DDX24 Negatively Regulates Cytosolic RNA Mediated Innate Immune Signaling

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DDX24 NEGATIVELY REGULATES CYTOSOLIC RNA MEDIATED INNATE IMMUNE SIGNALING

By

Zhe Ma

A DISSERTATION

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

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The requirements for the degree of
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DDX24 NEGATIVELY REGULATES CYTOSOLIC RNA MEDIATED
INNATE IMMUNE SIGNALING

Zhe Ma

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Innate immunity is characterized by production of type I interferon (IFN α/β) which is necessary for the stimulation of effective anti-viral host defense. Upon recognition of cytosol viral dsRNA species, RIG-I-Like Receptors (RLRs), as well as many co-regulators, are recruited to adaptor protein IPS-1 and trigger innate immune responses. FADD (Fas associated with death domain) and RIP1 (receptor-interacting protein 1), have been reported to be recruited to this IPS-1 complex during viral infection and essential for optimal RLR signaling.

Here we reported a novel type I interferon inducible DExD/H family helicase DDX24, which was found and confirmed to specifically associate with FADD through yeast two hybrid system and co-immunoprecipitation. Overexpression of DDX24 negatively regulates dsRNA induced type I IFNs signaling, while knockdown of DDX24 by siRNA has the opposite effect. Moreover, Plaque assays of virus titer consistently demonstrate that DDX24 also negatively regulates the cellular antiviral response. Further
studies demonstrated that DDX24 disrupted the recruitment of IRF7 to the signaling complex through RIP1 interaction, leading to the attenuated K63 linked ubiquitination of IRF7 and subsequently decrease IRF7 dependent type I IFN production. Correlated with this, DDX24 was found to be induced by IFN and blocked the type I IFNs signaling more likely through IRF7 but not IRF3, suggesting a negative feedback role of DDX24 in RLR antiviral signaling. To evaluate the in vivo role of DDX24, DDX24 knockout mice were generated by gene trap technology. Although DDX24\textsuperscript{+/−} mice appeared normal and were fertile, DDX24\textsuperscript{−/−} mice died at as early as E7.5. Collectively, these results suggest an important role of DDX24 as a negative regulator in RLR dependent type I interferon production.
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TABLE OF CONTENTS

LIST OF FIGURES ................................................................. vi
LIST OF TABLES ................................................................. viii
LIST OF ABBREVIATIONS ..................................................... ix

CHAPTER

1 INTRODUCTION ........................................................................... 1
  1.1 Innate Immunity ................................................................. 1
    1.1.1 Overview ............................................................... 1
    1.1.2 Signaling pathways for type I interferon induction ............. 2
    1.1.3 Transcription factors for Induction of type I interferons ....... 8
    1.1.4 IFNAR activation and induction of ISGs ........................... 12
    1.1.5 Negative regulation of innate immunity .......................... 13
  1.2 FADD and RIP1 ................................................................. 17
    1.2.1 Overview of FADD protein and conventional functions ...... 17
    1.2.2 Overview of RIP1 protein and conventional functions ......... 19
    1.2.3 FADD and RIP1 in RLR pathway .................................. 20
  1.3 DDX family ...................................................................... 22
    1.3.1 Overview ............................................................... 22
    1.3.2 DDX family members in RLR pathway ............................ 23
    1.3.3 DDX24 protein and its function ..................................... 26
  1.4 Specific aims ...................................................................... 28

2 RESULTS ................................................................................. 31
  2.1 Identification of DDX24 as a FADD associated protein ......... 31
    2.1.1 Characterization of DDX24 ........................................... 31
    2.1.2 Confirmation of FADD-DDX24 interaction by Yeast two hybrid system .......................................................... 34
    2.1.3 Confirmation of FADD-DDX24 interaction by Co-immunoprecipitation ........................................................................ 35
    2.1.4 DDX24 co-localized with FADD ..................................... 38
    2.1.5 Discussion ............................................................... 40
  2.2 DDX24 negatively regulates dsRNA triggered RLR signaling .... 44
    2.2.1 Overexpression of DDX24 blocks dsRNA triggered RLR signaling .................. 44
    2.2.2 Knockdown of DDX24 enhances dsRNA triggered RLR signaling .... 51
    2.2.3 Knockdown of DDX24 blocks VSV replications ................. 54
    2.2.4 Discussion ............................................................... 58
  2.3 Mechanism of DDX24's negative regulation of RLR pathway .... 60
    2.3.1 DDX24 blocks RLR antiviral signaling at TBK-1/IKKi level ....... 60
2.3.2 DDX24 forms a complex with FADD and RIP1..............................65
2.3.3 DDX24 disrupts the interaction of RIP1 and IRF7..........................67
2.3.4 DDX24 inhibits k63 linked ubiquitination of IRF7...........................69
2.3.5 DDX24 inhibits type I IFN activation through disrupting the RIP1-IRF7 synergistic effect...............................................................70
2.3.6 DDX24 selectively inhibits IRF7 dependent but not IRF3 dependent type I IFN activation.................................................................73
2.3.7 DDX24 served as feedback regulator................................................76
2.3.8 Alternate explanation of DDX24 mechanism, multifunctional protein?
............................................................................................................78
2.3.9 Discussion.......................................................................................80
2.4 Generation of DDX24 knock out mice..................................................86
2.4.1 Strategy of generating DDX24 knockout mice.................................86
2.4.2 Embryonic lethality of DDX24 knockout mice.................................88
2.4.3 Discussion.......................................................................................91
2.5 Concluding remarks............................................................................94

3 MATERIALS AND METHODS.................................................................98

REFERENCE ..........................................................................................106
LIST OF FIGURES

Figure 1.1 Pattern recognition receptors activate IFNβ under virus infection .......3
Figure 1.2 RIG-I like receptor pathway.........................................................6
Figure 1.3 Transcriptional factors for RLR dependent IFNβ activation.........9
Figure 1.4 IFNβ induced signaling through JAK-STAT pathway...............13
Figure 1.5 Schematic of FADD protein......................................................18
Figure 1.6 Schematic of RIP1 protein.........................................................19
Figure 1.7 Schematic of DDX24 protein.....................................................26
Figure 2.1 Whole sequence homolog of human DDX24 and mouse DDX24...32
Figure 2.2 DDX24 RNA and protein expression pattern............................34
Figure 2.3 Yeast two hybrid experiments testing DDX24 and FADD interaction....35
Figure 2.4 Co-immunoprecipitation experiments testing DDX24-FADD interaction.
..........................................................................................................36
Figure 2.5 Identification of DDX24 deletions interacting with FADD............37
Figure 2.6 Fractionation of endogenous DDX24 in 293T cells....................38
Figure 2.7 Locations of DDX24 deletions in 293T cells...............................39
Figure 2.8 Locations of endogenous DDX24 and FADD in HUVEC cells.....40
Figure 2.9 DDX24 inhibits poly I:C and VSVdM triggered IFNβ promoter activation
..........................................................................................................45
Figure 2.10 DDX24 inhibits poly I:C and VSVdM triggered endogenous ifnb RNA
induction...............................................................................................47
Fig 2.11 DDX24 inhibits poly I:C and VSVdM triggered RLR signaling in MEFs.
..........................................................................................................49
Fig 2.12 DDX24 inhibits poly I:C and VSVdM triggered endogenous IFNβ
production in MEFs.............................................................................50
Figure 2.13 SiRNA-mediated knockdown of DDX24 enhances poly I:C and VSVdM
triggered IFNβ promoter activation in 293T cells...............................51
Figure 2.14 SiRNA-mediated knockdown of DDX24 enhances poly I:C and VSVdM
triggered RLR signaling in MEF.........................................................53
Figure 2.15  SiRNA-mediated knockdown of DDX24 blocks VSV-luc replication in MEF ................................................................. 55
Figure 2.16  SiRNA-mediated knockdown of DDX24 blocks VSV-GFP replication in MEF ................................................................. 56
Figure 2.17  SiRNA-mediated knockdown of DDX24 blocks VSV-GFP replication in HUVEC ................................................................. 57
Figure 2.18  DDX24 inhibits RLR signaling ........................................... 62
Figure 2.19  DDX24 inhibits FADD dependent RLR signaling .................. 64
Figure 2.20  DDX24 interacts with RIP1 ................................................. 65
Figure 2.21  DDX24 and inhibits RIP1 dependent RLR signaling .............. 66
Figure 2.22  DDX24 compete with IRF7 for RIP1 binding ......................... 68
Figure 2.23  DDX24 attenuates K63 linked polyubiquitination of IRF7 ........ 70
Figure 2.24  DDX24 inhibits IRF7 dependent IFNα activation .................. 71
Figure 2.25  Loss of DDX24 enhanced FADD-IRF7 synergistic effect on IFNα activation ................................................................. 73
Figure 2.26  DDX24 inhibits IRF7 phosphorylation ................................ 74
Figure 2.27  DDX24 does not inhibit IRF3 phosphorylation or dimmerization .... 76
Figure 2.28  DDX24 is interferon inducible ............................................. 77
Figure 2.29  DDX24 binds to dsRNA and ssRNA .................................... 79
Figure 2.30  Generation of DDX24 deficient mice .................................... 87
Figure 2.31  Abnormal embryonic developments of DDX24 deficient embryos .... 90
LIST OF TABLES

Table 1.1 Negative regulators in RLR signaling......................................................15
Table 2.1 DDX24 shares homolog among species...............................................33
Table 2.2 Genotypes of embryos/mice derived from DDX24\textsuperscript{+/-} intercrosses.............88
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIM2</td>
<td>absent in melanoma</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein</td>
</tr>
<tr>
<td>ATF2</td>
<td>activating transcription factor</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>cFLIP</td>
<td>cellular FLICE like inhibitory protein</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP responsive element binding</td>
</tr>
<tr>
<td>CYLD</td>
<td>cylindromatosis gene</td>
</tr>
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<td>DBD</td>
<td>DNA binding domain</td>
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<tr>
<td>DDX</td>
<td>DEAD/H box helicase</td>
</tr>
<tr>
<td>DISC</td>
<td>death inducing signaling complex</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
</tr>
<tr>
<td>DUBA</td>
<td>de-ubiquitinating enzyme ovarian tumor type</td>
</tr>
<tr>
<td>FADD</td>
<td>fas associated death domain</td>
</tr>
<tr>
<td>IAD</td>
<td>IRF associated domain</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IFNAR</td>
<td>IFNα/β receptor</td>
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<tr>
<td>IKKα/β</td>
<td>inhibitory κB kinase kinase α/β</td>
</tr>
<tr>
<td>IKKi</td>
<td>IKK inducible</td>
</tr>
<tr>
<td>IPS-1</td>
<td>interferon promoter stimulator</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>ISG</td>
<td>interferon stimulated gene</td>
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<tr>
<td>ISGF-3</td>
<td>interferon stimulated gene factor</td>
</tr>
<tr>
<td>ISRE</td>
<td>interferon stimulated response element</td>
</tr>
<tr>
<td>IκBα</td>
<td>inhibitory κB kinase α</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>Mal</td>
<td>MyD88 adaptor like</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAVS</td>
<td>mitochondrial antiviral signaling adaptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MDA5</td>
<td>melanoma differentiation associated gene</td>
</tr>
<tr>
<td>MEF</td>
<td>murine embryonic fibroblast</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>cell natural killer cell</td>
</tr>
<tr>
<td>OTUB 1/2</td>
<td>ovarian tumor de-ubiquitination enzyme</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>Pin1</td>
<td>Peptidyl-prolyl cis-trans isomerase NIMA-interacting</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase R</td>
</tr>
<tr>
<td>Poly I:C</td>
<td>polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>PRD (I-IV)</td>
<td>positive regulatory domain</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>Rig-I</td>
<td>retinoic acid inducible gene</td>
</tr>
<tr>
<td>RIP1</td>
<td>receptor interacting protein</td>
</tr>
<tr>
<td>RLR</td>
<td>Rig-I like receptor</td>
</tr>
<tr>
<td>RNF125</td>
<td>Ring finger protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded RNA</td>
</tr>
<tr>
<td>STAT 1/2</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STING</td>
<td>stimulator of interferon genes</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF family member-associated NF-κB activator</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK binding kinase</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-IL-1R homology domain</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR associated protein containing death domain</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNFR associated factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNFR apoptosis inducing ligand</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor-inducing interferon-β</td>
</tr>
<tr>
<td>TRIM</td>
<td>tripartite motif</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction

1.1 Innate immunity

1.1.1 Overview

Innate immunity is the first and most rapid host defense against invading pathogens. Host cells trigger signals for innate immune responses upon recognition of conserved structures in microbial pathogens called pathogen associated molecular patterns (PAMPs) through different pattern recognition receptors (PRRs) specifically (1). Accordingly, signaling cascades are activated and culminated in the synthesis of multiple proinflammatory cytokines, which can upregulate transcription of many genes corresponding to protein synthesis, growth arrest, and apoptosis to create a non-specific antiviral state, along with the coordination of a secondary T-cell mediated adaptive immune response resulting in pathogen clearance and elimination (2-3).

Type I interferons (IFNα and IFNβ) are the most fundamental and important cytokines that are synthesized and secreted during innate immune response (4). Type I interferons are recognized by IFNα/β receptor (IFNAR) on the surface of its own cell as well as neighbor cells to activate JAK/STAT pathway, which leads to the production of stronger and broader antiviral responses. Most importantly, these autocrine and paracrine patterns of signal transduction greatly facilitate rapid and sufficient spread of the danger signals.
and collectively facilitate the building of a wider antiviral states within the host (5-7). It has been demonstrated that type I interferon coordinates activations and stimulations of dendritic cells as well as natural killer cells. Moreover, it also contributes to enhanced antibody production and differentiation of cytotoxic T lymphocytes, which lead to specific anti-pathogen adaptive immune responses (4,8-10).

Type I interferon is broadly produced among almost all type of cells. In contrast, type II interferon, IFNγ, is mainly produced in immune cells, such as NK cells and T lymphocytes. Although Type II interferon is serologically different from type I interferon and they share no structure homology as well, it also acts very importantly in modulating innate and adaptive immunity (11-12).

1.1.2 Signaling pathways for type I interferon induction

Pattern recognition receptors (PRRs) are classes of germ line encoded receptors in response to microbial signatures that are unique and rarely found in host cells, pathogen associated molecular patterns (PAMPs). PAMPs are usually conserved molecular components essential for pathogen survival such as nucleic acids, lipopolysaccharide (LPS), lipoproteins, lipopeptides, bacterial flagellin, and zymosan (13-15). Recognition of invasion of microbe is the first step of host defense, and therefore is extremely important for rapid and efficient innate immune response.
Pattern recognition receptors were extensively studied during last two decades. In the context of virus infection, the best characterized PAMPs are viral genome or accumulated viral nucleic acids presented during replication cycles in the host, such as double stranded RNA, single stranded RNA, DNA. Accordingly, three major PRRs participating in the recognition of viral PAMPs have been identified and studied, including Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors (RLRs) and cytosolic DNA receptors (16-18) (Figure 1.1).

![Pattern recognition receptors activate IFN$\beta$ under virus infection](image)

Viral nucleic acids serve as PAMPs to be recognized by TLR, RLR and cytosolic DNA sensors. TRIF, MyD88, IPS-1 and STING are key adaptor molecules that facilitate signal transduction from these PRRs to activate IFN$\beta$. 
The best characterized PRRs recognizing virus PAMPs are TLRs, which were initially identified in drosophila and later characterized in many different species. There are 10 human TLRs and 13 mouse TLRs identified so far (named TLRs 1-10 and TLRs 1-13 respectively) (16, 19-20). Toll-like receptors are type I integral transmembrane glycoproteins anchored on the cell surface or in endosome compartment. All TLRs share a common architecture consisting of extracellular leucine-rich repeats domain responsible for PAMP recognition and a cytoplasmic Toll/Interleukin-1 Receptor (TIR) domain capable of recruiting downstream adaptors for signal transduction (21). TLR3, 7, 8 and 9 are responsible for recognizing viral nucleic acid. Specifically, TLR3 recognizes dsRNA; TLR7 and TLR8 are responsible for recognizing ssRNA; TLR9 recognizes unmethylated CpG dsDNA (16). Upon viral nucleic acid binding, TLR3 recruits adaptor protein TRIF with other associated proteins such as FADD, RIP1 and TRAF6 to activate NF-κB pathway and TRAF3, TBK-1/Ikki for IRF3/7 activation (22). TLR 7, 8 and 9 all utilizing MyD88 as adaptor protein and recruiting DD containing serine threonine kinase interleukin-1 receptor-associated kinase 1 (IRAK-1) and interleukin-1 receptor-associated kinase 4 (IRAK-4) to transduce 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 (23-25). All of these signaling will culminate in production of proinflammatory cytokines or type I interferon (IFN).
The RLR family comprises three receptors so far, RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), each recognizing various viral PAMPs (26-28). RIG-I recognizes 5’-triphosphate RNA and short formed synthetic dsRNA analog poly I:C, whereas MDA5 is mainly responsible for long dsRNA, such as long formed poly I:C (29, 30). LGP2 is reported to work upstream of RIG-I and MDA5 and regulate RIG-I/MDA5 mediated viral recognition (31). The engagement of RIG-I to RNA causes conformational changes of RIG-I, leading to the exposure of RIG-I CARD domain to viral RNAs (32, 33). The active form of RIG-I is then recruited to the adaptor protein IPS-1 (also called MAVS, Cardif, VISA) located on the mitochondria. IPS-1 contains an N-terminal CARD-like domain which is responsible for the interaction with RLRs and a transmembrane domain at the C-terminal end that is required for mitochondrial targeting as well as triggering anti-viral responses (34-37). Subsequently, more molecules were recruited to IPS-1 and assemble a mitochondria-associated protein complex. Some of these molecules were proposed as adaptor or scaffold proteins, which are important for maintaining complex stability as well as help recruit downstream factors, such as RIP1 and FADD (38). It is reported that TRADD, RIP1 and FADD will form a complex called TRADDosome which is recruited to IPS-1 during virus infection for optimal RLR-dependent signaling (39). Furthermore, FADD recruits downstream factors Caspase-8 and caspase-10 to facilitate activation of NF-κB (40). On the other side, IPS-1 subsequently recruits downstream kinase TBK-1 and IKK-I, which phosphorylate IFN-regulatory factor 3 (IRF3) and 7
Phosphorylated IRF3 and IRF7 then dimerized and translocated into nucleus to trigger the production of type I interferons (41, 42). NF-κB and AP-1 are also activated in this pathway and required for the optimal IFNβ production (Figure 1.2).

**Figure 1.2 RIG-I like receptor pathway**

Intermediate viral RNAs are sensed by RLR. The signaling will then be transmitted to mitochondria localized IPS-1. FADD, RIP1, TBK-1, IKKi, DDX3X, LGP2 and many other molecules were recruited to IPS-1 and formed a signaling complex. The transcription factor IRF3 and IRF7 were recruited and phosphorylated to activate IFNβ production.

Cytosolic viral DNA sensing pathway is less characterized than the other two pathways even after proteins sensing cytosolic viral DNA were identified recently. One of the ER
located transmembrane protein named STING (TMEM173, MITA, ERIS, MPYS) has been identified as the adaptor for cytosolic DNA pathway and is critical for recognizing cytoplasmic DNA and activating innate immune response (43-45). Loss of STING completely abolished many DNA stimulus induced IFNβ production in MEFs. Correlated with this, STING deficient mice showed great susceptibility to HSV-1 infection, suggesting a STING dependent cytosolic DNA pathway (46, 47). Recently, two upstream cytosolic DNA sensors utilizing STING as adaptor are reported as IFI16 and DDX41 (48, 49). Both of them exerted direct interaction with IFNβ-inducing vaccinia virus (VACV) DNA and both could recruit STING during DNA stimulation. Knockdown of IFI16 using small interfering RNA showed reduced VACV DNA stimulated IFNβ signaling (48). Similarly, less DDX41 resulted in significant abolishment of poly dA:dT, poly dG:dC, HSV and influenza stimulated IFNβ production (49). However, to confirm the importance as well as detailed mechanism, knock out models of these genes are necessary. Besides these STING dependent DNA sensors, AIM2 could sense B-form DNA and activating inflammatory response (50, 51). Mover, B-form DNA has been shown to be transcribed by RNA polymerase III and recognized by RIG-I (52, 53). Although many detailed mechanisms are still under investigation, the cytosolic DNA pathway is drawing increased attention and is critical for the better understanding of the antiviral signaling pathway.
1.1.3 Transcription factors for Induction of type I interferons

The transcriptional induction of IFNβ is achieved through a cooperative binding of three distinct families of transcription factors, including NF-κB, Activating Transcription Factor 2 (ATF2)/c-Jun and IRF3/7, to the positive regulatory domain (PRD) of the promoter (pRD II, pRD IV, pRD III-I respectively) (Figure 1.3). Other transcriptional co-activators are also involved such as CBP or p300, which could bind to the enhancer region of IFN promoter to positively regulate type I IFN production (54-56).

NF-κB exists as a heterodimer composed of RelA and p50. Under unstimulated conditions, NF-κB is kept in the cytoplasm as an inactive form by a direct interaction with IκB proteins such as IκBα and IκBβ. After TLR or RLR signaling is activated, IκB proteins get phosphorylated by IKK complex, consisting of IKKa and IKKβ, and a regulatory component, NEMO (also known as IKKγ). This is followed by the polyubiquitination and proteasome dependent degradation of IκB, allowing the translocation of NF-κB from the cytoplasm to the nucleus for IFNβ activation (57, 58). ATF2/c-Jun is a component of AP-1, which are phosphorylated by MAP kinases (MAPK), such as c-jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) (59).
Figure 1.3 Transcriptional factors for RLR dependent IFNβ activation

The cooperative assembly of IRF3/7, NF-κB and ATF2-Jun transcription factors at the IFNβ promoter region (PRD I-IV) is required for optimal IFNβ production.

The interferon regulatory factor (IRF) family members also play important roles in controlling appropriate IFN signaling, especially IRF3 and IRF7 in RLR signaling. IRF3 and IRF7 share many similarities in their structures and functions. For example, IRF3/7 both contain a well-conserved N-terminal DNA-binding domain (DBD) of about 120 amino acids, which forms a typical helix-turn-helix domain and recognizes a DNA sequence corresponding to the IFN-stimulated response element (ISRE, A
The C-terminal regions carry an IRF association domain (IAD) that is responsible for homo- or heteromeric interactions with other family members or other transcription factors such as signal transducer and activator of transcription (STAT1, 2) (60). This structural homology shared by IRF3 and IRF7 implicate functional similarities between them. For example, they are both phosphorylated by TBK-1/IKKi at the serine/threonine clusters in the C-terminal regulatory region, followed by dimerization and translocation from the cytoplasm into the nucleus for IFNβ activation (61).

Although IRF3 and 7 share structural similarities and some overlapping functions, increasing evidence has elucidated the different roles for these two molecules in the RLR pathway as well. For example, IRF3 is a constitutively expressed protein, therefore is easily accessed by TBK-1/IKKi to be phosphorylated and initiates a quick and sufficient first round of IFNβ production followed by induction of various ISGs. On the other hand, IRF7, itself an ISG, exists at very low endogenous expression level in various unstimulated cells, but is highly induced by IFNβ, which is indispensable for the second round of IFNβ induction as well as triggering IFNα induction (62, 63). Moreover, although IRF3 and IRF7 could both bind to IFNβ promoter region, IRF7 has been reported to be specific for certain types of IFNα activation, such as IFNα11 (64). Therefore, IRF3 is critical for primary IFNβ production, whereas IRF7 could bind to both IFNβ and IFNα promoter region, which is necessary for sufficient induction of the second round of type I IFN. Besides, Taniguchi group recently reported that IRF7
deficient mice and pDC are extremely sensitive to VSV infection, while IRF3 deficient mice and pDC are hardly affected (65). These data indicate IRF7’s involvement in the primary response as well. Depending on the nature of the virus and stimulation, both IRFs might be activated to cooperate to ensure a robust antiviral response.

Many mechanisms are defined concerning the regulation of IRF3/7, which are important for appropriate RLR signaling. Besides phosphorylation, ubiquitination also plays important roles in IRF3 and 7 regulations. IRF3 and IRF7 have been reported to be ubiquitinated and degraded upon virus infection and dsRNA stimulation. For example, RAUL is an E3 ligase that can modify IRF3 and 7 with K48 linked poly-ubiquitination and results in their degradation (66). Similarly, RBCK1 and Pin1 are also reported to promote proteasome dependent degradation of IRF3 (67, 68). Ro52, associated with IRF7, is reported as the E3 ligase for IRF7’s K48 specific ubiquitination (69). The K48 linked ubiquitination is critical for controlling protein level of these transcription factors and is closely related to the negative regulation of IRF3/7 dependent type I interferon activation. On the other side, K63 linked ubiquitination of IRF7 by TRAF6 was also recently reported to play a positive role in the activation of IRF7 dependent type I IFN. Moreover, phosphorylation deficient mutants of IRF7 were still capable of being modified by K63 linked ubiquitination, suggesting ubiquitination of IRF7 is earlier than phosphorylation or independent of phosphorylation (70, 71). Furthermore, the Chen group reported that UBC5 is required for IRF3 activation and they demonstrated that replacement of
endogenous ubiquitin with its K63R mutant abolishes viral activation of IRF3, suggesting that K63 polyubiquitination plays a key role in IRF3 activation (72). However, no direct evidence about K63 conjugated poly-ubiquitination of IRF3 was reported so far.

1.1.4 IFNAR activation and induction of ISGs

After being transcribed and translated, type I interferon is secreted outside of the cell and recognized by the IFNα receptors located at the cell surface (73). Type I interferon engagement leads to the cross-phosphorylation of JAKs (JAK1, TYK2) that are recruited to IFNAR, thus promoting their further phosphorylation (74, 75). This process helps recruit further signal transducers and activators of transcription (STAT) and facilitate their phosphorylation which leads to the dissociation of the STAT1-STAT2 complex from IFNAR. Subsequently, IRF9 is recruited to STAT dimer and forms an interferon stimulated gene factor 3 (ISGF3) complexes. This complex is able to bind to the interferon sensitive responsive element (ISRE) located in the promoter regions of hundreds of interferon stimulating genes (ISGs), establishing an antiviral state in the host (76, 1) (Figure 1.4).

Many ISGs are identified to be involved in regulating innate immune responses as feedback regulators. Some ISGs serve as positive regulators to induce further and stronger interferon production for pathogen clearance. For example, RIG-I can be induced by IFNβ and thus recognizes more viral RNAs to strengthen antiviral effects (77).
IRF7 is induced dramatically after the primary response of innate immunity and initiates the second round of type I interferon production. Some ISGs, on the other hand, negatively regulate innate immunity to prevent inappropriate production of type I interferon, which can be detrimental to the host.

Figure 1.4 IFNβ induced signaling through JAK-STAT pathway

After IFNβ is produced and secreted outside of the cells, IFNβ engages the IFNα/β receptor (IFNAR) on the cell surface of its own or neighboring cells. This leads to the activation of the JAK-STAT pathway and formation of a STAT1-STAT2-IRF9 (ISGF3) complex to initiate the production of several hundred interferon stimulated genes (ISG) as part of an innate antiviral program. Some of the ISGs such as IRF7 and RIG-I will serve as a positive feedback of IFNβ production.

1.1.5 Negative regulation of innate immunity

The production of type I IFN is tightly regulated to prevent chronic inflammation and
autoimmunity caused by over responses of innate immunity. Sophisticated mechanisms are thought to underlie the proper regulation of the RLR signaling response to prevent unnecessary signaling. Recently, the RLR pathway has been extensively studied along with the identification of important regulators. So far, more than 20 negative regulators were identified which target various stages of RLR signaling with different mechanisms. In table 1.1, major negative regulators in RLR pathway published are summarized, based on their targets, proposed mechanisms and regulation models.

These negative regulators have a broad target range from the upstream RIG-I like receptors to the downstream transcription factors IRF3/7. Besides, some of them are even proposed to have multiple targets and tightly regulate the antiviral signaling at different level through variable strategies. For example, the RNF125 has been reported as an E3 ligase of RIG-I/MDA5 or IPS-1, promoting the degradation of RIG-I/MDA5 or IPS-1 (78). The proposed mechanism can be classified as following three ways. The first way is to cause instability of signaling complexes. These negative regulators usually sequestrate crucial molecules from their appropriate positions, or anchored into a signaling complex to disrupt the sufficient interactions between target proteins and other regulators, which will disrupt the signal transduction among the pathway. For example, NLRC5 could bind RIG-I and disrupt the interactions between RIG-I and IPS-1, causing insufficient signaling transduction (79).
Table 1.1 Negative regulators in RLR signaling

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Proposed mechanism</th>
<th>Regulation model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGP2</td>
<td>dsRNA, RIG-I, IPS-1</td>
<td>1. dsRNA sequestration 2. Competition with IKKi for IPS-1, 3. Inhibition of RIG-I dimerization</td>
<td>Negative feedback</td>
<td>83-87</td>
</tr>
<tr>
<td>Atg5-Atg12</td>
<td>RIG-I, MDA5, IPS-1</td>
<td>Sequestration of RLRs and IPS-1</td>
<td>Steady-state</td>
<td>88</td>
</tr>
<tr>
<td>RNF125</td>
<td>RIG-I, MDA5, IPS-1</td>
<td>Proteosomal degradation of RLRs and IPS-1</td>
<td>Negative feedback</td>
<td>78</td>
</tr>
<tr>
<td>ISG15</td>
<td>RIG-I</td>
<td>Sequestration of RIG-I</td>
<td>Negative feedback</td>
<td>89</td>
</tr>
<tr>
<td>CYLD</td>
<td>RIG-I, TBK1</td>
<td>Deubiquitination of RIG-I</td>
<td>Steady-state</td>
<td>90, 91</td>
</tr>
<tr>
<td>NLRC5</td>
<td>RIG-I</td>
<td>Competition with IPS-1 for RIG-I</td>
<td>Negative feedback</td>
<td>79</td>
</tr>
<tr>
<td>DAK</td>
<td>MDA5</td>
<td>Sequestration of MDA5</td>
<td>Steady-state</td>
<td>92</td>
</tr>
<tr>
<td>AIP4</td>
<td>IPS-1</td>
<td>K48 linked ubiquitination of IPS-1</td>
<td>Negative feedback</td>
<td>80</td>
</tr>
<tr>
<td>PCBP2</td>
<td>IPS-1</td>
<td>Adaptor protein of AIP4 to induce IPS-1 degradation</td>
<td>Negative feedback</td>
<td>80</td>
</tr>
<tr>
<td>NLRX1</td>
<td>IPS-1</td>
<td>Competition with RLRs for IPS-1</td>
<td>Steady-state</td>
<td>82</td>
</tr>
<tr>
<td>DUBA</td>
<td>TRAF3</td>
<td>Deubiquitination of TRAF3</td>
<td>Negative feedback</td>
<td>81</td>
</tr>
<tr>
<td>A20</td>
<td>Multiple targets</td>
<td>Deubiquitination of the target protein(s)?</td>
<td>Negative feedback</td>
<td>93-95</td>
</tr>
<tr>
<td>SIKE</td>
<td>TBK1 and IKKi</td>
<td>Sequestration of TBK-1/IKKi</td>
<td>Steady-state</td>
<td>96</td>
</tr>
<tr>
<td>Optineurin</td>
<td>TBK-1, TRAF3</td>
<td>Unknown</td>
<td>Negative feedback</td>
<td>97</td>
</tr>
<tr>
<td>ABIN1</td>
<td>TBK-1/IKKi</td>
<td>Disrupt the interaction between TRAF3 and TBK-1/lkki</td>
<td>Negative feedback</td>
<td>98</td>
</tr>
<tr>
<td>TAX1BP1</td>
<td>TBK-1/IKKi</td>
<td>Disrupt the interaction between TRAF3 and TBK-1/lkki</td>
<td>Negative feedback</td>
<td>99</td>
</tr>
<tr>
<td>Pin1</td>
<td>IRF-3</td>
<td>Phosphorylation-dependent isomerization</td>
<td>Steady-state</td>
<td>68</td>
</tr>
<tr>
<td>SENP2</td>
<td>IRF3</td>
<td>deSUMOylation, K48-specific ubiquitination and degradation of IRF3</td>
<td>Steady-state</td>
<td>100</td>
</tr>
</tbody>
</table>
Alternatively, some negative regulators control the protein levels of key components in the RLR pathway. These negative regulators modify target proteins with K48 linked ubiquitination, which leads to the degradation of the target protein. For example, AIP4 is responsible for the K48 linked polyubiquitination of IPS-1 to control the signaling strength (80). Furthermore, many negative regulators are able to block post translational modifications which are important for activation of RLR signaling, such as K63 linked ubiquitination and SUMOylation. Some regulators have deubiquitinase activity, which could potentially remove the K63 linked ubiquitination of a target protein, for example, DUBA. It has been reported that DUBA could deubiquitinate TRAF3, blocking type I interferon production (81).

Negative regulators can be classified based on the stage of signal transduction they regulate. The steady-state model of regulators control signaling in the early stage and the feedback model of regulators prevent over activation of signaling in a later stage. The steady-state negative regulators are usually non-inducible by type I IFN, and exhibit endogenous binding activity with their targets under unstimulated situations. One good example is NLRX1, which targets IPS-1 to block virus induced RLR signaling. It has been reported that endogenous NLRX1 associates with IPS-1 through putative
nucleotide-binding domain (NBD) and CARD domain interactions. This occupation of the CARD domain implicates a disruption of RIG-I and IPS-1 association, supporting the role of NLRX1 as a negative regulator of the RLR pathway (82). On the other hand, a feedback negative regulator is usually characterized as an interferon inducible protein, or shows either weak or little interaction with their targets, followed by stronger association with their targets after virus/dsRNA stimulations. PCBP2 is a typical negative regulator of the RLR pathway, which can be induced by SeV and VSV treatment. SeV stimulation greatly enhance the interaction between endogenous PCBP2 and IPS-1, which facilitates the K48-linked polyubiquitination of IPS-1 by E3 ligase AIP4 and promotes the degradation of IPS-1 through a proteasome dependent manner. This suggests the negative feedback mechanism of PCBP2 on RLR regulation to prevent over production of type I interferon by degradation of IPS-1 (80).

1.2 FADD and RIP1

1.2.1 Overview of FADD protein and conventional functions

FADD was originally identified as a FAS associated protein, nominated FAS associated with death domain. Human FADD gene is located at chromosome 11q13.3 and FADD protein is highly conserved from mouse to human. Human FADD contains 208 amino acids and has a predicted molecular weight of ~23 kDa (101, 102). There are two functional domains in FADD, death domain (DD) and death effector domain (DED)
The death domain is responsible for the interaction between FADD and death receptors (DRs) such as FAS and tumor necrosis factor receptor 1 (TNF-R1). Upon association with these DRs, FADD will further recruit variant functional partners such as Caspase 8 or c-FLIP, etc through its DED domain to transduce the signaling (103-105).

(Figure 1.5). FADD consists of a Death effector domain in its N term and a Death domain in its C term. Asterisk indicates phosphorylation site. Numbers indicate amino acid position.

Figure 1.5 Schematic of FADD protein

The first function described for FADD was its interaction with FAS, suggesting that FADD is located in the cytoplasm and involved in death signaling pathways. Indeed, FADD has been characterized as the main adaptor transmitting apoptotic signals mediated by many DRs. This suggests an indirect control of FADD on cellular homeostasis, elimination of auto-reactive cells, activated cells, infected cells, tumor cells, etc (106). However, emerging evidence indicate that FADD is a much more complicated molecule. Depending on its state of phosphorylation, sub-cellular localization as well as binding partners, FADD can be involved in apoptosis, survival, cell cycle progression, and cell
proliferation (107-111). In particular, FADD possesses a nuclear localization signal (NLS) and a nuclear export signal (NES) in DED domain, indicating possible location in both the cytoplasm and nucleus of most cells. Moreover, a specific serine phosphorylation site has been reported responsible for FADD protein trafficking and some non-apoptotic functions (105, 112).

1.2.2 Overview of RIP1 protein and conventional functions

![RIP1 Schematic](image)

**Figure 1.6 Schematic of RIP1 protein**

RIP1 protein contains three domains - kinase domain, intermediate domain and death domain. Amino acid position is marked by numbers.

RIP1 was also identified as a binding partner of FAS through a yeast two hybrid screen (113). RIP1 is constitutively expressed in most of tissues, while possessing the potential of being induced by TNFα and type I interferons. RIP1 has 671 amino acids and a molecular weight of ~75 kDa. RIP1 consists of a kinase domain (KD), intermediate domain (ID) and death domain (DD), which have different functions respectively (114,
115) (Figure 1.6). The DD domain is the best characterized for its function. Studies have described the capacity of the DD of RIP1 to be recruited to several DD receptors, such as FAS, TNF-R1, TRAIL-R1 and TRAIL-R2, which is important for DR induced apoptosis (116-119). In contrast, the intermediate region of RIP1 plays very important roles in cell survival by activating downstream NF-κB signaling. It has been reported that the ID domain of RIP1 is responsible for binding TRAF2 and recruits IKKs to the activated TNF-R1 complex via interaction with NEMO (NF-kB essential modulator; also called IKKγ) to facilitate NF-κB signaling (120, 121). Different from these two domains, the KD domain of RIP1 is responsible for its self-phosphorylation and plays an important role in necrosis process. Reconstitution of a RIP1-deficient Jurkat T-cell line with a kinase mutant form of RIP1 fails to restore the FasL-induced necrosis, suggesting RIP1 kinase activity is required for necrosis (122-124). Moreover, the Yuan group has successfully identified a chemical compound named necrostatin that could block RIP1’s kinase activity and inhibit necrosis. This inhibition, however, does not affect RIP1 dependent NF-κB, MAPK or JNK activation (125). Collectively, RIP1 plays important roles in many different pathways with variant binding partners and it is very tightly regulated.

1.2.3 FADD and RIP1 in RLR pathway

Although FADD and RIP1 were originally identified as crucial players in apoptotic and inflammatory signaling pathways, it has been realized that they are both multifunctional
proteins and are involved in many different signaling pathways. In 2004, the Barber group firstly reported that FADD and RIP1 are important for cytosolic dsRNA triggered antiviral signaling. FADD and RIP1 deficient MEFs exhibit defects in type I interferon production and therefore are rendered more susceptible to VSV infection (38). In the same year, RIG-I was identified as the RNA sensor of this pathway, leading to the definition of the RIG-I like receptor pathway (RLR) (26). In 2005, the mitochondria located adaptor IPS-1 was identified and RIP1/FADD were reported as IPS-1 associated protein (34). Nevertheless, RIG-I, MDA-5, and IPS-1 triggered IFNβ promoter activation are significantly reduced but not abolished in FADD deficient MEFs compared to FADD+/- MEFs, indicating FADD is dispensable for RLR signaling but is required for optimal RLR signaling (126). Moreover, the Tschopp group also reported the importance of FADD and RIP1 in facilitating RLR signaling and proposed that it is achieved by a complex of TRADD, RIP1 and FADD, called the TRADDosome. TRADDosome binds to IPS-1 through a TRADD-IPS1 interaction and then acts as a scaffold protein to recruit TRAF3, TANK and NEMO, which will eventually lead to the activation of NF-κB, as well as IRF3 for type I IFN production. Distinctively, they proposed that FADD and RIP1 are recruited to IPS-1 through TRADD rather than direct binding to IPS-1 (39). Although further experiments are needed to clarify this contradiction, the idea that an IPS-1 based signaling complex was assembled during virus infection is proposed. Furthermore, it was reported by the Pagano group that RIP1 associates with IRF7 in 293T and Raji cells, and promotes the K63 specific ubiquitination and activation of IRF7. Consistently, IRF7
activity is reduced in RIP1 deficient MEFs, shedding more light on RIP1’s positive role in the RLR dependent pathway (71). Although the importance and positive regulatory effect of FADD/RIP1 in RLR pathway have been clearly reported, the detailed mechanism such as how they are recruited to signaling complexes and how these process are regulated is still ill defined and need to be further addressed.

1.3 DDX family

1.3.1 Overview

The DEAD-box family contains a total of 59 members thus far. They are characterized by the presence of nine conserved motifs, Q-motif, motif I, motif Ia, motif Ib, motif II, motif III, motif IV, motif V, and motif VI. Motif II contains amino acid sequence D-E-A-D (Asp-Glu-Ala-Asp), which is the origin of this family’s name (127, 128). All of these 9 motifs are closely related to protein function. Generally, motifs Ia, Ib, III, IV and V are related to RNA binding activity and motifs I, Q motif and motif VI are responsible for ATP binding and hydrolysis activity (129). Besides DEAD-box family, DEAH, DExH and DExD families are very closely related families and they share eight conserved motifs except for motif II. These four families are generally referred as DExD/H family. Because of the ATPase and RNA binding activity, DEAD family helicases have been reported to be involved in RNA related biological process such as transcription, splicing, rRNA processing, RNA transportation, ribosomal biogenesis and RNA decay (130).
DEAD-box proteins are “motor proteins” that are activated and regulated by the partners they interact with in a biological process. Therefore, it is plausible that a given DEAD-box protein could be activated differently by different partners during different processes. So far, most of the studies were focused on structural information, and only a few DEAD-box proteins have been studied extensively.

1.3.2 DDX family members in RLR pathway

Four DDX family members are well characterized as key molecules in RLR signaling, which are RIG-I (DDX58), MDA5, LGP2 (DHX58) and DDX3X (26, 83-87). Although these helicases share similar helicase ATP binding domain and helicase C-term domain, they regulate RLR pathway at different stages and play different roles.

RIG-I and MDA5 both contain two extra CARD domains in the N terminus besides the conserved helicase domain and serve as RNA sensors in recognizing cytosolic viral RNA. They are responsible for different RNA species according to the studies of knockout animal experiments (83). RIG-I recognizes 5’-triphosphorylated, uncapped ssRNA, which is common in viral genomes, and short formed dsRNA, such as synthesized short poly I:C (30, 131). MDA5, on the other hand, is responsible for recognizing long dsRNA, such as long formed poly I:C or high molecular weight RNA (RNA web) (30, 132). Besides, RIG-I and MDA5 also showed different preferences in recognizing viral RNAs.
Knockout mouse experiments indicate that RIG-I is responsible for vesicular stomatitis virus (VSV), Sendai virus (SeV), Newcastle disease virus (NDV), Influenza A and B, hepatitis C virus (HCV), etc. (131, 133). MDA5 is important for recognizing EMCV and mengo virus (133, 134). Moreover, some virus such as dengue, West Nile virus, and reovirus activate both RIG-I and MDA5 dependent signaling (135).

LGP2 shares high homology with RIG-I and MDA5 in the helicase domain, and exhibits RNA binding activity as well. However, due to lack of CARD domains, LGP2 can not transduce the signal to IPS-1 through CARD-CARD domain interaction. Therefore, LGP2 was originally proposed as a RNA binding competitor of RIG-I/MDA5 that sequesters viral RNA from being recognized by the host to negatively regulate the RLR pathway (83). However, no direct evidence or experiments were performed to address this hypothesis. Although promoter assays indicate that expression of LGP2 negatively regulates Sendai virus and Newcastle disease virus (NDV)-mediated IFNβ gene activation, and RNAi-mediated knock-down of LGP2 can enhance anti-viral gene expression, additional suppressing mechanism by LGP2 are suggested. The second suggested mechanism is that LGP2 could bind to RIG-I through helicase-helicase domain interactions and therefore blocks RIG-I from contacting with viral RNA (85). In addition, the third mechanism is based on the experiment that LGP2 and IKKi share overlapping binding region on IPS-1, so LGP2 could potentially disrupt the recruitment of IKKi to the IPS-1 signaling complex (86). Despite these three proposals of LGP2’s function in the
RLR pathway, it was recently reported that LGP2 deficient mice and MEF shows greater susceptibility to many viruses such as EMCV, VSV and SeV, etc. Therefore, it is contradictory to previous results that LGP2 might serve as a positive regulator instead of negative regulator (31). Moreover, it was suggested that LGP2 functions upstream of RIG-I/MDA5. These disparate results are still under discussion and more experiments are needed to clarify the conflicts.

Another member of the DExD/H box RNA helicase family, DDX3X, has also recently been studied as a regulator in the RLR pathway. Schroder et al. found that DDX3X associate with IKKi to facilitate RLR signaling. A novel VACV protein, K7, can inhibit PRR-induced IFNβ induction by preventing TBK1/IKKepsilon-mediated IRF activation by targeting DDX3X (136). Another group reported the similar phenotype that DDX3X is important for IFNβ induction. However, they proposed that DDX3X acts further downstream and binds directly to the IFNβ promoter region to enhance ifnb transcription (137). Moreover, it was more recently reported that DDX3X works much more upstream. It binds to both poly I:C and viral RNA introduced into the cytosol and associates with MAVS/IPS-1 to upregulate IFNβ production (138). Collectively, these evidences indicate that DDX3X might work at different levels of the RLR pathway and plays multiple roles in innate immunity.
In all, although all these helicases belong to the same family and share homology in their helicase domain, they exert distinct roles and functions at different stages of signaling, which is either dependent or independent of their helicase binding activity. Moreover, some of the proteins even exhibit multiple functions in regulating RLR pathway. As increasing DExD/H family members have been characterized as important molecules in innate signaling, the underestimated potential of these helicases in regulating innate immunity is being realized. In this study, I report for the first time that a DExD/H family member, DDX24, serves as a negative regulator of RLR antiviral signaling.

1.3.3 DDX24 protein and function

![Figure 1.7 Schematic of DDX24 protein](image)

DDX24 protein contains two domains, helicase ATP-binding domain and helicase C-terminal domain. Amino acid position is marked by numbers.
Appropriate innate immune responses are critical to maintain homeostasis of the host. Therefore, the RLR signaling is tightly regulated by co-regulators, which associate with key molecules in the RLR pathway. One of the candidates, DDX24, is reported to associate with FADD through a yeast two hybrid screening analysis (139). DDX24 belongs to the DExD/H RNA helicase family, which was originally identified in a genome project (140). Similar to other family members, DDX24 contains one DExD/H-box helicase ATP binding domain and a helicase C-terminal domain (Figure 1.7). The DExD/H family is broadly involved in many RNA related enzymatic activities, such as RNA binding, RNA unwinding and RNA annealing.

Very limited functions of DDX24 have been reported since it was identified. It was demonstrated that yeast MAK5 protein, a DDX24 homolog, is essential to 60s ribosome biogenesis. Mutation of MAK5 lead to delayed protein translation (141). The only function related study demonstrated that human DDX24 interacts with HIV1 Rev and plays a role in facilitating HIV-1 viral RNA packaging, although the mechanism was not elucidated (142). Nevertheless, the involvement of DDX24 in innate immune signaling is unknown. Thus, in this study, my goal was to explore the involvement of DDX24 in RLR pathway. Moreover, by revealing the connection between DDX24 and FADD, as well as other key molecules in this pathway, I aim to characterize DDX24’s regulatory mechanism, as well as clarify FADD and RIP1’s role in RLR pathway.
1.4 Specific Aims

Innate immunity is characterized by rapid recognition of pathogen associated molecular patterns (PAMPs) from microbes through host pattern recognition receptors (PRRs) (1). RIG-I-like receptors (RLRs) are PRRs that sense double stranded RNAs (dsRNAs) derived from RNA viruses and trigger antiviral innate immune responses (5, 6). Upon recognition of these nucleic acid species, RLRs are recruited to a specific intracellular adaptor protein IPS-1 to initiate signaling pathways, culminating in activation of NF-κB and IRF-3 that control the transcription of genes encoding type I interferons (9). These primary type I IFNs induce expression of IFN regulatory factor (IRF)-7, required for production of a second cascade of IFNα subtypes and the further establishment of a complete antiviral state (5).

Recently, FADD has been characterized as a critical member of RLR signaling pathway. It has been demonstrated that TRADD, RIP1 and FADD can form a complex recruited to IPS-1 during VSV infection and activate RLR signaling. Loss of FADD leads to defective interferon production and increased susceptibility to RNA virus infection in MEF and animals (38, 39). Moreover, it was reported that RIP1 positively regulates K63-linked polyubiquitination of IRF7, which is important for IRF7 dependent type I IFN activation (71). However, the regulators that are involved in this FADD and RIP1 dependent RLR pathway and regulation of these processes are still ill defined.
To further unravel the FADD and RIP1 dependent mechanisms of innate immune signaling, I report here, the identification of an uncharacterized helicase DDX24, which was found to specifically associate with FADD. Given this, I aimed to unravel the importance of DDX24 in the regulation of innate immune signaling. Thus, this proposal encompasses a study to evaluate whether DDX24 is involved in the RLR pathway and to define its role in the RLR pathway, which will also help to clarify FADD and RIP1’s role in the RLR pathway.

Aim I: Analysis of the association of FADD with DDX24. (Section 2.1)

Yeast two-hybrid and co-immunoprecipitation experiments were performed to confirm DDX24-FADD interactions in this study. Moreover, the cellular distribution of FADD and DDX24 was investigated by immunofluorescence staining and fractionation experiments.

Aim II: Role of DDX24 in RLR mediated innate immune signaling. (Section 2.2)

Whether and how DDX24 regulates RLR mediated innate immune signaling was evaluated through overexpression of DDX24. After RLR signals are triggered by VSVdM infection or poly I:C transfection, differences of type I IFNs production were monitored through luciferase reporter assays. Furthermore, endogenous IFN was evaluated at mRNA level by RT-PCR and protein level by ELISA between overexpression group and
control group. Same experiments were performed by knocking down endogenous DDX24 using small interfering RNA to confirm the role of DDX24 in the RLR pathway.

**Aim III: Molecular mechanism of DDX24 in RLR mediated innate immune signaling (Section 2.3)**

While FADD and RIP1 are clearly essential for the optimal production of type I IFNs, they only modestly stimulate the IFNβ promoter. For this aim, the effects of DDX24 on FADD dependent RLR signaling were examined. Furthermore, the association of DDX24 with other key molecules in RLR pathway was examined, which could provide more information of DDX24’s regulatory mechanism in the RLR pathway.

**Aim IV: Analysis of DDX24−/− MEFs and animals (Section 2.4)**

DDX24−/− mice were generated using genetrap technology. If available, DDX24−/− MEFs and mice will be challenged by RNA virus or stimulated with dsRNA to evaluate the in vivo role of DDX24 in antiviral signaling. DDX24−/− MEFs will also be utilized to confirm the in vitro phenotypes that were observed. If DDX24 deficiency leads to embryonic lethality, experiments will be focused on clarifying the reason and time of embryonic lethal of DDX24−/− mice.
Chapter 2 Results

2.1 Identification of DDX24 as a novel FADD associated protein

2.1.1 Characterization of DDX24

Our work and others have demonstrated that FADD and RIP1 are critical for RLR signaling. Loss of FADD or RIP1 leads to defective interferon production and increased susceptibility to RNA virus infection in MEFs (38, 39). However, the regulatory mechanisms of FADD and RIP1 on RLR signaling are still unclear. In this study, we aimed to explore and characterize FADD or RIP1 associated proteins, which could potentially help better understand FADD and RIP1’s role in RLR signaling. DDX24 was identified as a FADD associated protein through a yeast two hybrid screening analysis by the Wanker group (139). In addition, increasing DDX family members are characterized as regulators in RLR pathway, which makes DDX24 an excellent candidate which might be potentially involved in the FADD dependent RLR pathway.

The DDX24 protein contains 859 amino acids in human cells and 857 amino acids in murine cells. Amino acid sequences of human DDX24 and mouse DDX24 shared over 90 percent homology, suggesting conserved DDX24 functions between these two species (Figure 2.1). Further homology analysis indicated that DDX24 was conserved among species from yeast to human. A series of sequence comparisons indicated that even the
yeast homolog of DDX24 (named MAK5) shares about 40% identity with human DDX24, indicating evolutionary conserved functions of DDX24 (Table 2.1).

Figure 2.1 Whole sequence showing homolog between human DDX24 and mouse DDX24
Human DDX24 RNA was found to be ubiquitously expressed in a variety of human tissues, as determined by RT-PCR profiling analysis (Figure 2.2a). The predicted molecular weight of human DDX24 was 98 KD, which is similar to its observed molecular weight in HUVEC cells following immunoblot analysis using a rabbit antibody for DDX24. RNAi studies confirmed that the observed 98 kDa band was indeed DDX24 (Figure 2.2b). We further determined the endogenous DDX24 protein level in several different primary cells or cell lines from human and mouse MEFs. Among all these cells/cell line, endogenous DDX24 was detected but exerted varied endogenous protein level, in which THP-1 contains the highest amount of DDX24 (Figure 2.2c).

### Table 2.1 DDX24 shares homolog among species

<table>
<thead>
<tr>
<th>Species (Homo sapiens Vs.)</th>
<th>Protein identity(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan troglodytes (Chimpanzee)</td>
<td>99.1</td>
</tr>
<tr>
<td>Canis familiaris (Dog)</td>
<td>85.4</td>
</tr>
<tr>
<td>Bos taurus (cow)</td>
<td>84.4</td>
</tr>
<tr>
<td>Mus musculus (Mouse)</td>
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<td>Rattus norvegicus (Rat)</td>
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<td>Gallus gallus (chicken)</td>
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<td>Danio rerio (zebrafish)</td>
<td>54.0</td>
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<tr>
<td>Drosophila melanogaster (fruit fly)</td>
<td>40.5</td>
</tr>
<tr>
<td>Caenorhabditis elegans (Worm)</td>
<td>39.0</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe (Baker yeast)</td>
<td>40.0</td>
</tr>
</tbody>
</table>
Figure 2.2 DDX24 RNA and protein expression pattern

(a) RNA expression profiling of ddx24 in different human organs by RT-PCR assays.
(b) Immunoblot analysis of DDX24 in HUVEC cells treated with DDX24 siRNA or control Non-Specific siRNA.
(c) Immunoblot analysis of DDX24 in various human cells or cell lines, normalized by human GAPDH.

2.1.2 Confirmation of FADD-DDX24 interaction by Yeast two hybrid systems

We first confirmed DDX24 as an interacting protein of FADD through yeast two hybrid experiments. Briefly, two plasmids expressing GAL4 binding domain (BD)-fused with FADD and GAL4 activation domain (AD)-fused with DDX24 were constructed and
transfected into yeast strain AH109 with appropriate control plasmids expressing only BD or AD as indicated (Figure 2.3). Transformed yeasts in every group grew in –Trp-Leu plate, indicating successful transformation as well as expression of the plasmids in each group. However, only the yeast with both BD-FADD and AD-DDX24 transformed grew on the His-Leu-Trp- plate but not the control group, indicating a specific interaction between DDX24 and FADD, which is consistent with a previous report (139).

Fig. 2.3 Yeast two hybrid experiments testing DDX24 and FADD interaction

GAL4 binding domain (BD) only or fused to the FADD were cotransfected respectively with GAL4 activation domain (AD) only or fused DDX24 in yeast two hybrid screening.

2.1.3 Confirmation of FADD-DDX24 interaction by co-immunoprecipitation

It was necessary to confirm the interactions in a mammalian system, where the protein could be folded and modified more properly than in the yeast system. Therefore, we performed co-immunoprecipitation experiments in HEK 293T cells by overexpressing amino terminal c-Myc-tagged human DDX24 and amino terminal FLAG-tagged human
FADD plasmids. Indeed, when overexpressed, c-Myc-tagged DDX24 exerted a strong binding to FLAG-tagged FADD when precipitated either by anti c-Myc or anti FLAG antibody (Figure 2.4a, b). To further confirm the interaction between FADD and DDX24, we examined the association between endogenous DDX24 and FADD in primary Human Umbilical Vein Endothelial Cells (HUVEC). Endogenous DDX24 were detected only when FADD was pulled down using anti FADD antibody but not in IgG control group, suggesting a physical interaction between these two molecules even under normal conditions in HUVEC cell (Figure 2.4c).

![Co-immunoprecipitation experiments testing DDX24-FADD interaction.](image)

(a,b) 293T cells were transiently transfected with cmyc-DDX24 and FLAG–FADD or proper control plasmid. Lysates were immunoprecipitated (IP) and immunoblotted (IB) using antibodies to c-Myc or FLAG.

(c) Endogenous human DDX24 associates with FADD in HUVEC. Lysates of HUVEC cells were immunoprecipitated with anti-FADD or mouse IgG serum. The immunoprecipitates were analyzed by immunoblot with anti-DDX24 or anti-FADD (top). The expression levels of the endogenous DDX24 and FADD were detected by immunoblot analysis (bottom).
In order to further detect which part of DDX24 mediates the interaction with FADD, c-Myc-tagged variants of DDX24 were constructed as described (Figure 2.5). Accordingly, variants of DDX24 were co-transfected with FLAG-tagged FADD in 293T cells, and the immunoprecipitation with FLAG antibody indicated that the DDX24-N expressing only the DExD/H box helicase ATP binding domain can bind to FADD. Collectively, these data confirm the interaction of DDX24 to FADD by two different methods and FADD could be a potential physiological target of DDX24 in regulating innate immune signaling.

**Figure 2.5 Identification of DDX24 deletions interacting with FADD**

(a) Schematic of human DDX24 (hDDX24) indicating helicase domains. 293T cells were transiently transfected with c-Myc-DDX24-FL, c-Myc-DDX24-N or control plasmid with FLAG-FADD, Lysates were immunoprecipitated (IP) using antibodies to FLAG and immunoblotted (IB) using antibodies to c-Myc.
2.1.4 DDX24 co-localized with FADD

To better evaluate the physiological interacting conditions of FADD and DDX24, the subcellular localizations of DDX24 and FADD were investigated. Fractionation experiments were performed to separate and check nuclear/cytosolic fractions in HUVEC cells. Clearly as shown in figure 2.6, both DDX24 and FADD were detected in both nuclear and cytosolic fractions. Human β-actin and Histone H3 were also detected in this experiment as marker of cytosol and nucleus accordingly.

![Figure 2.6 Fractionation of endogenous DDX24 in HUVEC cells.](image)

Fractionation of endogenous DDX24 and FADD in HUVEC cells. Histone H3 and β-actin are used as markers for nucleus and cytosol.

The localizations of endogenous DDX24 and FADD in HUVEC cells were further evaluated by immunostaining. As shown in figure 2.7a, DDX24 and FADD were detected in both nuclear and cytosolic region of the cell as determined by confocal microscopy,
which is consistent with fractionation results. Furthermore, we also investigated the localizations of DDX24 and FADD in MEF cells, which showed similar staining pattern, suggesting that endogenous DDX24 and FADD are colocalized (Figure 2.7b).

![Figure 2.7 DDX24 and FADD colocalized in nuclear and cytosolic regions](image)

**Figure 2.7 DDX24 and FADD colocalized in nuclear and cytosolic regions**

(a) HUVEC cells or (b) MEF cells were seeded on glass slides at 30% confluence. Twenty four hours later, cells were harvested, fixed and stained with anti DDX24 (Rabbit), anti FADD (mouse). DAPI staining indicated the nucleus localization.

Interestingly, we observed that DDX24 aggregated in many spots in the nuclear region in both HUVEC and MEFs. The immunostaining experiments in HUVEC indicated that endogenous DDX24 colocalized with fibrillarin (a nucleolus marker), suggesting DDX24 has a preferred location in the nucleolus (Figure 2.8). This is consistent with previous a report that DDX24 localized primarily in the nucleolus, with unknown functions (142). Collectively, our results confirmed the association and colocalization of DDX24 and FADD, potentially suggesting that DDX24 maybe involved in FADD-dependent signaling, possibly RLR signaling.
Figure 2.8 DDX24 primarily localized in nucleolus.
Confocal analysis of HUVEC cells labeled with both DDX24 and Fibrillarin (nucleolus marker). DAPI staining indicates the location of nucleus.

2.1.5 Discussion

FADD was first identified as a FAS associated protein which plays critical roles in apoptosis signaling pathway. Indeed, decades of studies indicate that FADD acts as an adaptor protein that transmit the signaling from upstream death receptors such as FAS and TNFR1 to the downstream effectors caspase 8 and 10 to induce apoptosis (143, 144). However, it is now better understood that FADD also plays underlying roles in many distinct cellular processes. This is largely dependent on the adaptor proteins that are associated with FADD, which determine the specificity of each different pathway that FADD participates. For example, it was reported that Atg5 binds to the DD of FADD, and that FADD deficiency blocked both apoptotic and autophagic cell death (145). V- and C-FLIP proteins associated with the DED of FADD, causing the activation of NF-κB signaling for cell survival (146). The interactions between RIP1 and DD of FADD are
critical for the necrosis signaling. In a FADD inducible cell line, it was shown that enhanced oligomerization of FADD DD only mutant led to necrosis. (147). Most recently, RIP1 and FADD were implicated in positively regulating RLR signaling, which paves the way for better understanding of RIP1 and FADD’s role in innate immunity.

DDX24, identified by Wanker group as a FADD associated protein, belongs to the DExH/D family, which contains 59 proteins so far. DExH/D helicases are broadly involved in many RNA related processes such as transcription, translation, ribosome biogenesis, RNA transportation and RNA modifications, which are critical in every cell or organism (139). Indeed, our preliminary studies indicated that human DDX24 RNA was widely expressed in a broad range of organs tested. Moreover, at the protein level, DDX24 was detected in all nine primary cell or cell lines derived from different organs in this study. This globally expressed pattern suggests general functions of DDX24 that might be required by most cell types. The reason why testes and brains contain the highest RNA level of DDX24 might be related to DDX24 functions that need to be further addressed. It is reported that DDX24 interacts with HIV1 Rev protein and suggested an important role in efficient HIV-1 Rev/RRE-dependent nuclear export of viral RNA, thus facilitating HIV-1 viral RNA packaging (142). This article indicated a connection between HIV-1 and DDX24, but no further detailed mechanism was described in this study, unfortunately. Recently, DExH/D family has gained more attention as being involved in the antiviral pathway. For example, RIG-I, MDA5, LGP2 and DDX3X were
all identified a few years ago for their critical functions in the RLR pathway, which support DDX24 as a good candidate for being potentially involved in FADD dependent RLR pathway.

The interaction between DDX24 and FADD was confirmed through several independent experiments, including yeast two hybrid assay, reciprocal overexpression based co-IP experiments in 293T cells and endogenous IP in HUVEC cells. Furthermore, intracellular localization of FADD and DDX24 also suggest colocalization of DDX24 and FADD in nuclear and cytoplasmic region, suggesting potential related functions between these two molecules.

Notably, our immunofluorescent staining in HUVEC cells revealed that endogenous DDX24 is localized in both the nucleus and cytoplasm, with a specific aggregation in nucleolus. This observation is different from conclusions from the Liang group which described that the nucleolus is the only distribution of DDX24 in their paper. However, the cytoplasmic DDX24 does exist, because DDX24 was clearly detected in cytoplasmic fractions in our fractionation experiments, which supports our staining results. One possible explanation of the contradictory observations of Liang’s group is possibly due to the different exposure time of the staining, because nucleolus staining pattern is very obvious after a very short exposure time.
The intracellular distribution of FADD is also under debate. Based on the primary role of FADD as an apoptosis adaptor protein, FADD was expected to be localized in the cytoplasm, where the death receptor signaling complex is located. However, both cytoplasmic and nuclear localization of FADD were reported by independent groups. In our study, endogenous FADD is primarily localized in the nucleus, with minor cytoplasmic FADD distribution, which is correlated with the finding of Huang group and Frisch group (148, 149). In other studies, putative nuclear import and export sequences were proposed in FADD and revealed that the nuclear localization is affected by phosphorylation at a single serine in its carboxyl terminus (150). It was also reported that exportin-5 might be responsible for the transportation of FADD, suggesting FADD’s shuttling moves between nucleus and cytoplasm. The localization of FADD in the nucleus might indicate additional uncharacterized nuclear functions of FADD, which need to be further addressed. In my study, we did not observe the change of FADD’s localization when DDX24 is present or absent (data not shown), suggesting that DDX24 might not affect FADD’s distribution related functions.

Collectively, my preliminary study indicated DDX24 as a globally expressed protein, which is high conserved among species. As a DExD/H family member, DDX24 was confirmed to be associated and colocalized with FADD in different experiments, suggesting DDX24’s potential role in FADD related functions. In the following sections, we will specifically investigate the underlying function of DDX24 in the RLR antiviral pathway.
2.2 DDX24 negatively regulates dsRNA triggered RLR signaling

2.2.1 Overexpression of DDX24 blocks dsRNA triggered RLR signaling

Thus far, we have confirmed the interactions between FADD and DDX24. Next we will determine whether DDX24 is involved in the FADD-related RLR pathway. We first tested if DDX24 could activate IFN\(\beta\) promoter by overexpressing a c-Myc-tagged DDX24 plasmid or control plasmid together with IFN\(\beta\) promoter driven luciferase plasmid in 293T cells. However, no significant activation was observed in the presence of DDX24 compared to the control group, indicating that DDX24 alone is not a strong stimulator of IFN\(\beta\) (data not show). We therefore tested if DDX24 could affect the signaling on the IFN\(\beta\) promoter after RLR signaling was stimulated in cells. We utilized a synthetic analog of double-stranded RNA, poly I:C to activate the RLR pathway, which is widely used as a dsRNA stimulus to trigger RLR signaling through MDA5 dependent pathway (29). 293T cells were co-transfected with DDX24, control plasmids and reporter plasmids for 24 hours, and then stimulated the cells with poly I:C transfection overnight before testing the activation of the IFN\(\beta\) promoter. As shown in figure 2.9a, poly I:C strongly activate IFN\(\beta\) promoter about 50 folds, but no synergistic effect by DDX24 with poly I:C stimulations was observed. Instead, overexpression of DDX24 significantly suppressed poly I:C triggered IFN\(\beta\) promoter activation in a dose dependent manner, indicating a negative regulatory role of DDX24 in the RLR pathway.
**Figure 2.9** DDX24 inhibits poly I:C and VSVdM triggered IFNβ promoter activation

(a) DDX24 inhibits poly I:C-induced activation of the IFNβ promoter in a dose-dependent manner in 293T. 293T cells were transfected with an IFNβ promoter reporter and increased amount of DDX24 expressing plasmid. Twenty four hours after transfection, cells were transfected with poly I:C or left untreated. Luciferase assays were performed 12 hours after transfection. Expression of c-Myc-DDX24 was monitored using c-Myc antibody, normalized by β-actin.

(b) DDX24 inhibits VSVdM-induced activation of the IFNβ promoter in a dose-dependent manner in 293T cells. 293T cells were transfected with an IFNβ promoter reporter and an increased amount of DDX24 expressing plasmid. Twenty four hours after transfection, cells were infected with VSVdM at MOI=1 or left uninfected overnight before luciferase assays. Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

To better evaluate the inhibitory effect of DDX24 on RLR pathway, we also selected another stimulus, vesicular stomatitis virus (VSV), which is reported to be sensed by RIG-I instead of MDA5 to initiate RLR signaling (83). Instead of WT VSV, we used VSVdM (a mutated version of VSV in which the M protein is removed from virus
genome to increase the potential of activating IFNβ), which has more potential to stimulate the RLR pathway than wt VSV. Briefly, we performed similar co-transfection experiments by infecting 293T cells using VSVdM at MOI=1 for overnight before testing the IFNβ promoter activation. Similarly, we observed a 10 fold activation of IFNβ promoter by VSVdM infection, and DDX24 also blocked this activation in a dose dependent manner (Figure 2.9b). A c-Myc-tagged DDX24 plasmid was used to overexpress DDX24 in these experiments in 293T, and the expression level of DDX24 were monitored using anti c-Myc antibody. β-actin was used as a loading control (Figure 2.9a).

To corroborate our findings that DDX24 negatively regulate IFNβ promoter activation, we further evaluated the effect of DDX24 on the endogenous ifnb gene using RT-PCR in 293T cells. DDX24 plasmid or control plasmids were transfected into 293T cells and 24 hours later, cells were treated with either poly I:C transfection or VSVdM at MOI=1 for 4 hours before endogenous ifnb RNA was detected by RT-PCR. As shown in Figure 2.10a and b, poly I:C treatment and VSVdM infection led to 6000 folds and 4000 fold induction of endogenous ifnb RNA respectively in 293T cells. Together with the reporter assay results, DDX24 could significantly inhibit both poly I:C and VSVdM induced transcription of the endogenous ifnb gene, which confirms the inhibitory effect of DDX24 on IFNβ production induced by either dsRNA poly I:C or VSVdM infection.
Figure 2.10 DDX24 inhibits poly I:C and VSVdM triggered endogenous *ifnb* RNA induction

(a) DDX24 inhibits poly I:C-induced endogenous *ifnb* in 293T cells. 293T cells were transfected with control plasmid or DDX24 expressing plasmid. Twenty four hours after transfection, cells were transfected with poly I:C or left untreated for 4 hours. Endogenous *ifnb* were analyzed by RT-PCR.

(b) DDX24 inhibits VSVdM-induced endogenous *ifnb* in 293T cells. 293T cells were transfected with control plasmid or DDX24 expressing plasmid. Twenty four hours after transfection, cells were infected with VSVdM at MOI=1 or left uninfected for 4 hours. Endogenous *ifnb* were analyzed by RT-PCR. Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

In order to rule out the possibility that the inhibitory effect is a global suppression of RNA transcription caused by DDX24, we performed experiments testing the transcription
of a non-host gene *luc*, driven either by an IFNβ promoter or Gal3 promoter. As indicated in Fig 2.10c and d, DDX24 could inhibit *luc* transcription only when it is under control of the IFNβ promoter, but not that driven by GAL3 promoter, suggesting a specific block of IFN transcription by DDX24. Moreover, a plasmid expressing GFP was also included in GAL3-luc experiments as a negative control plasmid. As expected, GFP had no effect on *luc* transcription in our experiment, suggesting DDX24 indeed suppressed IFNβ transcription. Collectively, these results suggest that overexpression of DDX24 blocks dsRNA induced MDA5 and RIG-I dependent RLR signaling in 293T cells and this suppression is not due to a global transcriptional suppression by DDX24.

To eliminate potential cell-specific effects and confirm DDX24’s role in RLR pathway, we repeated the same experiments in primary mouse embryonic fibroblast (MEF) cells. As indicated previously, mouse and human DDX24 share over 90% identity and have highly conserved helicase domains. Therefore, the same c-Myc-tagged human DDX24 plasmid was transfected using AMAXA electrotransfection systems and expressed in MEFs. As expected, DDX24 exerted inhibitory effects on poly I:C/VSVdM triggered IFNβ promoter activation in a dose dependent manner in MEFs (Figure 2.11a, b). Similarly, RT-PCR experiments indicated that DDX24 was able to significantly block poly I:C/VSVdM induced *ifnb* mRNA. These results correlated with the previous study in 293T cells (Figure 2.11c, d).
Fig 2.11 DDX24 inhibits poly I:C and VSVdM triggered RLR signaling in MEFs.

(a) DDX24 inhibits poly I:C-induced activation of the IFN-β promoter in a dose-dependent manner in MEF cells. Expression of c-Myc tagged DDX24 in MEFs was monitored using anti c-myc antibody, normalized by β-actin.

(b) DDX24 inhibits VSVdM-induced activation of the IFN-β promoter in a dose-dependent manner in MEF cells.

(c) DDX24 inhibits poly I:C-induced endogenous ifnb transcription in MEF cells.

(d) DDX24 inhibits VSVdM-induced endogenous ifnb transcription in MEF cells.

Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

Furthermore, DDX24’s effect on IFNβ at the protein level was also investigated through ELISA. Our results consistently demonstrated that ectopic overexpression of DDX24
could also lead to suppression of poly I:C and VSVdM induced IFNβ production in MEF cells (Figure 2.12a, b). Collectively, we have examined the inhibitory effect of DDX24 on both poly I:C and VSVdM triggered type I interferon signaling at the promoter activation level, RNA level and protein level in both 293T and MEFs. The inhibitory effects observed in these experiments suggest DDX24 as a potential negative regulator of RLR dependent antiviral signaling.

![DDX24 inhibits poly I:C and VSVdM triggered endogenous IFNβ production in MEFs.](image)

(a) DDX24 inhibits poly I:C-induced endogenous IFNβ protein level in MEF cells. MEF cells were transfected with control plasmid or DDX24 expressing plasmid. Twenty four hours after transfection, cells were transfected with poly I:C or left untreated overnight. Endogenous IFNβ were analyzed by ELISA.

(b) DDX24 inhibits VSVdM-induced endogenous IFNβ protein level in MEF cells. MEF cells were transfected with control plasmid or DDX24 expressing plasmid. Twenty four hours after transfection, cells were infected with VSVdM at MOI=1 or left uninfected overnight. Endogenous IFNβ were analyzed by ELISA.

Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.
2.2.2 Knockdown of DDX24 by siRNA enhanced dsRNA triggered RLR signaling

We have previously demonstrated that ectopic expression of DDX24 inhibits RLR signaling. To further confirm the negative effect of DDX24 on the RLR pathway, we next evaluated the effect of knocking down endogenous DDX24 on the RLR pathway using small interfering RNA (siRNA) that is specifically targeting DDX24. IFNβ promoter assay were performed and knock down efficiency of DDX24 was monitored using anti DDX24 antibody in 293T cells, normalized with β-actin expression. As shown in Figure 2.13a and b, poly I:C and VSVdM activate the IFNβ promoter and this was significantly enhanced after knocking down DDX24.

Figure 2.13 SiRNA-mediated knockdown of DDX24 enhances poly I:C and VSVdM triggered IFNβ promoter activation in 293T cells.

(a) Effects of DDX24 RNAi on poly I:C-induced activation of the IFNβ promoter in 293T cells. 293T cells were transfected with ns or human ddx24 siRNA. Forty eight hours after transfection, cells were left untreated or transfected with poly I:C overnight
before luciferase assays were performed. 293T cell lysates were analyzed by immunoblotting with indicated antibodies to ensure the knockdown of hDDX24.

(b) Effects of DDX24 RNAi on VSVdM-induced activation of the IFNβ promoter in 293T cells. 293T cells were transfected with ns or human ddx24 siRNA. Forty eight hours after transfection, cells were left untreated or infected with VSVdM overnight before luciferase assays were performed. Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

In agreement with the reporter assays in 293T cells, RT-PCR studies in MEFs indicated that knockdown of DDX24 by siRNA in MEFs could also enhance poly I:C or VSVdM induced transcription of the endogenous ifnb gene compared to the non-specific siRNA treated control group (Figure 2.14a, b). ELISA experiments also indicated that knocking down of DDX24 led to stronger poly I:C and VSVdM triggered IFNβ production in MEFs compared to control group (Figure 2.14c and d). Knock down efficiency was monitored using DDX24 antibody in MEFs (Figure 2.14a). Collectively, these data demonstrate that siRNA based knockdown of DDX24 enhance poly I:C or VSVdM activated type I IFN production in both 293T and MEFs, which further support our hypothesis that DDX24 plays a negative role in type I interferon production.
Figure 2.14 SiRNA-mediated knockdown of DDX24 enhances poly I:C and VSVdM triggered RLR signaling in MEF

(a) Effects of DDX24 RNAi on poly I:C-induced endogenous ifnb transcription in MEF cells by RT-PCR. MEF cell lysates were analyzed by immunoblotting with the indicated antibodies to ensure the knockdown of mDDX24.

(b) Effects of DDX24 RNAi on VSVdM-induced endogenous ifnb transcription in MEF cells by RT-PCR.

(c) Effects of DDX24 RNAi on poly I:C-induced endogenous IFNβ production in MEF cells by ELISA.

(d) Effects of DDX24 RNAi on VSVdM-induced endogenous IFNβ production in MEF cells by ELISA.

Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.
2.2.3 **Knockdown of DDX24 by siRNA blocks VSV replication**

IFNβ is critical to block virus replication. Therefore, we tested if DDX24 affected VSV replication, since it was shown to block IFNβ production. To monitor VSV replications, we utilized two different VSV mutants which are modified with either *luc* or *gfp* reporter gene. The insertion of these two genes will allow us to monitor virus replication not only by plaque assay, but also by measuring luciferase activity from cell lysate or observing GFP protein in living cells by microscopy.

After DDX24 was knocked down by siRNA treatment, MEFs were infected with VSV-luc at different MOI (MOI=0.1, 1) and different time point (8, 24 hours). VSV replication in MEFs was then monitored by plaque assay and luciferase assay. DDX24 siRNA treated MEFs showed less luciferase activation compared to the non specific siRNA treated group. It has been reported that RIG-I, a sensor of the RLR pathway, plays a positive role in the antiviral response. Therefore, we used RIG-I siRNA in these experiments as a control. Indeed, compared with the control siRNA group, MEFs transfected with RIG-I siRNA had increased virus replication, which was opposite to that of the DDX24 siRNA group (Figure 2.15a). Plaque assay using the supernatant taken from the same experiments of each group showed similar threads in each group in that less virus was detected in DDX24 siRNA treated group, and RIG-I siRNA treated group had the highest virus titer (Figure 2.15b).
Figure 2.15 SiRNA-mediated knockdown of DDX24 blocks VSV-luc replication in MEF

(a) MEF cells transfected with ns, mDDX24 or RIG-I siRNA for 72 hours were infected by VSV-luc at M.O.I.=0.1 or 1. Twenty four or 48 hours post infection, luciferase activity from infected MEF cell lysate were detected.

(b) Virus titers were measured from supernatant of (a) respectively.

Data are presented as means ± s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

We next performed similar experiments in MEFs using VSV-GFP. Compared to the control group, much less GFP positive cells, if any, were observed in DDX24 siRNA treated MEFs compared to ns siRNA treated MEFs, while the RIG-I siRNA group had the most GFP positive cells (Figure 2.16a). Virus titer in the supernatant of each group also...
showed consistent trends with previous experiments using VSV-luc. MEFs transfected with siDDX24 showed the lowest viral replication, while siRIG-I group exerted the highest viral titer, which further confirms the positive effect of DDX24 on VSV replication in MEF (Figure 2.16b). Knock down efficiency was confirmed by western blotting using DDX24 antibody (Figure 2.16c).

![Image](image.png)

**Figure 2.16 SiRNA-mediated knockdown of DDX24 blocks VSV-GFP replication in MEF**

(a) Fluorescence microscopy (GFP) of NS, mDDX24 or mRIG-I siRNA treated MEFs following with VSV-GFP infection 24 hours post infection at M.O.I 1. (b) MEF cells transfected with ns, mDDX24 or RIG-I siRNA for 72 hours were infected by VSV-GFP at M.O.I.=0.1 or 1. Twenty four hours post infection, plaque assays were performed using supernatants from infected MEF cells. Data are presented as means ± s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

Besides MEFs, we further evaluated endogenous human DDX24’s effect on VSV replication in HUVEC cells. Briefly, after DDX24 was knocked down by siRNA,
HUVEC cells were infected with VSV-GFP at different MOI (0.1, 1) and at different times (8, 24 hours) before virus titers were monitored by plaque assay. Knockdown efficiency was confirmed by western blotting using DDX24 antibody in HUVEC cells (Figure 2.17). Compared with the ns siRNA group, the DDX24 siRNA group showed significantly lower virus titer, indicating a stronger antiviral effect. Collectively, our data indicate that siRNA based knockdown of DDX24 renders both MEFs and HUVEC cells more resistant to VSV replication. These results correlate with the previous findings that DDX24 suppression leads to more type I IFN production, and further suggest the negative regulatory role of DDX24 in the RLR pathway.

Figure 2.17 SiRNA-mediated knockdown of DDX24 blocks VSV-GFP replication in HUVEC
HUVEC cells transfected with ns or hDDX24 siRNA for 72 hours were infected by VSV-luc at M.O.I.=0.1 or 1. Eight or 24 hours post infection, plaque assays were performed using supernatants from infected MEF cells. HUVEC cell lysates were analyzed by immunoblotting with the indicated antibodies to ensure the knockdown of hDDX24. Data from are presented as means ± s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.
2.2.4 Discussion

RIG-I and MDA5 are two receptors in the RLR pathway that initiate signaling recognizing viral nucleic acids stimulus. Structural studies reveal that RIG-I and MDA5 recognition preference might be determined by the length, the structure and the modification of viral RNAs. Depending on its length, poly I:C can be recognized by either RIG-I or MDA5. In particular, poly I:C that is about 300 bp rely on RIG-I for triggering RLR signaling, while poly I:C greater than 4 kb in length activates the MDA5 dependent pathway (30). FADD has been reported acting downstream of these two molecules in that FADD deficient MEF shows significantly decreased IFNβ promoter activation triggered by either RIG-I or MDA5 (126). Therefore, to better study the effect of DDX24 on RLR signaling, it was necessary to evaluate both RIG-I and MDA5 dependent pathways. In our experiments, long poly I:C was utilized for triggering MDA5 dependent pathway, while VSVdM was used to activate RIG-I dependent signaling.

In this study, VSVdM instead of wild type VSV was used as a stimulus of RLR signaling, because the matrix (M) protein of VSV was reported to suppress host RNA and protein synthesis including IFNβ (151-155). Moreover, the matrix protein was also reported to block host RNA transport by hijacking the Rea1/mrnp41 nuclear transport pathway, which also contributes to the defective innate immune responses (156). Consistently, a modified VSV with deleted M protein was reported to have a high potential of inducing interferon beta and therefore was used in our study for interferon induction (157).
Therefore, the wt VSV containing functional matrix (M) protein has a very weak ability to induce type I interferon production, which makes VSV a better candidate for triggering RLR signaling.

In our study, we have shown that overexpression of DDX24 blocks either poly I:C or VSVdM induced type I IFN promoter activation, endogenous IFNβ RNA and protein production in two different cells/cell line, 293T and MEFs. Correlated with this, siRNA based knockdown of endogenous DDX24 resulted in opposite effects on type I IFN production. These data clearly suggested DDX24 as a negative regulator of RLR pathway, which were further supported by the evidence that DDX24 suppression attenuated VSV replication in primary cells from mouse (MEF) or human (HUVEC). Based on these studies, in the next section, we specifically explored FADD and potential targets of DDX24 in the RLR pathway and further investigated the molecular mechanism of DDX24 dependent negative regulation of RLR signaling. In the meantime, we initiated the study of the in vivo role of DDX24 by generating DDX24 deficient mice, which will be discussed in chapter 2.4, as analysis of RLR signaling in DDX24 deficient MEFs is critical to confirm our siRNA based studies.
2.3 Mechanism of DDX24’s negative regulation of RLR pathway.

2.3.1 DDX24 blocks RLR dependent antiviral signaling at TBK-1/IKKi level

Others and our group have demonstrated that IPS-1 recruited many regulators during virus infection and formed a signaling complex. FADD is reported to be directly associated with IPS-1 by the Akira group (34). Although the Tschopp group has reported that FADD and IPS-1 interaction is indirect and their association is mediated by TRADD, it is agreed that FADD is an important component of the IPS-1 recruited signaling complex (39). Moreover, regulators like FADD, RIP1 and TRADD are reported to be dispensable for IFNβ induction, but loss of any component will lead to significantly attenuated signaling (39, 129). Therefore, it has been proposed that FADD and RIP1 functioned as scaffold protein to help maintain specific conformations for optimal recruitment of other regulators as well as supporting appropriate and stable contacts between these molecules. Based on previous publications and our preliminary data, we hypothesize that DDX24 targets FADD or other potential molecules within the signaling complex to inhibit RLR signaling, possibly through disrupting the connections among the molecules or attenuating stability of the complex.

To prove this hypothesis, we investigated the effect of DDX24 on IFNβ promoter activation mediated by the known signaling components from upstream RLR receptors to downstream transcription factors. We have previously reported in this study that
overexpression of DDX24 could block VSVdM as well as poly I:C induced IFNβ promoter activation. Since virus infection and dsRNA can activate IFN via the RLR family of PRRs, we initiated our study by determining if DDX24 inhibited RIG-I/MDA5 mediated activation of IFN. In this study, instead of the full length RIG-I/MDA5, we used dRIG-I and dMDA5 which contains only the two CARD domains located in the N terminus, and have more potential than full length proteins in activating IFNβ signaling (77, 83). We co-transfected dRIG-I/dMDA5 with DDX24 and measured IFNβ reporter activity. Indeed, DDX24 was able to block dRIG-I/dMDA5 mediated activation of IFNβ.

Downstream of RIG-I/MDA5, the adaptor IPS-1 functions as a critical adaptor protein to transduce the signaling. Similarly, we found that DDX24 overexpression also blocked IPS-1 triggered activation of the IFNβ promoter. The kinases TBK-1 and IKKi are also recruited to the IPS-1 based signaling complex during virus infection to initiate IRF3/7 phosphorylation, subsequently leading to the activation of type I interferon. We also evaluated the effect of DDX24 overexpression on TBK-1/IKKi dependent activation of IFNβ. DDX24 significantly blocked TBK-1 triggered IFNβ activation, and slightly inhibited IKKi mediated IFNβ activation (Figure 2.18a). We have also tested further downstream, and DDX24 did not block IRF3 or IRF7 superactive form (IRF3/IRF7 is constitutively phosphorylated) induced IFNβ activation, suggesting that DDX24 works upstream of IRF3/IRF7. Our data suggested that DDX24 functioned at the level of IPS-1 based signaling complex, which is correlated with our hypothesis (Figure 2.18b).
Figure 2.18 DDX24 inhibits RLR signaling

(a) DDX24 inhibits dRIG-I, dMDA5, IPS-1, TBK-1 and IKKi mediated IFNβ promoter activation. 293T cells were transfected with reporter plasmid and variant plasmids as indicated. Activations of IFNβ promoter were detected 36 hours post transfection.
(b) DDX24 does not inhibits IRF3(SA) or IRF7(SA) induced IFN promoter activation. (c) 293T cells were transfected with reporter plasmid and variant plasmids as indicated. Activations of p53 promoter were detected 36 hours post transfection.

Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

To confirm that the inhibition of IFNβ activation was indeed due to the block of signaling but not affecting the protein expression, immunoblotting experiments were performed to monitor protein expression. As indicated in Figure 2.18a, all plasmids were properly
expressed and loading amounts were normalized by β-actin. Furthermore, we also checked DDX24’s effect on an unrelated pathway to eliminate the possibility that DDX24 caused a global inhibitory effect. The P53 reporter plasmid, consisting of a P53 responsive element followed by luc gene, was utilized to activate the signaling. In this experiment, we included a plasmid expressing HTLV Tax-1 protein as control, which is reported as a negative regulator of P53 pathway (158). As shown in Figure 2.18c, Overexpression of DDX24 did not affect P53 signaling, while Tax-1 blocked the activation of P53 (Figure 2.18c). These evidences confirm that DDX24 does not cause a global inhibitory effect on all of the pathways, and further confirms the specificity of DDX24’s negative regulation on the RLR pathway.

As DDX24 is a FADD interacting protein, we next checked DDX24’s effect on FADD dependent RLR signaling. It has been reported that FADD alone is not a strong inducer of IFNβ, but is required for optimal RLR signaling. Therefore, we performed experiments by co-overexpressing FADD with RIG-I or dRIG-I with increasing DDX24 plasmid in 293T cells as indicated in Figure 2.19a and b. As expected, FADD alone could not induce IFNβ significantly in this experiment. While RIG-I and dRIG-I could activate IFNβ promoter about 200 and 300 fold, the fold activation was synergistically increased to 300 and 700 in the presence of FADD respectively, confirming a positive role of FADD in facilitating RLR pathway. Moreover, in the presence of increasing DDX24 protein, this synergistic activation was significantly inhibited in a dose dependent manner (Figure
Notably, we have also monitored renilla luciferase activities in all groups as controls for transfection efficiency. The renilla luciferase, however, is not affected by overexpression of DDX24, indicating the block of IFNβ-luc was indeed due to the inhibition of signaling without affecting expression of protein. Collectively, these results suggest that DDX24 indeed disrupts FADD dependent RLR signaling.

**Figure 2.19 DDX24 inhibits FADD dependent RLR signaling**

(a) 293T cells were transfected with an IFNβ-Luc plasmid and vector, RIG-I, FADD, RIG-I along with FADD in presence of increasing amounts of DDX24 (0, 200ng, 400ng, 800ng). Thirty six hours later, firefly luciferase and renilla luciferase were tested from cell lysates.

(b) 293T cells were transfected with an IFNβ-Luc plasmid and vector, dRIG-I, FADD, dRIG-I along with FADD in presence of increasing amounts of DDX24. Thirty six hours later, firefly luciferase and renilla luciferase were tested from cell lysates. Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.
2.3.2 DDX24 forms a complex with FADD and RIP1

Given the fact that RIP1 and FADD are both physiologically and functionally related, it is possible that RIP1 is also a potential target of DDX24. To determine if RIP1 interacts with DDX24, we performed co-IP experiments in 293T cells by overexpressing c-Myc tagged RIP1 and FLAG tagged DDX24. RIP1 was successfully detected when lysate were immunoprecipitated with anti-FLAG antibody, suggesting that RIP1 interacts with DDX24 (Figure 2.20).

![Figure 2.20: DDX24 interacts with RIP1.](image)

293T cells were transfected with either c-Myc-tagged RIP1 or FLAG-DDX24 as indicated. After 24h, cells were harvested, and lysates were subjected to co-IP and immunoblotting (IB) with the indicated antibodies.

Given that RIP1 interacts with DDX24, we further tested if DDX24 is also involved in RIP1-dependent RLR signaling. Hence, we co-overexpressed RIP1 with RIG-I or dRIG-I with increasing amounts of DDX24 in 293T cells as indicated in Figure 2.21. Indeed, while RIG-I and dRIG-I could activate IFNβ promoter about 200 and 300 fold, the fold
activation was synergistically increased to 900 and 2000 in the presence of RIP1 respectively. Moreover, in the presence of increasing DDX24 protein, this synergistic activation was significantly attenuated in a dose dependent manner, suggesting the involvement of RIP1 on the DDX24 dependent regulation of RLR (Figure 2.21). Furthermore, renilla luciferase activity was not affected by overexpressing DDX24, suggesting that the attenuated IFNβ-luc was indeed due to the inhibition of signaling.

Fig 2.21 DDX24 interacts with RIP1 and inhibits RIP1 dependent RLR signaling

(a) 293T cells were transfected with an IFNβ-Luc plasmid and vector, RIG-I, RIP1, RIG-I along with RIP1 in presence of increasing amounts of DDX24. Thirty six hours later, firefly luciferase and renilla luciferase were tested from cell lysates.

(b) 293T cells were transfected with an IFNβ-Luc plasmid and vector, dRIG-I, RIP1,
dRIG-I along with FADD in presence of increasing amounts of DDX24. Thirty six hours later, firefly luciferase and renilla luciferase were tested from cell lysates. Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

2.3.3 DDX24 disrupts the interaction of RIP1 and IRF7

RIP1 was identified as a positive regulator in RLR signaling. It was reported by the Pagano group that RIP1 associated with IRF7 and could synergistically foster the IRF7 dependent type I interferon activation, suggesting that RIP1 is required for optimal IRF7 activity (71, 159). This prompted us to examine whether DDX24 could disrupt the interaction between RIP1 and IRF7, which could potentially explain the mechanism of DDX24 dependent RLR negative regulation.

To test this hypothesis, we performed a competition binding assay by co-transflecting c-Myc-tagged DDX24, c-Myc-tagged IRF7 and FLAG-tagged RIP1 in 293T cells. Lysates were subsequently immunoprecipitated with anti FLAG antibody followed by immunoblotting using anti c-Myc antibody. As shown in Fig 2.22a, IRF7 was co-precipitated with RIP1 in the absence of DDX24, but the interaction between RIP1 and IRF7 was disrupted in the presence of DDX24. Besides, we also detected endogenous FADD interaction with RIP1 was not affected by DDX24, suggesting a specific disruptions of RIP1-IRF7 interactions by DDX24. To further confirm the competition results, reciprocal co-IP experiments were also performed using c-Myc-tagged DDX24, c-Myc-tagged RIP1 and FLAG-tagged IRF7 as indicated in figure.
2.22b. Consistently, the interactions between RIP1 and IRF7 were significantly reduced when DDX24 was present (Figure 2.22b). Moreover, siRNA experiments were performed to test endogenous DDX24’s effect on the RIP1/IRF7 interaction. As shown in figure 2.22c, increased RIP1 was co-precipitated with IRF7, when DDX24 was knocked down, thus correlating with the overexpression experiment results. Collectively, these data corroborate our hypothesis that DDX24 competes with IRF7 for RIP1 binding, which better explains DDX24’s inhibitory role in antiviral signaling.

Figure 2.22 DDX24 disrupts RIP1-IRF7 interactions.

(a)(b) 293T cells were transiently transfected with variant plasmids as indicated and proper control plasmids. Twenty four hours post transfection, cell lysates were immunoprecipitated (IP) and immunoblotted (IB) using antibodies to c-Myc, FLAG or FADD.

(c) 293T cells were transfected with ns siRNA or DDX24 siRNA as indicated. Forty eight hours later, cells were transfected with variant plasmids for 24 hours before IP and IB using antibodies to c-Myc, FLAG.
2.3.4 DDX24 inhibits k63 linked ubiquitination of IRF7

It has been previously reported that RIP1 promoted the assembly of K63-linked polyubiquitin chains on IRF7, which is important in IRF7 dependent IFN activation. Knockout experiments indicated that IRF7 ubiquitination was diminished in RIP1 deficient MEFs (71). Therefore, we tested if the disruption of RIP1-IRF7 interactions by DDX24 could lead to attenuated RIP1 dependent ubiquitination of IRF7, specifically K63-linked polyubiquitination. Briefly, DDX24, IRF7 and HA-UB were cotransfected in 293T cells as indicated in Figure 2.23. Indeed, overexpression of DDX24 inhibited IRF7 polyubiquitination. Furthermore, to investigate whether overexpression of DDX24 specifically targeted k63-linked polyubiquitination chains conjugated on IRF7, we also used the HA-UB-K63 plasmid, in which all the lysines were mutated to arginines except for k63. As shown in lane 5-6, IRF7 was subject to K63-linked polyubiquitination, but the K63-linked polyubiquitination of IRF7 was significantly reduced in the presence of DDX24. Moreover, immunoblot of precipitated IRF7 indicated that the attenuated ubiquitination is caused by the presence of DDX24, but not by varied expression level of IRF7 (Figure 2.23). This reveals the detail on how DDX24 inhibits RIP1-IRF7 synergistic signaling, and further explains the mechanism of DDX24 regulation on RLR signaling.
**Figure 2.23 DDX24 attenuates K63 linked polyubiquitination of IRF7**

Co-IP using 293T cells transfected with c-Myc-tagged DDX24, FLAG-tagged IRF7, HA-tagged UB and HA-tagged UB-K63 as indicated. Cell lysate were IP with FLAG and IB with anti HA, FLAG and c-Myc antibody.

**2.3.5 DDX24 inhibits type I IFN activation by disrupting the RIP1-IRF7 synergistic effect**

Previously, we have shown that DDX24 could inhibit K63-linked ubiquitination of IRF7, a modification shown to be important for IFN activation. Therefore, we proposed that DDX24 could specifically block IRF7 dependent type I IFN activation. Because both IRF3 and IRF7 could activate IFNβ promoter, in order to distinguish IRF7 dependent IFN activation, we used IFNα4 promoter based reporter assay, which has been previously reported to be activated only by IRF7, but not IRF3 (160).
As shown in Figure 2.24a, IRF7 could activate the IFNα4 promoter by about 10 fold, but it was not affected by overexpression of DDX24, indicating that DDX24 works upstream of IRF7. Furthermore, DDX24 inhibited RIG-I-IRF7 synergistic activation of IFNα, suggesting that DDX24 blocks the signaling between RIG-I and IRF7 (Figure 2.24a). To validate the overexpression experiments, we also performed knock down experiments and observed that DDX24 suppression in 293T cells resulted in stronger synergistic effects between RIG-I and IRF7 (Figure 2.24b). Collectively, these data indicate that overexpression of DDX24 indeed blocked IRF7 dependent IFN activation. Moreover, it further provided evidence that DDX24 works between RIG-I and IRF7, correlated with our hypothesis that DDX24 regulates signaling complex, containing FADD and RIP1.

**Figure 2.24** DDX24 inhibits IRF7 dependent IFNα activation

**(a)** 293T cells were transfected with IFNα4-luc reporter plasmid and variant plasmids as indicated. Activation of IFNα4 promoter was detected 36 hours post transfection.
(b) 293T cells were transfected with ns or DDX24 siRNA. Forty eight hours post transfection, cells were transfected with IFNα4-luc reporter plasmid and variant plasmids as indicated. Activation of IFNα4 promoter was detected 36 hours post transfection. Data from (a)(b) are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

Previously, we have shown that DDX24 associates with both FADD and RIP1, without disrupting the interaction between FADD and RIP1. Therefore, it was plausible to test if FADD also play a positive role in IRF7 dependent IFNα4 activation and if DDX24 also negatively regulated this activity. IFNα4 promoter assay in 293T cells were performed whereby FADD was co-overexpressed, with or without IRF7, to detect the synergistic effect on IFNα4 activation, in presence or absence of DDX24 siRNA. Indeed, overexpression of FADD could not activate IFNα4 by itself, but could synergistically enhance IRF7 activated IFNα4. Moreover, this FADD-IRF7 synergistic activity was significantly enhanced after DDX24 was knocked down, indicating a negative role of DDX24 on FADD-IRF7 dependent IFNα4 activation (Figure 2.25). We also examined the association between FADD/DDX24 and IRF7 by co-IP experiments. However, no interactions of FADD/IRF7 or DDX24/IRF7 were detected (Data not shown), indicating that FADD and DDX24 might regulate IRF7 activation indirectly through its partner RIP1 without direct contact with IRF7.
293T cells were transfected with ns or DDX24 siRNA. Forty eight hours post transfection, cells were transfected with IFNα4-luc reporter plasmid and vector or FLAG-FADD plasmids as indicated. Activations of IFNα4 promoter were detected 36 hours post transfection. Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.

2.3.6 DDX24 selectively inhibits IRF7 dependent but not IRF3 dependent type I IFN activation

Previously, we have shown that DDX24 could block the RIP1-IRF7 synergistic effect, leading to attenuated IFNα4 activation. Although the details are not reported yet clear on how the RIP1 and IRF7 interactions lead to the ultimate activation of type I interferon, it has been proposed that RIP1, as an adaptor protein, recruits IRF7 to the signaling complex. This will enable the kinases TBK-1/IKKi to gain enough proximity to IRF7 for
sufficient IRF7 phosphorylation. According to this hypothesis, if DDX24 disrupts the RIP1 and IRF7 synergistic effect, the TBK1/IKKi dependent phosphorylation would be attenuated by DDX24. Therefore, we stimulated IRF7 phosphorylation by overexpressing TBK-1 or IKKi in 293T cells, and tested if overexpression of DDX24 could block the phosphorylation of overexpressed FLAG tagged IRF7. As shown in Fig 2.26, TBK-1 and IKKi induced strong phosphorylation of IRF7, and DDX24 abolished IRF7 phosphorylation without affecting the expression level of IRF7.

**Figure 2.26 DDX24 inhibits IRF7 phosphorylation**

Immunoblot using 293T cells transfected with c-Myc-tagged DDX24, FLAG-tagged IRF7, TBK-1 and IKKi as indicated for 24 hours before the lysates were analyzed by IB. GAPDH is used as a loading control.
Since IRF3 and IRF7 are two major IRFs responsible for IFNβ activation, we then evaluated DDX24’s effects on IRF3 activation, specifically IRF3 phosphorylation and dimerization. Briefly, 293T cells were stimulated by RIG-I overexpression and examined the endogenous phosphorylated IRF3 in the presence or absence of DDX24. Although DDX24 could significantly block RIG-I triggered IFNβ promoter activity in 293T cells as we previously reported, RIG-I stimulated IRF3 phosphorylation was not affected by overexpression of DDX24 (Figure 2.27a). Consistent with the phosphorylation experiments, our IRF3 dimerization assay showed that overexpressing DDX24 did not inhibit RIG-I stimulated IRF3 dimerization in 293T cells. Moreover, A20, a positive control in this assay, significantly attenuated IRF3 phosphorylation as previous reported, suggesting that DDX24 indeed played little role in IRF3 dimerization (94) (Figure 2.27b). We further tested phosphorylation and dimerization of IRF3 using poly I:C and VSVdM as stimulus. As shown in figure 2.27c, overexpression of DDX24 did not affect poly I:C/VSVdM triggered either phosphorylation or dimerization status of IRF3, which is consistent with previous experiments with overexpressed RIG-I. Collectively, our data suggested that DDX24 inhibited RLR dependent antiviral signaling, likely through IRF7 activation rather than IRF3 activation.
Figure 2.27 DDX24 does not inhibit IRF3 phosphorylation or dimerization

(a) 293T cells were transfected with GFP-RIG-I and increasing doses of c-Myc-DDX24. Cell lysates were then prepared, and the phosphorylation of IRF3 was analyzed by native PAGE. (b) 293T cells were transfected with plasmids as indicated. Twenty-four hours post transfection, IRF3 dimerization was checked by native gel and plasmid expression was checked by SDS-PAGE. (c) 293T cells were transfected with plasmids as indicated. Twenty-four hours post transfection, cells were transfected with 2μg/ml poly I:C or infected by VSVdM at MOI=5. Four hours later, IRF3 dimerization was checked by native gel, IRF3 phosphorylation and plasmid expression was checked by SDS-PAGE.

2.3.7 DDX24 served as feedback regulator

Previous data indicated that DDX24 negatively regulated type I interferon production with minor effect on IRF3 activation, which is responsible for early innate immune responses. Instead, DDX24 played an inhibitory role in the RLR signaling mediated by IRF7, which is critical for late innate immune responses, suggesting DDX24 as a feedback regulator. Correlated with this, promoter analysis predicted two STAT1 binding sites at 130bp upstream of DDX24 transcription initiation site, suggesting that DDX24 may be a potential interferon inducible protein, which further indicated a feedback role of DDX24 in innate immune response (Figure 2.28a).
Figure 2.28 DDX24 is interferon inducible

(a) Schematic of human DDX24 promoter region, indicating STAT1 binding site.
(b) MEFs cells were left untreated or treated with mIFNβ at 100 U/ml. Mouse ddx24 and rig-i mRNA were analyzed by RT-PCR.
(c) MEF cells were left untreated or treated with poly I:C for the indicated time. Mouse ddx24 mRNA were analyzed by RT-PCR.
(d) MEF cells were left untreated or treated with poly I:C, mIFNβ for the indicated time. Mouse DDX24 and β-actin were detected by immunoblot analysis.
(e) HUVEC cells were left untreated or treated with hIFNβ at increasing does. Human DDX24 and β-actin were detected by immunoblot analysis.

Data are presented as means±s.e. from three independent experiments. * indicates P<0.05. ** indicates P<0.01.
To confirm that DDX24 is indeed interferon inducible, we performed an RT-PCR assay to monitor endogenous DDX24 RNA level in primary MEFs, with or without IFNβ treatment for 6 hours. Indeed, the endogenous DDX24 was significantly induced by about 5 fold, comparable to RIG-I which is known as an interferon inducible gene (26) (Figure 2.28b). Additionally, Poly I:C, a strong interferon stimulator, could also induce DDX24 gene by 4 fold after 8 hours treatment (Figure 2.28c). We further investigated the protein level of DDX24 upon IFNβ treatment and poly I:C transfection. In MEFs, mDDX24 protein level was elevated following poly I:C or IFNβ overnight treatment (Figure 2.28d). Collectively, these data confirm that DDX24 is IFN inducible, which further supports that DDX24 plays a negative feedback role in RLR signaling.

### 2.3.8 Alternate explanation of DDX24 mechanism, multifunctional protein?

DExD/H family members are characterized as RNA helicases that are able to bind RNA, or even DNA. This is closely related to the function of many DExD/H family members in the RLR pathway (161). Therefore, it is also plausible to explore the nucleic acid binding activity of DDX24 that might be related to its function as in regulating RLR signaling. To evaluate nucleic acid binding activity of DDX24, biotin labeled poly I:C (dsRNA), poly G (ssRNA), poly dA:dT (dsDNA), ISD90 (dsDNA) and ISD90 (ssDNA) were prepared for pull down assay. As shown in Figure 2.29a, c-Myc-tagged DDX24 was specifically pulled down with poly I:C and poly G, but not by others, suggesting a binding specificity of DDX24 to RNA species, but not DNA species (Figure 2.29a). Moreover, competition
experiments using increasing amounts of unlabeled poly I:C further confirmed the binding activity of DDX24 to poly I:C (Figure 2.29b). In addition, we also tested if DDX24 could bind to RNA from VSV. As shown in figure 2.29c, overexpressed c-Myc tagged DDX24 could also bind to the VSV-G RNA transcripts VSV-G in 293T cells.

Figure 2.29 DDX24 binds to dsRNA and ssRNA.

(a) 293T cells were transfected with c-Myc-tagged DDX24 for 24 hours before lysed. Pull-down assays were performed by incubating 293T lysate with various biotin-conjugated polynucleotides, and then precipitated with streptavidin beads. Bound proteins were analyzed by immunoblotting with anti-c-myc antibody.

(b) The mixture of cmyc-DDX24 and 1µg/ml biotin-poly I:C were incubated without poly I:C or with poly I:C at 1 and 2 µg/ml concentration. Bound proteins were analyzed by immunoblotting with anti-c-Myc.

(c) ssRNA transcribed from VSV-G cDNA were conjugated with biotin and utilized in pull-down assays similar to (a).

(d) 293T cells were transfected with cmyc-tagged DDX24 or FLAG-RIG-I as indicated for 24 hours before being lysed. Pull-down assay were performed using biotin-VSV-G, and precipitated proteins were subjected to immunoblotting.
Because DDX24 was shown to negatively regulate RLR pathway, it is plausible to hypothesize that DDX24 might compete with RIG-I for binding to RNA from pathogens, thus attenuating sufficient recognition of viral RNA by RIG-I. To test this hypothesis, we performed a competition binding assay to test if DDX24 could affect binding of VSV-G-ssRNA by RIG-I. As shown in figure 2.29d, RIG-I that was successfully precipitated with VSV-G RNA. Moreover, decreased RIG-I was detected in the presence of DDX24, indicating a binding competition between RIG-I and DDX24 (Figure 2.29f). The competition assay results indicated a possible explanation of DDX24’s negative role in RLR signaling, but further studies are required for better understanding this mechanism by DDX24.

2.3.9 Discussion

In this section, we focused on answering how DDX24 could affect RLR signaling. Because DDX24 is associated with FADD, we first investigated if DDX24 could affect FADD’s function in RLR signaling. 293T experiments indicated that FADD-RIG-I synergistic effect was blocked by overexpression of DDX24 in a dose dependent pattern, suggesting DDX24 affected FADD’s function in RLR pathway. In addition, experiments of co-overexpressing DDX24 with essential components in RLR signaling further supported that DDX24 works upstream of IRF3/IRF7, possibly at the signaling complex level where FADD is located.
In order to clarify the mechanism of DDX24 dependent RLR regulation, other potential targets of DDX24 in the signaling complex besides FADD were explored through co-IP assays. Among all the candidates tested (RIG-I, MDA5, IPS-1, RIP1, TBK-1, IKKi, IRF3 and IRF7), only RIP1 was co-precipitated with DDX24 when overexpressed in 293T cells, suggesting RIP1 as a potential target of DDX24 in RLR signaling (Data not shown). Furthermore, it has been reported that RIP1, FADD and TRADD formed a complex termed TRADDosome, which is recruited to IPS-1 during virus infection and is critical for antiviral responses (39). Therefore, it is plausible to hypothesize that DDX24 is incorporated in an IPS-1 based signaling complex, and possibly regulates RLR signaling by disrupting the protein interactions within this complex. Therefore, our original hypothesis was that DDX24 may disrupt the interactions between RIP1 and FADD, thus inhibiting IFN signaling by abrogating proper formation of the TRADDosome. However, in our experiments, both DDX24 and FADD can be co-precipitated with RIP1 without affecting each other, suggesting DDX24 might block the recruitment of other proteins rather than FADD and RIP1.

An alternate hypothesis is proposed based on the facts that RIP1 could physiologically associate with IRF7 in 293T and Raji cells and synergize IRF7 dependent activation of type I interferon (71). In our co-IP experiments, both IRF7 and DDX24 were found to be associated with RIP1. Moreover, competition Co-IP experiments confirmed that DDX24 was able to disrupt interactions between RIP1 and IRF7, and attenuate K63-linked
ubiquitination of IRF7. IFNα4 promoter assay and IRF7/3 phosphorylation experiments indicated that DDX24 could specifically block IRF7, but not IRF3-dependent type I interferon activation, which further supports our hypothesis that DDX24 negatively regulates type I interferon signaling through disruption of RIP1-IRF7 interaction.

In addition to RIP1, we also explored FADD involvement in the regulation of IRF7. Although no direct interactions between FADD and IRF7 were observed (data not shown), FADD was able to greatly facilitate IRF7 dependent IFNα promoter activation as well in our study. Moreover, this synergistic effect by FADD-IRF7 was significantly enhanced in the absent of DDX24, indicating this positive effect of FADD on IRF7 is also negatively regulated by DDX24. Given the fact that DDX24 associates with both FADD and RIP1 without disrupting FADD-RIP1 interaction, it is plausible to propose that both FADD and RIP1 are possibly involved in regulating IRF7. In support of this idea, previous studies also suggested that FADD and RIP1 are functional partners and might act in the same level in the RLR pathway. First, FADD and RIP1 interact with each other through the death domain and are both recruited to the IPS-1-based signaling complex directly, or indirectly by TRADD. Second, FADD-/- and RIP1-/- MEFs both show greater susceptibility to VSV infection and impaired interferon production when challenged with VSV or poly I:C (38). Third, both FADD and RIP1 could facilitate the signaling triggered by RIG-I, and have been proposed to maintain appropriate conformation of the IPS-1-based signaling complex (126). Therefore, a cooperative role between FADD and
RIP1 is suggested in our model and the FADD-RIP1 complex may act as a scaffold that facilitates the recruitment of IRF7 to the IPS1 based signaling complex. Lose of either FADD or RIP1 could lead to a change in the structure or stability of the complex, which would potentially lead to insufficient recruitment of DDX24, anchored into this complex through FADD and RIP1, disrupts the binding of IRF7 to this complex, and plays an important role in controlling the amount of IRF7 recruited to the signaling complex. Recently, it has also been published that TRAF6 is the E3 ligase which is responsible for K63 specific polyubiquitination of IRF7. It is possible that TRAF6 might be recruited to the RIP1-FADD-DDX24 complex, which might play a role in DDX24’s regulating RLR signaling.

Since DDX24 is a protein that is poorly studied, it is plausible to obtain a comprehensive understanding of this protein by exploring alternative functions and mechanisms. Indeed, DDX24 is an RNA helicase and its function could be linked to its RNA binding activity. DDX24 belongs to the DExH/D family, which contains around 59 proteins conserved from bacteria/viruses to humans. DExH/D helicases are broadly involved in many RNA related processes such as transcription, translation, ribosome biogenesis, RNA transportation and RNA modifications (162-164). The majority of DExH/D members have characterized functions related to RNA processing, including some of the members which play important roles in the antiviral pathway. Besides the best known RNA sensor helicases RIG-I, MDA5 and LGP2 in the RLR pathway, many other RNA helicases also
regulate innate immune signaling based on their helicase activity. DHX9 is associated with IPS1 and acts as a dsRNA sensor in myeloid dendritic cells (mDCs) (165). DHX9 and DHX36 are able to sense cytosolic CpG-DNA via MyD88 in pDCs (166). The DDX1/DDX21/DHX36 complex was able to sense both short and long poly I:C via TRIF in mDCs (167). DDX41 has been found to be associated with STING and senses intracellular DNA in dendritic cells (49). DDX60 can bind to RIG-I, but not MDA5 or downstream IPS-1, and is involved in RLR-dependent pathways (169). DDX3x binds to IPS-1 and enhances IPS-1 dependent ifnβ inducing potential (138).

Although the majority of these helicases are identified as RNA or DNA sensors based on their ability to bind to RNA or DNA, some other members exert distinct functions independent of RNA binding activities. For example, DDX3x works with Ikki to positively regulate IFNβ production, which is independent of its ATPase/helicase activity (136). Another group indicated that DDX3 works further downstream as a stimulator of IFNβ promoter (137). Although LGP2 has been previously reported as a negative regulator of the RLR pathway in vitro by sequestering dsRNA away from RIG-I, it has also been proposed that LGP2 could bind RIG-I, or block IPS-1 binding to TBK-1, which is unrelated to its helicase activity (83-87). These studies indicate that DExD/H family members might have multiple different roles and distinct functions involving the same or different pathways. Indeed, our IFNβ reporter assay indicated that CARD domain only RIG-I or MDA5 dependent IFNβ activation could also be blocked by DDX24, suggesting
an RNA-binding-independent inhibitory role of DDX24 (Figure 2.18). However, the binding of VSV-G single strand RNA provides some evidence that DDX24 was able to compete with RIG-I for the binding of VSV-G RNA. Further experiments are needed in order to better address these questions. So far, it is unclear whether DDX24 could disrupt the recognition process of RLR signaling, which provides an additional role of DDX24’s negative regulation.

Collectively, in this section, our current findings reveal that DDX24 works in the FADD signaling complex, which led to the identification of RIP1 as a potential target of DDX24 in RLR signaling. Hereby, we propose the following model on how DDX24 negatively regulates RLR signaling. After virus infection or dsRNA stimulation, FADD, RIP1 and many other regulators are recruited to IPS-1 to form a signaling complex. To ensure efficient and sufficient signaling transduction, appropriate conformation and compact structures of the complex is important. FADD and RIP1 are adaptors rather than activators in this pathway, which are responsible for appropriate formation of this complex. As one of the important activators, IRF7 has been demonstrated to be recruited by RIP1 prior to activating IFN production. DDX24 competes with IRF7 for RIP1 binding, resulting in attenuated RIP1 dependent K63 specific polyubiquitination of IRF7 and subsequently decreased IRF7 dependent type I interferon activation. FADD is positively involved in IRF7 dependent type I interferon activation by forming a FADD-RIP1-DDX24 complex, whereas DDX24 disrupts the cooperative role of
FADD-RIP1 in IRF7 regulation. Because IRF7 is the major transcription factor responsible for the second round of type I interferon production, DDX24 is considered to be a negative regulator that functions in the late phase of innate immunity. Correlated with this, DDX24 is identified as interferon inducible protein, which supports the negative feedback model.

2.4 Generation of DDX24 deficient mice

2.4.1 Strategy of generating DDX24 knockout mice

Previous experiments suggested that DDX24 acts as a negative regulator of the RLR pathway in vitro. RNAi based experiments indicate that knocking down DDX24 led to significantly increased type I interferon production upon stimulation by either poly I:C transfection or VSV infection. The in vitro evidence prompted us to study the in vivo role of DDX24, specifically how the loss of DDX24 will affect the RLR-based innate immunity upon challenge by foreign pathogens, such as virus or bacteria.

To study the function of DDX24 in vivo, DDX24 knockout mice were generated by a gene trapping strategy. Mouse embryonic stem (ES) cells with the DDX24 genomic locus disrupted by insertion of a β-galactosidase/neomycin cassette between exons 6 and 7 were purchased from BayGenomics (cell line RRK059) (Figure 2.30a). First, DDX24 mutant ES cells were microinjected into C57BL/6 blastocysts to create chimeric mice.
Male chimeras were then bred with female C57BL/6 mice and pups were screened for germline transmission of the mutant allele. We observed successful transmission to the next generation in all 5 male chimeras, and then we screened for the heterozygous mice using the genomic DNA based genotyping method as indicated in figure 2.29a. The DDX24<sup>+/−</sup> mice were then intercrossed with each other to obtain DDX24<sup>−/−</sup> mice.

**Figure 2.30 Generation of DDX24 deficient mice**

(a) Genomic organization of the mouse DDX24 locus. PCR primers for genotyping are indicated by arrows. Primers F1&R are for WT detections. Primers F2 and R are for Genetrap detections.

(b) Genomic DNA-based PCR genotyping strategy for mice using primers described in materials and methods.

(c) Genotyping of mouse embryos at E8.5.

(d) Genotyping of mouse embryos at E3.5.
2.4.2 Embryonic lethality of DDX24 knockout mice

Although DDX24\(^{+/−}\) mice appeared normal and were fertile, no viable homozygous mutant mice were identified in the first 300 pups derived from DDX24\(^{+/−}\) intercrosses, suggesting that DDX24 deficiency results in embryonic lethality (Figure 2.30b)(Table 2.1). To investigate the stage and cause of embryonic lethality as well as evaluate the endogenous role of DDX24, MEFs were derived from early mutant embryos from E13.5. However, no DDX24\(^{−/−}\) embryos were identified within the first 200 embryos, suggesting an embryonic lethal stage that is earlier than 13.5 days. Therefore, MEFs were subsequently isolated from E12.5 to as early as E9.5. However, no DDX24\(^{−/−}\) embryos were identified even at E9.5 as determined using genome DNA PCR based genotyping method (Figure 2.30c)(Table 2.2). Furthermore, MEF isolation at E8.5 was attempted, but unfortunately no MEFs were successful recovered or cultured in vitro. Collectively, these results indicate developmental failure of DDX24\(^{−/−}\) embryos earlier than E8.5 and it is difficult to recover DDX24\(^{−/−}\) MEFs under our current strategy.

Table 2.2 Genotypes of embryos/mice derived from DDX24\(^{+/−}\) intercrosses

<table>
<thead>
<tr>
<th>stage</th>
<th>+/+</th>
<th>+/-</th>
<th>−/−</th>
<th>Resorptions</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>E9.5</td>
<td>14</td>
<td>27</td>
<td>0</td>
<td>12</td>
<td>53</td>
</tr>
<tr>
<td>E10.5</td>
<td>14</td>
<td>25</td>
<td>0</td>
<td>7</td>
<td>46</td>
</tr>
<tr>
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<td>11</td>
<td>23</td>
<td>0</td>
<td>2</td>
<td>36</td>
</tr>
<tr>
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<td>8</td>
<td>16</td>
<td>0</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Postnatal</td>
<td>112</td>
<td>189</td>
<td>0</td>
<td></td>
<td>301</td>
</tr>
</tbody>
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(4 weeks)
Due to the unavailability of DDX24/− MEFs, we aimed to further investigate the embryonic stage and cause of lethality. It is known that embryos will implant in the uterus after E3.5 and it is very difficult to successfully isolate them from uterus. Therefore, we isolated embryos at E3.5 to characterize DDX24-/− embryos by genotyping. Indeed, DDX24+/− embryos were clearly detected at E3.5, suggesting an embryo lethal stage between E3.5 and E8.5 (Figure 2.30d). We then utilized immuno-histological staining to analyze the embryos from E3.5 to E8.5. An anti-DDX24 antibody recognizing only the C term of DDX24 was used to ensure that the antibody only stains the wild type cells, but not the DDX24 deficient cells. Although this antibody shows specificity in recognizing human DDX24, it does not recognize mouse DDX24 as well as human DDX24 (Data not shown). Therefore, this antibody is not applicable for charactering DDX24 deficient embryos. Hence, we stained all the embryos from intercrosses of DDX24+/− mice using Dab2 antibody to identify any severe damage that might be caused by DDX24 deficiency. Indeed, we were able to detect multiple embryos with severe embryogenesis problem at a Mendel ratio at E7.5 (Figure 2.31). Interestingly, no developmental defects were observed in all isolated E6.5 embryos, narrowing down the embryonic lethal stage between E6.5 to E8.5. Correlated with this, an increased embryo resorption rate was observed from E9.5-10.5, which indicated a DDX24+/− embryonic lethal stage that is 2-3 days earlier (Table 1). Collectively, these data suggest that DDX24/− embryos die at ~E7.5 due to the early embryonic developmental failure, but the mechanism of how DDX24 deficiency affected early embryonic development was still
open to questions. Unfortunately, it is impossible to explore the in vivo role of DDX24 due to the unavailability of DDX24 deficient mice and MEFs. Therefore, to clarify the in vivo role of DDX24 as well as confirm the in vitro data generated by siRNA based experiments, a conditional knockout strategy will need to be considered in the future.

Figure 2.31 abnormal embryonic development of DDX24 deficient embryos
(Courtesy of Dr. Robert Moore)

Embryos were stained with Dab2 antibody with 1:1000 dilution. DDX24 deficient embryos exhibit abnormal development at E7.5. Gastrulation & mesoderm induction have occurred normally in the +/- embryo. However, the +/- embryos are unable to withstand a major pressure imbalance. They burst & rupture the uterine tissue
2.4.3 Discussion

To explore the in vivo role of DDX24 in RLR signaling and further confirm the in vitro data obtained by siRNA based knockdown experiments, DDX24-/ mice were generated using gene trapping strategy. Unfortunately, no DDX24-/ mice were identified and DDX24/- MEF cells were not able to be isolated due to early embryonic lethality of DDX24 deficient embryos. Notably, we have observed several resorbed embryos around E7.5 at Mendel ratios, which indicated developmental failure. Although we were unable to confirm the deficiency of DDX24 in these destroyed embryos due to lack of a valid IHC antibody (data not shown), the severe embryonic phenotypes indeed suggests that DDX24 is crucial to early embryogenesis. The detailed mechanism of embryonic lethality caused by DDX24 deficiency is still open to questions.

Notably, FADD knockout mice are also embryonic lethal, which was independently reported by two groups. The Winoto group reported that FADD-/- embryos died at around day 9 of gestation, suggesting an essential role of FADD in embryonic development. In their study, although FADD has been shown to be essential for Fas dependent apoptosis, Fas-deficient mice are viable, indicating the lethality of FADD deficient mice is due to defects of other signaling pathways, rather than Fas-dependent apoptosis. Additionally, FADD appears to play a role in T-cell proliferation and lymphocyte development, which might be an alternate explanation of embryonic lethality (169). Another group reported a later stage of embryonic lethality, ranging from 10.5E to 12.5E. FADD deficient embryos
were underdeveloped and displayed signs of abdominal hemorrhage. Subsequent im-
munohistological analysis revealed that FADD is important for normal cardiac
development (170). While DDX24 or FADD deficiency leads to embryonic lethality, loss
of RIP1, another target of DDX24 in RLR pathway, does not cause embryonic lethality.
However, RIP1 knockout mice die at 1-3 days after birth due to extensive apoptosis in
both lymphoid and adipose tissue. RIP1 deficient MEFs are highly susceptible to TNFα
induced apoptosis, accompanied by a failure of NF-κB activation (171). Interestingly, it
has been reported recently that FADD−/− embryos contain increased levels of RIP1 and
exhibit massive necrosis. Furthermore, RIP1 deficiency rescued the embryonic lethality
of FADD−/− mice, suggesting a critical compensation of these two genes in the regulation
of apoptosis and necrosis during embryogenesis (172). Although DDX24’s function in
pathways other than RLR is not investigated, it might be worthwhile to study the
involvement of DDX24 in apoptosis and necrosis pathway in the future, which could
potentially explain the reason of embryonic lethality caused by DDX24 deficiency.
Collectively, although DDX24, FADD and RIP1 are closely associated in the RLR
pathway, the defects caused by loss of genes are quite different. So far, no direct evidence
indicates the embryonic lethality due to loss of one of these three genes are related to
abnormal RLR signaling. It is possible that DDX24 is involved in other signaling
pathways which are critical for early embryonic stage development.
Furthermore, the analysis of knocking out DExD/H family members in RLR signaling also indicated differences among these family members. RIG-I knockout mice were successfully generated by the Akira group by homologous recombination of ES cells. However, the ratio of RIG-I deficient mice that were born was very low, as most of the RIG-I knockout embryos died at E12.5 to E14 due to massive liver degeneration related to aberrant apoptosis. Furthermore, RIG-I knockout mice have a very short life span of 3 weeks after birth (133). Notably, deficient mice of other RLR stimulators such as RelA, IKK-β and TBK1 are also reported to be embryonic lethal because of liver apoptosis (173-175). In contrast to RIG-I deficient mice, MDA5 deficient mice were born and grew normally and were healthy until 24 weeks of age, suggesting a different role in development (176). LGP2 deficient mice were first generated by our group through homologous recombination. Similar to MDA5, knockout mice of LGP2 grew similarly to wild type mice without any sign of developmental abnormalities (87). The Akira group also generated LGP2 knockout mice. However, they reported a much lower ratio of LGP2 deficient mice that were born, suggesting embryonic lethality caused by lack of LGP2 (31). Nevertheless, they found that adult female LGP2 deficient mice accumulated more fluid in their uterus, possibly due to vaginal atresia. These contradictory results between these two groups might be attributed to the different backgrounds of the mice, as our group used a mixed 129SvEv/C57BL6J (50%/50%) genetic background, while Akira group used C57BL6 background. Little is known for the in vivo role of DDX3X, as knockout DDX3X mice have not yet been reported. Collectively, RIG-I, MDA5 and
LGP2 deficient mice were all born alive, but loss of DDX24 resulted in embryonic developmental failure as early as E7.5. Compared with other DExD/H family members in the RLR pathway, DDX24 seems to play a more important role in early embryonic development.

Collectively, we have confirmed the embryonic lethality at the stage of ~E7.5 due to DDX24 deficiency. However, to further address if the role of DDX24 as negative regulator of the RLR signaling pathway could potentially contribute to the embryonic lethality of DDX24-/- mice, more studies and direct evidence are needed in the future. Unfortunately, due to the unavailability of DDX24-deficient embryos and MEFs, we were not able to evaluate the impact of RLR signaling and analyze cytokine production after infection with pathogens upon loss of DDX24. In order to verify the in vitro data obtained from siRNA experiments as well as explore the in vivo role of DDX24 in antiviral signaling, conditional knockout strategy will be necessary in future studies.

2.5 Concluding remarks

The discovery of cytosolic RNA sensing pathways opens a new field in innate immunity and attracts much attention and studies. Identification of novel regulators and characterization of mechanisms in the antiviral pathway greatly enriched our acknowledgement of RLR signaling. FADD and RIP1 were identified among the earliest key components of the RLR pathway, and were reported as adaptor proteins which
facilitate sufficient RLR signaling. However, so far, few host regulators targeting FADD or RIP1 were reported, resulting in a lack of detailed mechanism on how FADD and RIP1 are controlled and regulated in RLR signaling. Identification of DDX24 as a FADD associated protein has offered a unique opportunity to reveal the molecular mechanism of DDX24 in innate immunity.

We first confirmed the interactions between DDX24 and FADD using yeast two hybrid and co-immunoprecipitation experiments by overexpression and with endogenous protein. Immunofluorescence studies also indicated the co-localization of endogenous DDX24 and FADD in both the nucleus and cytosol, suggesting physiological association of these two molecules, indicating a possible involvement of DDX24 in FADD’s function.

In the functional evaluation of DDX24, we examined the effect of overexpressing or knocking down DDX24 on RLR signaling at three different levels, IFNβ promoter activation, endogenous ifnb RNA induction and endogenous IFNβ protein. In these studies, both 293T and MEFs were tested to eliminate cell specific effects. Our results clearly showed that ectopic overexpression of DDX24 inhibits poly I:C/VSVdM induced type I IFNs signaling, while knockdown of endogenous DDX24 by siRNA results in the opposite effect. Virus replication experiments revealed that DDX24 suppression results in attenuated VSV replication in MEFs and HUVECs. Collectively, our studies for the first time reveal the function of DDX24, a negative regulator in RLR signaling.
We further explored the mechanisms of DDX24 in regulation of RLR signaling. As a FADD associated protein, DDX24 indeed regulated FADD-dependent activation of RLR signaling by disrupting the FADD-RIG-I synergistic activation of IFNβ promoter. Nevertheless, RIP1 was also identified as a potential target of DDX24 in the RLR pathway in addition to FADD. A competition assay revealed that DDX24 competed with IRF7 for RIP1 intermediate domain binding, which disrupted the connection between IRF7 and RIP1. This led to the attenuated K63 linked polyubiquitination and phosphorylation of IRF7, which subsequently resulted in decreased IRF7 dependent type I IFN production. Notably, there was little impact of overexpressing DDX24 on IRF3 dimerization or phosphorylation, suggesting a specific inhibition of IRF7 dependent late RLR signaling by DDX24. This is corroborated by the fact that DDX24 is induced by type I interferon, which suggests a negative feedback role of DDX24. On the basis of our own data, as well as the published work of our group and the Pagano group, the relationship between FADD, RIP1, IRF7 and DDX24 has become clearer. This not only confirmed the importance of FADD and RIP1 in IRF7 dependent signaling, but also demonstrated how the positive effects of FADD and RIP1 are opposed by DDX24.

In order to learn the in vivo role of DDX24, DDX24 knockout mice were attempted to be generated by gene trap technology. Although DDX24<sup>+/−</sup> mice appeared normal and were fertile, DDX24<sup>−/−</sup> mice died as early as E7.5 due to severe developmental failure. DDX24<sup>−/−</sup> embryos were identified at E3.5 but no later than E7.5, thus preventing the
obtaining of DDX24+/− MEFs. This was the first time that DDX24-deficient mice were attempted to be generated and we were the first to report the embryonic lethality stage of DDX24 deficient mice. Although the detailed mechanism causing the embryonic lethality is still open to questions, we have clearly shown an important role of DDX24 in early embryonic development, which might provide useful information for the future study of DDX24 by other researchers. A conditional knockout strategy will be considered in the future study to further unravel the in vivo role of DDX24.
Chapter 3 Materials and Method

Yeast two-hybrid assay

To verify FADD binding protein, a yeast two-hybrid assay was performed using the Matchmaker Gal4 two-hybrid system (Clontech) according to the protocols provided by the manufacturer. Briefly, the PCR product of the full length hFADD was cloned into the yeast bait vector, pGBT9. The pGBT9-FADD plasmid or pGBT9 vector were then transformed into yeast strain AH109 using the lithium acetate method. pGBT9-FADD transformed yeasts were selected on the SD-Trp plates. Single colony was picked up, grow and transfected with another plamid pGAD-hDDX24 or pGADT7 vector. Yeasts with both plasmids transfected were selected on SD-Leu/-Trp plates. Single colonies on the plates were picked up and seeded on SD-His/-Leu/-Trp (low stringency) and SD-Ade/-His/-Leu/-Trp (high stringency) plates for verification of interactions between molecules.

Generation of DDX24 deficient mice

Gene trap mutated ddx24 embryonic stem cells (RRK059) were purchased from BayGenomics. Chimera mice were produced by microinjection of heterozygous ES cells into E3.5 C57BL/6 blastocysts that were subsequently transferred to pseudo-pregnant foster mothers. Chimera male mice were bred with control female C57BL/6 mice to transmit the mutated ddx24 allele to germline. Heterozygous mice were interbred to
obtain ddx24⁻/⁻ mice. Genotyping was performed by genomic DNA based PCR. The primer 5’-GCTAATCCTGCCTGTATGACCTT-3’ was used in combination with either 5’-ATTCAGAGCAGGTTAACCCAGGAC-3’ for the wild type ddx24 allele, or the primer 5’-GACTGGTGAGTACTCAACCAAGTC-3’ for the mutant allele. Animals were generated at the University Of Miami School Of Medicine Transgenic Core Facility (Miami, FL). Mice were allowed to access food and water freely and were housed at an ambient temperature of 23°C and at a 12 hour light/dark cycle. Animal care and handling was performed as per Institutional Animal Care and Use Committee guidelines.

**Isolation of murine embryonic fibroblasts (MEFs)**

Timed matings were performed between mature DDX24 heterozygote mice and MEFs were obtained using a standard procedure (177). Briefly, embryos from E8.5 to E12.5 days were dissected free of surrounding tissues, washed in PBS and the heads and livers were removed. Each individual embryo was completely trypsinized for 15 minutes and cultured separately. MEFs were genotyped using same gDNA based method as described above. Primary DDX24⁺/+ and DDX24⁺/- MEFs before passage 6 were used for all experiments.

**Histology and Immunohistochemistry**

Uteri from timed matings were formalin fixed and embedded in paraffin. Sections were cut at 6 μm. After dewaxing in xylene and rehydration in a series of graded ethanols,
intermittent sections were stained with hematoxylin and eosin (H&E) in order to identify mutant embryos. Immuno-staining was performed after a heat and citrate based antigen retrieval of sections. The primary antibody used to detect Disabled-2 (Dab2) was a mouse monoclonal antibody from BD Transduction Laboratories. The secondary antibody applied was a peroxidase conjugate (Vector Labs, CA) and sections were counterstained with hematoxylin. (Courtesy of Dr. Robert Moore)

**Isolation of ES cells**

After timed matings of DDX24 heterozygotes, preimplantation embryos were flushed at E3.5. The embryos were cultured upon an irradiated fibroblast feeder layer in ES cell media (DMEM with 15% FBS, 1000 units/ml ESGRO, 1x non-essential amino acids, 2 mM L-glutamine, 50 IU/ml penicillin and 50 mg/ml streptomycin) until they had attached. They were then trypsinized, routinely fed and assayed for the appearance of ES cell clones. (Courtesy of Dr. Robert Moore)

**Cell Culture, Reagents, and Antibodies**

293T cells (ATCC), WT MEFs, DDX24+/+ and DDX24+-/ MEFs were grown in DMEM supplemented with fetal bovine serum (10%) and penicillin/streptomycin (1%). HUVEC cells were purchased from ATCC and cultured in EGM-2 media supplied with growth factors obtained from EGM Bullet kit. All cells were maintained at 37°C in a 5% CO₂ laboratory incubator subject to routine cleaning and decontamination. Poly I:C
(Amersham) was reconstituted in PBS at 2 mg/ml, denatured at 55°C for 30 min, and allowed to anneal at room temperature before use. Antibodies were obtained from following sources: rabbit anti-DDX24 (A300-697A Bethyl); mouse anti-FLAG M2 antibody, rabbit anti-c-myc, mouse anti-HA (Sigma); rabbit anti-IRF3, rabbit anti-GFP, mouse anti-GAPDH (Santa Cruz Biotechnology, Inc.); rabbit anti-phospho-IRF3 (Upstate); rabbit anti-phospho-IRF7(Cell signaling); rabbit anti-Fibrillarin (ab5821), mouse anti β-actin (Abcam); mouse anti-ubiquitin (Stressgen); mouse anti-RIP1 (BD Science); mouse anti-FADD(cell signaling). Control scrambled (D-001206-01-80), mRIG-I (L-065328-01), mDDX24 (L-042299-01) and hDDX24 (L-010397-01) smart pool siRNAs were purchased from Dharmacon/Thermo Scientific.

**Transfections and virus infections**

Plasmid or poly I:C transfection in 293T cells or MEFs were conducted using Lipofectamine 2000 (Invitrogen) transfection reagents in Opti-MEM (Invitrogen) according to the manufacturer’s manual. HUVEC and MEFs siRNA transfections were performed using AMAXA HUVEC and MEF Nucleofectin Kit 1 according to the manufacturer’s recommendations (AMAXA Biosystems). Indiana strain of VSV was used in all experiments. Constructed VSVs (VSV-GFP, VSV-luc and VSVdM) were constructed in our lab. Briefly, keep the medium serum free during first two hours post infection and change the medium back to full medium until harvest.
**Plasmid constructs**

Expression vectors (pcDNA3.1, Invitrogen) FLAG or GFP tagged RIG-I, MDA-5, dRIG-I, dMDA5, IPS1, TBK1, RIP1, FADD, TRAF6 were generated in our lab by polymerase chain reaction. N-terminal cmyc-tagged or FLAG-tagged plasmids DDX24, DDX24-N (AA 1-577), DDX24-C (AA 578-859) were generated using pCMV-tag system from stratagene. Same fragment of DDX24 were also ligated into the EcoRI and SalI site of pGADT7 vector to generate pGADT7-DDX24 for yeast two hybrid experiments. Other plasmids used in this study, cmyc-RIP1, cmyc-IRF7, FLAG-IRF7, and IFNα4-luc (Dr Ning); FLAG-IRF3, and IFN-β Luc (J Hiscott); PRD-III-I Luc (T. Maniatis); NF-κB-Luc (Stratagene); IRF3 (SA) (Invivogen),

**Real-Time PCR**

Total RNA was isolated by using RNeasy RNA extraction kit (Qiagen) and cDNA synthesis was performed with random hexamer primers using 5μg of total RNA (Invitorgen) Real-time PCR was performed using a LightCycler 2.0 instrument and the TaqMan Gene Expression Assays (Applied Biosystems): mIFNβ (Mm00439546), mIFNα2 (Mm00833961), mIRF7 (Mm00516788), mRIG-I (Mm00554529), mDDX24 (Mm00517454), hIFNβ (Hs00185375), hIRF7 (Hs00185375), Luciferase (4331348 customized). Relative amount of mRNA was normalized to the 18S ribosomal RNA level in each sample. Alternatively, SYBR green systems from New England Biolabs (DyNAmo SYBR Green qPCR Kit) were used for human ddx24 detection. The human RNA samples
used for ddx24 profiling were purchased from Ambion (AM6000 FirstChoice Human Total RNA Survey Panel). Primers used for human ddx24 were: Forward 5’-GCCGAATTACAGGAATTTAAAACCTG-3’; Reverse 5’-GTCATCCACTACCAGGGCACCTGAGC-3’. Primers used for human gapdh were Forward 5’-atgacatcaagaaggtggtg-3’; Reverse 5’-cataccaggaaatgacctg-3’. Relative amount of mRNA was normalized to the gapdh RNA level in each sample.

**Reporter assays and Immunofluorescence**

Reporter assays were performed as previously described (38). Briefly, 293T cells or MEF cells were seeded on 24-well plates and were transiently transfected with 50 ng/100 ng of the luciferase reporter plasmids together with a total of 600 ng of various expression plasmids or empty control plasmids. As an internal control, 10 ng/20 ng pRL-TK plasmids expressing Renilla protein was transfected simultaneously. Twenty four or 36 hours later, cells were lysed by adding 100 μl/well of Cell culture lysis buffer (CCLR), and luciferase activity in the total cell lysate was measured by illuminometer.

Immunofluorescence experiments were performed as previously described (43). Briefly, cell monolayers were fixed in 4% paraformaldehyde for 10 min, washed with PBS and permeabilized with 0.2% Triton-X 100 in PBS for 5 min. After blocking in PBS containing 10% FBS for 20 min, samples were incubated 1 hour at 37°C or overnight at 4°C with appropriate primary antibody. After PBS washing for three times, samples were
incubated 1 hour with secondary antibodies conjugated with Cy3, Cy5 or FITC at 1: 200 dilution. Cells were washed again and incubated with 0.5 μg/ml DAPI solution for 5 min. Samples were then washed with PBS and mounted using prolong gold antifade reagent from invitrogen. Pictures were taken using an Olympus fluorescent microscope equipped with a digital camera and a Zeiss LSM-510 Confocal Laser Scanning Microscope.

**Immunoblotting, Co-immunoprecipitations, and Ubiquitination Assays**

Whole cell lysates were generated by lysing cells in RIPA buffer on ice, followed by centrifugation. Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and subjected to immunoblotting. For co-immunoprecipitation, expression vectors were transfected into 293T cells for 36 to 48 hours, cells were lysed in ice-cold NP40 IP buffer (50 mM Tris, pH8.0, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 0.1 mM Na3VO4, 1 mM DTT) or RIPA buffer with protease inhibitors (100 mM PMSF, Leupeptin, Aprotinin, Pepstatin), and cell lysates were precipitated with appropriate amount of FLAG-M2 antibody (SIGMA) or endogenous antibodies overnight at 4°C. Following day, 30 μl of Protein G was added and incubated for 3 hours. All precipitates were washed with lysis buffer 3 times and proteins were released by 2X Sample Buffer after boiling and analyzed by SDS-PAGE. For ubiquitination detection co-IP, similar co-IP process was performed using RIPA buffer. Moreover, potential ubiquitinated target-interacting proteins were removed by stringent washes using RIPA buffer containing 1 M urea.
Native PAGE Gel Dimerization Assay

293T cells recovered from 6-well dishes were lysed in 100 μl of native lysis buffer (50 mM Tris-Cl, pH 8.0, 1% NP40, 150 mM NaCl, 100 mg/ml leupeptin, 1 mM PMSF, 5 mM orthovanadate). Ten μg of protein was mixed with 2x native PAGE sample buffer (125 mM Tris-Cl, pH 6.8, 30% glycerol, bromphenol blue) and subjected to electrophoresis on non-denaturing 7.5% polyacrylamide gels as described (178).

ELISA

ELISAs for mouse IFNβ were performed using supernatants from cells where values are expressed as pg/ml±S.E. as calculated from a standard curve derived from recombinant IFNβ provided in the ELISA kit (PBL Interferon Source).

Statistical analysis

Statistical significance of differences in cytokine levels, mRNA levels, viral titers, and luciferase intensity in reporter assay and VSV-Luc-infected cells were determined using Student’s t-test.
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