FUNCTIONAL CHARACTERIZATION OF NLRP4 AND ATG13 IN TYPE I INTERFERON SIGNALING AND INNATE ANTIVIRAL IMMUNE RESPONSE

A Dissertation

by

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ABSTRACT

The innate immune system is considered as the first line of host defense during infection to recognize the pathogen-associated molecular patterns (PAMPs) by several classes of germline-encoded pattern-recognition receptors (PRRs) and induce the activation of NF-κB, type I interferon (IFN) and inflammasome signaling pathways, which subsequent trigger proinflammatory response to invading pathogens. Although type I interferon is required for viral clearance, aberrant production of type I interferon can have a role in immunopathology and autoimmune disorders. Thus, tight regulation of these key signaling pathways is essential for both innate and adaptive immunity to maintain the homeostasis. However, the molecular mechanisms for regulation of type I interferon are still poorly understood.

In this study we have demonstrated that the pattern-recognition receptor NLRP4 played a negative role in regulation of type I interferon signaling and have shown detail molecular mechanisms of NLRP4-mediatde activated TBK1 degradation through K48-linked ubiquitination via the E3 ubiquitin ligase DTX4. Ectopic expression of NLRP4 inhibited type I interferon signaling induced by ligand stimulation, whereas knockdown of either NLRP4 or DTX4 abrogated TBK1 K48-linked ubiquitination and degradation and thus enhance the antiviral response. Our findings identify the NLRP4-DTX4 axis as an additional signaling cascade for TBK1 degradation to maintain immune homeostasis during antiviral innate immunity.

Autophagy plays a key role in the innate and adaptive immune system by elimination of pathogens and the induction of acquired immune response. However, the

molecular mechanism of how autophagy affects the innate immunity to keep host homeostasis is still a mystery. We have identified Autophagy-related protein 13(Atg13) as a positive regulator in type I IFN signaling and antiviral response by interacting with RIG-I through Beclin1 during the initial stage of autophagy. We found the induction of autophagy can enhance the innate immune signaling and antiviral response. Our study will provide us a hint to deep understand how this ancient self-defense machinery functions in immunity.

In summary, this study characterized the function of NLRP4 and Atg13 in the regulation of type I IFN signaling and innate immune antiviral response, which provided potential therapeutic targets for enhancing host immunity against pathogen infection and inflammation associated disease.

DEDICATION

This dissertation is dedicated to

My husband

Hao Shen

And

Our family

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NOMENCLATURE

3-MA	3-Methylamphetamine
AMPK	5' AMP-activated protein kinase
Atg13	Autophagy-related protein 13
BIR	Baculovirus inhibitor of apoptosis protein repeat
CARD	Caspase activation and recruitment domain
CCL5	Chemokine (C-C motif) ligand 5
CHX	Cycloheximide
CQ	Chloroquine
DAPI	4',6-diamidino-2-phenylindole
DDX41	Probable ATP-dependent RNA helicase 41
DMSO	Dimethyl sulfoxide
DTX4	Deltex 4
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GSK3b	Glycogen synthase kinase 3 beta
IFI-16	Gamma-interferon-inducible protein Ifi-16
IKK	IκB kinase
IL-1β	Interleukin-1 beta
IL18	Interleukin-18
IRF3	Interferon regulatory factor 3
ISGs	IFN-inducible genes

ISREs	IFN-stimulated response elements
Jak1	Janus kinase 1
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MEF	Mouse Embryonic Fibroblast
MDA5	Melanoma Differentiation-Associated protein 5
mTOR	Mammalian target of rapamycin
MYD88	Myeloid differentiation primary response gene (88)
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	Nod-like receptors
NLRP4	NACHT, LRR and PYD domains-containing protein 4
NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen-associated molecular patterns
PBMCs	Human peripheral blood mononuclear cells
PCBP2	Poly(rC)-binding protein 2
PRRs	Germline-encoded pattern-recognition receptors
PYD	Pyrin domain
RD	Repressor domain
RLR	RIG-I-like receptors
RIG-I	Retinoic acid-inducible gene 1

RING	Really interesting new gene
ROS	Reactive oxygen species
STAT1	Signal Transducers and Activators of Transcription 1
STAT2	Signal transducer and activator of transcription 2
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
TLRs	Toll-like receptors
TRAF6	TNF receptor associated factor 6
TRIF	TIR-domain-containing adapter-inducing interferon-β
Tyk2	Tyrosine kinase 2
USP3	Ubiquitin-specific protease
VSV	Vesicular stomatitis virus

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1. INTRODUCTION*

1.1 Innate immune defense and PRRs

The innate immune system is considered as the first line of host defense during infection to recognize the microorganisms at the early phase and subsequent trigger of a proinflammatory response to invading pathogens[1], while the adaptive immune system is responsible to eliminate of pathogens in the late and to generate the immunological memory. The host 'senses' pathogen infection is dependent on the recognition of pathogen-associated molecular patterns(PAMPs) by several classes of germline-encoded pattern-recognition receptors(PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors, Nod-like receptors (NLRs) and sensors of DNA[2, 3]. Upon the PAMPs stimulation, these PRRs trigger activation of the transcription factor NF-κB, type I interferons and inflammasome signaling pathways, which leads to the production of proinflammatory cytokines and induction of adaptive immune responses.

The family of TLRs is the major class of PRRs, which is most extensively studied in past years. TLRs were originally discovered based on homology to the Drosophila melanogaster Toll protein, which is essential in dorso-ventral patterning during embryogenesis and in the antifungal response in Drosophila as well [4]. Based on TLR-mediated recognition of nucleic acids in intracellular compartments, TLR3 are activated by double stranded RNA produced during viral replication [5], while TLR7 and

*Part of this chapter is reprinted with permission from "NLRP4 negatively regulates type I interferon signaling by targeting the kinase TBK1 for degradation via the ubiquitin ligase DTX4". Jun Cui, Yinyin Li, Liang Zhu, Dan Liu, Zhou Songyang,Helen Y Wang &Rong-Fu Wang, 2012. Nat Immunol. 4;13(4):387-95.

TLR8 can sense single-stranded RNA (ssRNA)[6, 7]. TLR9 can recognize unmethylated CpG DNA present in both viruses and bacteria[8] as opposed to methylated DNA present in mammalian cells.

While the TLRs are mostly function at either the cell surface or the luminal aspect of endolysosomal membranes, RLRs and NLRs play a major role in recognition of intracellular cytosolic pathogens [9, 10]. The RNA helicases RIG-I and Mda5 are IFN inducible RNA helicases that play an important role in sensing of cytoplasmic RNA. After ligand stimulation, it will trigger the activation of downstream molecular protein called MAVS (VISA, IPS-1 or Cardif) which is the mitochondrial signaling adaptor. Studies have shown that RNA polymerase III can serve as an intracellular viral DNA sensor by transcribing viral AT-rich dsDNA into dsRNA, which in turn stimulates RIG-I and initiates the activation of MAVS and induce consequent type I IFN signaling[11, 12]. NLRs include a large family of cytosolic pattern-recognition receptors which have a conserved nucleotide-binding-and-oligomerization domain (Nod), a leucine-rich repeat (LRR) domain and a variable amino-terminal effector domain. Many NLRs have been studied as pattern-recognition receptors that trigger relevant signaling pathways after recognizing their pathogen-associated molecular pattern or sensing a danger signal[13].

Furthermore, people identified several DNA sensors during past years, such as IFI16 and DDX41, which function as cytosolic sensors of DNA and interact with the membrane-associated adaptor STING to activate the type I interferon signaling pathway [14, 15].

1.2 Type I IFNs

Nearly 50 years ago, IFNs was discovered as the first cytokine produced in viral-infected cells, which has the ability to induce resistance to infection with a different virus[16, 17]. Based on receptor usage, molecular structure, and sequence homology, IFNs are classified into type I IFNs and type II IFNs. Type I IFN subtype is mainly presented by IFNα and IFNβ, which interact with a heterodimeric receptor composed of IFNAR1 and IFNAR2 that signal through two Janus family kinases, Tyk2 and Jak1 to recruit STAT1 to receptor-bound STAT2 and form STAT1-STAT2 heterodimers. Then STAT1-STAT2 heterodimers dissociate from the receptors and translocate into the nucleus to activate the transcription of IFN-inducible genes (ISGs) [18-20]. The type II IFN subtype is represented by IFN-γ, which directly binds to a heterodimeric receptor composed by IFNGR1 and IFNGR2, which signal through Jak1 and Jak2 to phosphorylate STAT1 homodimers [21] , resulting to the transcription of IFNγ-induced genes.

Almost any cell type in the body can produce type I IFNs by recognition of pathogens by different transmembrane and cytosolic receptors. Generally type I IFNs play important roles in both innate immunity and adaptive immunity. First, types I IFNs induce the ISGs to initiate an intracellular antimicrobial response in infected and neighboring cells to control the spread of infectious agents. Second, they also trigger the function of innate immune cells, such as antigen presentation and natural killer cells, to produce the cytokine and chemokine to modulate innate immune responses in a balanced manner. Third, they activate the adaptive immune system. Type I IFNs enhance the

function of B cells to produce specific antibodies and development of antigen-specific T cells responses and generate immunological memory.

1.3 The role of type I IFNs in antiviral response

While both type I and type II IFNs can induce an antiviral state to prevent the replication of virus in host cells, type I IFN are considered as the most natural mediators to trigger the antiviral activity in humans. Because the intracellular environment is critical for virus life cycle, the host needs to develop efficient antiviral mechanisms to interfere with cellular functions or eliminate the infected cells to block or limit the replication of virus. Some well-characterized intrinsic antiviral factors can be induced by IFN receptor signaling, such as RNA-activated protein kinase(PKR), the Myxovirus Resistance (Mx) protein, and the 2′–5′ oligoadenylate synthetase/RNase L system(2-5A system)[22]. PKR plays an important role in mediating signal transduction in response to dsRNA and other ligands or mediating the apoptosis to clear virus infection. The Mx proteins, which are tightly regulated by type I IFN, mainly contribute to the host antiviral activity, while the 2-5A system may also cause apoptosis of infected cells to induce the antiviral activity of IFN.

On the other hand, type I IFN can inhibit the growth of target cells or induce cell apoptosis to limit the virus spread. The major role of IFN is to ensure that the infected cells are triggered to undergo apoptosis[23] by inducing a pro-apoptotic state in uninfected cells [24]. Moreover, it has been demonstrated that IFN also can induce caspase1[25], caspase3[26] and caspase8[27] to cause apoptosis of the viral infected cells.

Finally, IFNs have profound immunomodulatory effects and trigger the adaptive immune response. The type I IFNs can not only enhance the cytotoxicity of NK cells [28, 29] but also stimulate the proliferation, through the induction of IL-15 secreted by monocytes/macrophages[30-33]. At last, IFNs can stimulate the division of memory T cells and B cells to induce the adaptive immune response.

1.4 Type I interferon in autoimmune disorder

More than 40 years ago, it has shown that interferon plays important role in the immune system. Type I interferon plays an important role in viral clearance, but its aberrant production can have pathological role in immunopathology and autoimmune disorders. In 1979, interferon response was found in the patients' serum with several autoimmune diseases[34], which were confirmed mainly in Systemic Lupus Erythematosus (SLE)[35]. SLE is a systemic autoimmune disease which can cause effect on any part of the body. SLE patients mostly are women with chronic nonspecific symptoms, such as inflammation, tissue damage, weight loss, fever. It has demonstrated that a critical pathogenic event in SLE might be a disorder of peripheral tolerance mechanisms induced by activated myeloid dendritic cells in response to an aberrant of IFN-α and IFN-β[36]. An excessive production of IFN-α was also found in the pancreas of patients suffering insulin-dependent diabetes mellitus (IDDM)[37]. The high expression of IFN-α and IFN-β are also involved in the dermatomyositis, which targets the skin and proximal muscle groups [38]. Sjogren's syndrome, an autoimmune disease affecting salivary and lacrimal glands, also seems to be related with aberrant production of IFN-αβ [39]. From these observations, a chronic activation of the type I IFN system

seems to be a major role in an autoimmune process leading to inflammation and tissue damage. Thus, the tightly regulation of type I IFN response will provide us the significant insight in the controlling of autoimmune disease and inhibition of cancer development.

1.5 TANK-binding kinase 1 (TBK1) in type I IFN signaling

TBK1 plays a key role in type I interferon signaling, which can be activated by various DNA and RNA sensors. After RNA recognition, RIG-I-like receptors (RLRs) interact with a scaffold molecule named MAVS, which leads to activation of downstream kinases TBK1. The activated TBK1 then further phosphorylates transcription factor IRF3, resulting the expression of type I interferon-responsive gene [40-42]. While upon DNA stimulation, TBK1 can be activated by an important adaptor STING to activate the IRF3 pathway and induce type I IFNs. It has been shown that after viral infection, the kinase GSK3β interacts with TBK1 and enhance self-association and autophosphorylation of TBK1 at Ser172, resulting the activation of IRF3 and induction of IFN-β [42]. In addition, TBK1 can also be activated by ubiquitination at K63 linkages for the LPS and RLR induction. The E3 ligases Mind Bomb 1 and 2 (MIB1 and MIB2) cause K63-linked ubiquitination of TBK1 after RNA virus infection, while Ndrp1 ubiquitinates TBK1 after lipopolysaccharide (LPS) stimulation[43, 44]. It seems that TBK1 functions as a converging point for IRF3-mediated type I interferon signaling and interferon-responsive genes induction. Although type I interferon is critical for viral clearance, aberrant production of type I interferon can interfere with basic cellular function and induce autoimmune disorders. Thus, the activation of TBK1 needs to be

tightly regulated.

1.6 The role of ubiquitination in protein degradation and other function

Ubiquitin is a highly evolutionarily conserved molecule, which contains 76 residue amino acid polypeptides. Ubiquitination is post-translational modification where ubiquitin is attached to one or more lysine (K) residues of protein substrates. The process of ubiquitination is carried out in three major steps, and each step is facilitated by different classes of enzymes. Initially, ubiquitin is activated by E1 (ubiquitin-activating enzyme), in an ATP-requiring reaction. Next, E2 (ubiquitin-Conjugating enzymes) transfers the activated ubiquitin from E1 to a member of the ubiquitin-protein ligase family, E3 as well as a protein subtract. In humans, there are only two E1 enzymes and 38 E2 enzymes, but around 600 E3 ubiquitin ligases. According to the catalytic domains, the E3 family can be divided into three groups: HECT (homologous to E6-associated protein [E6AP] C terminus)[45] and those containing a RING (“really interesting new gene”) domain [46] or U box[47].

Initially, ubiquitination is recognized as a signature mark for protein degradation by the 26S proteasome [48]. However, based on the different internal linkage between ubiquitin moieties, it can serve a variety of non-proteolytic functions, such as receptor endocytosis, enzyme activation, DNA damage repair, protein trafficking, cell cycle, autophage and activation of certain signaling pathways, such as NF-KB and type I IFN signaling pathways[49].

Currently, there are three major types of ubiquitination, including monoubiquitination, mono-ubiquitination at multiple sites as well as poly-ubiquitination.

Monoubiquitination takes place for the attachment of a single ubiquitin to its substrate[50]. When ubiquitination occurs on several lysine residues, it is named multiple-ubiquitinations. The initial of monoubiquitination is typically followed by the formation of polyubiquitin chains. During this process, there are mainly seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) involved in polyubiquitin chain formation[51]. Different types of ubiquitination determine the different fate of ubiquitinated protein. The K48-linked polyubiquition is identified for protein degradation by the 26S proteasome[48]. In contrast, the K63-linked polyubiquition is important for non-proteolytic functions including DNA damage repair, protein endocytosis, stress response and inflammatory response. It has been reported that TBK1 can be activated by ubiquitination at K63 linkages to trigger the downstream pathway after the LPS and RLR induction[43, 44]. Thus, the post modification on key protein plays an important role in the regulation of signaling pathway.

1.7 The role of NOD- like receptor

The NOD-like receptors (NLRs) family contains 22 members sharing a typical central NACHT domain (NAIP, CIITA, HET-E, and TP1) and a leucine-rich repeat (LRR) receptor domain and a variable amino-terminal effector domain[10, 52-54]. NLR protein family members can be classified into several subfamilies based on the type of the effector domains. For example, the NLRC proteins contain caspase activation and recruitment domain (CARD), the NAIP protein has baculovirus inhibitor of apoptosis protein repeat (BIR) domain, while NLRP proteins contain PYRIN domain[53]. Many NLRs have been well studied as pattern-recognition receptors, which can recognize

pathogen-associated molecular pattern or sense a danger signal to trigger relevant signaling pathways. In addition to recognizing bacteria structure, NOD1 and NOD2, which contains CARD domain, can activate NF-κB through an adaptor, RIP2/RICK. NOD2, but not NOD1, is involved in type I IFN production induced by 5′-triphosphate RNA and host immune defense against the infection of respiratory syncytial virus[55]. NLRX1 has been reported to inhibit both the type I interferon signaling pathway and TLR-induced NF-κB activation by binding to MAVS and dynamically interacting with TRAF6 and IKK complex, respectively[56-58]. NLRC5 plays an important role in negative regulating both the NF-kappaB and type I interferon signaling pathways by blocking the phosphorylation of IKK complex and interacting with RIG-I and MAD5 [59, 60]. NLRP3, which is well-characterized in inflammasome response, activates caspase1 and leads to the pro-inflammatory cytokines IL-1beta and IL-18 processing and secretion. NLRP4, containing NACHT domain, LRR domain and PYD domain, can negatively regulate the autophagy process by interacting with Beclin1 upon bacterial infections[61]. In addition, it has been reported that NLRP4 plays a negative role in regulating NF-κB signaling through interaction with the kinase IKK[62]. However, the role of NLRP4 in the regulation of type I interferon signaling and antiviral immunity is still unknown.

1.8 The autophagy process

In eukaryotic cells, there is a well-known mechanism, which functions to dispose of intracellular large protein aggregates and organelles that cannot be degraded by the proteasome, called autophage. Autophagy (Greek words, self-eating), is an essential

cellular catabolic process that induces cell degradation of their own unnecessary or dysfunctional components through lysosomal degradation[63]. The most primordial function of autophage is to ensure cellular survival during nutrient deprivation through maintaining cellular energy levels[63].

There are three different types of autophagy, including microautophagy, chaperone-mediated autophagy as well as macroautophagy. Microautophagy involves the engulfment of a small portion of cytoplasmic material into the lysosome lumen by invagination. Chaperone-mediated autophagy medicates the translocation of cytosolic proteins across the lysosomal membrane by chaperone proteins. The most well-studied is macroautophagy. Macroautophagy, usually referred to as autophagy, is conserved from yeast to mammals. After induction, a portion of cytoplasm is enclosed by a small vesicular sac named the isolation membrane or phagophore, which results in the formation of autophagosome, a double-membrane structure. The outer membrane of the autophagosome fuses with the lysosome to form an autolysosome, resulting in the degradation of enclosed contents as well as the inner membrane of the autophagosome by lysosomal enzymes. Autophagic degradation generates amino acids as well as other small molecules, which are delivered back to the cytoplasm for energy production or recycling.

Autophagy can be activated by different stimulation, besides the most well-known inducer, nutrient starvation, other stimulation also can cause autophagy, such as physiological stress stimuli, pharmacological agents (e.g., rapamycin), innate immune signals, as well as viral, bacterial, and parasitic infections. Under physiological

conditions, autophagy plays a number of roles to maintain the cellular homeostasis. Besides the main function of maintaining cellular energy levels during starvation, autophagy also involves in suppression of tumor development, prevention of neurodegeneration, antiaging and regulation of innate and adaptive immune response [64-68].

1.9 Key proteins involved in mammalian autophagy process

A typical autophagy process is mainly involved in three stages, membrane initiation stage, elongation stage, and completion of the autophagosome. The ULK complex, which includes ULK1, Atg101, Atg13 and FIP200, plays an important role in the initiation of autophagy. Autophagy is induced through the inhibition of mammalian target of rapamycin (mTOR) by nutrient starvation, which leads to the translocation of the ULK1 complex from the cytosol to early autophagic structures. This results in the recruitment of the PI(3)K (class III phosphatidylinositol-3-OH kinase) complex to the ER[69, 70]. The PI(3)K complex, which at least includes Beclin1, VPS34, Atg14, UVRAG, produces phosphatidylinositol-3-phosphate (PtdIns(3)P) to recruit double WD repeat domain phosphoinositide-interacting (WIPI) family proteins and FYVE-containing protein 1 (DFCP1), resulting in generation of omegasomes and isolation membranes or autophagosomes, respectively[71, 72].

VMP1, an ER-associated protein, is critical for autophagosome formation by interacting with Beclin 1 at an early stage[70, 73]. At the last step of autophagosome formation, two ubiquitin-like conjugates are required for isolation membrane elongation and/or enclosure completion. ATG12–ATG5 conjugate is the first conjugate, which is

produced by ATG7 and ATG10 enzymes. Together with ATG16L1, it functions as a dimeric complex[74]. The second conjugate is the phosphatidylethanolamine (PE)-conjugated LC3, which is produced by ATG7 and ATG3 enzymes [75, 76].

1.10 Protein phosphorylation as a key mechanism for autophagy induction

The induction of autophagy is mainly regulated by posttranslational modifications of autophagy related genes protein, such as phosphorylation. There is significant evidence that the phosphorylation status of ULK1 complex dramatically changes under different nutrient condition. Ulk1 and Ulk2, are involved in starvation induced autophagy, which forms a stable complex with Atg13, FIP200[77-82] (73-77), and Atg101 as well. In mammals, under the nutrient conditions, activated mTOR phosphorylated ULK1 and Atg13 at several serine residues, which resulting in the inhibition of kinase complex. In response to starvation, Ulk1/2 are rapidly dephosphorylated in the mTORC1- dependent phosphorylation sites, and then Ulk1/2 autophosphorylates and also phosphorylates Atg13 and FIP200, which triggers the induction of autophagy. AMPK is an evolutionarily conserved energy-sensing kinase, which can be activated by ATP consumption or metabolic stress. According to its catabolic function, AMPK could also be involved in the regulation of autophagy. AMPK phosphorylates raptor to inhibit mTORC1 activation, leading to autophage induction. In addition, AMPK directly phosphorylates and activates Ulk1/2[82] to induce the autophagy thereby. VPS34 complex (VPS34, Beclin1, Atg14, UVRAG) is also critical for autophagesome formation. It has been reported that under amino acid starvation condition, the activated ULK1 will directly phosphorylates Beclin-1 at Ser14 and

activates the VPS34 complexes to trigger autophagy induction and maturation [83].

1.11 Inhibition of autophagy activity

To fully understand a biological process, it is essential to perform experiments to regulate the activity of the process. Besides the genetic approaches, different pharmacological approaches have been utilized to modulate autophagy process. PI3-kinase inhibitors are the most commonly used to inhibit the autophagy process, including LY294002, wortmannin, or 3-MA[84-86]. However, these inhibitors are not so specific that can both affect class I PI3-kinase activity, which can inhibit autophagy, as well as class III PI3-kinase activity, which is required for autophagy process. For 3-MA, it also can target other kinases and has effect on other cellular processes, endocytosis[87], lysosomal acidification[88], and the mitochondrial permeability transition [89]. Recently, people has reported that there is an autophagy specific inhibitor called specific and potent autophagy inhibitor-1(spautin-1), which can cause the degradation of PI3 kinase complexes to inhibit autophagy activity[90]. Besides PI3-kinase inhibitors which functions in autophagsome formation, the other major used pharmacological inhibitors functions to block the later stages of autophagy (Figure 1). Bafilomycin A1 is an inhibitor that blocks the autophagosome-lysosome fusion[91], it also affects intralysosomal degradation through inhibiting acidification[92]. And chloroquine(CQ) can also cause inhibition of the lysosome acidification or inhibit fusion of autophagosome-lysosome.

Compared with pharmacological approaches, genetic approaches are much more specific to inhibit the autophagy pathway, which can be achieved by knockout different

ATG genes. The deficiency of autophagy has been verified in cells those are lacking essential autophagy related genes, such as Atg3[93], Atg5 [94], Beclin 1[95, 96], Atg7[97], Atg9a[98], Atg16L1 [99, 100], FIP200[101] and Ambra1[102].

1.12 The role of autophagy in innate immunity and inflammation

Recent studies demonstrated that autophagy plays a key role in the innate and adaptive immune system by elimination of pathogens and the induction of acquired immune response. Several autophagy proteins have been identified as positive or negative regulators in innate immune signaling[103]. The autophagy process plays a direct antiviral role against the mammalian viral pathogen vesicular stomatitis virus (VSV) in the model organism Drosophila[103]. Moreover, autophagy activates type I IFN production by mediating ssRNA virus detection and interferon-a secretion in plasmacytoid dendritic cells[104]. In contrast, several other autophagy proteins play a negative role in RIG-I-like receptor mediated activation of type I IFN response. Atg5-Atg12 conjugate interacts with the CARD domains of RLR and MAVS to inhibit the production of type I IFN signaling pathway[105]. Consistently, knockout Atg5 enhances type I IFN production after VSV infection and dsRNA stimulation. Knockout Atg7, an essential requirement for the Atg5-Atg12 conjugate, also results in enhancement of type I IFN production after dsRNA treatment[105]. Another group shows that in Atg5-deficient cells, the damaged mitochondria accumulated because of the loss of autophagy, resulting in the elevated expression of MAVS which triggers ROS production to active the innate immune respons. Moreover, it has been reported the negative regulation of autophagy protein Atg9a in the activation of STING which is required for the production

of type I IFN and pro-inflammatory[98]. In addition, deficiency of Atg16L1, which forms a complex with Atg5-Atg12 conjugate, enhances endotoxin-induced inflammatory immune responses. Macrophages from Atg16L1-deficient mice show high amounts of IL-1b and IL-18 after LPS treatment. Macrophages lacking Atg7 also show enhanced IL-1b production, indicating that Atgs play an important role in the regulation of the inflammatory response and its relevance to inflammatory disease [100]

2. MATERIALS AND METHODS*

2.1 Cell culture

HEK293T, THP-1, BxPC-3 and RAW264.7 cells were maintained in DMEM (Mediatech) or RPMI-1640 medium (Invitrogen) containing 10% heat-inactivated FCS. Mouse embryonic fibroblasts were prepared from embryos of C57BL/6 mice at day 15 and were cultured in DMEM supplemented with 10% FBS as described29. Buffy coats of blood from healthy donors (from the Gulf Coast Regional Blood Center) were used for isolation of PBMCs by density-gradient centrifugation with Lymphoprep (Nycomed Pharm).

2.2 Antibodies and reagents

Anti-NLRP4 (C-20; sc-50623), anti-IRF3 (sc-9082), anti-GFP (FL; sc-8334), anti-ubiquitin (sc-8017), anti-ULK1 (H-240, sc-33182), were from Santa Cruz Biotechnology. Horseradish peroxidase–anti-Flag (M2) and anti-β-actin (A1978) were purchased from Sigma. Horseradish peroxidase–anti-hemagglutinin (3F10), horseradish peroxidase–anti-c-Myc (11814150001) and unlabeled anti-c-Myc (11667203001) were from Roche Applied Science. Antibody to IRF3 phosphorylated at Ser396 (4947), anti-IKKi (2690), anti-TBK1 (3013), anti-Sting (3337s), anti-MDA5 (D74E4) and anti-RIG-I(D14G6) were from Cell Signaling Technology. Anti-Atg13 (M183-3), anti-LC3 (PM036), anti-Atg14 (PD026), anti-UVRAG (M160-3) were from MBL. Anti-VPS15

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was from BETHYL. Anti-h-VPS34 was from echelon. MAVS (ALX-804-847-C100) was from Enzo Life Sciences. Anti-PI 3 Kinase Class 3 antibody was from abcam.

NLRP4-specific, DTX4-specific, Atg13-specific, Beclin1-specific, VPS34-specific, Atg101-specific, UVRAG-specific and control (2-scramble mix) siRNA oligonucleotides, were obtained from Invitrogen. Two NLRP4-specific shRNA plasmids, four DTX4-specific shRNA plasmid, three Atg13-specific shRNA plasmids and control shRNA plasmids were obtained from Openbiosystems.

2.3 Plasmid DNAs

A complete open reading frame of human NLRP4 was obtained from human PBMC cDNA by RT-PCR and subsequently subcloned into pcDNA-HA, pcDNA-Flag and pEGFP-C2 vectors. The full-length and deletion domains of human NLRP4, Flag-tagged and GFP-tagged DTX4, deletion domains of TBK1 and DTX4 were generated by PCR using the following primers:

NLRP4 forward primer,

5’ CGATATGTTTAAACATGGACTACAAAGACGAT;

NLRP4 reverse primer,

5’ CGATATCTCGAGTCAGATCTCTACCCTTG;

NLRP4-GFP forward primer,

5’ AATTCTCGAGCGCAGCCTCTTTC;

NLRP4-GFP reverse primer,

5’ GGCCGAATTCTCAGATCTCTACCCT;

NLRP4(PYD) forward primer,

5’ GTGGTACCGCAGCCTCTTTCTTCTCT

NLRP4(PYD) reverse primer,

5’CGCTCGAGTCACTGTTTCCCAGTTTCCTT

NLRP4(Nod) forward primer,

5’ GTGGTACCCAGCCACGTACAGTGATT

NLRP4(Nod) reverse primer,

5’ CCGCTCGAGTCAAAAACAGAGTTTCCTCAA

NLRP4(LRR) forward primer,

5’ GTGGTACCTGCTCCAGCTTGAGGAAA

NLRP4(LRR) reverse primer,

5’ CGCTCGAGTCAGATCTCTACCCTTGT

TBK1-(1-301) forward primer,

5’ CGATATGGATCCATGCAGAGCACTTCTAA

TBK1-(1-301) reverse primer,

5’ CGATATCTCGAGCTATTCTGCAAAAAACT

TBK1-(1-383) forward primer,

5’ CGATATGGATCCATGCAGAGCACTTCTAA

TBK1-(1-383) reverse primer,

5’ CGATATCTCGAGCTAGCTTACTACAAATA

TBK1-(383-730) forward primer,

5’ CGATATGGATCCATGCGGGAACCTCTGAATA

TBK1-(383-730) reverse primer,

5’ CGATATCTCGAGCTAAAGACAGTCAACGTTG

DTX4 forward primer,

5’ CGATATAAGCTTGAAGTGGGCATCACCAT

DTX4 reverse primer,

5’ CGATATCTCGAGGTCCTTCTCCTGGGCAG

DTX4-GFP forward primer,

5’ CGATATCTCGAGCGAAGTGGGCATCACCAT

DTX4-GFP reverse primer,

5’ CGATATAAGCTTTCAGTCCTTCTCCTGGGCAG

DTX4-(1-301) forward primer,

5’ CGATATAAGCTTGAAGTGGGCATCACCAT

DTX4-(1-301) reverse primer,

5’CGATATCTCGAGTCACTCATCTGGTGGGTGCCGGA

DTX4-(302-513) forward primer,

5’CGATATAAGCTTGACTGCATGCACCATCTGATGGAA

DTX4-(302-513) reverse primer,

5’ CGATATCTCGAGGTCCTTCTCCTGGGCAG

Mouse-Atg13 forward primer,

5’ TATCTCGAGcGAAACTGAACTCAGCTCCCAGGA

Mouse-Atg13 reverse primer,

5’ TATGGATCCTTACTGCAGGGTTTCCACAAAG

Human-Atg13 forward primer,

5’ TATCTCGAGCGAAACTGATCTCAATTCCCAGGACA

Human-Atg13 reverse primer,

5’TATGGATCCTTACTGCAGGGTTTCCACAAAGGCAT

VPS34 forward primer,

5’ TATGAATTCTGGGGAAGCAGAGAAGTTTCACTACAT

VPS34 reverse primer,

5’ TATCTCGAGTCATTTTCTCCAGTACTGGGCAAACTTGTGA

AMPKa-attb forward primer,

5’GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAACCATGCGCAGACTCAGTTCCTGGAGAAA

AMPKa-attb reverse primer,

5’GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTGTGCAAGAATTTTAATT

Beclin1-GFP forward primer,

5’ TATGAATTCGAAGGGTCTAAGACGTCCAACAA

Beclin1-GFP reverse primer,

5’ TATGGATCCTCATTGTTATAAAATTGTGAGGAT

2.4 Transfection and reporter assays

HEK293T cells were transfected with plasmid encoding an NF-κB, IFN-β or ISRE luciferase reporter (firefly luciferase; 100 ng) and pRL-TK (renilla luciferase plasmid; 10 ng) together with 100 ng specific plasmids through the use of Lipofectamine 2000 (Invitrogen). Luciferase activity was measured with a Dual-Luciferase Assay (Promega) with a Luminoskan Ascent luminometer (Thermo Scientific) according to the manufacturer’s protocol. Reporter gene activity was determined by normalization of the firefly luciferase activity to renilla luciferase activity. An Amaxa nucleofector kit V was used according to the manufacturer’s protocols (Lonza Amaxa) for transfection of plasmids or siRNAs into THP-1 cells and Raw 264.7 cells.

2.5 Immunoprecipitation and immunoblot analysis

For immunoprecipitation, whole-cell extracts were prepared after transfection or

stimulation with appropriate ligands, followed by incubation overnight with the appropriate antibodies plus Protein A/G beads (Pierce). For immunoprecipitation with anti-Flag or anti-hemagglutinin, anti-Flag or anti-hemagglutinin agarose gels (Sigma) were used. Beads were then washed five times with low-salt lysis buffer, and immunoprecipitates were eluted with 3x SDS Loading Buffer (Cell Signaling Technology) and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad) followed by further incubation with the appropriate antibodies. LumiGlo Chemiluminescent Substrate System (KPL) was used for protein detection.

2.6 Immunofluorescence staining

Cells in culture plates or chamber slides were fixed for 20 min at −20 °C with methanol and nonspecific receptors were blocked with 10% normal goat serum. IRF3 was stained with polyclonal rabbit anti-IRF3 (sc-9082; Santa Cruz Biotechnology), followed by rabbit antibody to Texas red (A-6399; Invitrogen). Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Immunofluorescence staining was visualized and cells were photographed with an Olympus 1X71S1F fluorescence microscope.

2.7 Cytokine-release assay

Human IFN-β was detected with ELISA kits according to the manufacturer's protocols (PBL Biomedical Laboratories).

2.8 Real-time PCR analysis

Total RNA was isolated from cells or tissues, and first-strand cDNA was generated from total RNA using oligo-dT primers and reverse transcriptase II (Invitrogen). Real-time PCR was conducted with the SYBR Green qPCR Super Mix

Universal (Invitrogen) and specific primers. The values of the target geneexpression were normalized to GAPDH. The following primers were used for real-time PCR:

hNLRP4 forward primer, 5’ AGAAAGGATCTCTGCATGAAGGT

hNLRP4 reverse primer, 5’GCGGTCCAAATGGTCACATTC

hGAPDH forward primer, 5’ TCAAGAAGGTGGTGAAGCAG

hGAPDH reverse primer, 5’GAGGGGAGATTCAGTGTGGT

hISG54 forward primer, 5’ GGAGGGAGAAAACTCCTTGGA

hISG54 reverse primer, 5’ GGCCAGTAGGTTGCACATTGT

hISG15 forward primer, 5’ TCCTGGTGAGGAATAACAAGGG

hISG15 reverse primer, 5’ GTCAGCCAGAACAGGTCGTC

hISG56 forward primer, 5’ TCAGGTCAAGGATAGTCTGGAG

hISG56 reverse primer, 5’ AGGTTGTGTATTCCCACACTGTA

hRANTES forward primer, 5’ ATCCTCATTGCTACTGCCCTC

hRANTES reverse primer, 5’ GCCACTGGTGTAGAAATACTCC

hIFN-β forward primer, 5’ CATTACCTGAAGGCCAAGGA

hIFN-β reverse primer, 5’ CAATTGTCCAGTCCCAGAGG

hDTX4 forward primer, 5’ TTAAGGCAGCCGTGGTCAATG

hDTX4 reverse primer, 5’ CTTCAGTGGGCCTCGAATGG

hAtg13 forward primer, 5’ TCGGGAGGTCCATGTGTGT

hAtg13 reverse primer, 5’ TGGTGTCACCCTAGTTATAGCAA

mISG54 forward primer, 5’ CAGCAAGATGCAACCAAGATG

mISG54 reverse primer, 5’ TCTCCAGTGACTCCTTACTC

mISG56 forward primer, 5’ TGCGATCCACAGTGAACAAC

mISG56 reverse primer, 5’ ACTTCCGGGAAATCGATGAG

mβ-actin forward primer, 5’ AGATCTGGCACCACACCTTCT

mβ-actin reverse primer, 5’ CTTTGATGTCACGCACGATTT

mAtg13 forward primer, 5’TCTCTTCTCGCTATTACAAGGGT

mAtg13 reverse primer, 5’ CCATTCAGTTGAACTTCCCCAAA

2.9 Cycloheximide-chase assay

Cells were treated for various periods of time with cycloheximide (100 μg/ml) after virus infection, then were collected and analyzed by immunoblot.

2.10 Viral infection

VSV-eGFP was provided by S. Balachandran. Cells were infected at various multiplicities of infection as described.

2.11 RNA interference

Specific siRNA or shRNA plasmids were transfected into 293T or THP-1 cells or Raw 264.7 cells using Lipofectamine 2000 (Invitrogen) or Nucleofector kit V respectively according to the manufacturer’s instruction

3. NLRP4 NEGATIVELY REGULATES TYPE I INTERFERON SIGNALING BY TARGETING THE KINASE TBK1 FOR DEGRADATION VIA THE UBIQUITIN LIGASE DTX4*

3.1 Introduction

The innate immune response provides the first line of defense against invading microorganisms, such as bacteria and viruses, by recognizing pathogen-associated molecular patterns (PAMPs). Recognition of such PAMPs relies on several classes of germline-encoded pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), RIG-I-like receptors, Nod-like receptors (NLRs) and sensors of DNA[2, 3]. After stimulation with PAMPs, these PRRs trigger activation of common downstream signaling pathways, such as NF-κB, type I interferons and inflammasome, which leads to the production of proinflammatory cytokines and induction of adaptive immune responses to facilitate the pathogen clearance. Whereas TLR3, TLR7, TLR8 and TLR9 are important sensors, which detect viral DNA or RNA, and induce TIR domain-containing adaptor-inducing interferon-beta (TRIF)- and MYD88-mediated signaling pathway, activation of RIG-I and MDA-5 by double-stranded RNAs or certain viruses recruit the mitochondrial signaling adaptor MAVS (VISA, IPS-1 or Cardif). Studies have shown that RNA polymerase III can serve as an intracellular viral DNA sensor by transcribing viral AT-rich dsDNA into dsRNA, which in turn stimulates RIG-I and

*Part of this chapter is reprinted with permission from "NLRP4 negatively regulates type I interferon signaling by targeting the kinase TBK1 for degradation via the ubiquitin ligase DTX4". Jun Cui, Yinyin Li, Liang Zhu, Dan Liu, Zhou Songyang,Helen Y Wang &Rong-Fu Wang, 2012. Nat Immunol. 4;13(4):387-95.Copyright 2012 by Nature Publishing Group.

initiates the MAVS-dependent signaling cascade. Furthermore, IFI16,DDX41,DAI,AIM2 function as cytosolic DNA sensors and recruit the membrane-associated adaptor STING to activate the type I interferon signaling pathway[14, 15].

Type I interferon plays an important role in viral clearance, but its aberrant production can have pathological role in immunopathology and autoimmune disorders. Thus, tight regulation of these key signaling pathways is essential for both innate and adaptive immunity to maintain the homeostasis. NLRs represent a large family of intracellular pattern-recognition receptors that are characterized by a conserved nucleotide-binding-and-oligomerization domain (Nod), a leucine-rich repeat (LRR) region and a variable effector domain. Several NLRs have been extensively studied and shown to activate signaling pathways after stimulated by different PAMPs. NLRC5 negatively regulates type I IFN and NF-κB by interaction with RIG-I and Mda5 after viral infection[59, 60], whereas NLRX1 has been characterized as a negative inhibitor in the type I interferon signaling pathway by binding to MAVS[56-58]. NLRP4, a member of the NLR family of proteins, contains an pyrin domain (PYD), a nucleotide binding oligomerization domain (NOD) and a C-terminal leucine-rich repeat domain (LRR).Although studies have shown that NLRP4 plays a negative role in regulation of NF-κB signaling and autophagic processes[61, 62], its physiological role in the regulation of type I interferon signaling and antiviral response still remains unclear. In our study we showed that NLRP4 negatively regulated the type I interferon signaling pathway by targeting TBK1 for degradation. NLRP4 can recruit the E3 ubiquitin ligase DTX4 for Lys48 (K48)-linked polyubiquitination and caused the degradation of TBK1.

3.2 Results

3.2.1 NLRP4 negatively regulates type I interferon signaling pathway

To determine whether the members of NLR family play possible roles in type I interferon signaling and antiviral immunity, we transfected HEK293T human embryonic kidney cells (293T cells) with an IFN-β luciferase reporter and the internal control renilla luciferase, as well as expression vectors containing candidate genes encoding NLRs. Cells were then treated with the synthetic RNA duplex poly(I:C) for 24 hours to trigger type I interferon signaling; and the results indicate that NLRP4 plays a negative role for activation of the IFN-β luciferase reporter(Figure 3-1a). Since IFN-β activation requires coordinated signaling from both IRF3- and NF-κB-mediated pathways, we used an interferon-stimulated response element (ISRE) luciferase reporter (which requires activation by IRF3 only) to determine whether the inhibition of type I interferon by NLRP4 requires the participation of NF-κB signaling. We found that NLRP4 significantly inhibited the ISRE activation induced by intracellular poly(I:C) (Figure3-1a), which suggested that NLRP4 directly inhibits IFN-β activation. The similar results were obtained when we transfected NLRP4 plasmid in 293T-TLR3 cells (293T cells that express TLR3) treated with poly(I:C) (Figure3-1b) or 293T cells treated with poly(dA:dT) (Figure3-1c) or infected with vesicular stomatitis virus tagged with enhanced green fluorescent protein (VSV-eGFP) (Figure3-1d). These results indicated that NLRP4 played a negative role in the type I interferon signaling pathway.

Since NLRP4 specifically inhibits type I IFN signaling, we next sought to determine how NLRP4 inhibits the type I interferon signaling. We found that

overexpression of NLRP4 potently inhibited the phosphorylation of endogenous IRF3 induced by RIG-I, Mda5, MAVS or TRIF in 293T cells (Figure 3-1e). As activation of IFN-β is also associated with the translocation of IRF3 from the cytoplasm into the nucleus, we next examined the translocation of endogenous IRF3 in cells with or without expression of NLRP4. IRF3 rapidly translocated from the cytoplasm to the nucleus of the cells transfected with empty vector after poly(I:C) treatment. By contrast, in the cells expressing GFP-tagged NLRP4, IRF3 remained in the cytoplasm after stimulation (Figure 3-1f). Taken together, these results suggested that NLRP4 inhibits the activation of type I interferon induced by various stimulation by blocking the phosphorylation and translocation of IRF3.

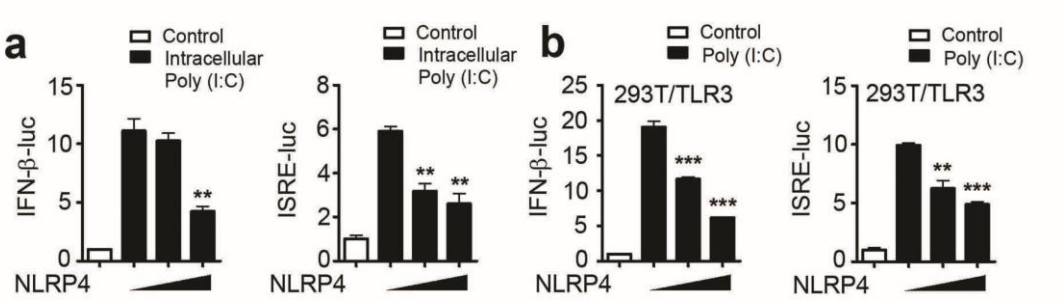


Figure 3-1. NLRP4 negatively regulates type I interferon signaling pathway

(a–d) Luciferase activity in 293T cells (a,c,d) or 293T-TLR3 cells (b) transfected with plasmid encoding a luciferase reporter for IFN-β(IFN-β–luc) or ISRE (ISRE-luc; 100 ng each), together with empty vector (no wedge) or an expression vector for NLRP4 (0, 50 and 100 ng; wedge), followed by no treatment (control (Ctrl)) or treatment with intracellular (IC) poly(I:C) (1 μg/ml; a), poly(I:C) (10 μg/ml; b), poly(dA:dT) (1 μg/ml; c) or VSV-eGFP (MOI, 0.01; d). (e) Immunoblot analysis (IB) of total and phosphorylated (p-) IRF3 in 293T cells transfected with various combinations (above lanes) of plasmid for Flag-tagged RIG-I, Mda5, MAVS or TRIF plus vector for HA-tagged NLRP4, probed with antibodies (α-) along left margin. (f) Fluorescence microscopy of IRF3 in 293T cells transfected with empty vector (EV) or vector for GFP-tagged NLRP4, then left untreated (top row) or treated with intracellular poly(I:C). DAPI, DNA-intercalating dye.

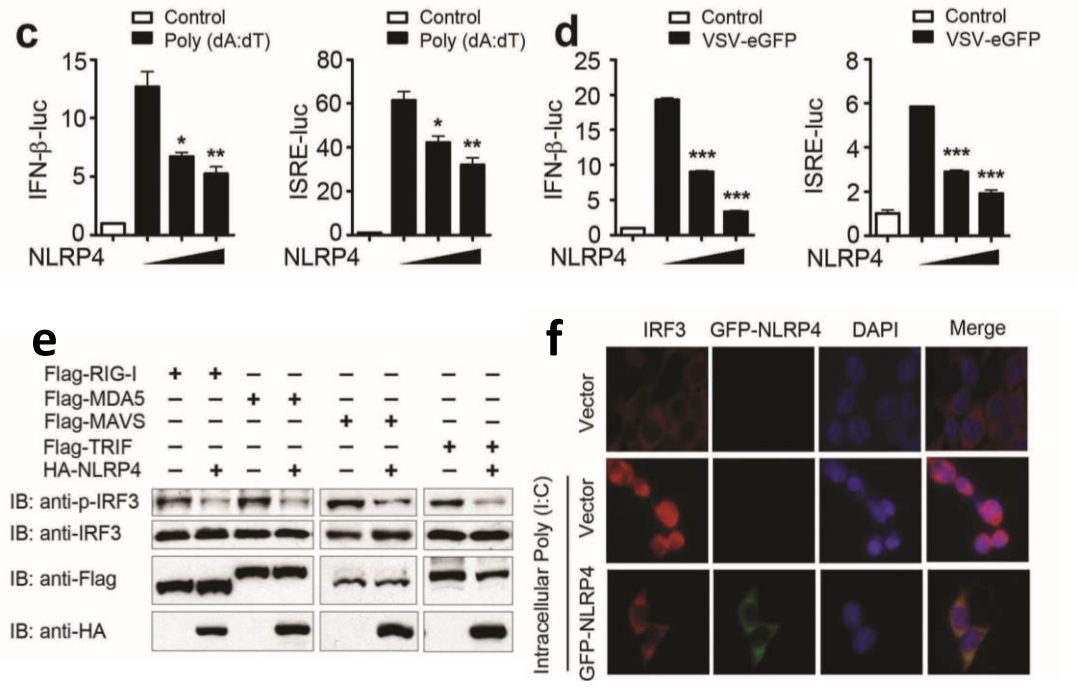


Figure 3-1. Continued

3.2.2 Knockdown of NLRP4 enhances IFN-β expression and antiviral responses

To determine whether specific knockdown of endogenous NLRP4 would increase antiviral responses under physiological conditions, we selected two NLRP4-specific lentivirus short hairpin RNA (shRNA) constructs and an NLRP4-specific small interfering RNA (siRNA) to knock down the expression of NLRP4. The NLRP4 siRNA and two NLRP4 shRNA efficiently inhibited the expression of transfected and endogenous NLRP4 in 293T cells and THP-1 cells (Figure 3-2a). Using the ISRE luciferase reporter assay, we showed that knockdown of NLRP4 markedly increased the ISRE-luc activity induced by poly(I:C), intracellular poly(I:C), poly(dA:dT) or infection with VSV-eGFP in 293T cells or 293T-TLR3 cells (Figure 3-2b). Consistent with this observation, knockdown of NLRP4 resulted in markedly increased IFN-β protein expression or mRNA abundance of interferon-stimulated cytokines, such as *ISG15*, *IFIT1*, *IFIT2* and *CCL5* in THP-1 cells (Figure 3-2c,d). We obtained similar results with human peripheral blood mononuclear cells (PBMCs) transfected with NLRP4-specific siRNA or scrambled siRNA (Figure 3-2e). To further determine whether the enhanced type I interferon response is correlated with antiviral immunity, we knocked down NLRP4 expression in THP-1 cells and then infected cells with different dose of VSV-eGFP (MOI =1 or10). Knockdown of NLRP4 rendered the cells resistant to viral infection and resulted in considerably fewer GFP+ (virus infected) cells than those treated with control siRNA (Figure 3-2f). Flow cytometry analysis revealed that 1% or 11% of cells were infected (GFP+) in cells transfected with USP3-specific siRNA, compared to 55.83% or 87% of GFP+ cells transfected with control siRNA (Figure 3-2g).

Taken together, these results suggest that NLRP4-specific knockdown markedly enhances the type I interferon response and antiviral immunity.

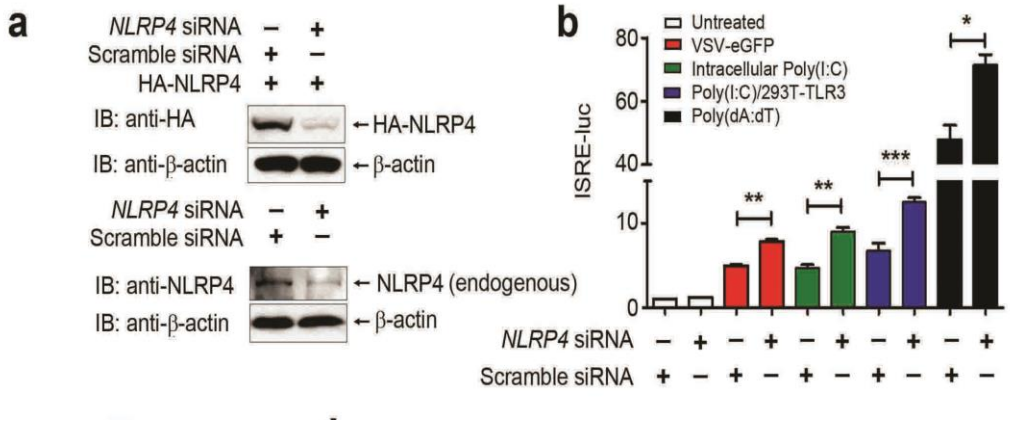


Figure 3-2. Knockdown of NLRP4 enhances IFN-β expression and antiviral responses

(a) Immunoblot analysis of the knockdown of exogenous NLRP4 in 293T cells expressing HA-NLRP4 (top) or endogenous (endo) NLRP4 in 293T cells (bottom) treated with NLRP4-specific siRNA or scrambled (Scr) siRNA. β-actin serves as a loading control throughout. (b) Luciferase activity in 293T or 293T-TLR3 cells transfected with NLRP4-specific or scrambled siRNA, together with an ISRE luciferase reporter, then left untreated (UT) or treated with VSV-eGFP, intracellular poly(I:C), poly(I:C) or poly(dA:dT).(c,d) Real-time PCR analysis of IFNB mRNA and enzyme-linked immunosorbent assay of IFN-β protein (c) and real-time PCR analysis of ISG15, IFIT1, IFIT2and CCL5mRNA (d) in THP-1 cells treated with NLRP4-specific or scrambled siRNA, followed by no infection (−) or infection (+) with VSV-eGFP (MOI, 1); results for mRNA are relative to those of untreated cells.(e) Enzyme-linked immunosorbent assay of IFN-βprotein and real-time PCR analysis of ISG15mRNA in PBMCs treated as in c,d. (f,g) Phase-contrast (PH) and fluorescence microscopy (f) and flow cytometry (g) assessing the infection of THP-1 cells left untreated or treated with NLRP4-specific or scrambled siRNA, and then infected with VSV-eGFP at an MOI of 1 or 10.

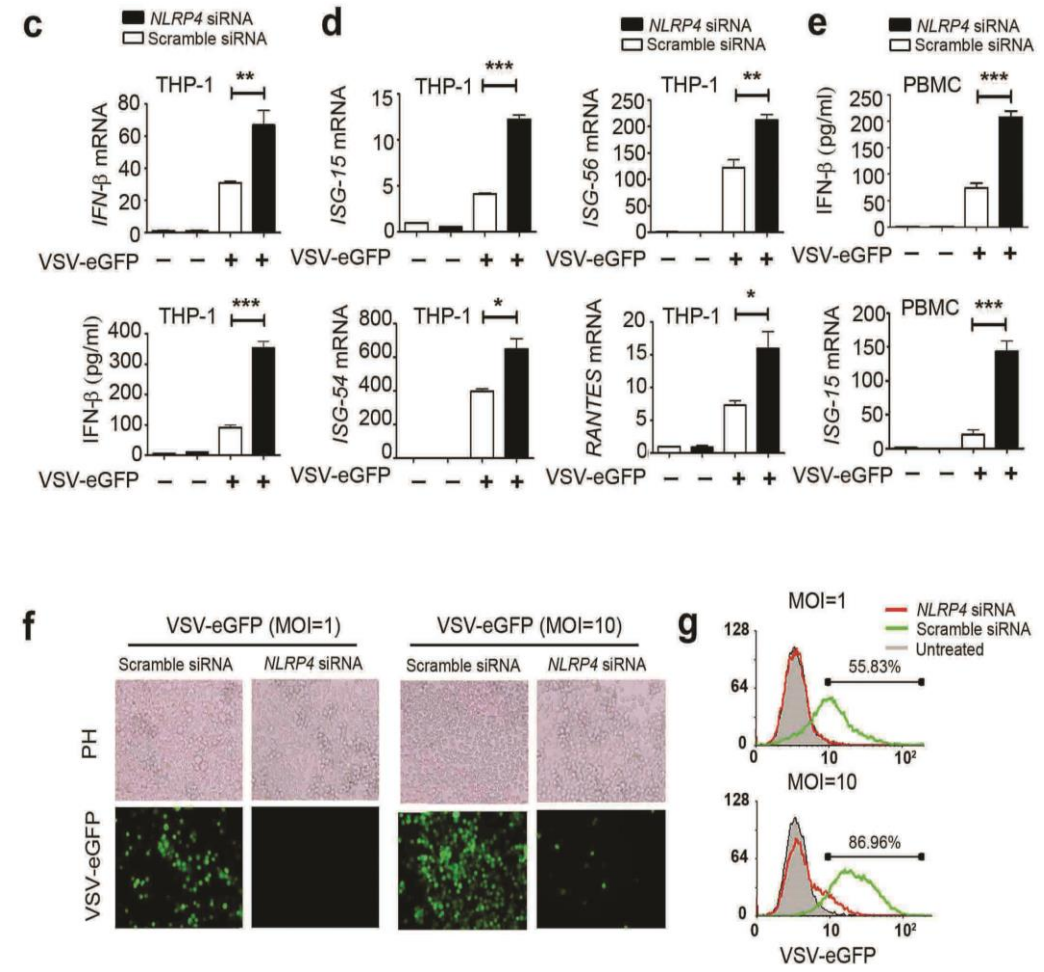


Figure 3-2 Continued.

3.2.3 NLRP4 directly associates with TBK1 to inhibit IRF3 phosphorylation

Since NLRP4 specifically inhibits type I IFN signaling, we next sought to determine the molecular mechanisms by which NLRP4 inhibits type I interferon signaling. 293T cells were transfected with TRIF, RIG-I, Mda5, MAVS, TBK1, IKKi together with increasing amounts of NLRP4 plus the IFN-β or ISRE luciferase reporter. We found that NLRP4 markedly inhibited activation of the luciferase reporters induced by TRIF, RIG-I, Mda5, MAVS, STING and TBK1 but showed weak or no inhibition of activity of either luciferase reporter induced by IKKi(Figure3-3a,b), which suggested that NLRP4 may inhibit type I interferon signaling by interacting with TBK1.We next sought to determine whether NLRP4 could directly interact with TBK1. Coimmunoprecipitation and immunoblot analyses showed that NLRP4 interacted with TBK1 but not with IKKi, IRF3 or IRF7 (Figure 3-3c). To determine the physiological relevance of these findings, we treated the 293T cells with VSV-eGFP, and then collected cells at various time points. We found that NLRP4 had little or no interaction with TBK1 in unstimulated 293T cells, but the interaction between NLRP4 and TBK1 increased considerably at 8 h and 10 h after VSV infection. In contrast, we detected neither IKKi nor IRF3 in samples immunoprecipitated with anti-NLRP4 (Figure 3-3d). The similar results were obtained with VSV-eGFP–infected THP-1 cells and PBMCs (Figure 3-3e, f). These results suggested that NLRP4 interacted with the activated form of TBK1 but not with IKKi or IRF3 after viral infection. To address that possibility, we generated four deletion mutants of TBK1 containing various combinations of the TBK1 domains (Figure 3-3g). We found that NLRP4 interacted with the TBK1 mutant

containing only the kinase domain, but not TBK1 mutants containing only the coiled-coil domain or the ubiquitin-like domain plus the coiled-coil (Figure 3-3g), which indicated that NLRP4 binds to the kinase domain of TBK1.

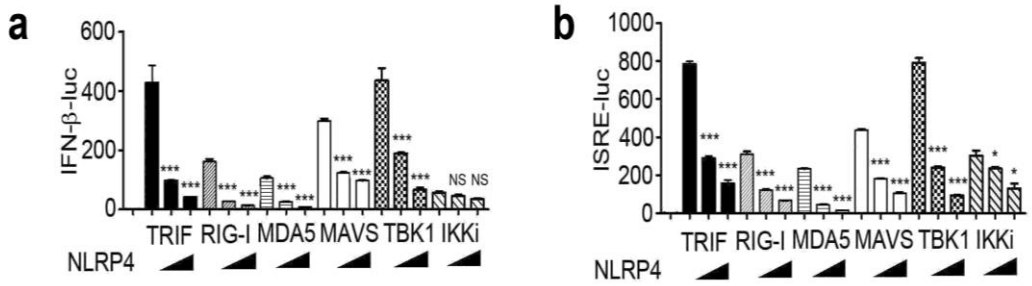


Figure 3-3. NLRP4 directly associates with TBK1 to inhibit IRF3 activation

(a,b) Luciferase activity of 293T cells transfected with an IFN-β (a) or ISRE (b) luciferase reporter, together with vector for TRIF, RIG-I, Mda5, MAVS, TBK1 or IKKi, along with empty vector (no wedge) or with increasing amounts (wedge) of expression vector for NLRP4.(c) Immunoassay of 293T cells transfected with vector for HA-NLRP4 together with plasmid for Flag-tagged TBK1, IKKi, IRF3 or IRF7, followed by immunoprecipitation (IP) with anti-Flag beads and immunoblot analysis with anti-HA. WCL, immunoblot analysis of whole-cell lysates without immunoprecipitation. (d) Immunoassay of extracts of 293T cells infected for various times (above lanes) with VSV-eGFP, followed by immunoprecipitation with anti-NLRP4 or anti-IRF3 and immunoblot analysis. (e,f) Immunoassay of extracts of THP-1 cells (e) or PBMCs (f) infected for various times (above lanes) with VSV-eGFP, followed by immunoprecipitation with anti-NLRP4 and immunoblot analysis (antibodies, left margin).(g) Coimmunoprecipitation and immunoblot analysis (bottom) of 293T cells transfected with deletion mutants of TBK1 (top) along with vector for HA-NLRP4. WT, wild-type; KD, kinase domain; ULD, ubiquitin-like domain; CC, coiled-coil domain.

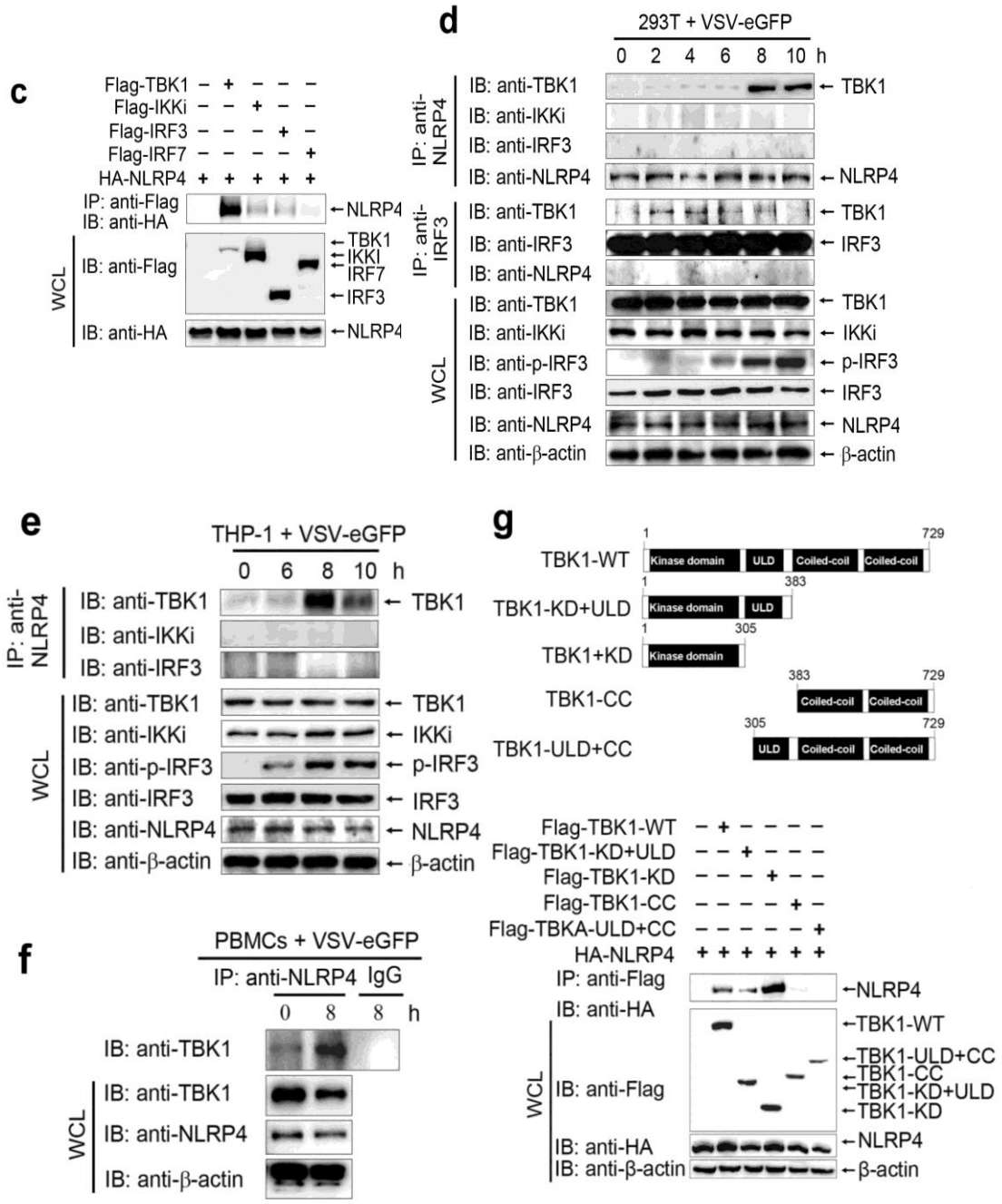


Figure 3-3 Continued.

3.2.4 NLRP4 mediates the degradation of TBK1

Since we found that NLRP4 specifically interacts with TBK1, we next want to check how NLRP4 inhibits type I IFN signaling through its interaction with TBK1. We found that the concentration of TBK1 protein diminished remarkably with increasing NLRP4 expression, when we transfected with 293T cells with plasmid encoding TBK1 and NLPR4 (Figure3-4a). To exclude the possibility that the decrease in TBK1 protein was caused by lower expression of the gene (TBK1), we used RT-PCR to analyze the same 293T cells expressing various genes and found that the abundance of TBK1 mRNA did not change with increasing expression of NLRP4 (Figure3-4a). To determine the specificity of the NLRP4-mediated degradation of TBK1, we did similar experiments with cells expressing IKKα, IKKβ or IKKi with increasing NLRP4 expression and found that NLRP4 specifically induced the degradation of TBK1 but did not affect the concentration of IKKα, IKKβ or IKKi (Figure 3-4b). Additionally, knockdown of NLRP4 not only resulted in much more Flag-tagged TBK1 but also enhanced the TBK1-induced activity of the ISRE luciferase reporter relative to that in cells transfected with the control shRNA (Figure 3-4c,d). Since endogenous NLRP4 interacted with TBK1 after viral infection, we hypothesized that NLRP4 induces TBK1 degradation only when type I interferon signaling is activated. To test that hypothesis, 293T cells were transfected with HA-NLRP4 or empty vector and infected with VSV-eGFP. We found much less TBK1 protein in HA-NLRP4–expressing cells infected with VSV-eGFP than in HA-NLRP4–expressing cells without VSV-eGFP infection or in cells transfected with empty vector and infected with VSV-eGFP (Figure 3-4e).We got the similar result when

we knockdown of NLRP4 in THP-1 cells, which resulted in much more endogenous TBK1 in cells infected with VSV-eGFP but not in uninfected cells (Figure 3-4f). These results suggested that overexpression or knockdown of NLRP4 was able to change the abundance of TBK1 only in cells infected with virus. Since previous data showed that NLRP4 interacts with the kinase domain of TBK1, we further determined whether NLRP4 bound to phosphorylated (activated) TBK1 to mediate its degradation. We generated a mutant of TBK1 with substitution of alanine for the serine at position 172 (Ser172) in the kinase domain of TBK1 (S172A) and found that NLRP4 did not bind the mutant TBK1or mediate its degradation, in contrast to its binding to the wild-type TBK1 construct (Figure 3-4C).Taken together, these results indicated that the phosphorylation of TBK1 at Ser172 was critical for its interaction with NLRP4.

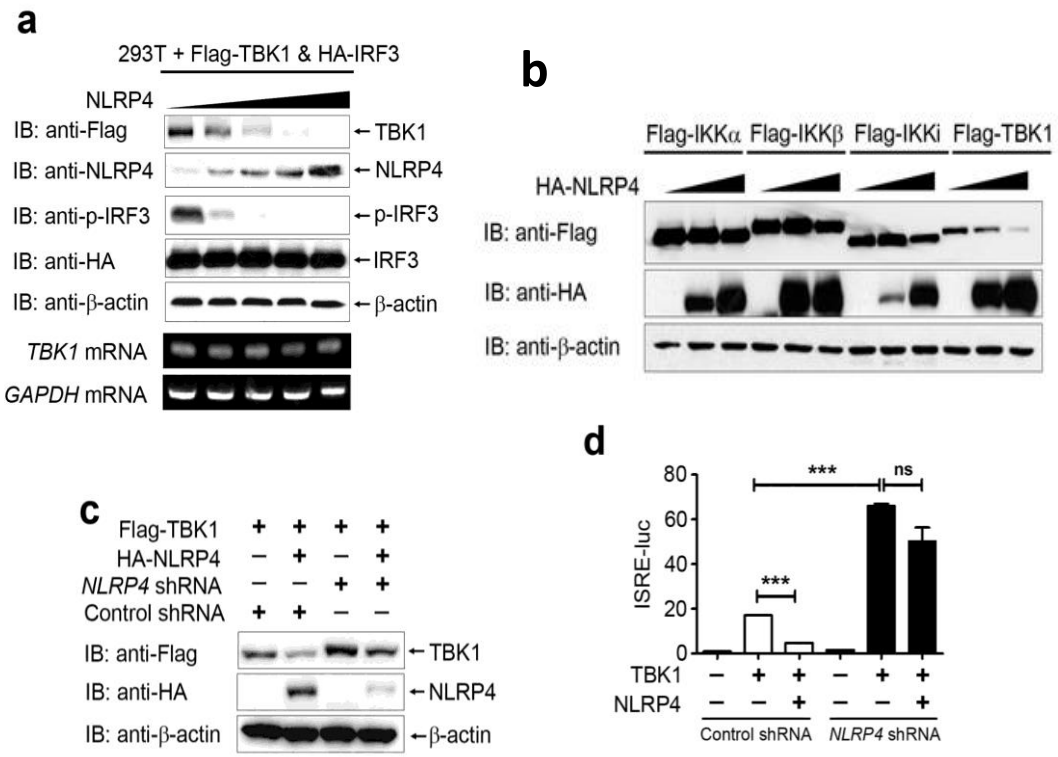


Figure 3-4. NLRP4 mediates the degradation of TBK1

(a)Immunoblot analysis (top) of extracts of 293T cells transfected with plasmid for Flag-TBK1 and HA-IRF3 and increasing doses of plasmid for NLRP4 (wedge). Below, RT-PCR analysis of TBK1 mRNA; GAPDH mRNA(encoding glyceraldehyde phosphate dehydrogenase) serves as a loading control.(b)293T cells were transfected with an empty vector or HA-NLRP4, together with Flag-IKKα, Flag-IKKβ, Flag-TBK1 or Flag-IKKi. Cell extracts were analyzed by immunoblot with the indicated antibodies. Results are representative of three independent experiments. (c,d) Immunoblot analysis (c) and luciferase activity (d) of 293T cells transfected with plasmids for Flag-TBK1 and HA-NLRP4, as well as NLRP4-specific or control shRNA (c,d), together with an ISRE luciferase reporter(e) Immunoblot analysis of extracts of 293T cells transfected with HA-tagged empty vector (HA-EV) or vector for HA-NLRP4, followed by no infection or infection with VSV-eGFP.(f) Immunoblot analysis of extracts of THP-1 cells transfected with NLRP4-specific or scrambled siRNA, followed by no infection or infection with VSV-eGFP. (g,h) Immunoblot analysis of NLRP4 (g) and total TBK1(S172A) and phosphorylated TBK1 (h) in 293T cells transfected with various combinations (above lanes) of expression vector for NLRP4 and plasmid for Flag-tagged TBK1 or TBK1(S172A), followed by immunoprecipitation with anti-Flag beads.

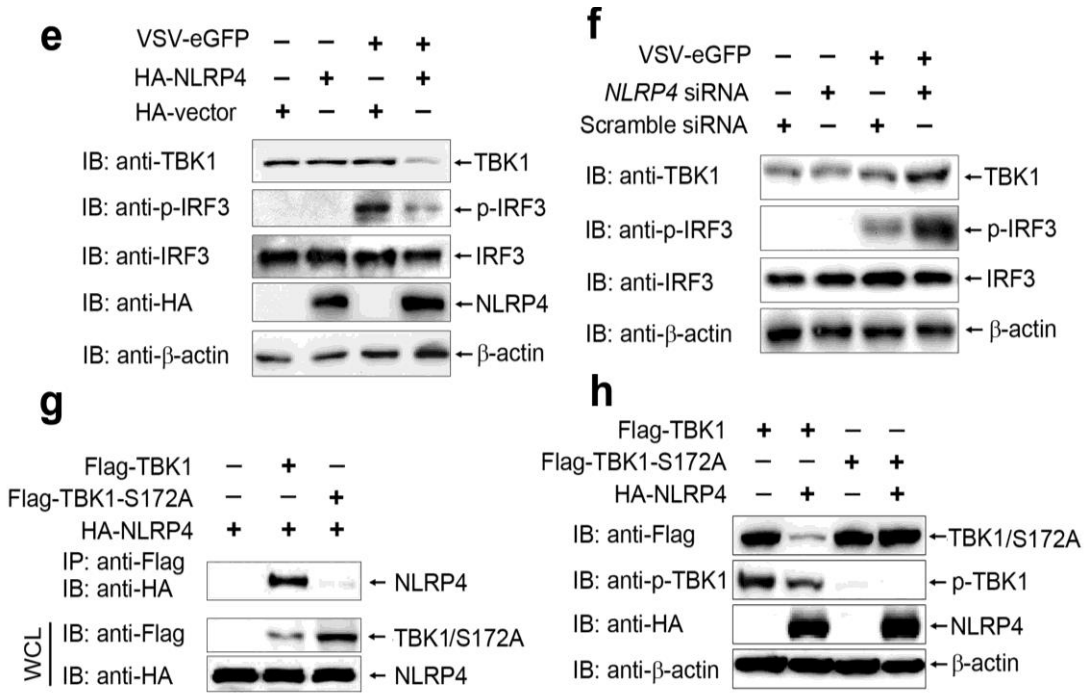


Figure 3-4 Continued.

3.2.5 NLRP4 induces K48-linked ubiquitination of TBK1after viral infection

To identify the molecular mechanisms by which NLRP4 targets TBK1 for degradation by viral infection, a cycloheximide-chase assay was performed to determine by the time course of TBK1 degradation after viral infection. 293T cells and THP-1 cells were treated with cycloheximide for 2 h after VSV-eGFP infection to block protein synthesis. We found that viral infection accelerated TBK1 degradation in both cell types (Figure 3-5a,b). Previous studies have shown that viral infection induces TBK1 K63-linked ubiquitination, which is important for activation of the type I IFN signaling pathway (13,14). In our case, TBK1 was ubiquitinated with K48 and K63 linkage after infection with VSV-eGFP (Figure3-5c). In investigate whether NLRP4 was required for TBK1 ubiquitination, we found more K48-linked ubiquitination of TBK1 in cells with coexpression of NLRP4 and TBK1, whereas the amount of K63-linked ubiquitination of TBK1 remained unchanged, compared to that in cells transfected with TBK1 alone (Figure 3-5d). Consistently, knockdown of NLRP4 resulted in much less K48-linked ubiquitination of TBK1, whereas the K63-linked polyubiquitination of TBK1 was not affected (Figure 3-5e). These results suggested that NLRP4 specifically induced K48-linked polyubiquitination of TBK1, thus facilitating its degradation after viral infection.

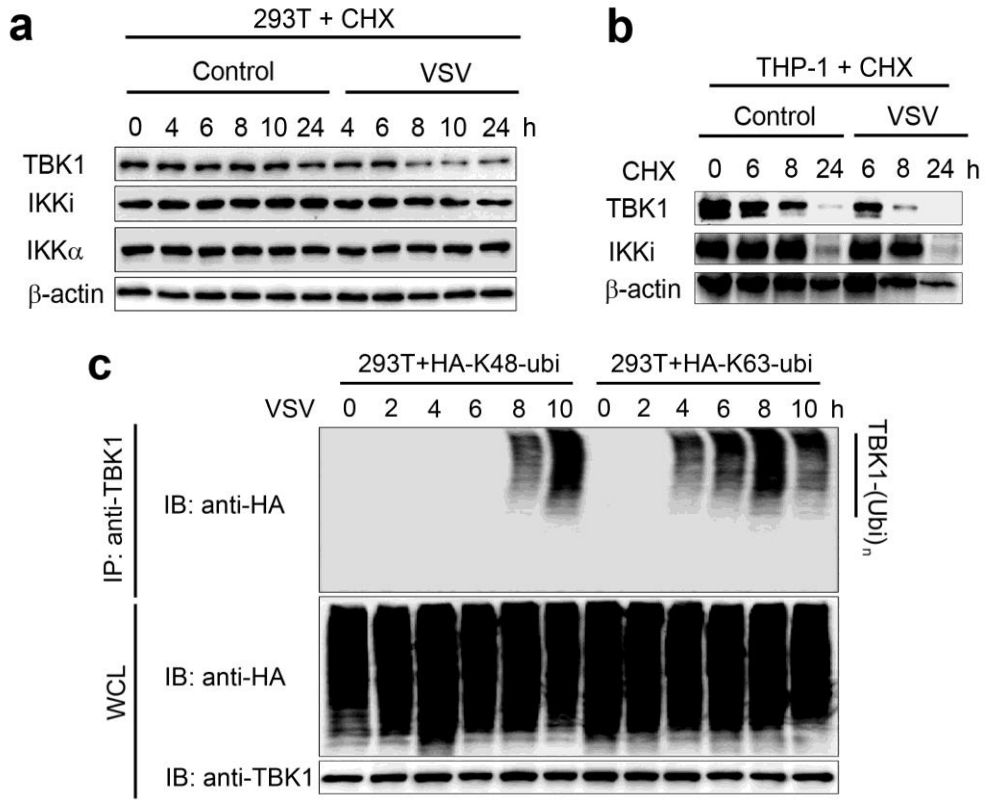


Figure 3-5. NLRP4 induces K48-linked polyubiquitination of TBK1 after viral infection

(a,b) Immunoblot analysis of extracts of 293T cells (a) or THP-1 cells (b) left uninfected (control (Ctrl)) or infected for 2 h with VSV-eGFP (VSV), then treated for various times (above lanes) with cycloheximide (CHX). (c) Immunoassay of lysates of 293T cells transfected with plasmid for HA-tagged K48-linked ubiquitin (HA–K48-ubi) or HA-tagged K63-linked ubiquitin (HA–K63-ubi) and infected with VSV-eGFP, followed by immunoprecipitation with anti-TBK1, probed with anti-HA. (d) Immunoassay of extracts of 293T cells transfected with various combinations of plasmid for Flag-TBK1, GFP-tagged NLRP4, or HA-tagged K48-linked or K63-linked ubiquitin, followed by immunoprecipitation with anti-Flag beads and immunoblot analysis with anti-HA. (e) Immunoassay of extracts of 293T cells transfected with plasmid for Flag-TBK1 and HA-tagged K48-linked or K63-linked, together with NLRP4-specific or scrambled siRNA, assessed as in d.

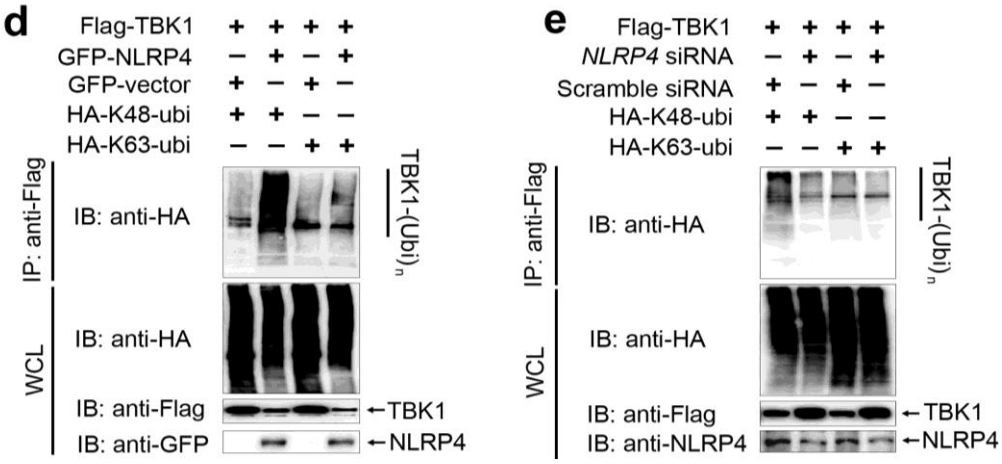


Figure 3-5 Continued.

3.2.6 Nod is required for NLRP4-mediated inhibition of type I interferon signaling

NLRP4 contains three conserved protein domains: a pyrin domain (PYD), nucleotide-binding-and-oligomerization domain (Nod) and a leucine-rich repeat (LRR) region. To identify which domain of NLRP4 is responsible for TBK1 ubiquitination and degradation, we generated three deletion mutants only containing the PYD, Nod or LRR domains of NLRP4, and assessed their ability to inhibit the TBK1-induced signaling pathway (Figure3-6a). Like full length NLRP4, we found that NLRP4 (NOD) inhibited the TBK1-induced activity of the IFN-β or ISRE luciferase reporter, but not other two deletion mutants (Figure 3-6b), which suggested that Nod of NLRP4 was responsible for the observed inhibition of TBK1 activity by NLRP4.We further found that NLRP4 (NOD) can interact with and cause degradation of TBK1(Figure 3-6c). In addition, NLRP4 (NOD) interacted with TBK1 kinase domain and enhanced the K48-linked ubiquitination of TBK1 (Figure 3-6d), which indicated that NLRP4 (NOD) is critical for the ubiquitination and degradation of TBK1.

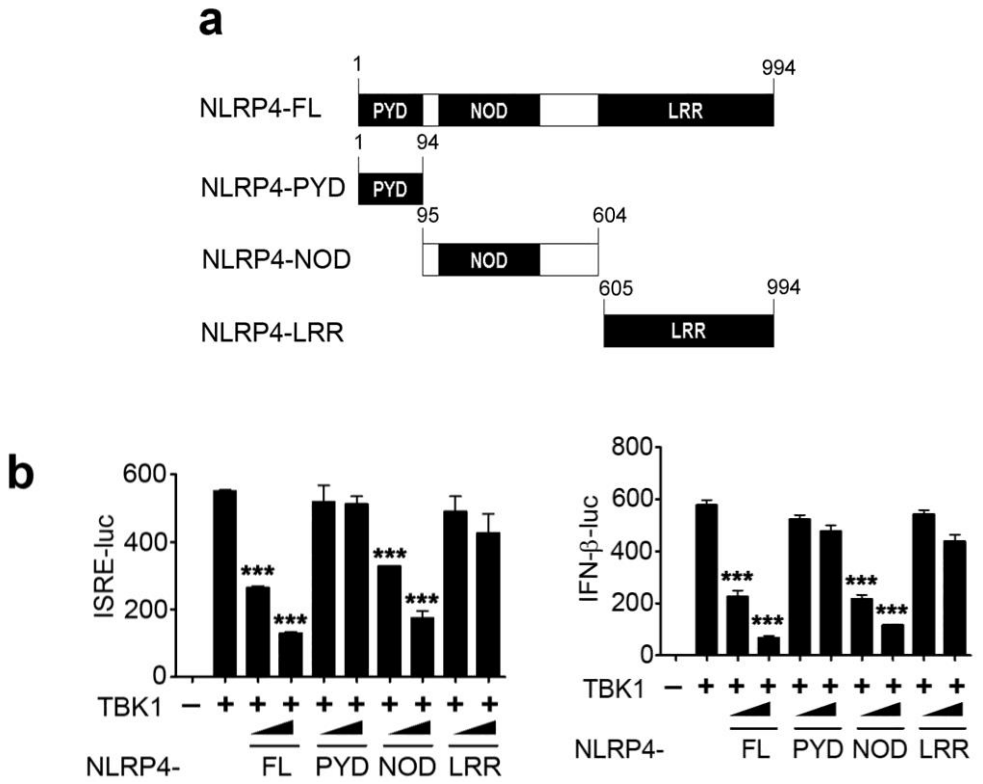


Figure 3-6. Nod is required for NLRP4-mediated inhibition of type I interferon signaling

(a) Constructs of full-length NLRP4 (NLRP4(FL)) or NLRP4 containing only PYD (NLRP4(PYD)), Nod (NLRP4(Nod) or the LRR domain (NLRP4(LRR)). (b) Luciferase activity of 293T cells transfected with expression vector for TBK1 and an ISRE or IFN-βluciferase reporter, together with empty vector (no wedge) or increasing concentrations (wedge) of vectors for the NLRP4 constructs in a. (c)Coimmunoprecipitation and immunoblot analysis of 293T cells transfected with various combinations (above lanes) of plasmid for Flag-TBK1 and the HA-tagged NLRP4 constructs in a. (d) Immunoassay of extracts of 293T cells transfected with plasmid for c-Myc-tagged TBK1, Flag-tagged NLRP4(Nod) and HA-tagged K48-linked or K63-linked ubiquitin, followed by immunoprecipitation with anti-c-Myc beads and immunoblot analysis with anti-HA.

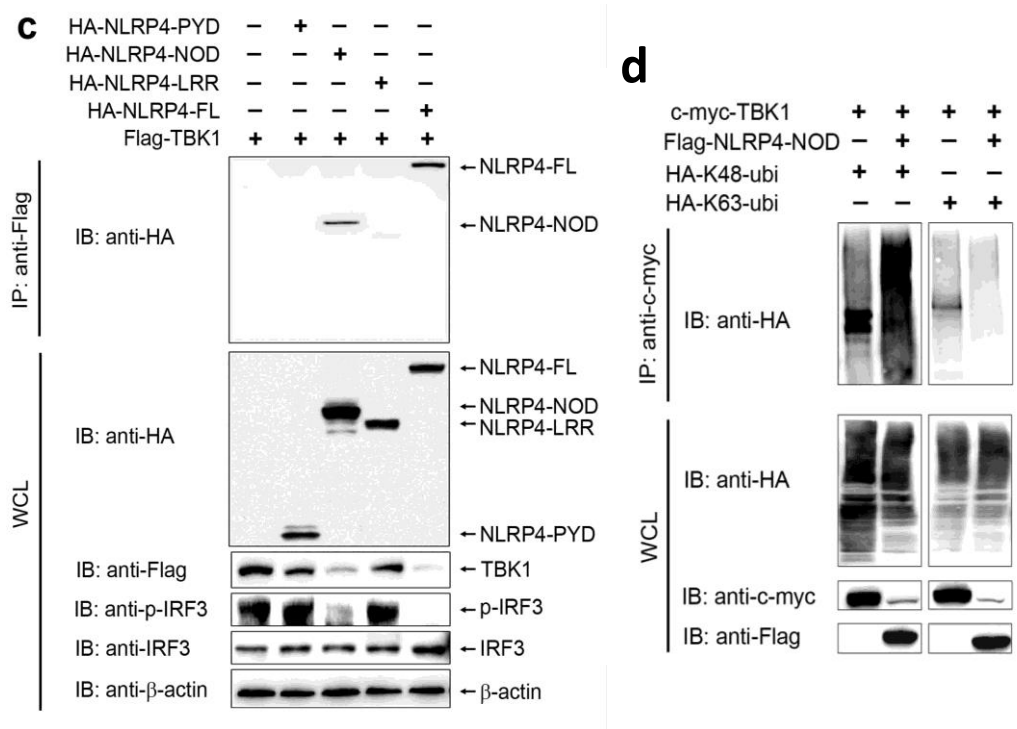


Figure 3-6 Continued.

3.2.7 DTX4 is an E3 ubiquitin ligase for TBK1 ubiquitination

To identify the E3 ubiquitin ligase(s) responsible for TBK1 ubiquitination, we designed a screen assay for the activity of the ISRE luciferase reporter. 293T cells were transfected to express TBK1 and the ISRE luciferase reporter, as well as shRNA constructs from a sub-library of shRNAs for human E3 ubiquitin ligases containing a RING domain (a ligase domain that promotes ubiquitination). Among an initial screening of about 900 shRNAs, we identified shRNA that targeted the E3 ubiquitin ligase DTX4, which resulted in much more activity of the ISRE luciferase reporter than control shRNA (Figure 3-7a). To demonstrate the involvement of DTX4 in type I interferon signaling, we first checked whether specific knockdown of DTX4 restored the TBK1-induced ISRE activation inhibited by NLRP4. Knockdown of endogenous DTX4 by four DTX4-specific shRNAs markedly abrogated the inhibition of TBK1-induced activity of ISRE reporter by NLRP4 (Figure 3-7b). Consistently, the degradation of TBK1 induced by NLRP4 was completely or partially blocked when DTX4 was knocked down (Figure 3-7b). We also found that DTX4 expression alone did not cause TBK1 degradation, but coexpression of DTX4 and NLRP4 resulted in more TBK1 degradation than did expression of NLRP4 alone(Figure 3-7c). These results demonstrated that DTX4 plays a critical role in the ubiquitination of TBK1 for degradation in a NLRP4-dependent manner.

To determine the sequence of events in the interaction among NLRP4, TBK1 and DTX4 under physiological conditions, 293T cells were transfected with Flag-DTX4 and then infected with VSV-eGFP. Cell lysates were collected at various time points and

followed by immunoprecipitation with anti-Flag (for DTX4), anti-TBK1 or anti-NLRP4. Immunoblot analysis showed that DTX4, TBK1 and NLRP4 did not interact in resting cells. We detected interaction between NLRP4 and DTX4 at 6 h; this increased by 8 h after infection. At 8 h after infection, we detected interaction of TBK1 with NLRP4 and DTX4 (Figure 3-7d). We also found that after knockdown of NLRP4, it inhibited the interaction between DTX4 and TBK1 induced by viral infection (Figure 3-7e). These results suggested that NLRP4 recruits DTX4 to interact with activated TBK1 after viral infection.

To investigate the role of endogenous NLRP4 and DTX4 in the ubiquitination of TBK1 during viral infection, 293T cells were transfected with control siRNA, NLRP4- or DTX4-specific siRNA, and treated with VSV-eGFP for various time points. We observed that K48-linked polyubiquitination of TBK1 (but not K63-linked TBK1 polyubiquitination) was remarkably abolished at 8 and 10 h after viral infection after knockdown of NLRP4 or DTX4, but not in cells transfected with control siRNA (Figure3-7f). Taken together, these data suggested that both NLRP4 and DTX4 were required for K48 ubiquitination of TBK1 and its degradation to inhibit the type I IFN signaling.

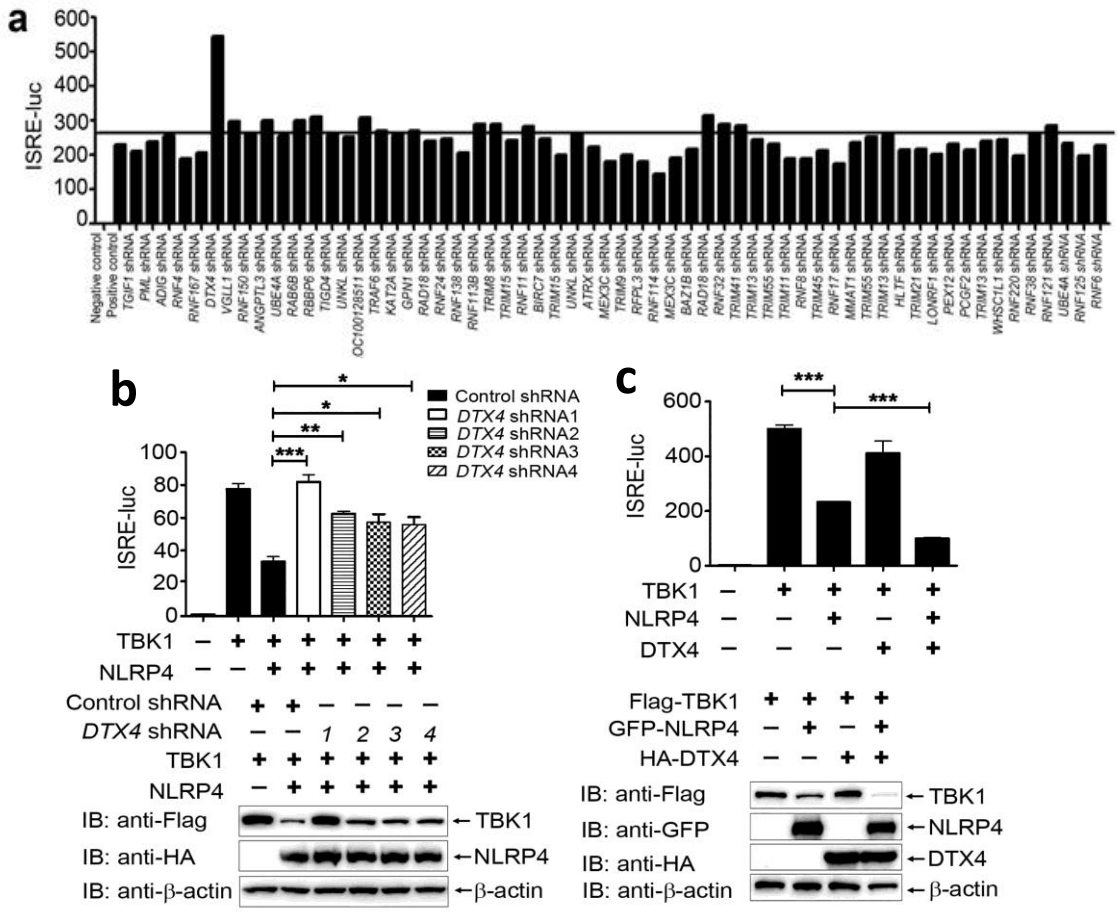


Figure 3-7. DTX4 is an E3 ubiquitin ligase for TBK1 ubiquitination

(a)HEK293 cells were transiently transfected with shRNA plasmids derived from a human RING domain-containing E3 ubiquitin ligase shRNA sub-library along with TBK1 and ISRE-luc reporter plasmid, followed by measurement of luciferase activity by a reporter assay. (b,c) Luciferase activity (top) and immunoblot analysis of TBK1 (below) in 293T cells transfected with various plasmids (below graph and above lanes) along with DTX4-specific or control shRNA (b) or various plasmids (c). (d) Immunoprecipitation and immunoblot analysis of extracts of 293T cells transfected with plasmid for Flag-DTX4 and infected for 0, 6 or 8 h (above lanes) with VSV-eGFP. (e) Immunoprecipitation (with anti-Flag) and immunoblot analysis of extracts of 293T cells transfected with plasmid for Flag-DTX4 and control or NLRP4-specific shRNA, then infected for 0, 6 or 8 h (above lanes) with VSV-eGFP. (f) Immunoassay of extracts of 293T cells transfected with scrambled NLRP4-specific or DTX4-specific siRNA and infected for 0, 8 or 10 h (above lanes) with VSV-eGFP, followed by immunoprecipitation with anti-TBK1 and immunoblot analysis with antibody to K48-linked or K63-linked ubiquitin.

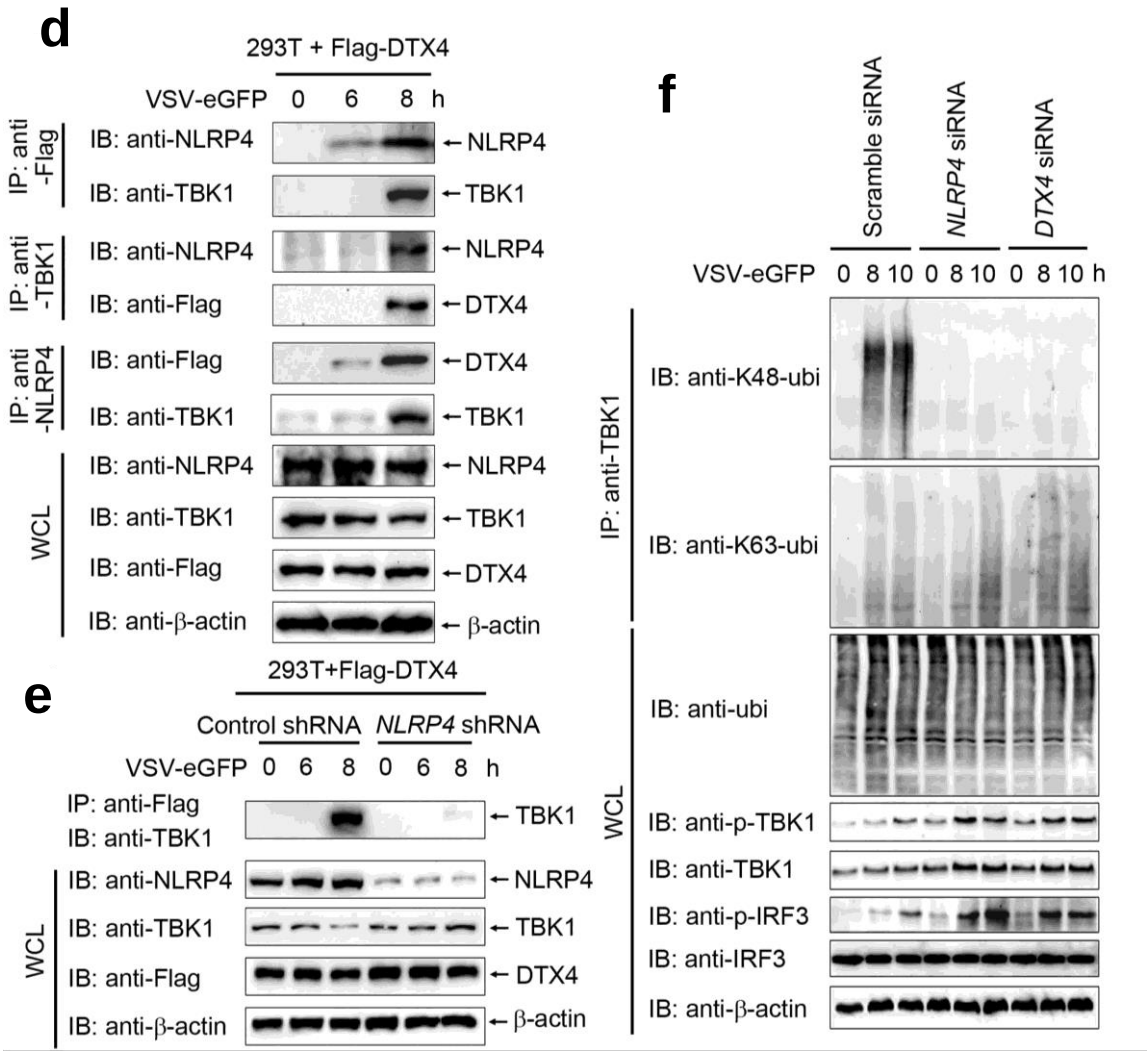


Figure 3-7 Continued.

3.2.8 TBK1 ubiquitination at Lys670 is essential for NLRP4-DTX4–mediated inhibition of type I interferon signaling

Although NLRP4 bound to the kinase domain of activated TBK1, we found that neither TBK1(KD) nor TBK1(KD+ULD) was ubiquitinated or degraded (Figure 3-8a), which suggested that the coiled-coil domain at the carboxyl terminus of TBK1 may be critical for ubiquitination. Using computed-assisted algorithms[21, 22], we identified three key ubiquitination sites in the coiled-coil domain of TBK1 and created K504R, K661R and K670R mutants of TBK1 after substituting Lys504, Lys661 and Lys670 with arginine respectively(Figure 3-8b). The result showed that the K670R TBK1 mutant almost completely blocked the degradation of TBK1, but not the K504R and K661R TBK1 mutants (Figure 3-8c). Consistently, there was no NLRP4-mediated K48-linked ubiquitination of the K670R TBK1 mutant (Figure3-8d). Although it enhanced the ISRE activation in cells transfected with express wild-type TBK1 or the K504R or K661R TBK1 mutant after knockdown of DTX4, we did not observe any effect of DTX4 knockdown on the activation of ISRE reporter in cells transfected to express the K670R TBK1 mutant (Figure 3-8e). Finally, we found that NLRP4 did not induce K48-linked ubiquitination of the S172A TBK1 mutant (Figure 3-8f), which indicated that phosphorylation of TBK1 was critical for NLRP4-mediated ubiquitination. Taken together, these results indicated that Lys670 in TBK1 was an essential residue for NLRP4-DTX4–mediated K48-linked ubiquitination and degradation of activated TBK1.

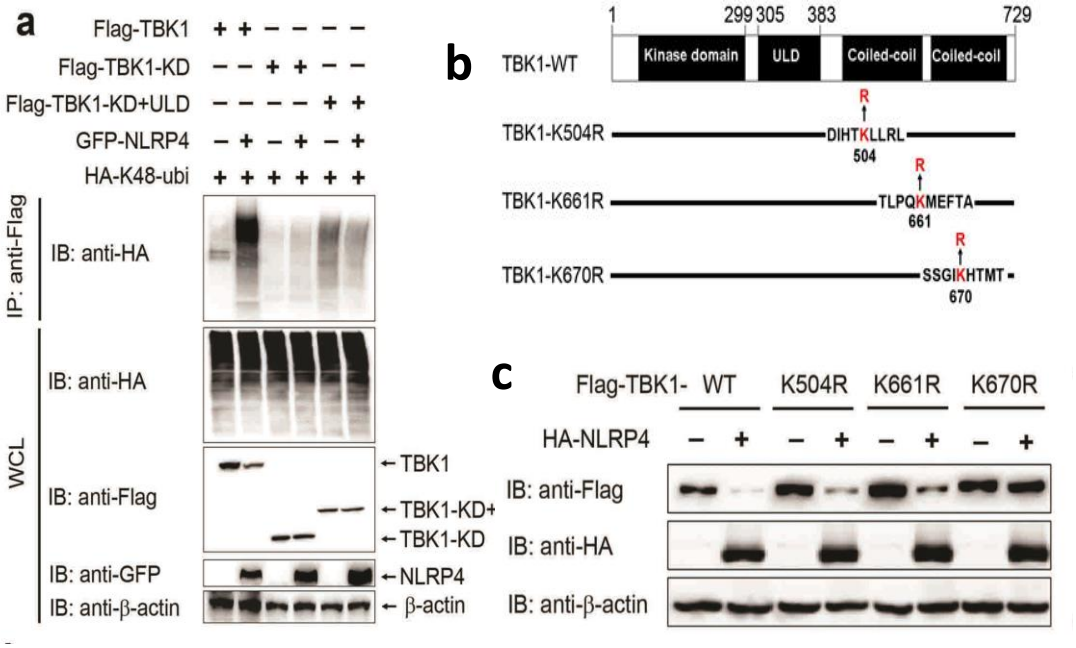


Figure 3-8. TBK1 ubiquitination at Lys670 is essential for NLRP4-DTX4–

mediated inhibition of type I interferon signaling

(a) Immunoassay of extracts of 293T cells transfected with various combinations (above lanes) of plasmid for GFP-tagged NLRP4 and HA-tagged K48-linked ubiquitin together with Flag-tagged TBK1 constructs. (b) Generation of TBK1 point mutations. (c) Immunoblot analysis of extracts of 293T cells transfected with empty vector or vector for HA-NLRP4, together with plasmid for Flag-tagged wild-type TBK1 or K504R, K661R or K670R mutant of TBK1 (top). (d) Immunoprecipitation and immunoblot analysis as in (a) of 293T cells transfected with various combinations (above lanes) of plasmid for GFP-tagged NLRP4 and HA-tagged K48-linked ubiquitin together with plasmid for Flag-tagged TBK1 constructs. (e) Luciferase activity of 293T cells transfected with plasmid for Flag-tagged TBK1 constructs and DTX4-specific or control shRNA, together with an ISRE luciferase reporter. (f)Immunoprecipitation and immunoblot analysis of 293T cells transfected with various combinations (above lanes) of vector for GFP-NLRP4, HA-tagged K48-linked ubiquitin and Flag-tagged wild-type TBK1 or the S172A mutant TBK1.

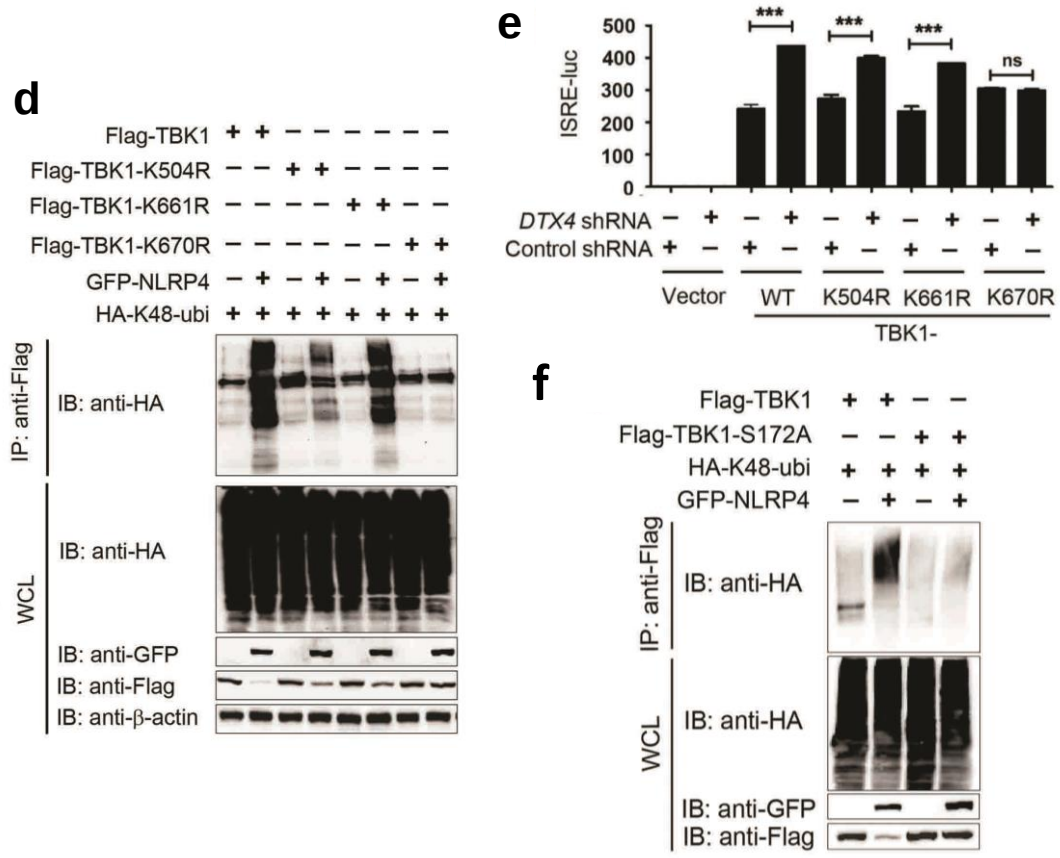


Figure 3-8 Continued.

3.2.9 NLRP4-DTX4 specifically inhibits TBK1-dependent type I IFN signaling

Most of the cell types use TBK1-IRF3–dependent type I interferon signaling pathways (for IFN-β production) with viral infection, however, plasmacytoid dendritic cells use MyD88-IRF7–dependent (TBK1-independent) type I interferon signaling pathways (for IFN-α production) with the dinucleotide CpG stimulation and viral infection. In macrophages, spatiotemporal regulation of MyD88–IRF7 signaling leads to robust production of IFN-α by liposomes containing CpG-A and DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate) but not CpG-A16. To investigate whether NLRP4-DTX4 inhibited type I interferon signaling through TBK1, but not MyD88-IRF7 pathway, we first found there's no interaction between NLRP4 and MyD88 (Figure 3-9a). Next PBMCs and THP-1 cells were transfected with NLRP4-specific siRNA, DTX4-specific siRNA, or scrambled siRNA, then cells were treated with VSV-eGFP, Sendai virus, poly(dA:dT), CpG-A–DOTAP or CpG-A. The result showed that VSV-eGFP, Sendai virus and poly(dA:dT) induced IRF3 phosphorylation, but CpG-A–DOTAP did not (Figure 3-9b). In contrast, it induced considerable IRF7 expression with CpG-A–DOTAP, VSV-eGFP and Sendai virus treatment, but not with poly(dA:dT) treatment (Figure3-9b). Moreover, knockdown of endogenous NLRP4 or DTX4 expression enhanced the phosphorylation of IRF3 with viral infection or poly(dA:dT) stimulation. We detected very little IRF7 expression level in resting PBMCs, but it induced much more IRF7 expression by infection with VSV-eGFP or Sendai virus after knockdown of NLRP4 or DTX4, whereas it induced high IRF7 expression with CpG-A–DOTAP treatment regardless of the status of NLRP4 or DTX4

(Figure 3-9b). These results indicated that NLRP4-DTX4 negatively regulated Sendai virus– and VSV-eGFP–stimulated TBK1-IRF3–dependent type I interferon signaling, which in turn induced the expression of IRF7. In contrast, it did not affect IRF7 expression after knockdown of NLRP4 or DTX4 in cells with CpG-A–DOTAP treatment. Indeed, in PBMCs, IRF7 mRNA expression was induced at 15 h after VSV-eGFP treatement. We observed that knockdown of NLRP4 resulted in higher IRF7 expression than that in cells transfected with scrambled siRNA (Figure 3-9c). However, there's no such differences in IRF7 expression in cells transfected with NLRP4-specific siRNA and treated with CpG-A–DOTAP relative to its expression in cells transfected with scrambled siRNA and treated with CpG-A–DOTAP (Figure 3-9c), which indicated that the expression of IRF7 induced by CpG-A–DOTAP was independent of NLRP4.

We next assessed the expression of IFN-α, IFN-β, IL-6 and IL-1β after various treatments by ELISA. After knockdown NLRP4 or DTX4 in PBMCs, there's much more secretion of IFN-α and IFN-β after VSV-eGFP or Sendai virus infection than that in cells transfected with scrambled siRNA (Figure 3-9d,e). It resulted in more production of IFN-β, but not of IFN-α after knockdown of NLRP4 or DTX4 after poly(dA:dT) treatment. In contrast, there's considerable IFN-α production and little IFN-β production in cells treated with CpG-A–DOTAP but there is no any difference between cells transfected with NLRP4- or DTX4-specific siRNA and those transfected with scrambled siRNA in their production of IFN-α and IFN-β (Figure 3-9d,e). As expected, CpG-A did not activate type I interferon signaling in PBMCs. And we obtained similar results with THP-1 cells (Figure 3-9f). Moreover, we found that it induced considerable production

of IL-6 and IL-1β in PBMCs with Sendai virus infection, but there was no appreciable difference between cells transfected with NLRP4 or DTX4 siRNA and those transfected with scrambled siRNA (Figure 3-9d,e). Our results suggested that NLRP4-DTX4 specifically inhibited TBK1-IRF3–dependent type I interferon signaling but not MyD88-IRF7–dependent type I interferon signaling.

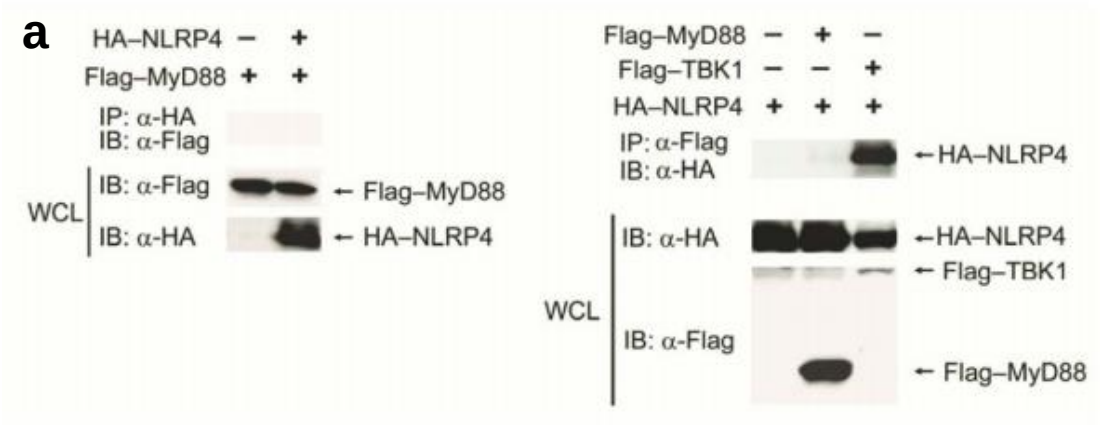
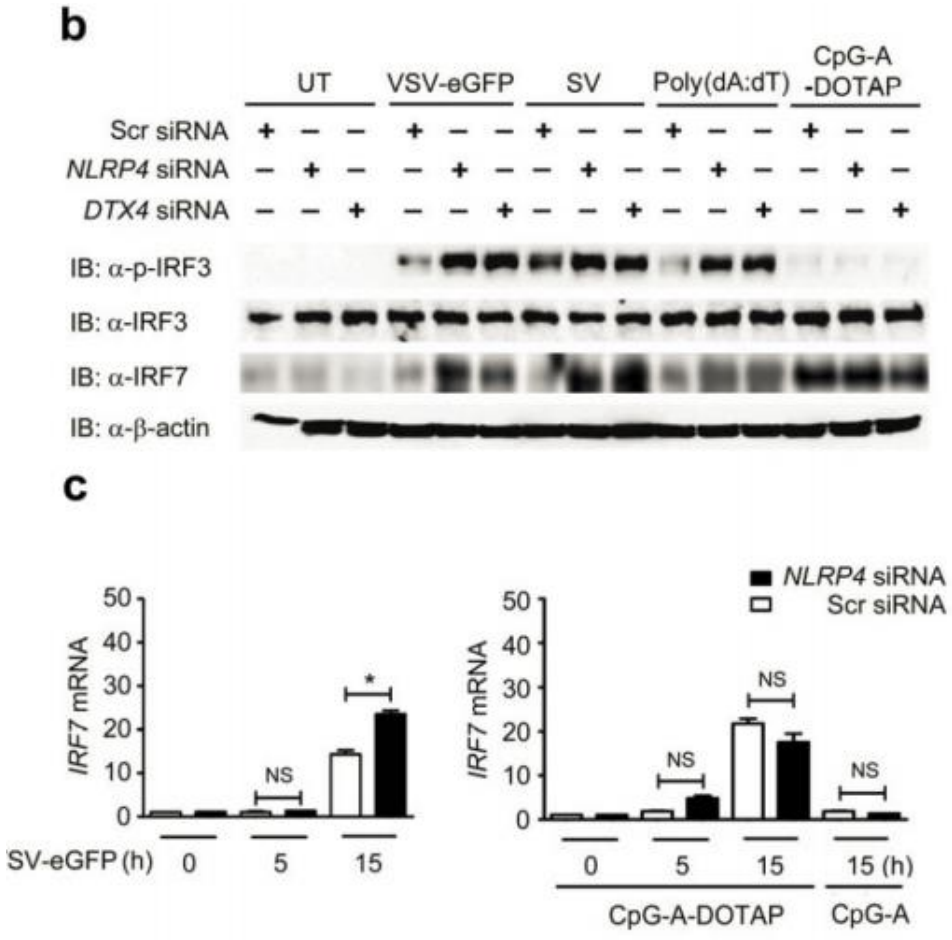


Figure 3-9. NLRP4-DTX4 specifically inhibits TBK1-dependent type I IFN signaling

(a) 293T cells were transfected with HA-NLRP4 together with Flag-TBK1, Flag-MyD88 or empty vector. After immunoprecipitation with anti-HA oranti-Flag beads, Flag-MyD88 or HA-NLRP4 was analyzed by immunoblot using anti-Flag or anti-HA. Results are representative of three independent experiments (b) PBMCs were transfected with NLRP4-specific siRNA, DTX4-specific siRNA or scrambled (scr) siRNA. After VSV-eGFP,Sendai virus (SV), ploy(dA:dT) or CpG-A-DOTAP treatment, cell extracts were harvested and analyzed by immunoblot with the indicated antibodies. UT, untreated. (c) PBMCs were transfected with NLRP4-specific siRNA or scrambled (scr) siRNA, followed by VSV-eGFP, CpG-A-DOTAP or CpG-A treatment and total RNA was collected at different time points (0, 5, 15 h) for real-time PCR analysis. (d-f) PBMCs or THP-1 cells were transfected with NLRP4-specific siRNA, DTX4-specific siRNA or scrambled (scr) siRNA, followed by VSV-eGFP, Sendai virus (SV), ploy(dA:dT) or CpG-A-DOTAP treatment. The concentrations of IFN-α, IFN-β, IL-6 and IL-1β were determined by ELISA.



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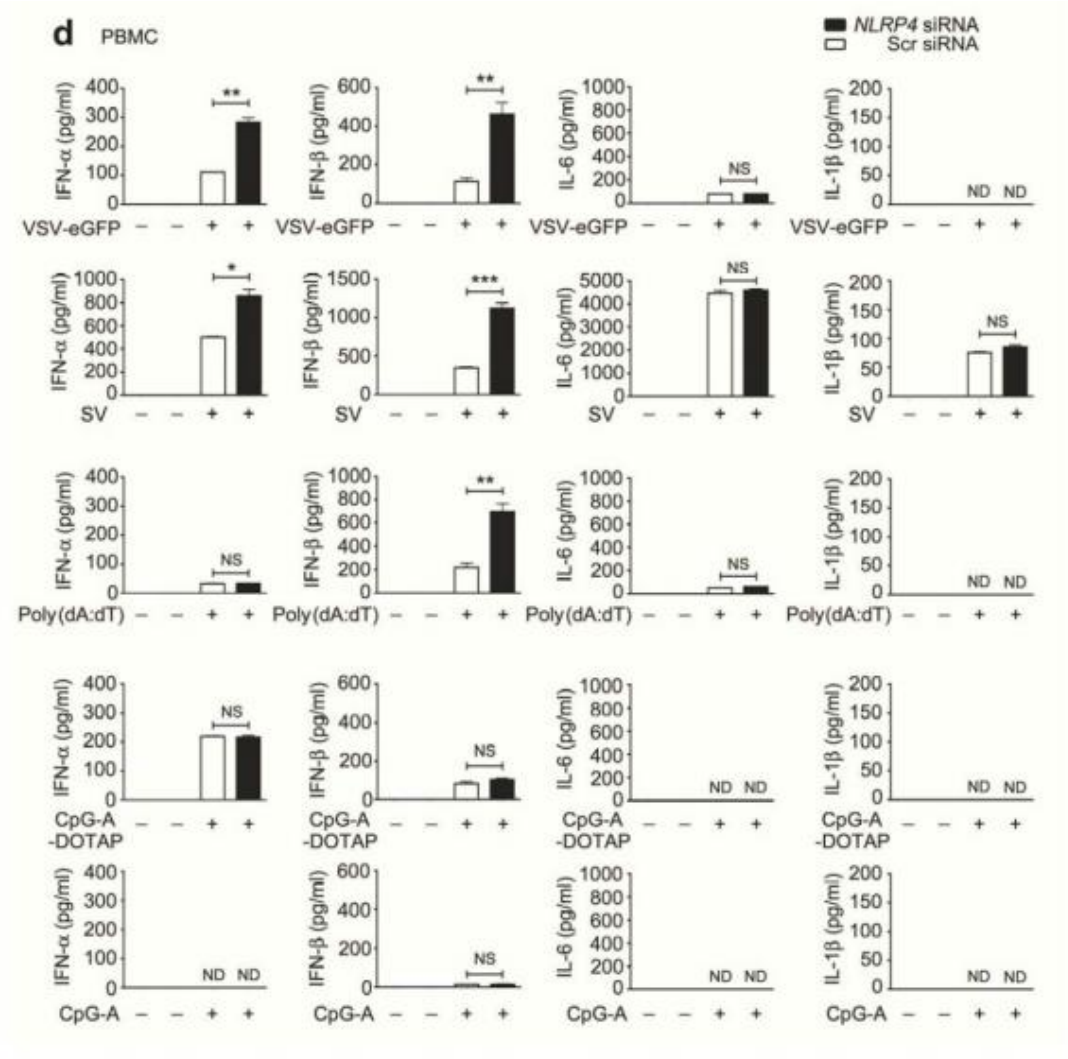

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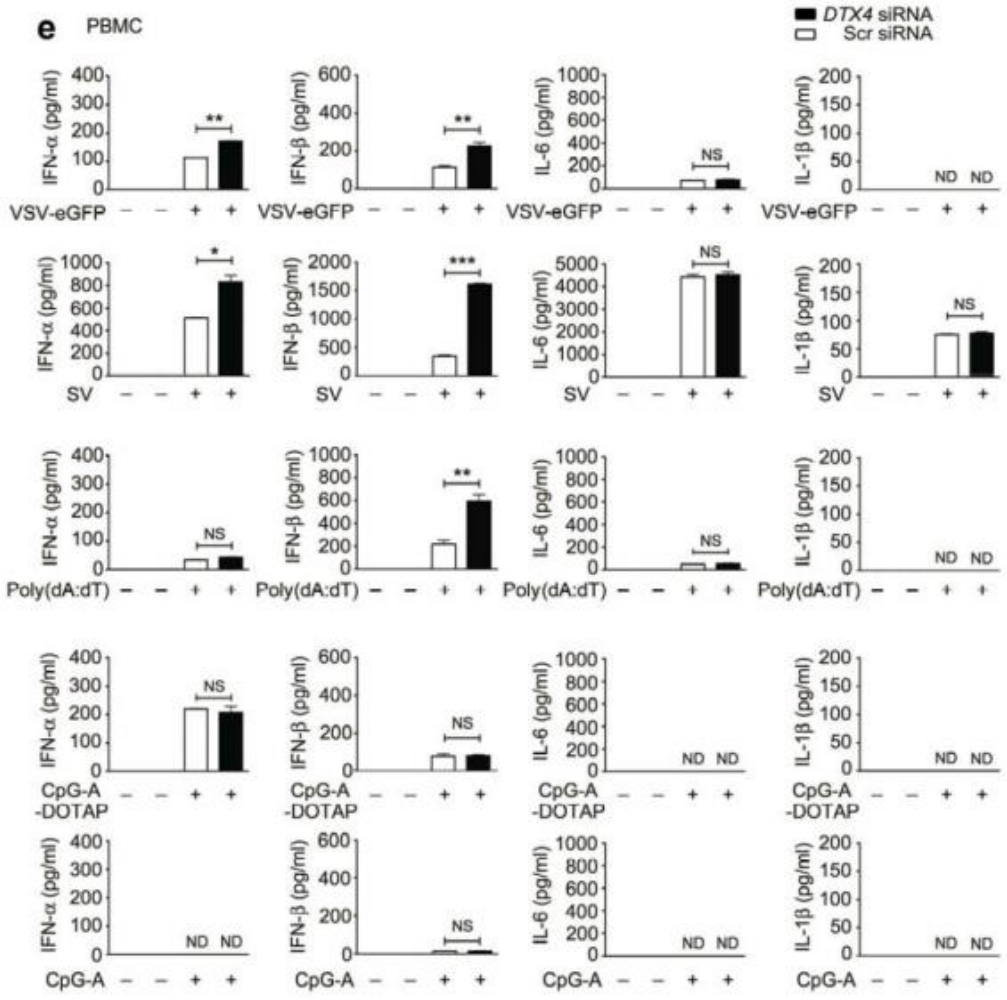

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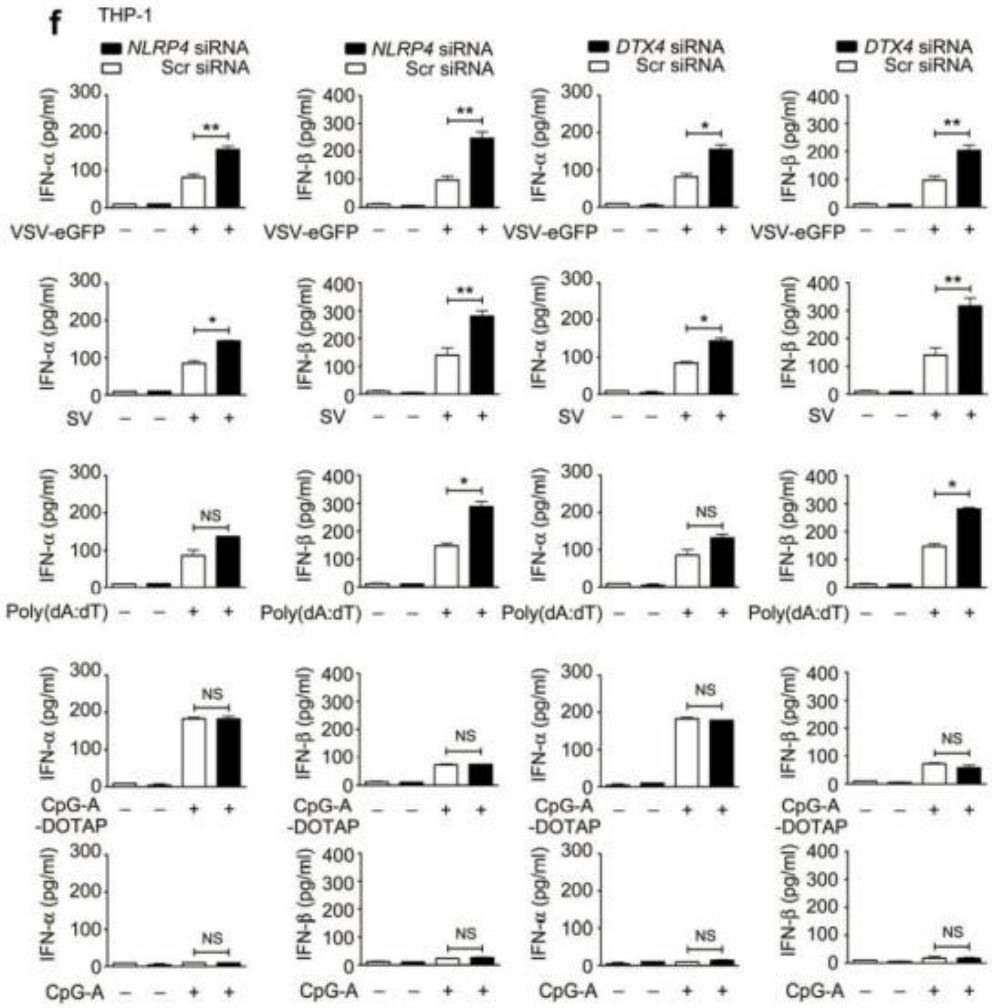


Figure 3-9 Continued.

3.3 Summary

In this study we have defined that NLRP4 plays a negative role in regulation of type I interferon signaling and have shown detail molecular mechanisms of NLRP4-mediatde activated TBK1 degradation through K48-linked ubiquitination via the E3 ubiquitin ligase DTX4. We found that ectopic expression of NLRP4 inhibited type I interferon signaling activated by ligand stimulation, whereas knockdown of NLRP4 enhanced type I interferon signaling and antiviral immune response. TBK1 is a key component of type I interferon signaling that is activated by various DNA and RNA sensors, which induce the phosphorylation of IRF3 and type I interferon–responsive gene expression as a converging point. Because aberrant production of type I interferon can have a role in immunopathology and autoimmune disorders, thus TBK1 activation must be tightly controlled. However, the mechanism by how activated TBK1 is inhibited remains poorly understood. Our findings have identified an unrecognized role for NLRP4 in the negative regulation of type I interferon signaling in which NLRP4 induce the degradation of TBK1 to maintain innate immune homeostasis in response to viral infection. NLRP4 enhanced Lys48 (K48)-linked polyubiquitination at Lys670 of TBK1 and caused degradation of TBK1 via the E3 ubiquitin ligase DTX4. Consistently, knockdown of either DTX4 or NLRP4 abolished degradation of TBK1 and type I IFN signaling. In conclusion, my thesis studies have identified a previously unrecognized role for NLRP4 in negative regulation of type I IFN signaling by targeting TBK1 for K48 polyubiquitination and degradation to keep the homeostasis of innate immune signaling and antiviral response (Figure 3-10).

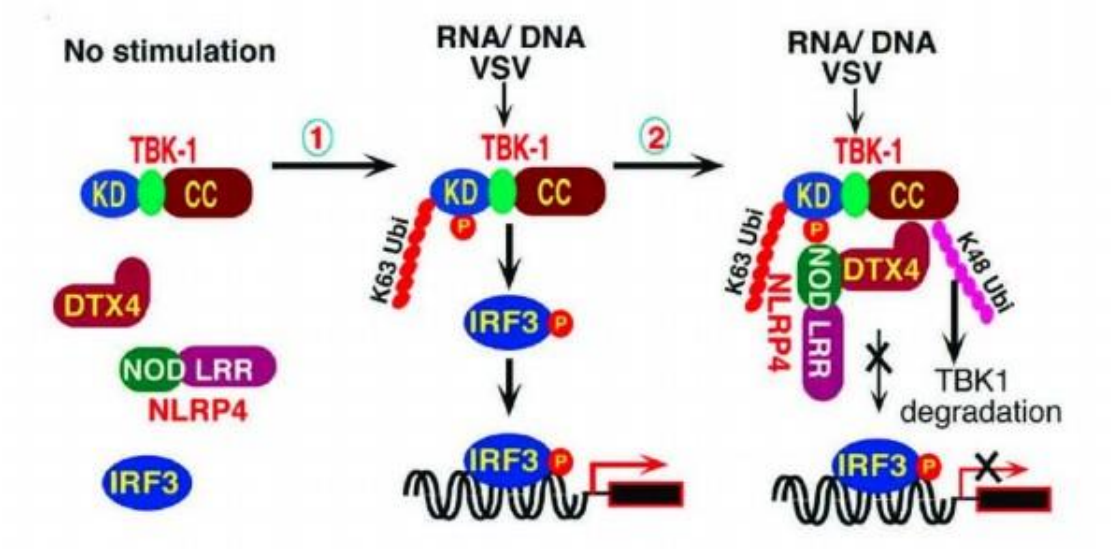


Figure 3-10. Proposed model indicating how NLRP4 negatively regulates type I IFN signaling pathways by degradation of activated TBK1 through DTX4

4. ATG13 POSTIVELY REGULATES TYPE I INTERFERON SIGNALING THROUGH BECLIN1

4.1 Introduction

Recent studies demonstrated that autophagy plays a key role in the innate and adaptive immune system by elimination of pathogens and the induction of acquired immune response. Several autophagy proteins have been identified as positive or negative regulators in innate immune signaling[103]. The autophagy process plays a direct antiviral role against the mammalian viral pathogen vesicular stomatitis virus (VSV) in the model organism Drosophila[103]. Moreover, autophagy activates type I IFN production by mediating ssRNA virus detection and interferon-a secretion in plasmacytoid dendritic cells[104]. In contrast, several other autophagy proteins play a negative role in RIG-I-like receptor mediated activation of type I IFN response. Atg5-Atg12 conjugate interacts with the CARD domains of RLR and MAVS to inhibit the production of type I IFN signaling pathway[105]. Consistently, knockout Atg5 enhances type I IFN production after VSV infection and dsRNA stimulation. Knockout Atg7, an essential requirement for the Atg5-Atg12 conjugate, also results in enhancement of type I IFN production after dsRNA treatment[105]. Another group shows that in Atg5-deficient cells, the damaged mitochondria accumulated because of the loss of autophagy, resulting in the elevated expression of MAVS which triggers ROS production to active the innate immune response. Moreover, it has been reported the negative regulation of autophagy protein Atg9a in the activation of STING which is required for the production of type I IFN and pro-inflammatory[98]. Although it has been reported that Atg13

interacts with Atg1 (ULK1/2), FIP200 and Atg101 as a complex, which plays an important role in the initiation stage of autophagy process, its role in the innate immune system remains unknown. Our studies showed that Atg13 positively regulates the ISRE-luc activation using cDNA screening assay. Therefore, we postulate that Atg13 may be involved in the regulation of the type I IFN signaling to maintain immune homeostasis during antiviral innate immunity. Our study will provide important insight into the understanding of the regulation and crosstalk of autophagy and antiviral immunity upon pathogen invasion.

4.2 Results

4.2.1 Atg13 positively regulates type I interferon signaling pathway

To identify possible roles of autophagy-related proteins in antiviral immunity, we used an ISRE-luciferase (ISRE-luc) assay to screen all of the Atg proteins for their ability to regulate ISRE activity. We transfected HEK293T cells with a ISRE-luc reporter, an internal control Renilla luciferase, with or without the candidate genes, and then treated them with intracellular poly (I:C) for 24 hours to trigger type I IFN signaling. Among them, we identified Atg13 as a positive regulator of ISRE-luc activation. Similar results were observed in 293T cells transfected with poly(dA:dT) or infected with VSV-eGFP (Figure4-1a). We got the similar results that Atg13 can enhance the IFN-β activity with luciferase assay (Figure4-1b). These results indicate that Atg13 positively regulates the type I IFN signaling after different stimulation. To further determine whether Atg13 positively regulates type I IFN pathway, we assessed the phosphorylation of IRF3 in 293T cells expressing myc-Atg13 or empty vector after treatment with intracellular

poly(I:C), poly(dA:dT) or infected with VSV-eGFP, and found that myc-Atg13 significantly enhance the phosphorylation of endogenous IRF3 (Figure 4-1c). To establish a link between reduced type I IFN response mediated by Atg13 and antiviral immunity, we cotransfected expression vector of Atg13 or empty vector in 293T cells, then infected the cells with VSV-eGFP (MOI = 0.01), and monitored viral infection based on GFP expression. Overexpression of Atg13 enhanced the antiviral response and resulted in considerably less GFP+ (virus-infected) cells than those transfected with empty vector at different time courses (Figure 4-1d). Flow cytometry analysis revealed that 68% of cells were infected (GFP+) in cells transfected with empty vector, compared to much less GFP+ cells transfected with myc-Atg13 24 h post infection(Figure 4-1e).These results suggested that ectopic expression of Atg13 positively regulates type I interferon response and thus enhances antiviral immunity.

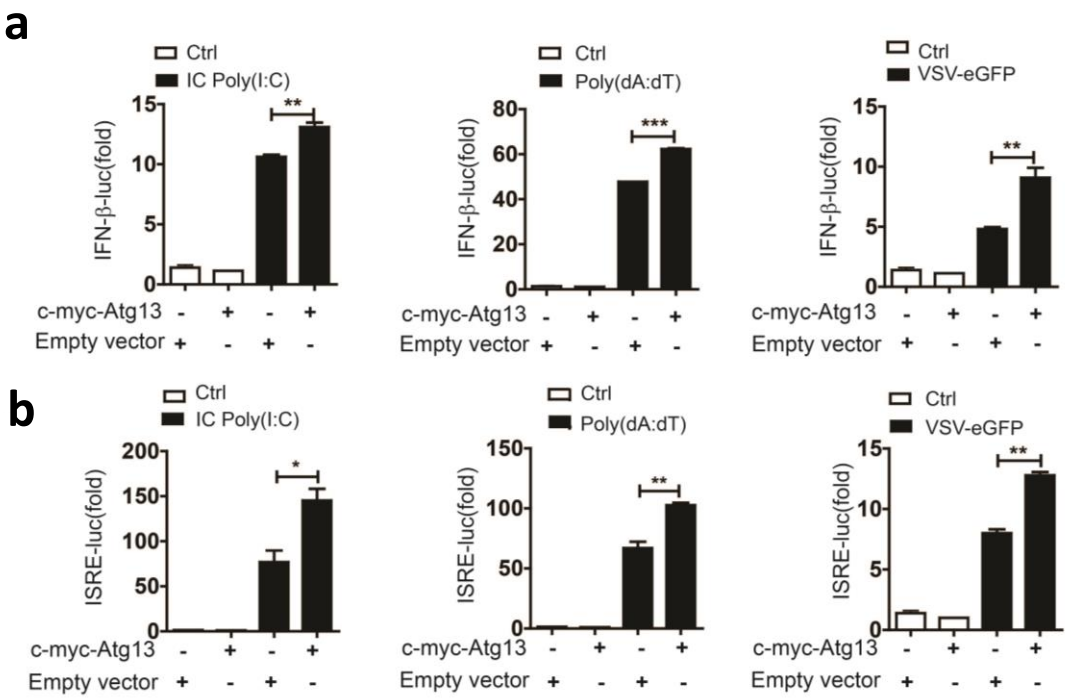


Figure 4-1. Atg13 positively regulates type I interferon signaling pathway.

(a). Luciferase activity in 293T cells transfected with plasmid encoding a luciferase reporter for IFN-β (IFN-β–luc) (a) or ISRE (ISRE-luc; 100 ng each) (b), together with empty vector or an expression vector for Atg13, followed by no treatment or treatment with intracellular (IC) poly(I:C) (1 μg/ml;), poly(dA:dT) (1 μg/ml;) or VSV-eGFP (MOI, 0.1). Data are presented relative to Renilla luciferase activity. (c) Immunoblot analysis of total and phosphorylated (p-) IRF3 in 293T cells transfected with empty vector or Myc-tagged Atg13 and followed by the same treatment. (d,e) Phase-contrast (PH) and fluorescence microscopy (d) and Flow cytometry analysis (e) of 293T cells transfected with empty vector or an expression vector of Atg13, and then infected with VSV-eGFP at an MOI of 0.01 at indicated time course.

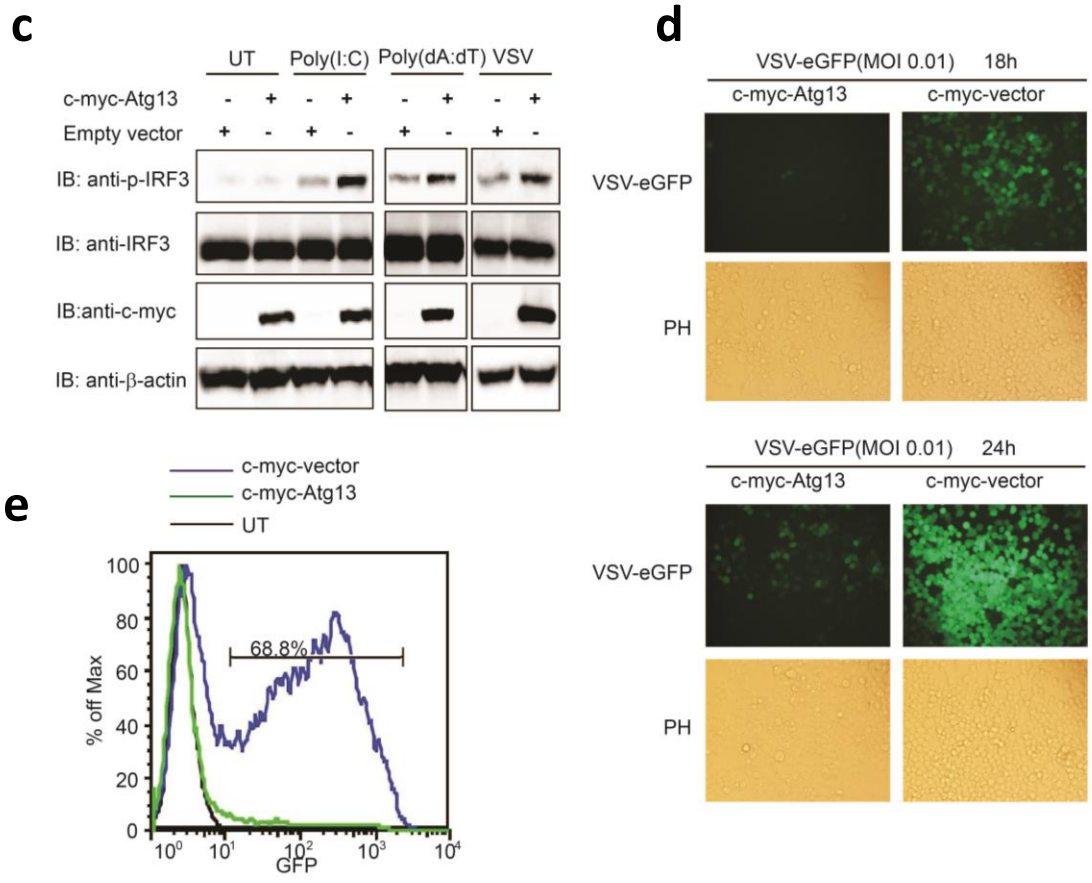


Figure 4-1 Continued.

4.2.2 Knockdown of Atg13 inhibits type I IFN signaling and antiviral responses

We next determined whether specific knockdown of endogenous Atg13 would inhibit the type I IFN signaling under physiological conditions. We selected three *Atg13*-specifc lentivirus short hairpin RNA (shRNA) constructs to knock down the expression of Atg13. All three efficiently inhibited the expression of transfected and endogenous Atg13 in 293T cells (Figure4-2a). We next assessed the effects of Atg13 knockdown on the activation of type I interferon. Using the IFNβ-luc or ISRE-luc reporter assay, we found that knockdown of Atg13 markedly decreased IFNβ-luc and ISRE-luc activity triggered by intracellular poly(I:C), poly (dA:dT) or VSV-eGFP in 293T cells(Figure4-2b). Further experiments showed that overexpression of myc-Atg13 could rescue this inhibition in knockdown cells (Figure4-2c). We next tested the effect of Atg13 knockdown on the phosphorylation of transcription factor IRF3. As shown in Figure4-2d, the phosphorylation of IRF3 (p-IRF3) in the Atg13 knockdown cells was decreased compared those in the control shRNA-transfected cells after intracellular poly(I:C), poly(dA:dT) treatment or the VSV infection, although the total amounts of IRF3 proteins were comparable between Atg13 knockdown and control cells. To further demonstrate the effects of Atg13 knockdown on the expression of interferon-responsive genes, we knocked down Atg13 in 293T cells and then treated the cells with poly(I:C) or poly(dA:dT); we found that knockdown of Atg13 resulted in less expression of *IFNB*, or interferon-stimulated cytokines, such as *IFIT1* and *IFIT2*, mRNA in cells after stimulation(Figure4-2e). Consistent with this observation, knockdown of Atg13 in THP-1 cells with *Atg13* specific siRNA also decreased the endogenous phosphorylation of

IRF3 and IFNβ protein secretion after different stimulation (Figure4-2f,g). These results indicated that Atg13 knockdown inhibited IFN-β activation and the expression of interferon-stimulating genes in different cell types. To further determine whether the inhibition of type I interferon response mediated by Atg13 knockdown is correlated with antiviral immunity, we knocked down Atg13 expression in 293T cells and then infected the cells with VSV-eGFP. Knockdown of Atg13 rendered the cells susceptible to viral infection and resulted in considerably much more GFP+ (virus infected) cells than those treated with control siRNA in 18 or 24 h post infection (Figure4-2h). Flow cytometry analysis revealed that 90% of cells were infected (GFP+) in cells transfected with *Atg13*-specific siRNA, compared to much less GFP+ cells transfected with control siRNA after 24h post infection (Figure4-2i). Taken together, these results suggested that Atg13 specific knockdown markedly inhibited the type I interferon response and antiviral immunity.

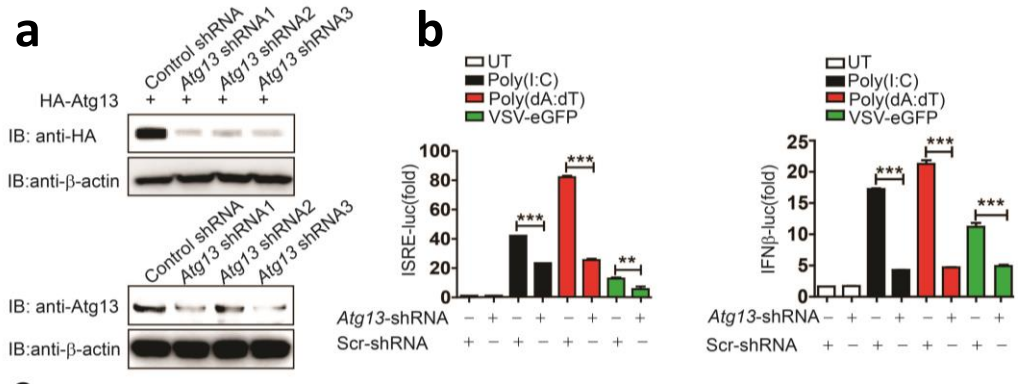
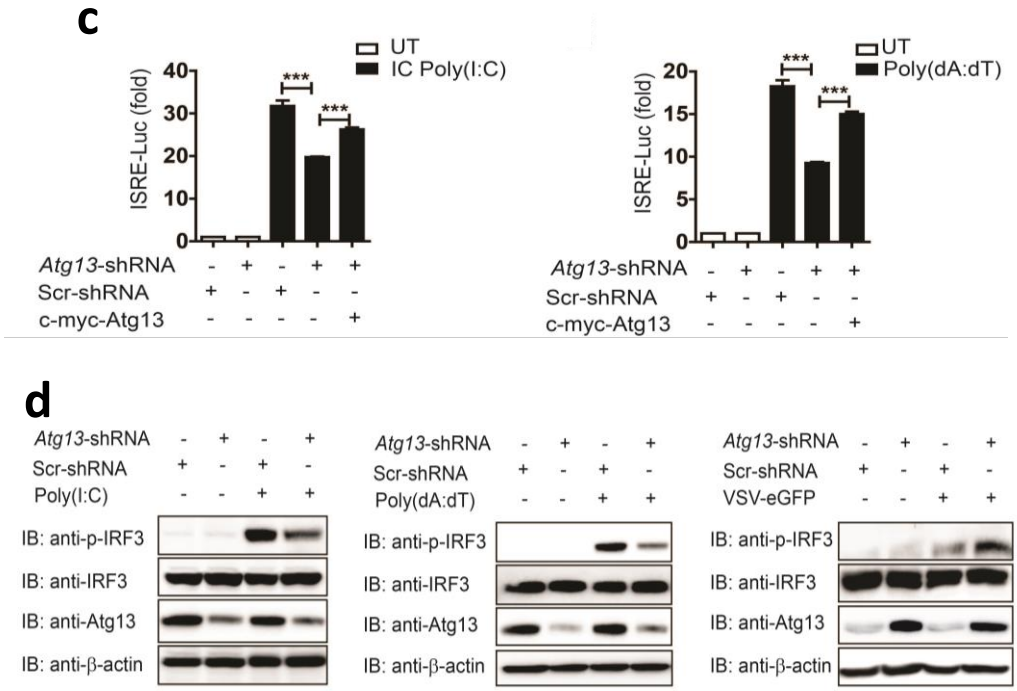


Figure 4-2. Knockdown of Atg13 inhibits type I IFN signaling and antiviral response

(a)Immunoblot analysis of the knockdown of exogenous Atg13 in 293T cells expressing HA-Atg13 (top) or endogenous (endo) Atg13 in 293T cells (bottom) treated with Atg13-specific shRNAs or control (Ctrl) shRNA. β-actin serves as a loading control throughout. (b)Luciferase activity in 293T cells transfected with Atg13-specific or ctrl shRNA, together with an ISRE or IFNβ luciferase reporter, then untreated (UT) or treated with intracellular poly(I:C), poly(dA:dT) or VSV-eGFP. (c) Luciferase activity in 293T cells transfected with Atg13-specific or ctrl shRNA, together with an ISRE luciferase reporter, with or without myc-Atg13, then untreated (UT) or treated with intracellular poly(I:C), poly(dA:dT). (d) Immunoblot analysis of total and phosphorylated (p-) IRF3 in 293T cells transfected with control or Atg13 specific shRNA and followed by treatment with intracellular poly(I:C), poly(dA:dT) or infection with VSV-eGFP. (e) Real-time PCR analysis of IFNB, IFIT1 and IFIT2 mRNA in 293T cells treated with Atg13-specific or control shRNA, followed by poly(I:C) and poly(dA:dT). (f) Immunoblot analysis of total and phosphorylated (p-) IRF3 in THP-1 cells transfected with control or Atg13 specific siRNA and followed by treatment with intracellular poly(I:C), poly(dA:dT) or infection with VSV-eGFP. (g) Enzyme-linked immunosorbent assay of IFN-β protein in THP-1 cells treated with Atg13-specific or control siRNA, followed by poly(I:C) treatment or VSV-eGFP infection. (h,i) Phase-contrast (PH) and fluorescence microscopy (h) and flow cytometry (i) assessing the infection of 293T cells left untreated or treated with Atg13-specific or control siRNA, and then infected with VSV-eGFP at an MOI of 0.001. Original magnification (h), ×10. Numbers above bracketed lines (i) indicate the percentage of cells expressing eGFP (infected cells).



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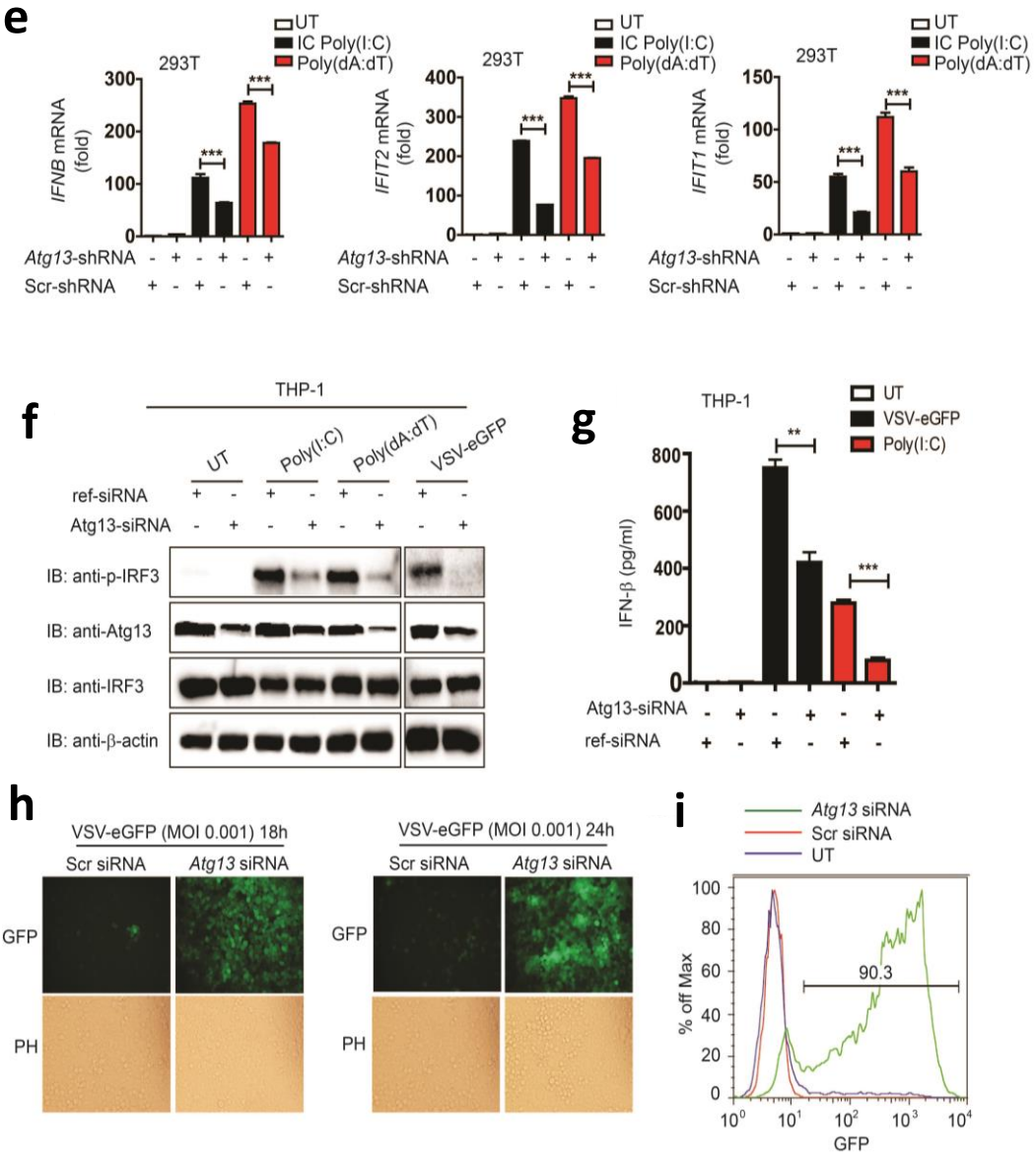


Figure4-2 Continued.

4.2.3 Atg13 physiologically enhances type I IFN signaling in primary cells

To further demonstrate the physiological role of Atg13 in primary cells, we knocked down Atg13 in PBMCs with *Atg13*-specific siRNA, and then treated with poly(I:C), poly(dA:dT) or VSV-eGFP. The RT-PCR results showed that it markedly decreased mRNA abundance of *IFNB*, *IFIT1* and *IFIT2* in the knockdown cells compared those in the control siRNA-transfected cells after intracellular poly(I:C), poly(dA:dT) treatment or the VSV infection(Figure4-3a). Moreover, the expression of IFN-β protein in cells transfected with *Atg13*-specific siRNA is much lower than in those transfected with control siRNA (Figure4-3b). We next tested the function of Atg13 in mice primary cells. Mouse peritoneal macrophages were transfected with *Atg13*-specific siRNA or control siRNA, and followed with VSV-eGFP treatment. We found that knockdown of Atg13 resulted in the decreased p-IRF3 expression and the mRNA and protein level of mouse *IFNB* after VSV-eGFP infection (Figure4-3c,d,e).The similar results were obtained from bone marrow derived dendritic cells(Figure4-3c,d,e). These results suggested that Atg13 played the conserved physiological role in type I IFN signaling in both human and mouse, and in various cell types as well.

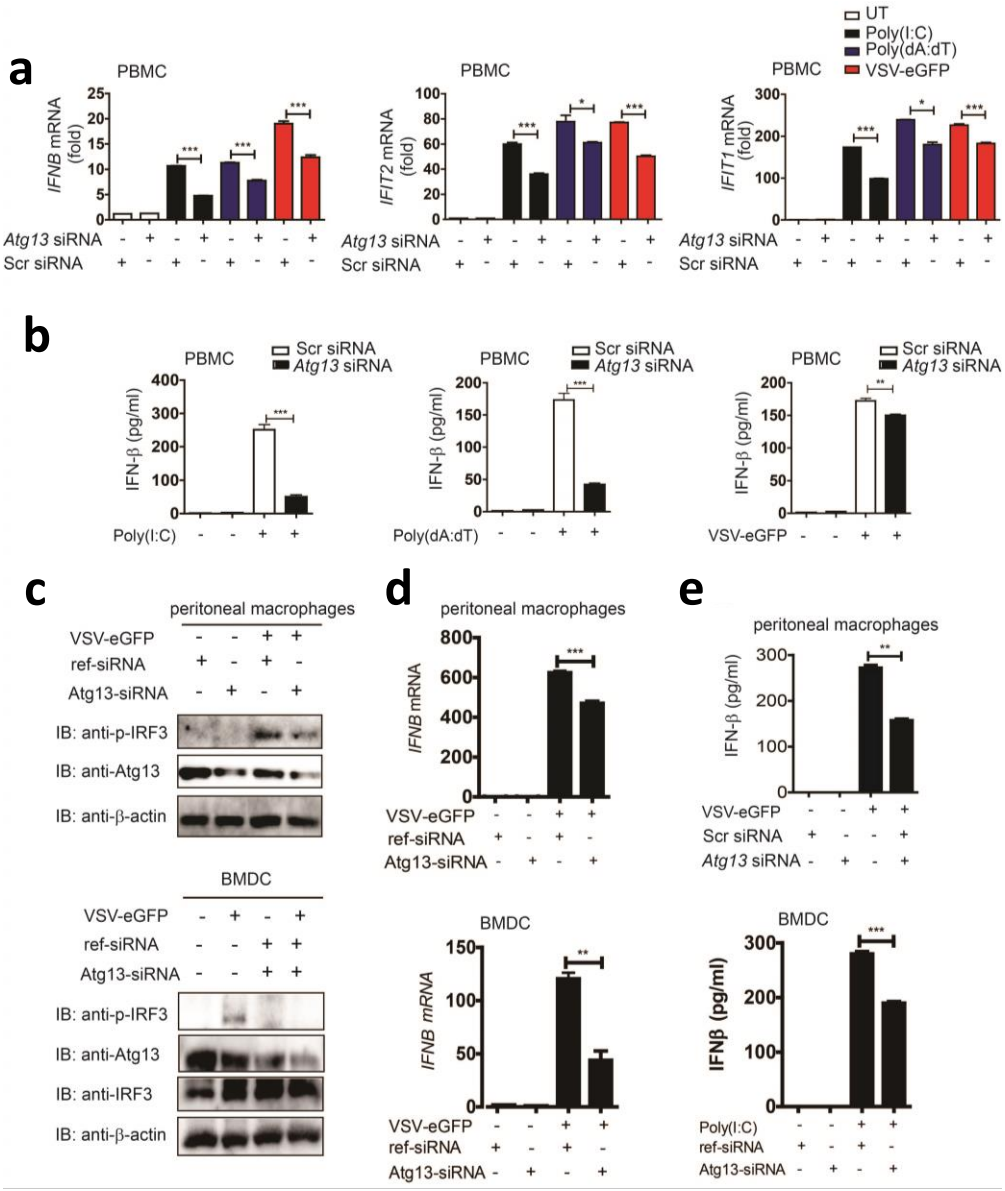


Figure 4-3. Atg13 positively regulates type I IFN in primary cells

(a)Real-time PCR analysis of *IFNB*, *IFIT1* and *IFIT2* mRNA in PBMCs cells treated with *Atg13*-specific or control siRNA, followed by the different treatment. (b) Enzyme-linked immunosorbent assay of IFN-β protein in PBMCs treated with *Atg13*-specific or control siRNA, followed by the same treatment set as in (a). (c,d,e) Immunoblot analysis of total and phosphorylated (p-) IRF3(c), Real-time PCR analysis of *Atg13* and *IFNB* mRNA (d) and Enzyme-linked immunosorbent assay of IFN-β protein (e) in peritoneal macrophages or bone marrow dendritic cells treated with *Atg13*-specific or control siRNA, followed by VSV-eGFP treatment.

4.2.4 Atg13 interacts with RIG-I after ligand stimulation

Since Atg13 can potently activate ISRE luciferase reporter activation triggered by different stimuli, we next sought to investigate the detail mechanism by which Atg13 enhances type I IFN signaling. To determine the molecular targets for Atg13, 293T cells were cotransfected with ref-shRNA and *Atg13*-shRNA, together with Flag-tagged RIG-I, MDA5, MAVS, and IRF3 plus with ISRE luciferase plasmid. Surprisingly, we found that knockdown of Atg13 had no effect on the ISRE activity induced by any of these molecular protein (Figure 4-4a), which indicated Atg13 may play important role in the upstream signaling pathway. To test whether Atg13 interacts with RIG-I-like receptors, 293T cells were transfected with myc-Atg13, together with Flag-RIG-I or Flag-MDA5, and then were treated with intracellular poly(I:C). Coimmunoprecipitation and immunoblot showed that the interaction between Atg13 and RIG-I became much more stronger after poly(I:C) treatment compared those in the unstimulated cells (Figure4-4b), but showed weak interaction between Atg13 and MDA5 (Figure 4-4c). To determine the endogenous interaction under the physiological conditions, THP-1 cells were treated with intracellular poly(I:C) at different time points. After immunoprecipitation by anti-Atg13 antibody, immunoblot was performed to check the expression level of RIG-I and MDA5. The result showed that Atg13 did not interact with either RIG-I or MDA5 in the rest cells. However, Atg13 strongly interacted with RIG-I after ligand stimulation, but not MDA5 (Figure 4-4d). To further access whether Atg13 interacts with RIG-I in primary cells, we freshly isolated PBMCs, and treated them with poly(I:C) or VSV-eGFP. We found that Atg13 interacted with RIG-I, but not with MDA5 after stimulation (Figure

4-4e). We obtained the similar results in mouse peritoneal macrophages after viral infection (Figure 4-4f). Taken together, these results suggested that Atg13 interacted with the activated form of RIG-I after stimulation. To address these possibility, we generated three deletion mutants of RIG-I containing various combinations of the RIG-I domains (Figure 4-4g). Among them, we found that Atg13 only interacted with RIG-I mutant without repressor domain (RD), whereas RIG-I mutant containing only CARD domain or Helicase plus RD showed no interaction with Atg13 (Figure 4-4g). These results indicated that Atg13 bound to CARD plus Helicase domain of RIG-I. To further confirm that the activation of RIG-I is essential to the interaction with Atg13, we generated K172R mutant of RIG-I, which could eliminate the majority of K63-linked ubiquitination and cause the inactivation of RIG-I. We cotransfected 293T cells with myc-tagged Atg13 and Flag-tagged RIG-I-WT or Flag-tagged RIG-I-K172R, and then treated the cells with intracellular poly(I:C). As we expected that Atg13 strongly interacted with wild type RIG-I after stimulation. However there's no interaction between Atg13 and RIG-I-K172R mutant even after poly(I:C) stimulation (Figure 4-4h). We got the similar result that Atg13 did not interact with RIG-I-CARD+HD-K172R (Figure 4-4i). Furthermore, to detect the colocalization between Atg13 and RIG-I, we transfected Hela cells with dsRed-RIG-I and GFP-Atg13, and then treated them with intracellular poly(I:C). Under the fluorescence microscopy, we observed that Atg13 partially co-localized with RIG-I after stimulation (Figure 4-4j). Taken together, these results indicated that Atg13 interacted with RIG-I after ligand stimulation to enhance the type I IFN signaling.

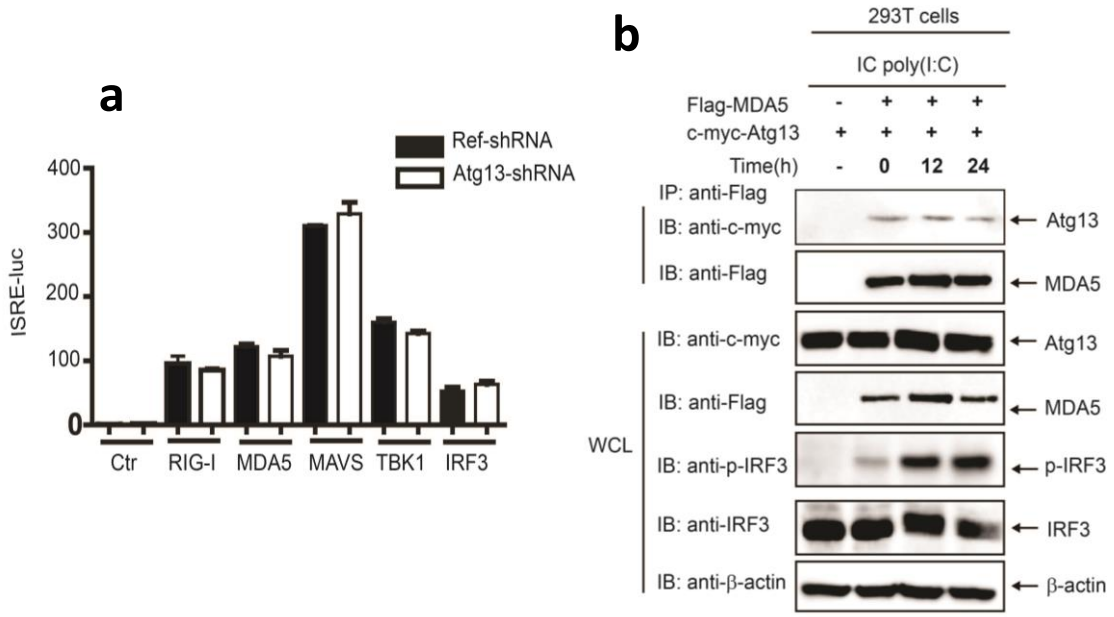
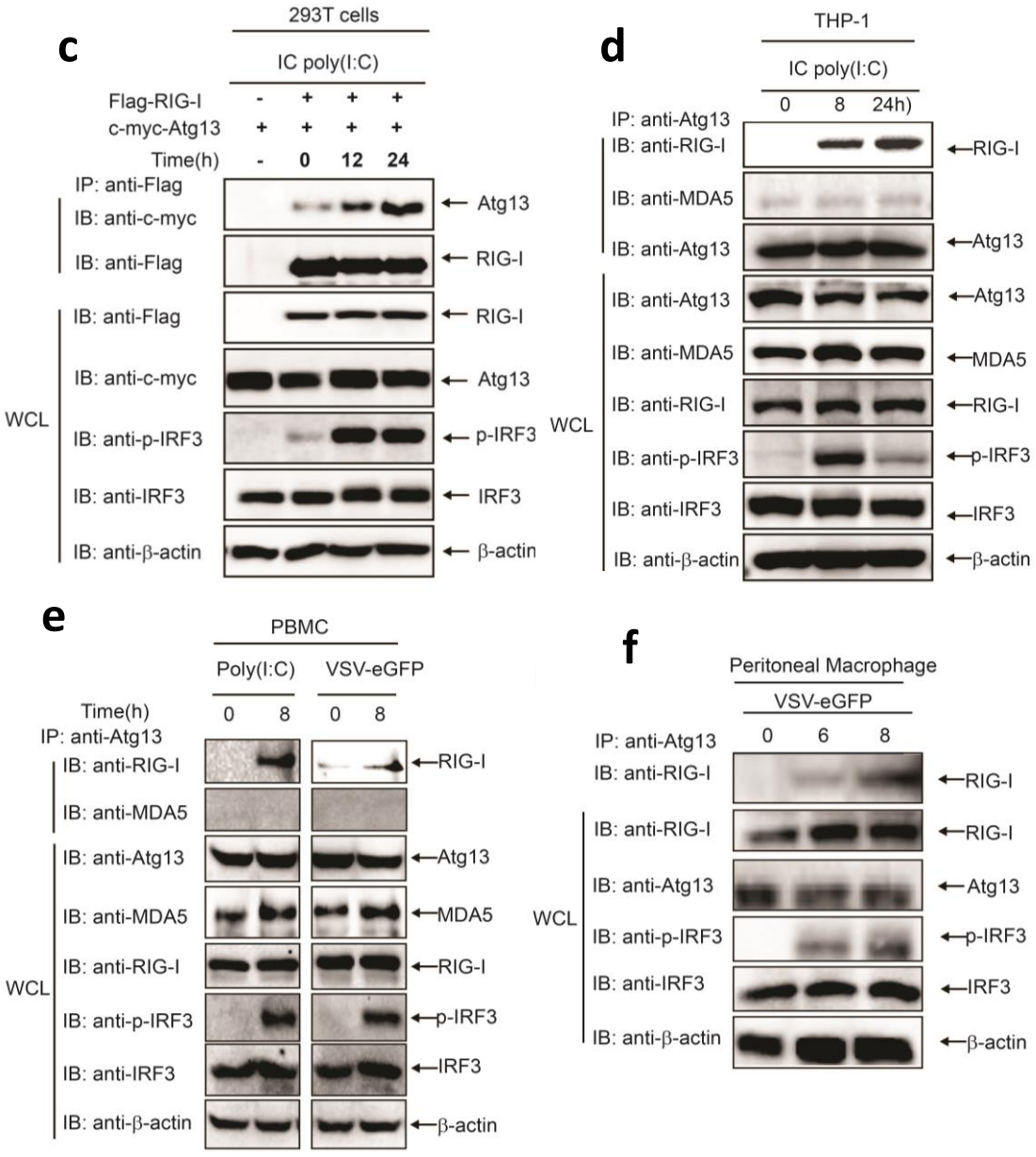


Figure 4-4. Atg13 interacts with RIG-I after ligand stimulation

(a) Luciferase activity in 293T cells transfected with ref-shRNA or Atg13-shRNA together with Flag-RIG-I, MDA5, MAVS, TBK1, IRF3, plus with plasmid encoding a luciferase reporter for ISRE (ISRE-luc; 100 ng each). (b,c)Immunoassay of 293T cells transfected with vector for c-myc-Atg13 and Flag-RIG-I (b), Flag-MDA5(c), followed by the treatment with intracellular poly(I:C) and immunoprecipitation (IP) with anti-Flag beads and immunoblot analysis with anti-c-myc. WCL, immunoblot analysis of whole-cell lysates without immunoprecipitation (throughout). (d) Immunoassay of extracts of THP-1 cells infected for various times (above lanes) with VSV-eGFP or treated with IC poly(I:C) followed by immunoprecipitation with anti-Atg13 and immunoblot analysis (antibodies, left margin).(e,f) Immunoassay of extracts of PBMCs (e) or mouse peritoneal macrophages (f) infected for various times (above lanes) with VSV-eGFP or treated with IC poly(I:C) followed by immunoprecipitation with anti-Atg13 and immunoblot analysis (antibodies, left margin). (g) Coimmunoprecipitation and immunoblot analysis of 293T cells transfected with various combinations of plasmids for c-myc-Atg13 and different Flag tagged RIG-I truncates (above panel).(h) Immunoassay of lysates of 293T cells transfected with plasmids for Flag-RIG-I-WT or Flag-RIG-I-K172R and c-myc-Atg13, then treated with poly(I:C), followed by immunoprecipitation with anti-Flag, probed with anti-c-myc. (i) Immunoassay of lysates of 293T cells transfected with plasmid for Flag-RIG-I-CARD+HD-WT or Flag-RIG-I-CARD+HD-K172R and c-myc-Atg13, then treated with poly(I:C), followed by immunoprecipitation with anti-Flag, probed with anti-c-myc. (j)Immunofluorensence colocalization between RIG-I and Atg13 in Hela cells transfected with Ds-Red-RIG-I and GFP-Atg13 and treated with poly(I:C).



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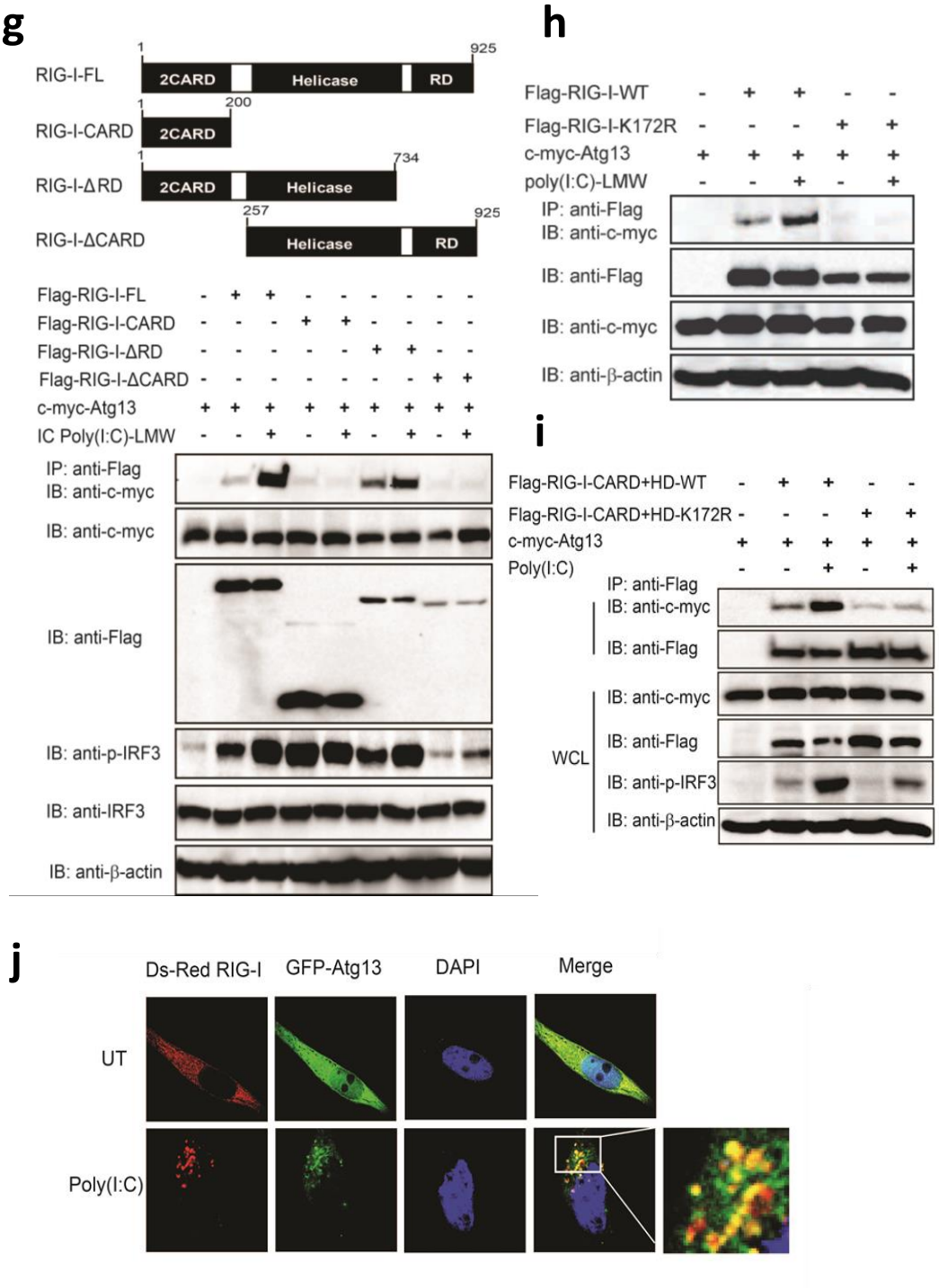


Figure 4-4 Continued.

4.2.5 The initiation stage of autophagy process is critical for type I IFN signaling regulated by Atg13

To investigate whether autophagy induction can enhance the type I IFN signaling, THP-1 cells were cultured under starvation for 16 hours, followed by VSV-eGFP infection. Immunoblot results showed that starvation can enhance the p-IRF3 expression level (Figure 4-5a). We got the similar results in RAW 264.7 cells (Figure 4-5b), which indicated that autophagy induction can enhance the type I IFN signaling after stimulation. It has been reported that Spautin-1 functions as an autophage inhibitor to promote the degradation of Vps34 PI3 kinase complexes in the initiation stage of autophay process, and chloroquine (CQ) inhibits autophagy in the late stagy as it raises the lysosomal pH, leading to inhibition of both formation of autolysosome and lysosomal protein degradation. To check which stage of autophagy is important to the type I IFN signaling, THP-1 cells were treated with Spautin-1 or CQ for 6 hours under starvation condition, followed with VSV-eGFP infection. Western blotting results showed that Spautin-1 treatment, but not CQ, can abolish the enhancement of p-IRF3 induced by starvation (Figure 4-5c). To establish a link between reduced type I IFN response mediated by starvation and antiviral immunity, we treated RAW 264.7 cells with DMSO, Spautin-1 as well as CQ under starvation, then infected cells with VSV-eGFP (MOI = 0.1), and monitored viral infection based on GFP expression. As we expected, starvation treatment enhanced the antiviral response and resulted in considerably less GFP+ (virus-infected) cells than those cultured under normal condition, while the Spautin-1 treatment abolished the antiviral response, but not CQ (Figure 4-5d).Furthermore, Atg5 WT and

KO MEF cells were cultured under starvation condition, followed with VSV-eGFP infection. We found it enhanced the antiviral response in Atg5 KO MEFs after starvation treatment (Figure 4-5e), which suggested that the induction of autophagy is sufficient to enhance the type I IFN signaling.

To investigate whether the function of Atg13 in type I IFN signaling is dependent on the autophagy, we transfected 293T cells with ISRE-luc, TK-luc as well as myc-Atg13 or control vector. After 6 hours treatment with DMSO, Spautin-1, CQ respectively, cells were transfected with poly(I:C), poly(dA:dT) or infected with VSV-eGFP followed by luciferase assay. The results showed that overexpression of Atg13 enhanced the ISRE activity, while these enhancement were abolished after treatment with spautin-1 but not CQ (Figure 4-5f), which suggested that the function of Atg13 may be dependent on the initial stage of autophagy. To check whether Atg13 functions properly in Atg5 deficiency cells, we transfected *Atg5*-/- MEF cells with ISRE-luc, TK-luc, together with or without mouse Atg13, and luciferase assay was performed after poly(I:C) stimulation or VSV-eGFP infection. The results showed that Atg13 still can enhance the ISRE activity in *Atg5*-/- MEF cells (Figure 4-5g). Consistently, knockdown of Atg13 in *Atg5*-/- MEF cells remarkably decreased the IFNβ mRNA expression after VSV infection (Figure 4-5h,i). Taken together, these results suggested that Atg13 can function properly in type I IFN signaling pathway depend on the initial stage, but not the late stage of autophagy. To further check whether spautin-1 can abolish the interaction between Atg13 and RIG-I, 293T cells were cotransfected with Flag tagged RIG-I and myc-tagged Atg13. After treatment with Spautin-1 or CQ for 6 hours, cells were then transfected with poly(I:C).

Coimmunoprecipitation and immunoblot analysis was performed to check the interaction between Atg13 and RIG-I. As we expected that Atg13 strongly interacted with RIG-I after stimulation in the cells treated with DMSO, while the interaction became much weaker after spautin-1 treatment, but not CQ (Figure 4-5j). We did further experiments in THP-1 cells to check endogenous interaction after spautin-1 or CQ treatment. The results showed that after spautin-1 treatment, it can inhibit the endogenous interaction between Atg13 and RIG-I after stimulation (Figure 4-5k), which indicated the initiation stage of autophagy process is critical for type I IFN signaling regulated by Atg13.

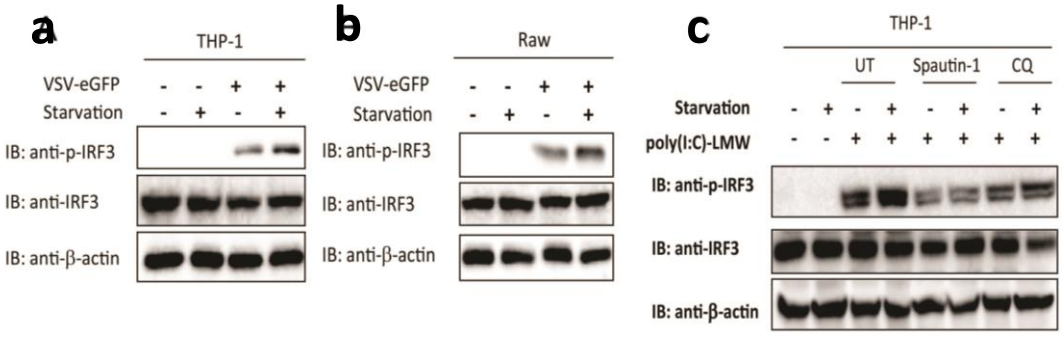
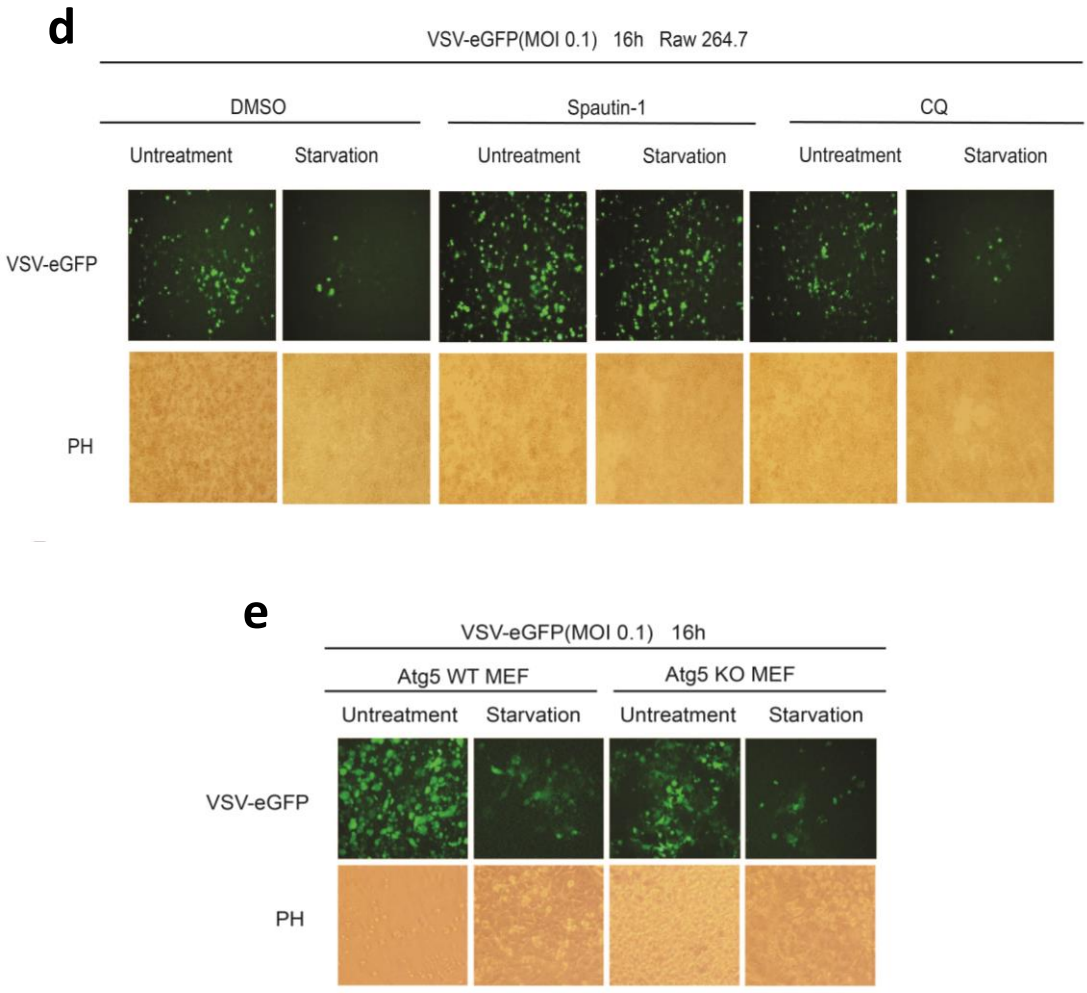


Figure 4-5. The initiation stage of autophagy process is critical for type I IFN signaling regulated by Atg13

(a,b) Immunoblot analysis of total and phosphorylated (p-) IRF3 in THP-1 cells(a) or RAW 264.7 cells (b) cultured under normal or starvation condition and followed by infection with VSV-eGFP.(c) Immunoblot analysis of total and phosphorylated (p-) IRF3 in THP-1 cells treated with Spautin-1 or CQ(6 hours) under normal or starvation condition and followed by infection with VSV-eGFP.(d) Phase-contrast (PH) and fluorescence microscopy (h) in the infection of RAW 246.7 cells treated with or without Spautin1 and CQ under normal or starvation condition , and then infected with VSV-eGFP at an MOI of 0.1.(e) Phase-contrast (PH) and fluorescence microscopy (h) in the infection of Atg5 WT and KO cells under normal or starvation condition , and then infected with VSV-eGFP at an MOI of 0.1 (f) Luciferase activity in 293T cells transfected with plasmid encoding a luciferase reporter for ISRE (ISRE-luc; 100 ng each), together with empty vector or an expression vector for Atg13. Cells were treated with DMSO, Spautin-1, CQ, followed by no treatment or treatment with intracellular (IC) poly(I:C) (1 μg/ml;) or VSV-eGFP (MOI, 0.1). (g) Luciferase activity in Atg5 KO cells transfected with mouse Atg13, together with an ISRE luciferase reporter, then untreated (UT) or treated with intracellular poly(I:C), or VSV-eGFP.(h,i) Real-time PCR analysis of mouse *Atg13* and *IFNB* mRNA in Atg5 KO MEFs treated with Atg13-specific or control shRNA, followed by VSV-eGFP infection. (j) Immunoassay of 293T cells transfected with vector for c-myc-Atg13 and Flag-RIG-I. Cells were treated with DMSO, Spautin-1, CQ, followed by the treatment with intracellular Poly(I:C) and immunoprecipitation (IP) with anti-Flag beads and immunoblot analysis with anti-c-myc. WCL, immunoblot analysis of whole-cell lysates without immunoprecipitation (throughout).(k) Immunoassay of THP-1 cells treated with DMSO, Spautin-1, CQ, followed by the treatment with intracellular Poly(I:C)-LMW and immunoprecipitation (IP) with anti-Atg13 and immunoblot analysis with anti-RIG-I. WCL, immunoblot analysis of whole cell lysates without immunoprecipitation (throughout).



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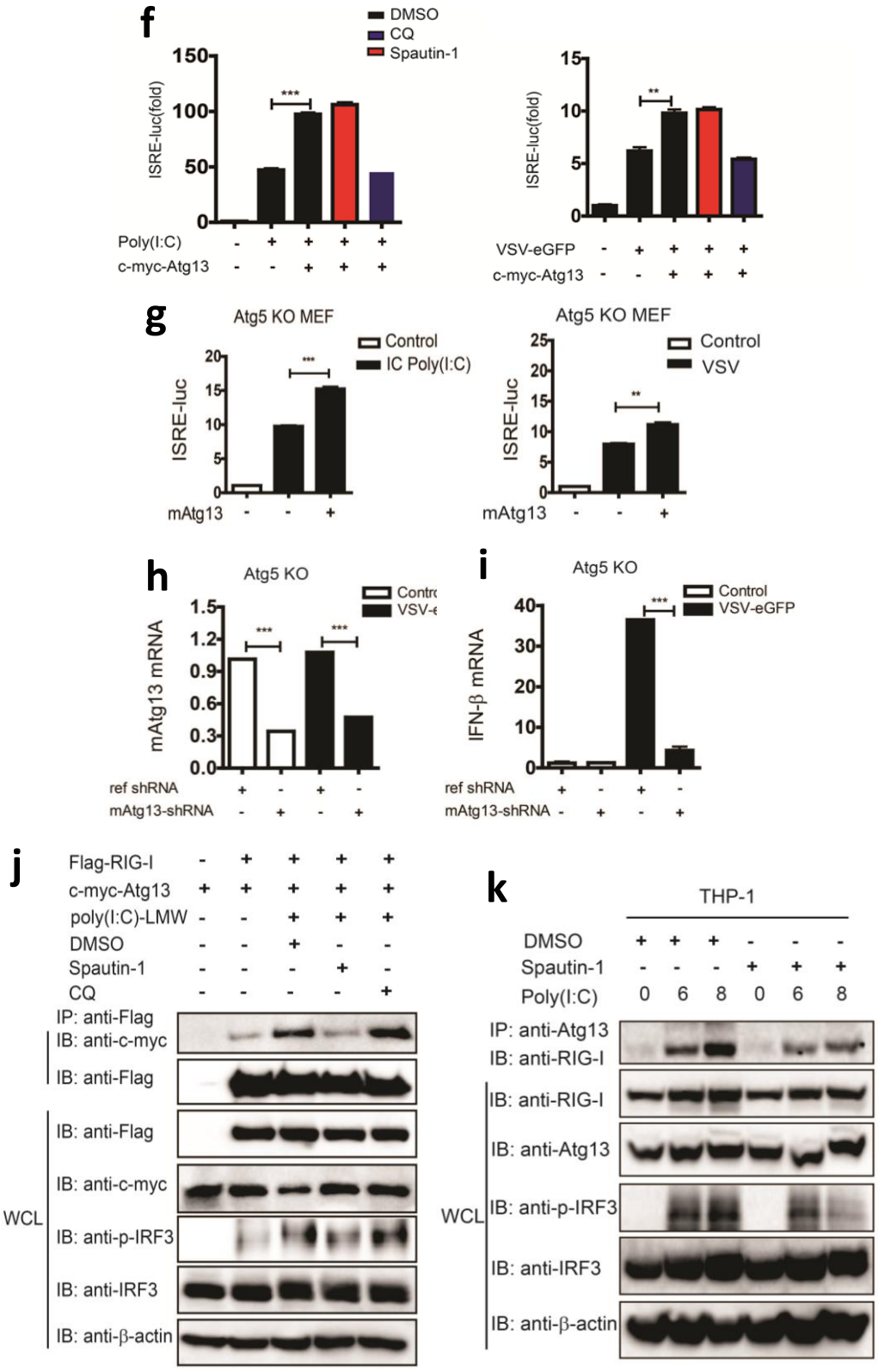

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4.2.6 Atg13 positively regulates type I IFN signaling through Beclin1

Since autophage initial stage is critical for type I IFN signaling regulated by Atg13, we next check whether the components in Atg13 and VPS34 complex have effect on the function of Atg13. After knockdown of Atg13, ULK1, Atg101, FIP200, Beclin1, VPS34, Atg14, UVRAG using specific shRNA, 293T cells were cotransfected with ISRE-luc, TK-luc and Flag-Atg13 followed by poly(I:C) stimulation. We found that knockdown of Atg101, Beclin1, VPS34, but not other components, could abolish the enhancement of ISRE activity induced by Atg13 (Figure 4-6a). Furthermore, to investigate the mechanism by which these proteins could affect Atg13's function in type I IFN signaling, we first check the interaction between different components and Atg13 or RIG-I by coimmunoprecipitation and immunoblot in overexpression system. The results showed that after stimulation, Atg13 interacted with Beclin1, but not VPS34, while RIG-I strongly interacted with Beclin1 even without stimulation (Figure 4-6b,c). Next, we check the relationship between Atg13, Beclin1, RIG-I under physiological condition in THP-1 cells and Flag-RIG-I stable expression 293T cells. Consistently, endogenous Atg13 interacted with Beclin1 after poly(I:C) stimulation(Figure 4-6d) and RIG-I constitutively combined to Beclin1, but not VPS34(Figure 4-6e). Furthermore, to investigate whether Beclin1, VPS34, Atg101 could affect the interaction between Atg13 and RIG-I, we knocked down Atg101, Beclin1, VPS34 in 293T cells coexpressed with Flag-RIG-I and myc-Atg13 followed by poly(I:C) treatment. The results showed that only after knockdown of Beclin1 inhibited the interaction, while knockdown of Atg101, VPS34 have no effect (Figure 4-6f). To confirm this result, Beclin1 wild type and knock

out mouse peritoneal macrophages were infected with VSV-eGFP. After immunoprecipitation by anti-Atg13 antibody, western blot was performed to check RIG-I expression level. Compared to the wild type group, there's no interaction between Atg13 and RIG-I in Beclin1 KO cells, which is consistent to the knockdown result (Figure 4-6g). Taken together, these results suggested that Atg13 enhanced the RIG-I mediated type I IFN signaling through Beclin1.

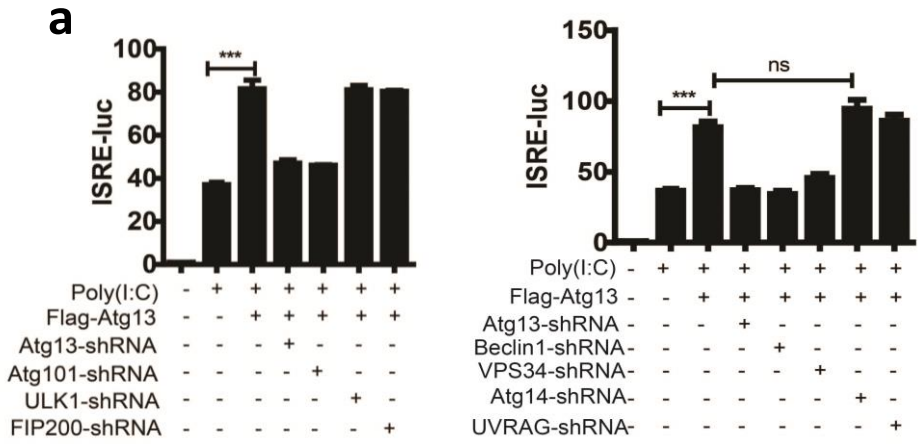
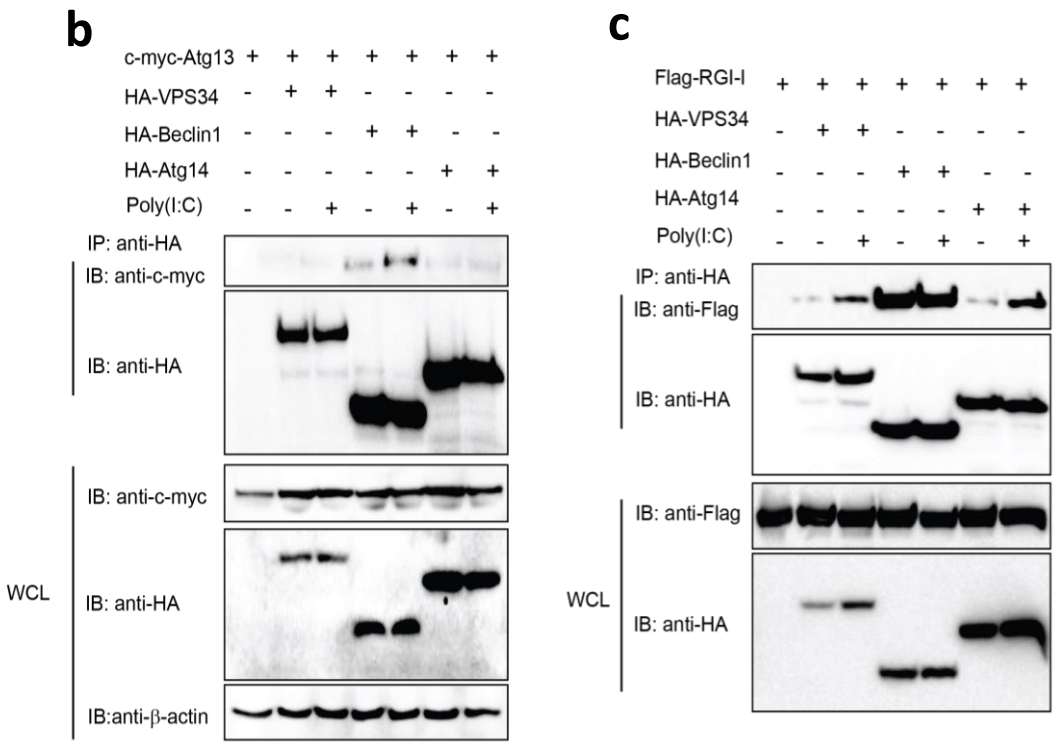


Figure 4-6. Atg13 positively regulates type I IFN signaling through Beclin1

(a)Luciferase activity in 293T cells transfected with plasmid encoding a luciferase reporter for ISRE (ISRE-luc; 100 ng each), together with empty vector or an expression vector for Atg13, as well as different shRNA followed by no treatment or treatment with intracellular (IC) poly(I:C).(b,c) Coimmunoprecipitation and immunoblot analysis of 293T cells transfected with various combinations of plasmids for c-myc-Atg13 (b) or Flag-RIG-I(c) and HA-VPS34, HA-Beclin1, HA-Atg14. (d) Immunoassay of extracts of THP-1 cells treated for various times with poly(I:C) followed by immunoprecipitation with anti-Atg13 and immunoblot analysis (e) Immunoassay of extracts of Flag-RIG-I stable expressed 293T cells treated for various times with poly(I:C) followed by immunoprecipitation with anti-Flag and immunoblot analysis. (f) Immunoassay of 293T cells transfected with ref-shRNA and Beclin1-shRNA, together with c-myc-Atg13 and Flag-RIG-I, followed by the treatment with intracellular poly(I:C) and immunoprecipitation (IP) with anti-Flag beads and immunoblot analysis with anti-c-myc. (g) Immunoassay of extracts of Beclin1-WT and Beclin1-KO mouse peritoneal macrophages treated for various times with poly(I:C) followed by immunoprecipitation with anti-Atg13 antibody and immunoblot analysis with anti-RIG-I



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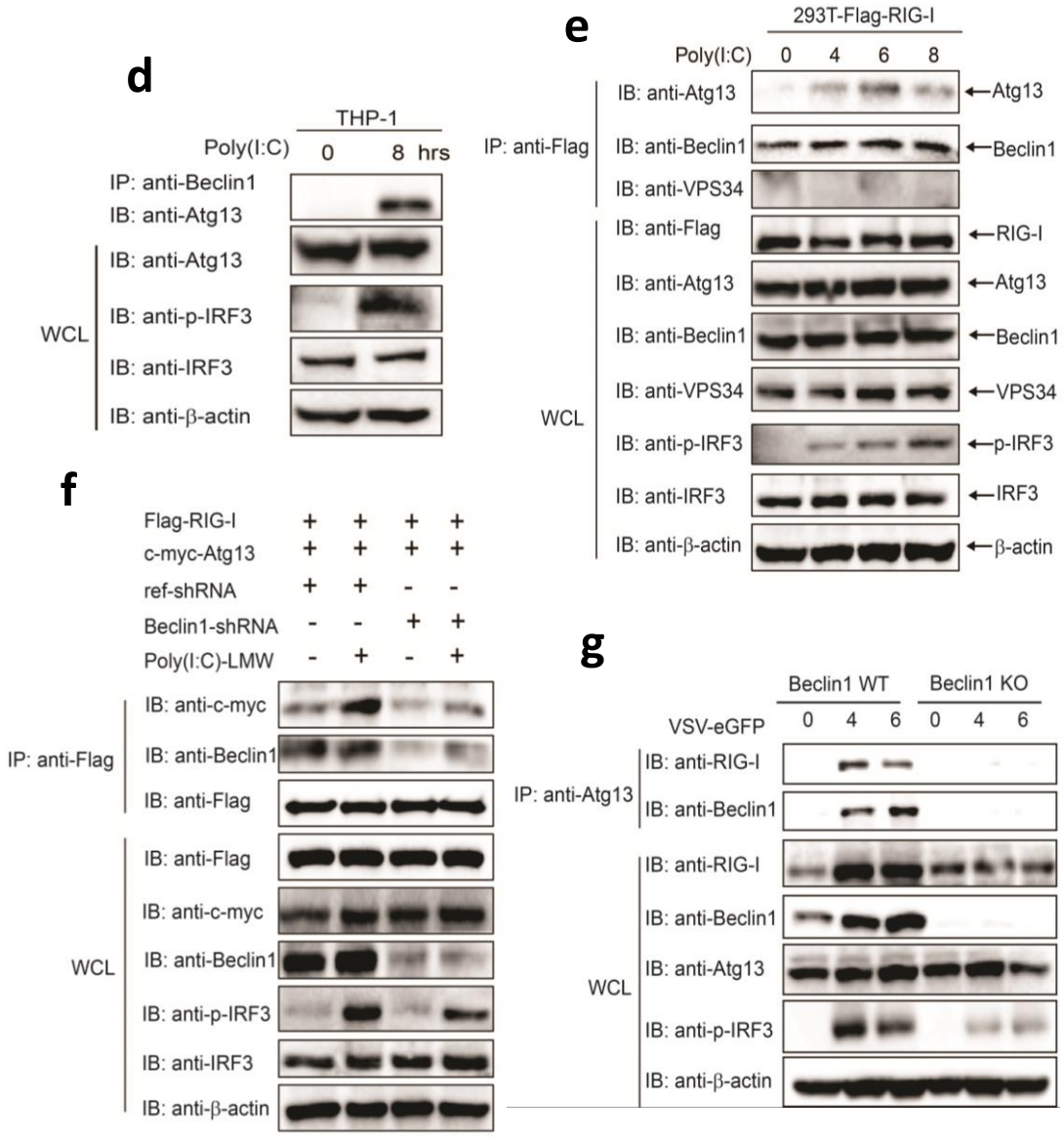

Figure 4-6 Continued.

4.3 Summary

In this study, we identified Atg13 as a positive regulator in type I IFN signaling and antiviral response by interacting with RIG-I through Beclin1 on an autophagy-independent manner. Our results showed that ectopic expression of Atg13 enhanced type I IFN signaling after different stimuli treatment. Consistently, knockdown of Atg13 decreased the type I IFN signaling and antiviral response. And we got the similar results both in human and mouse primary cells after Atg13 knockdown. Coimmunoprecipitation and immune bot experiments revealed that Atg13 interacted with activated RIG-I after stimulation under physiological condition. Using Beclin1 deficiency peritoneal macrophages, we found the interaction between Atg13 and RIG-I was impaired, and it consequently decreased the p-IRF3 and IFNβ expression, thus inhibited the type I IFN signaling response. Autophagy plays a key role in the innate and adaptive immune system by elimination of pathogens and the induction of acquired immune response. To investigate whether the autophagy process is involved in the role of Atg13 for enhancement of type I IFN signaling, we used Spautin-1 and CQ to block the autophagy at different stages and then check the activity of type I IFN signaling. The results showed Spautin-1, but not CQ, abolished the enhancement of type I IFN response, which indicated the late stage of autophagy process was not required within Atg13's function. In conclusion, our study demonstrated the mechanism of how Atg13 positively regulates innate immune signaling and antiviral response through Beclin1 on an autophagy-independent manner.

5. SUMMARY*

5.1 Significance

My thesis study is functionally characterizing the positive and negative regulators in type I IFN signaling and antiviral response to maintain innate immune homeostasis. TBK1 is a key component of type I interferon signaling that is activated by various DNA and RNA sensors, which induce the phosphorylation of IRF3 and type I interferon–responsive gene expression as a converging point. Because aberrant production of type I interferon can have a role in immunopathology and autoimmune disorders, thus TBK1 activation must be tightly controlled. However, the mechanism by how activated TBK1 is inhibited remains poorly understood. My first part of thesis studies identify that NLRP4 induced TBK1 K48 polyubiquitination and degradation, which specifically inhibited the TBK1-dependent type I interferon signaling, but had no effect on MyD88-IRF7–dependent type I IFN pathway. Additionally, it has reported that TBK1 plays a critical role in tumor development by activating the kinase Akt signaling pathway with oncoprotein KRAS medication[106-108]. Thus our study indicated that NLRP4-DTX4 may play an important role in inhibiting cancer development in NLRP4 high expression tissues.

Recent studies demonstrated that autophagy plays a key role in the innate and adaptive immune system by elimination of pathogens and the induction of acquired

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immune response. However, the molecular mechanisms of how autophagy related proteins play functions in immune-related processes are still unclear. My second part of thesis study identified Atg13 as a positive regulator in type I IFN signaling and antiviral response by interacting with RIG-I through Beclin1 in the autophagy initial stage. It provides important insight into the understanding of the regulation and crosstalk of autophagy and antiviral immunity upon pathogen invasion.

In conclusion, my thesis study identified positive and negative regulators in type I IFN signaling and antiviral response. It provides the molecular insight into the mechanisms by which NLRP4-DTX4 targets degradation of TBK1, and the interrelationship between autophagy and innate immunity by the regulation of Atg13 in type I IFNs. The studies make a significant advance in inhibition of cancer development and control of autoimmune disease.

5.2 NLRP4 negatively regulates type I IFN signaling by targeting TBK1 for degradation

Type I interferon plays an important role in viral clearance, but its aberrant production can have pathological role in immunopathology and autoimmune disorders. Thus, tight regulation of those key signaling pathways is essential for both innate and adaptive immunity to maintain the homeostasis. The first part of my thesis study identified NLRP4, which belongs to NOD like receptor family, negatively regulates type I IFN signaling by targeting TBK1. We found that ectopic expression of NLRP4 inhibited type I interferon signaling activated by ligand stimulation. Consistently, knockdown of NLRP4 enhanced type I interferon signaling and antiviral immune

response. Next we found NLRP4 inhibit type I interferon signaling by interacting with TBK1. NLRP4 enhanced Lys48 (K48)-linked polyubiquitination at Lys670 of TBK1 and caused TBK1 degradation. To investigate the molecular mechanisms of how NLRP4 negatively regulated type I interferon signaling, we found that NLRP4 strongly interacted with TBK1 after viral infection but not with TBK1 in resting cells, which indicated that the interaction between NLRP4 and TBK1 is signal dependent. That idea was further supported by several evidence. First, the Nod domain of NLRP4 specifically interacted with the kinase domain of TBK1. Second, NLRP4 interacted only to the phosphorylated (activated) form of TBK1 but did not interact to the S172A TBK1 mutant, which was unable to activate IRF3. To identify the E3 ubiquitin ligase(s) responsible for TBK1 ubiquitination, we designed a screen assay for the activity of the ISRE luciferase reporter and found the E3 ligase DTX4 as being involved in this. Notably, the NOD domain of NLRP4 directly bound to the DTX4 RING domain after stimulation; the NLRP4-DTX4 complex then interacted with the activated form of TBK1 and caused the K48-linked polyubiquitination of TBK1. A published study has shown that PCBP2 interacts with MAVS and ubiquitinates it via the E3 ligase AIP4, which leads to MAVS degradation (112). In our working mode, NLRP4 does not bind to TBK1 or DTX4 under normal conditions. However, after viral infection or TLR stimulation, activation of TBK1 triggers IRF3 phosphorylation and induce the type I interferon signaling. Then, NLRP4 and DTX4 form a complex to bind to the activated form of TBK1, and DTX4 catalyzes TBK1 K48-linked ubiquitination and causes its proteasomal degradation.

5.3 NLRP specifically inhibits TBK1-dependent type I IFN signaling

It has been reported that most of the cell types use TBK1-IRF3–dependent type I interferon signaling pathways (for IFN-β production) with viral infection, however, plasmacytoid dendritic cells use MyD88-IRF7–dependent (TBK1-independent) type I interferon signaling pathways (for IFN-α production) with the dinucleotide CpG stimulation and viral infection[109]. In macrophages, spatiotemporal regulation of MyD88–IRF7 signaling leads to robust production of IFN-α by liposomes containing CpG-A and DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate) but not CpG-A[110]. To investigate whether NLRP4-DTX4 plays a role in MyD88-IRF7–(TBK1-independent) type I interferon signaling pathway, we found that NLRP4 did not interact with MyD88 or IRF7 and did not affect MyD88-IRF7–dependent production of IFN-α. Thus, NLRP4-DTX4 specifically negatively mediated the TBK1-dependent type I interferon signaling pathway after viral infection and RNA and DNA stimuli. Since people have shown that TBK1 plays an essential role in tumor development mediated by the oncoprotein KRAS and in activating the kinase Akt signaling pathway, we believe that negative regulation of TBK1 by NLRP4-DTX4 may play an important protective role in cancer development in tissues with NLRP4 high expression. Further studies are needed to investigate the role of NLRP4 in tumor development. In conclusion, our studies have identified a previously unrecognized role for NLRP4 in negative regulation of type I IFN signaling by targeting TBK1 for K48 polyubiquitination and degradation to keep the homeostasis of innate immune signaling and antiviral response.

5.4 Atg13 positively regulates type I IFN signaling by interacting with activated RIG-I

Recent studies demonstrated that autophagy plays a key role in the innate and adaptive immune system by elimination of pathogens and the induction of acquired immune response. Several autophagy proteins have been identified as positive or negative regulators in innate immune signaling. Atg13, a component from ULK complex, which includes ULK1, Atg101, Atg13 and FIP200, plays an important role in the initiation of autophagy. Atg13 can be phosphorylated and activated under starvation condition, which triggers the induction of autophagy[111-113]. However, the role of Atg13 in type I IFN signaling remains unknown. Here my second part of thesis study identified Atg13 as a positive regulator in type I IFN signaling and antiviral response by interacting with RIG-I through Beclin1 on an autophagy-independent manner. Our results showed that ectopic expression of Atg13 enhances type I IFN signaling after different stimuli treatment. Consistently, knockdown of Atg13 decreases the type I IFN signaling and antiviral response. And we got the similar results both in human and mouse primary cells after Atg13 knockdown. Coimmunoprecipitation and immune bot experiments revealed that Atg13 interacted with RIG-I only after stimulation under physiological condition. Next, we found that Atg13 only interact with RIG-I mutant without repressor domain (RD), whereas RIG-I mutant containing only CARD domain or Helicase plus RD showed no interaction with Atg13, which indicated that Atg13 interacted with activated RIG-I at Helicase domain. It has been reported that RIG-I K172 is important for TRIM25-mediated RIG-I k63 polyubiquitination and MAVS binding to

induce the downstream signaling pathway[114]. Our results showed that Atg13 did not interact RIG-I K172R mutant even after poly(I:C) stimulation. Taken together, my thesis study indicates that Atg13 positively regulates type I IFN signaling and antiviral response by interacting with activated RIG-I after stimulation.

5.5 Beclin1 is required for the function of Atg13 in type I IFN signaling and antiviral response

Beclin1, which belongs to class III PI(3)K complex, plays an important role in initial stage of autophagy. Beclin1 is involved in tumorigenesis, development, and neurodegeneration[115]. It has been reported that Beclin 1knockout mice die early in embryogenesis, Beclin 1+/- mutant mice spontaneously develop a high incidence of tumors. Beclin 1 knockout embryonic stem cells have an altered autophagic response. These results indicate that Beclin 1 is a critical for mammalian autophagy and plays an important role for autophagy in tumor development[96]. To investigate the molecular mechanisms of how Atg13 negatively regulated type I interferon signaling, we found that Beclin1 is critical for the function of Atg13 in type I IFN signaling. Endogenous Atg13 interacted with Beclin1 after poly(I:C) stimulation and RIG-I constitutively combined to Beclin1. Knockdown of Beclin1 blocked the interaction between Atg13 and RIG-I, thus consequently inhibited downstream signal response. Furthermore, our results showed that Atg13 did not interact with RIG-I even after stimulation in Beclin1 -/- peritoneal macrophages, which suggested that Atg13 interacted with RIG-I to enhance the type I IFN signaling through Beclin1. In conclusion, my thesis study demonstrates Beclin1 is critical for the function of Atg13 in type I IFN signaling, which provides a

crosstalk between autophagy and innate immunity in antiviral response and tumor suppression

5.6 Atg13 positively regulates type I IFN signaling on autophagy-independent manner

A typical autophagy process is mainly involved in three stages, membrane initiation stage, elongation stage, and completion of the autophagosome. To further investigate whether autophagy is involved in type I IFN signaling regulated by Atg13, my thesis study found that starvation, the induction of autophagy, can enhance the type I IFN response and antiviral immunity. To fully understand a biological process, it is essential to perform experiments to regulate the activity of the process. Besides the genetic approaches, different pharmacological approaches have been utilized to modulate autophagy process. I use different autophagy inhibitors to block the autophagy process to investigate which stage of autophagy is critical for Atg13's function. Spautin-1, which can cause the degradation of class III PI3 kinase complexes to specifically inhibit the initial stage of autophagy, abolished the enhancement of type I IFN induced by Atg13 and inhibited the interaction between Atg13 and RIG-I. However, chloroquine(CQ), which can block the late stage of autophagy by inhibition of the lysosome acidification or fusion of autophagosome-lysosome, had no effect. These results suggested that the function of Atg13 dependents on the early stage of autophagy, but is not through the whole autophagy process. Atg5 plays an important role in autophagosome formation. In Atg5 deficient cells, Atg13 still can enhance the type I IFN response, which further indicated that the function of Atg13 is independent on the late

stage of autophagy. Since the early stage of autophagy is involved in the type I IFN response regulated by Atg13, we need to further investigate the detail mechanism that how the induction of autophagy, regulates type I IFN signaling through Atg13 and Beclin1, while the late stage of autophagy is not required.

6. FUTURE DIRECTION

6.1 What is the role of NLRP4 in tumor development

The first part of my thesis study has identified NLRP4 as a negative regulator in type I IFN signaling by targeting TBK1 through E3 ligase DTX4. Since TBK1 is essential for KRAS mutant tumors, one outstanding question is raised to be answered: what is the function of NLRP4 in tumor development? We speculate that NLRP4-DTX4 may play an important role in inhibiting cancer development in NLRP4 high expression tissues. In the future direction, it will be very interesting to detect the expression of NLRP4 in the different types of tumors to find out whether NLRP4 is down regulated. My study showed that NOD domain of NLRP4 is the functional domain which interacted with the kinase domain of TBK1 and inhibited the type I IFN signaling. We could sequence the genomic DNA of NLRP4 to detect whether there's mutation occurred in the NLRP4 functional domain from those tumor cells, which will provide us new molecular insight into tumor suppression.

My thesis study also showed that the biological function of NLRP4 is conserved in human and mouse, as well as in different cell types, and it appears to play an important role in maintaining immune homeostasis during antiviral innate immunity. Hence, NLRP4 may provide a potential therapeutic target for enhancing host immunity against pathogen infection and inflammation associated disease.

6.2 What's the detail mechanism of how the induction of autophagy is essential for enhancement of type I IFN through Atg13 and Beclin1

My second part of thesis study identified that Atg13 plays a positive role in type I IFN signaling through Beclin1 on autophagy-independent manner. We showed that Atg13 enhanced antiviral response by interacting with activated RIG-I after stimulation, and this interaction requires Beclin1 participation. It has reported that starvation somehow can enhance the type I IFN activity, which is consistent with our results that the initiation stage of autophagy is critical for the Atg13 function and the starvation induction can enhance the antiviral response as well. However, the molecular mechanism by which autophagy induction, like starvation, can enhance the type I IFN response remains unknown. It is believed that posttranslational modifications, such as phosphorylation, on autophagy related genes protein is the key mechanism for autophagy induction. After starvation, activated AMPKα will directly phosphorylate and activate ULK1. Then ULK1 will cause phosphorylation of both Atg13 and Beclin1 to induce the autophagy activation and maturation. So in the future direction, to figure out the relationship between autophagy induction and type I IFN signaling, and what's the mechanism by which Atg13 function in type I IFN through Beclin1, several key questions are raised to be answered: Whether the modification status of autophagy related genes, such as Atg13, Beclin1, have correlated effect on the type I IFN signaling after starvation treatment. What's the dynamic interaction between Atg13, Beclin1 and RIG-I after starvation followed by viral infection? Furthermore, Atg13 knockout mice will be generated to investigate the in vivo role of Atg13 in innate immunity and

antiviral response. There's also potential tumor development in Atg13 KO mice. Although recent studies demonstrated that autophagy plays a key role in the innate immune system, it is still a mystery how autophagy affects the innate immunity to keep host homeostasis. The future studies will provide us a hint to deep understand how this ancient self-defense machinery functions in immunity.

6.3 The in vivo role of Atg13 in immunity and antiviral response using Atg13 knockout mice model

Furthermore, Atg13 knockout mice will be generated to investigate the in vivo role of Atg13 in innate immunity and antiviral response. It will be very exciting to investigate the function of Atg13 in the inflammation related disease, such as inflammatory bowel disease, or cancer development in mouse model after different treatment. The function of Atg13 is conserved from mouse to human. Thus, the studies on mouse model will shed light on the potential role of Atg13 in human cancer development and other related disease.

6.4 Therapeutic implication of autophagy and autophagy genes

It has been reported that the dysregulation of autophagy gene function may result in the Crohn's disease or other inflammatory disorders. Depletion of Atg5 in the thymus causes autoreactive CD4+T cells as well as intestinal inflammatory infiltrates[116]. Loss of Atg16L1 in macrophages enhances endotoxin-induced inflammatory response[100], and lack of Atg16L1in Paneth cells causes transcriptional alterations in molecules, which regulate inflammation, and contributes to the pathogenesis of Crohn's disease[117]. These functions of autophagy related genes in immunity provide us opportunities to

manipulate autophagy therapeutically. For example, targeting antigens to the autophagic pathway may enhance CD4+T cell–dependent vaccines. My thesis studies demonstrate that Atg13 can enhance the type I IFN signaling and antiviral response through Beclin1in various cell types from mouse to human. It has been reported that mice with heterozygous depletion of Beclin1 can induce an increased frequency of spontaneous cancers, lung cancers and lymphomas [95, 96]. Thus, it will be very interesting to take the potential beneficial effects of autophagy in immunity into consideration in the clinical therapeutic development for cancer and other inflammation related disease. The further investigation on the detail molecular mechanisms of how induction of autophagy affects the innate immunity as well as antiviral response will open new doors for prevention in a variety of diseases without adverse potential immunological consequences.

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