(\alpha\) were reported to be decreased in Tax expressing cells. The underlying mechanism reveals that HTLV Tax protein interacts with CBP/p300 and promotes competition with STAT2 for its transactivation\textsuperscript{178}. Another paper reported that Tax consistently activates IRF3 phosphorylation via TAK1. However, IFN-inducible genes such as CXCL10, IRF4, and IFN-\(\beta\) are suppressed by the TLR negative regulator IRF4 which is overexpressed in HTLV-1 transformed cells\textsuperscript{179}. Recently, HTLV-1 induced SOCS1 (suppressor of cytokine signaling) has been reported to inhibit antiviral signaling by degrading IRF3\textsuperscript{180}.

The novelty of our study is also from our results on the function of RIP1. As described in previous sections, we reported that RIP1 functions as a scaffold protein which increases RIG-I activity for optimal IFN-\(\beta\) production through the IRF3 and IRF7 signaling axis. Based on these data, RIP1 and IRF7 interaction is essential for IFR7 activation since Tax abrogates these associations and results in a defect in IRF7 phosphorylation and IFN-\(\beta\) induction. Since RIP1 is essential for the ‘innateosome’ complexes viral proteins target RIP1 and disrupt RIP1 mediated signaling. For instance, cytomegalovirus M45 which contains a RHIM domain reportedly interacts with RIP1 and
inhibits NF-κB activation and RIP1 mediated apoptosis\textsuperscript{181,182}. However, an effect of M45 on IFN regulation to IRF3 and IRF7 has not been reported yet.

IRF7 is a pivotal regulator for innate immunity. Therefore, similar to HTLV-1, other viruses have developed strategies to impede IRF7 activation. For example, EBV LF2 tegument inhibits dimerization of IRF7 by interacting with a central inhibitory domain of IRF7\textsuperscript{183}. Ebola virus VP35 reportedly induces IRF7 SUMOylation which leads to inactivation of IRF7\textsuperscript{102}. KSHV ORF45 abrogates IRF7 phosphorylation and nuclear localization. KSHV vIRF3 associates with IRF7 and blocks IRF7 binding to IFN-β promoter regions\textsuperscript{184}. Additionally, KSHV RTA reportedly degrades IRF7 in a proteasome dependent pathway by acting as an E3 ligase\textsuperscript{185}.

The rapid production of type I Interferon is a hallmark of host defense against virus infection. To subvert the host immune system viruses develop immune evasion mechanisms by blocking IFN induction or IFN responses. Here, we showed that HTLV-1 Tax protein inhibits RLR and TLR3 antiviral signaling by binding to RIP1 and inhibiting IRF7 activation. Thus, this evasion mechanism may ultimately contribute to Tax mediated T cell leukemogenesis.
Chapter 2:

In this study we illustrated that RIP1 associates with the CARD domains of RIG-I and MDA-5 and facilitates RLH-mediated IFN-β inducing signaling. We reported that RIP1 was recruited to RLRs upon viral infection because RIP1 was pulled down with Poly (I:C) beads after treating with Poly (I:C) or IFN-β although no dsRNA binding motif exists in RIP1. Additionally, co-immunoprecipitation analysis indicates that CARD domains of RLRs associate with the ID of RIP1. To analyze outcome of the interactions, we performed a luciferase reporter assay under control of IFN-β promoter region. Our data indicate that RIP1 positively modulates RIG-I signaling mainly through ID and slightly through DD. We also confirmed the FADD requirements for RLH signaling. It appears that the RIG-I and IPS1 activity are synergized mainly through DED of FADD in IFN-β luciferase reporter assay. Additionally, DED of FADD is responsible for the IRF3 and IRF7 activation rather than NF-κB. Based on these data we concluded that RIP1 and FADD are scaffolding proteins in the ‘innateosome’. They form as a signaling point upon viral infection by interacting with RLRs through RIP1 ID and linking other proteins. FADD may help form higher ordered ‘innateosome’ by self-oligomerization.

Chapter 3:

We analyzed FADD SUMOylation in this section. We found that FADD undergoes polySUMOylation at lysine residue 110. SUMOylated FADD is located in the nucleus in immunofluorescence analysis. These observations lead us to hypothesize that FADD
SUMOylation might serve as a negative regulatory mechanism because lysine 110 of FADD is important for DD homophilic interaction and FADD functions as an adaptor in the cytoplasm. While we performed these experiments we detected specific Ubc9 SUMOylation by FADD. These results raise interesting question about the relationship between Ubc9 and FADD. FADD may require Ubc9 SUMOylation for its SUMO modification or FADD is part of the SUMOylation machinery.

Chapter 4:

Here, we inquired whether HTLV-1 Tax inhibits RLR mediated antiviral signaling. We observed that Tax inhibits RLR mediated IFN-β production. Endogenous IFN-β mRNA levels were also reduced in Poly (I:C) treated MEFs or VSVΔM infected Jurkat T cells in the presence of Tax. The defect of IFN production by Tax renders MEFs vulnerable to VSV mediated oncolysis. These data indicate that Tax inhibits IFN signaling by preventing IFN production. We also found that Tax inhibits the IRF3 and IRF7 signaling axis in IFN-β signaling by PRD III-I or PRD II promoter driven luciferase assays, however Tax persistently activates NF-κB and AP-1 for tumorigenesis. We found that Tax associates with RIP1 by co-immunoprecipitation assays. Further studies indicate that Tax abrogates IRF7 interaction with RIP1 and that this defect leads to IRF7 inactivation and suppression of IFN signaling.
MATERIAL and METHODS

Cell and Recombinant Virus

293T cells, BHK cells, and mouse embryonic fibroblasts (MEFs) and human telomerase fibroblast were grown in Dukbecco’s modified Eagle’s medium supplemented with 10% FBS and antibiotics. Jukat Tet-on Tax, Jurkat E6.1 and Primary ATL cells were grown in RPMI 1640 medium with 10%FBS, 2mM Glutamine and antibiotics. Indiana strain of VSV was used in all experiments. Recombinant viruses were generated by introducing XhoI and NheI sites of VSVXN2 along with compatible enzyme site harboring cDNA. Constructed VSV was obtained as described previously. (Femandez et al 2002)\textsuperscript{186} and (Lawson et al 1995)\textsuperscript{187}.

Transfections, Reagents, and Antibodies.

Transfections were conducted by using Lipofectamine 2000 (Invitrogen) transfection reagents in Opti-MeM (Invitrogen) according to thr manufacturer’s manual. MEF transfection was carried out using AMAXA MEF Nucleofectin Kit 1 according to the manufacturer’s recommendations (AMAXA Biosystems) Jurkat Transfections were performed with Fugene HD (Roche) as described in the manufacturer's instructions. Poly (I:C) (1ug/ml) was purchased from Invivogen. Doxycycline (1ug/ml), G418, and Polybrene were obtained from Sigma. Other reagents were purchased from; Quick change site directed mutagenesis kit (Stratagene) SUMOylation kit and SENP1 (BIOMOL), IRF3 TRANS AM Kit (Active motifs) and chromatin immunoprecipitation kit (Upstate). Polyclonal VSV antiserum was produced from Balb/c mice after
immunizing against wild-type VSV. Monoclonal anti-Tax antibody was a gift from Dr Harhaj. Other antibodies used in this study were: FLAG (Sigma); IRF3, IRF7 (Santa Cruz Biotechnology); Myc (Stratagen); HA, SUMO-1(Cell Signaling) TBK1 (Abcam); RIP1 (BD Science):

Plasmids and Mutagenesis

Expression vectors (pcDNA3.1, Invitrogen) FLAG or HA-tagged RIG-I, MDA-5, ΔRIG-I, ΔMDA5, IPS1, TBK1, RIP1, RIP1 variants, TRIF, STING, PIAS1 were generated by polymerase chain reaction. FADD-Ubc9 was cloned into pcDNA3Ubc9 after polymerase chain reaction. All lysine mutants of FADD Ubc9 by arginine were constructed by site-directed mutagenesis kit (stratagene). Other plasmids were obtains from the following sources: HTLV Tax, TaxM22, and Tax M47 (E. Harhaj), Myc-IRF7,PCLXN FLAG-IRF7(Dr Ning); FLAG-IRF3, FLAG-IRF7, and IFN-β Luc (J Hiscott) PRD II Luc and PRD-III-I Luc (T. Maniatis), pcDNAUbc9 (R. Niedenthal). T7-SUMO-1 (J. Chernoff), IRF3 (SA) and IRF7 (SA) (Invivogen),

Real-Time PCR

Total RNA was isolated by using RNasy RNA extraction kit (Qiagen) and cDNA synthesis was performed with random primers using 5ug of total RNA (Invitorgen) Real-time PCR was performed using a LightCycler 2.0 instrument and TaqMan Gene Expression Assay. Each sample was normalized to 18S ribosomal RNA level.
**In-vitro SUMOylation and DeSUMOylation assay**

A total of 1μg of Ubc9 and FADD-Ubc9 was added to the TNT T7 *in-vitro* transcription/translation system with S35-methionine in the absence and presence of SUMO-1/E1 activating enzyme (BIOMOL) and incubated 90 minutes at 30 degree Celsius. The reactions were stopped with SDS-PAGE loading buffer containing 5% β-mercaptoethanol and resolved on a 12% SDS-PAGE gel. Subsequently, the gel was dried and autoradiographed using an X-ray film. In order to perform DeSUMOylation assay, standard *in-vitro* SUMOylation was performed and the half of SUMOylated reaction mixture was subjected to 0.5μg SENP1 (BIOMOL) with 10mM DTT for 75 minutes at 30 degree Celsius. Then, the reaction was terminated by SDS loading buffer with the reducing agent and was analyzed by SDS-PAGE and autoradiography.

**Reporter gene assays and Immunofluorescence and Co-immunoprecipitations**

Reporter gene assays and Immunofluorescence were performed as described in (Balachandran et al, Harhaj et al) respectively. For co-immunoprecipitation, expression vectors were transfected into 293T cells for 36 to 48 hr, cells were lysed in EBC10 (50mM Tris,pH8.0, 150mMNaCl, 0.1% NP-40, 50mMNaF, 0.1mMNa3VO4, 1mMDTT) or RIPA buffer with protease inhibitors (100 mM PMSF, Leupeptin, Aprotinine, Pepstatin), and 1 mg of lysates were precipitated with 1 ug of FLAG mAb(SIGMA) overnight at 4°C. Following day, 30 ul of Protein G was added and incubated for 3 hr. All precipitates were washed with lysis buffer 5 times and proteins were released by 2X Sample Buffer after boiling and analyzed by SDS-PAGE.
Native PAGE Gel

Native PAGE Gel was carried out as described by Iwamura et al\textsuperscript{189}.

IRF3 TRANS AM Kit and chromatin immunoprecipitation (ChIP) analysis

Empty vector, ∆RIG-I and ∆RIG-I with Tax were expressed in 293T cells for 36 hrs and nuclear extracts from those cells were used for the ELISA based TRANS AM kit (Active motif). Chromatin was isolated from control vector, ∆RIG-I, and ∆RIG-I with Tax expressing cells, and precipitated with anti-IRF7 antibody, as described in Upstate protocol and the ifn-β promoter was amplified by PCR. Primer sequences for PCR are:

IFN-β forward: (CAVAGTTTGTAAACTTTTTCCC),
IFN-β reverse: (ATGGGTATGGCCTATTTATATGA)

Retrovirus production and Transduction

AmphoPack 293 cells (Clontech) were transfected with the following retroviral vectors (pLNCX Flag-IRF7, PCLXN-Tax) by Calcium Phosphate transfection method. After 48 hr later, the viral supernatant was used to infect Jurkat E.6 or 293T cells with polybrene (8ug/ml). The following day the infected cells were washed with PBS and added fresh media was added for 24hr. Initially, G418 (400-800ug/ml) was used for maintained cells for 1 week and used for further assay.

IRF7 Phosphorylation

Retrovirus infected IRF7 infected 293T cells was transfected with Tax for 36 hr by using Lipofectamine 2000 according to the manual. Next, VSV ΔM was used to infect the cells
at an MOI of 10 for 6 hr. Cells were lysed with native gel lysis buffer and subjected to SDS-PAGE.
REFERENCE


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