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Mechanisms of Type I Interferon Induction via the Retinoic Acid Inducible Gene-Like
Receptor and DNA-Sensor Pathways

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor
of Philosophy in Microbiology, Immunology, and Molecular Genetics

by

Anna Devi Reichardt

2015

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ABSTRACT OF THE DISSERTATION

Mechanisms of Type I Interferon Induction via the Retinoic Acid Inducible Gene-Like
Receptor and DNA-Sensor Pathways

by

Anna Devi Reichardt

Doctor in Philosophy in Microbiology, Immunology, and Molecular Genetics

UNIVERSITY OF CALIFORNIA, Los Angeles, 2015

Professor Genhong Cheng, Chair

Cells are equipped with a comprehensive set of sensors that permits rapid detection of viral infection and initiation of an antiviral immune response through secretion of type I interferons (IFNs). This series of studies focuses on elucidating in molecular detail the mechanisms of type I IFN induction via the retinoic acid inducible gene (RIG-I)-like receptor (RLR) and DNA-sensor pathways. A clearer understanding of how viral nucleic acids are recognized at the molecular level should aid development of novel antiviral therapies.

We report the crystal structures of the TRAF domains of TRAF5 and of TRAF3 bound to a peptide from the TIM of Cardif. We identify two residues in TRAF3 that allow TRAF3 to bind to Cardif, and we show that mutation of these two residues in TRAF5 to the corresponding TRAF3 residues confers TRAF3-like antiviral activity on the mutated TRAF5 proteins. Our results provide a structural basis for the critical role of TRAF3 in activating RIG-I-mediated IFN production.

We also present data showing that Optineurin is a positive regulator of non-canonical NF κ B. We propose that on stimulation of the non-canonical NF κ B pathway, Optineurin is recruited to the TRAF-clAP-NIK complex, wherein it acts to promote TRAF3 degradation and support NIK accumulation. In light of our lab's data showing that TRAF3 functions to inhibit the cytosolic IFN β response to DNA, we hypothesize that Optineurin should function to promote IFN β induction in the cytosolic DNA pathway.

Finally, we present results from a screen for genes capable of activating the type I IFN antiviral response. We focused our studies on the kinase LATS1, which we propose functions to promote IFN β activation in response to cytosolic RNA and DNA pathways. We show that LATS1 promotes IFN β activation in response to both pIC and BDNA-transfection. Mechanistically, we show that LATS1 associates with the TBK1/IRF3 complex after activation of either cytosolic RNA or DNA pathways, and that LATS1 acts to promote phosphorylation of TBK1 and IRF3, thereby so enhancing downstream IFN β induction. We propose that LATS1 could serve as a bridge between viral infection and Hippo signaling.

The dissertation of Anna Devi Reichardt is approved.

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2015

To my parents, for their constant support.

To Sam, for her love of life's simple pleasures.

To my donut support group members, Gayle and Maxime.

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Chapter two is a version of the published manuscript titled: Single amino acid substitutions confer the antiviral activity of the TRAF3 adaptor protein onto TRAF5 (Sci. Signal. 5, ra81. 2012). Constructs, protein expression and purification, and crystallization assays were performed by Dr. Peng Zheng. Data analyses were performed by Dr. Huanhuan Liang.

Chapter four is in preparation for publication. LATS1 KO cells were provided by Dr.

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Posters

1. Reichardt AD, Zhang P, Liang H, Aliyari R, Cheng D, Wang Y, Xu F, Cheng G, Liu Y. Single Amino Acid Substitutions Confer the Antiviral Activity of the TRAF3 Adapter Protein onto TRAF5. Presented at immunologyLA, Los Angeles, CA, April 2012.
2. Tang ML, Reichardt AD, Siegrist T, Bao Z. Trialkylsilylethynyl substituted tetraceno[2,3-b]thiophene and anthraceno[2,3-b]thiophene molecules for OTFTs. Presented at the spring meeting of the Materials Research Society, San Francisco, CA, March 2008.
3. Reichardt AD, Tang ML, Okamoto T, Miyaki N, Bao Z. Ambipolar tetraceno[2,3-b]thiophene derivatives for Organic TFTs. Presented at the spring meeting of the Materials Research Society, San Francisco, CA, March 2008.

CHAPTER 1

Introduction

The first step to activate any sort of immune response against an infectious pathogen is the recognition of that pathogen. The innate immune system has evolved numerous sensor molecules called PRRs (pattern recognition receptors), which recognize microbe-specific ligands called PAMPs (pathogen-associated molecular patterns).¹ PRRs recognize a diverse repertoire of pathogen-associated molecular patterns (PAMPs), including proteins, lipoproteins, glycans, and, most crucially for viral infection, nucleic acids.²

Currently, three primary groups of PRRs have been identified as responsible for detection of viral nucleic acids.¹ These include the endosomal Toll-like receptors (TLRs), the cytosolic retinoic acid inducible gene (RIG-I)-like receptors (RLRs), and finally a less-well characterized group of cytosolic DNA sensors.³ These nucleic acid-sensing PRRs all commonly activate proinflammatory cytokines and, crucially for their role in recognition of viral infection, type I interferons (IFNs).¹

Type I IFNs were first described as a secreted substance that ‘interfered’ with influenza infection; they are in fact an extensive family of cytokines produced by cells in order to combat viruses.^{4,5} For simplicity, type I IFNs are often considered to consist of IFN- α and IFN- β subtypes.⁶ Activation of the IFN α/β genes is regulated by transcription factors, including NF κ B and IRF3/7. These activators form an ‘enhanceosome’ that cooperatively activates the transcription of IFN α/β .¹ In canonical type I IFN signaling, IFN- α/β engage the IFN- α receptor complex (IFNAR), activating a Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway that induces

transcription of several hundred IFN-stimulated genes (ISGs).⁶ ISGs comprise a wide range of activities, but as a group they promote many antiviral processes.⁷ These include the amplification of IFN signaling, the production of the costimulatory molecules and cytokines required to initiate an adaptive immune response, and the production of many cell-intrinsic viral restriction factors (targeting all steps of viral replication, including viral entry, viral protein translation, and virion release).⁷

TLRs are the most widely studied class of PRRs, capable of recognizing PAMPs from diverse classes of microorganisms, including viruses, bacteria, fungi and protozoa.⁸ TLRs are type I membrane glycoproteins, that contain an extracellular/luminal ligand-binding domain consisting of leucine-rich repeat (LRR) motifs, and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain that is required for downstream signal propagation.⁸ TLRs can be broadly divided into two classes: those that engage ligand from the cell-surface (including TLR1, TLR2, TLR4, TLR5 and TLR6), and those that engage ligand from within endosomes (TLR3, TLR7, TLR8 and TLR9).⁹

The endosomal TLRs are responsible for recognition of viral nucleic acids. TLR3 recognizes viral dsRNA; TLR7/8 recognize viral ssRNA, and TLR9 recognizes viral dsDNA.¹ The endosomal TLRs can be further subgrouped according to signal propagation from the TIR domain. Endosomal TLR3 signals via the adapter protein TIR domain-containing adapter inducing IFN- β (TRIF); TLR7/8/9 use the adapter protein myeloid differentiation factor-88 (MyD88).¹ Both the TRIF- and MyD88-dependent pathways activate the proinflammatory transcription factor NF κ B.¹ Both the TRIF- and

MyD88-dependent pathways also induce type I IFNs.¹ TLR3 utilizes a TRIF-dependent pathway to activate IRF-3 and IRF-7. This TRIF pathway is dependent upon activation of TNF receptor associated factor 3 (TRAF3), which recruits the protein kinases TANK-binding kinase 1 (TBK1) and IKK ϵ , which in turn mediate phosphorylation of IRF3 and IRF7.¹⁰ TLRs7/8/9 utilize the MyD88-dependent pathway to activate IRF7; while this pathway is also dependent upon TRAF3, it utilizes the kinases IRAK1 and IKK α to phosphorylate IRF7.¹⁰

In contrast to TLRs that are expressed predominantly in immune cells, RLRs are widely expressed in the cytosol of most cell types.^{10,11} There are three RLRs – RIG-I (retinoic acid-inducible gene I), MDA5 (melanoma differentiation factor 5), and LGP2 (laboratory of genetics and physiology 2); they belong to the DExD/H-box family of helicases.^{12,13} The helicase domain binds nucleic acids; in particular, RIG-I and MDA5 detect ssRNA and dsRNA with a 5' triphosphate group.^{12,14}

RIG-I and MDA5 contain N-terminal caspase activation and recruitment (CARD) domains that are required for downstream signaling propagation.¹² At baseline, RIG-I inhabits an autoinhibited state in which the CARD domains are sterically blocked from downstream signaling.¹² Upon ligand binding, RIG-I adopts a more open conformation that frees the CARD domains.¹² Activated RIG-I/MDA5 binds to the adapter protein CARD-adaptor-inducing IFN β (CARDIF) via homotypic CARD-CARD-mediated interactions.¹² The association of RIG-I/MDA5 with CARDIF triggers the assembly of a protein complex that transduces downstream signaling to activate the IFN response.^{15–}

¹⁸ Activated CARDIF recruits the E3 ubiquitin ligase TRAF3.^{10,19} Similar to its role in TRIF-dependent TLR-signaling, TRAF3 in turn recruits and activates TBK1/IKKε to phosphorylate IRF3/7.¹⁰

The pathway of IFN induction to cytosolic DNA is not as well-defined as those for TLR- and RLR- mediated responses.²⁰ The IFN response to cytoplasmic DNA can be subdivided into RLR-dependent and –independent pathways.²⁰ Some reports have suggested a role for RNA polymerase III (Pol III) as a sensor that operates through the RIG-I/CARDIF pathway.^{20,21} Pol III can transcribe poly(dA-dT) to dsRNA; the dsRNA in turn is recognized by and activates RIG-I.^{20,22} However, this Pol III pathway might be an artifact specific to poly(dA-dT) DNA; and the pathway's functional significance remains unclear.²⁰ The RLR-independent IFN response to cytoplasmic DNA, though, is recognized as functionally relevant. This pathway is dependent on the adaptor protein stimulator of IFN genes (STING).²³ Activated STING recruits both TBK1 and IRF3, and promotes the phosphorylation of IRF3 by TBK1.²⁴

Upstream of STING, though, multiple putative and possibly redundant DNA sensors have been identified. These include DNA-dependent activator of IRFs (DAI), DDX41, IFN-γ-inducible protein 16 (IFI16), and cyclic GMP-AMP synthase (cGAS).²⁰ DAI was the first reported cytoplasmic DNA PRR.²⁵ It binds DNA and interacts with both TBK1 and IRF3.²⁰ DDX41 is a member of the DExD/H helicase family. It uses the DEADc domain both to bind DNA and interact with STING.²⁰ DDX41 has also been shown to bind cyclic dinucleotides, and on binding to promote formation of a complex with STING

that activates IFN.²⁰ IFI16 is a PYHIN family member that contains two DNA-binding HIN200 domains. Uniquely among the proposed DNA sensors, IFI16 is capable of sensing DNA in both the nuclear and cytosolic compartments.^{20,26} cGAS is an enzyme that synthesizes the second-messenger cyclic GMP-AMP (cGAMP) in response to binding DNA; in turn, cGAMP is capable of binding STING and activating the STING-TBK1-IRF3 axis.²⁰

The TRIF-, MyD88, and RLR-dependent IFN responses all converge at TRAF3, where TRAF3 functions as a positive factor to promote IFN induction.¹⁰ However, recent, unpublished work in our laboratory suggests that in contrast to its role promoting IFN induction in response to TLR- and RLR- pathways, TRAF3 plays a negative role in the antiviral response of the cytoplasmic DNA pathway. Our work suggests that TRAF3's function as a negative regulator of the IFN response to cytosolic DNA is linked to its more well-established role as a negative regulator of the non-canonical nuclear factor- κ B (NF κ B) pathway.

NF κ B signaling is classified into the canonical and non-canonical pathways.²⁷ The non-canonical NF κ B, the p52-RelB dimer, is activated by a subset of the Tumor Necrosis factor receptor superfamily (TNFRSFs) – including cluster of differentiation 40 (CD40), lymphotoxin β receptor (LT β R), B-cell activating factor receptor (BAFFR), receptor activator of NF κ B (RANK), fibroblast growth factor-inducible 14 (Fn14), and cluster of differentiation 27 (CD27).²⁷

Control of NF κ B-inducing kinase (NIK) post-translational stability is the primary regulation point of non-canonical signaling.²⁸ NIK is continuously synthesized, but at baseline its expression level is maintained at low to undetectable levels via proteasome-mediated degradation. This basal NIK degradation is controlled by a TRAF-cIAP (cellular inhibitor of apoptosis proteins) E3 ubiquitin ligase complex, composed of the proteins TRAF2, TRAF3, and cIAP1/2.²⁹ TRAF3 binds the complex to NIK.³⁰ TRAF2 functions as a linker, connecting TRAF3/NIK to cIAP1/2, which in turn mediate the K48-linked polyubiquitination of NIK that in turn targets it for degradation.^{29,31}

On receptor ligation, this process of constitutive NIK degradation is blocked. Receptor ligation triggers the recruitment of the NIK-containing complex to the receptor, where TRAF2-mediated K63-linked polyubiquitination of cIAP1/2 redirects their K48-ubiquitin ligase activity towards TRAF3 instead of NIK.²⁸ This causes TRAF3 degradation, and releases NIK from the TRAF-cIAP complex, allowing accumulation of de novo synthesized NIK.²⁷ Once allowed to accumulate, NIK undergoes autophosphorylation, and then phosphorylates IKK α , which in turn phosphorylates p100, marking p100 for partial proteasomal digestion and release of the p100 N-terminus, p52, in complex with RelB.²⁸

Our data suggests that TRAF3-mediated suppression of the non-canonical NF κ B signaling apparatus prevents these proteins from interacting with the TBK1-IRF3 axis to promote IFN induction to cytosolic DNA. Our work suggests that TRAF3's regulation of the cytoplasmic-DNA IFN response is actually independent of the p100 transcription

factor. Data from our lab suggests that NIK, IKK α , TBK1 and IRF3 form a complex that promotes a synergistic induction of IFN. This would not be the first instance of crosstalk between non-canonical NF κ B and the canonical IFN signaling pathways: non-canonical NF κ B was recently shown to be a negative regulator of TLR- and RLR-dependent IFN responses.

Cells are equipped with a comprehensive set of sensors that permits rapid detection of viral infection and initiation of an antiviral immune response. The major viral PAMPs are nucleic acids. On recognition of viral nucleic acids, IFN α/β are transcriptionally activated and the secreted type I IFNs initiate an antiviral response. There are three broad groupings of nucleic-acid PRRs: TLRs, RLRs, and DNA-sensors. TLRs detect viral RNA/DNA in endosomal compartments in immune cells. Non-immune cells, though, are still equipped to detect virus. RLRs detect viral RNA in the cytoplasmic compartment, and DNA sensors detect viral DNA in the cytoplasm. Our current understanding of viral detection is by no means comprehensive. This series of studies focuses on elucidating in molecular detail the mechanisms of type I IFN induction via the RLR and DNA-sensor pathways. A clearer understanding of how viral nucleic acids are recognized at the molecular level should aid development of novel antiviral therapies.

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CHAPTER 2

Single Amino Acid Substitutions Confer the Antiviral Activity of the TRAF3

Adaptor Protein onto TRAF5

ABSTRACT

The TRAF [tumor necrosis factor receptor–associated factor] family of cytoplasmic adaptor proteins link cell-surface receptors to intracellular signaling pathways that regulate innate and adaptive immune responses. In response to activation of RIG-I (retinoic acid–inducible gene I), a component of a pattern recognition receptor that detects viruses, TRAF3 binds to the adaptor protein Cardif [caspase activation and recruitment domain (CARD) adaptor–inducing interferon-beta (IFN β)], leading to induction of type I IFNs. We report the crystal structures of the TRAF domain of TRAF5 and that of TRAF3 bound to a peptide from the TRAF-interacting motif of Cardif. By comparing these structures, we identified two residues located near the Cardif binding pocket in TRAF3 (Tyr440 and Phe473) that potentially contributed to Cardif recognition. In vitro and cellular experiments showed that forms of TRAF5 with mutation of the corresponding residues to those of TRAF3 had TRAF3-like antiviral activity. Our results provide a structural basis for the critical role of TRAF3 in activating RIG-I–mediated IFN production.

INTRODUCTION

The tumor necrosis factor (TNF) receptor–associated factor (TRAF) family of adaptor proteins promotes the activation of several transcription factors to induce immune and inflammatory responses, including nuclear factor κ B (NF- κ B), activator protein-1 (AP-1), and interferon (IFN) regulatory factors 3 and 7 (IRF3/7), which are especially important for innate immune antiviral function.^{1, 2} Mammalian cells have six TRAF proteins, TRAF1 to TRAF6, all of which contain a conserved TRAF domain at the C terminus consisting of a coiled-coil domain followed by a TRAF-C domain; five (TRAF2 to TRAF6) also have an N-terminal RING domain and zinc finger motifs.

Despite high sequence homology, TRAF proteins associate with different ligands and carry out nonredundant functions.³ TRAF proteins are recruited directly or indirectly by various pattern recognition receptors, whereupon they recruit additional factors that activate the inhibitor of κ B kinase (IKK) complex to trigger NF- κ B activation.¹ In addition, TRAF2, TRAF5, and TRAF6 can promote the activation of mitogen-activated protein kinase and c-Jun N-terminal kinase, and TRAF3, TRAF5, and TRAF6 can mediate secretion of type I IFNs.^{2, 4-6} The molecular specificities that determine the individual TRAF protein functions, including the role of TRAF3 in stimulating IFN secretion, however, still remain incompletely defined.

On recognition of viral infection, TRAF3 is activated and primes the TRAF family member–associated NF- κ B activator (TANK)–binding kinase 1 (TBK1) and IKK complex to phosphorylate IRF3/7.^{1, 7} Two major antiviral pathways activate TRAF3 (and type I IFN secretion): a Toll-like receptor (TLR)–dependent pathway and a TLR-independent pathway that are active in immune and nonimmune cells, respectively. In

nonimmune cells, upon recognition of intracellular double-stranded RNA, cytoplasmic RNA helicases including retinoic acid–inducible gene I (RIG-I), helicard, and melanoma differentiation–associated protein 5 (MDA5) recruit the adaptor protein Cardif, which in turn recruits and activates TRAF3.^{6, 8}

Cardif functions as a scaffolding protein, helping to coordinate the assembly of larger protein complexes that trigger downstream production of IFN and various other cytokines. The TRAF-interacting motif (TIM) of Cardif has been mapped to three regions, including the TRAF2 binding motif (T2BM) 143PVQDT147, TRAF6 binding motif 1 (T6BM1) 153PVENSE159, and TRAF6 binding motif 2 (T6BM2) 431PEENEY437.⁹ Multiple TRAF-family proteins, including TRAF2, TRAF3, TRAF5, and TRAF6, have been identified as possible Cardif-binding partners and have been suggested to mediate IFN secretion downstream of Cardif. Previous studies from our group demonstrated direct interactions between the TRAF domain of TRAF3 and the T2BM of Cardif; subsequently, TRAF3 was also shown to interact with Cardif through the T6BM2 region.^{8, 10}

In addition, TRAF6 is critical for IFN gene activation and has been suggested to be another regulator of IFN activation.^{5, 11, 12} However, we have shown that no other TRAF family-member TRAF domain, including TRAF5, can functionally replace the antiviral activity of TRAF3.⁸ A hybrid form of TRAF5 containing the TRAF domain of TRAF3 gains antiviral activity, and conversely, substitution of the TRAF domain of TRAF5 into TRAF3 abrogates the antiviral function of TRAF3.⁸ These data suggest that the functional specificity between TRAF3 and TRAF5 is determined by the TRAF domain. Crystal structures of the TRAF domains of TRAF2, TRAF3, and TRAF6 in association

with various TIMs have been determined, including crystal structures of the TRAF domain of TRAF3 bound to five ligands: CD40, lymphotoxin-beta receptor (LT β R), B cell-activating factor receptor (BAFFR), TANK, and latent membrane protein 1 (LMP1).¹³⁻²⁰ Within the common TRAF protein fold, TRAF domains form trimers with an aggregate structure that is often compared to a mushroom. N-terminal coiled-coil motifs form the mushroom's "stalk"; high sequence conservation within these coiled-coil motifs allows TRAF proteins to form both homo and heterotrimers with other TRAFs. C-terminal TRAF-C domains form the mushroom's "cap" by folding into eight-stranded β -sandwiches that contain solvent-restricted binding crevices. These crevices serve as binding surfaces and measure about 15 Å~ 19 Å~ 22 Å, which can accommodate peptides about 20 amino acids long.²¹ Within this β -sandwich region, TRAF crystal structures have defined three structural "hotspots" shared within the TRAF family: a hydrophobic pocket, a set of serine fingers, and a grouping of polar residues.²¹ Subtle structural differences in this β -sandwich region and the hotspot residues account for the selective recruitment of various TRAF family members to binding partners. The TRAF domains of TRAF3 and TRAF5 show up to 65% sequence homology, and the hotspot residues are the same in these two proteins. However, the mechanisms and the specific amino acids responsible for the specificity of TRAF3 in the Cardif-mediated induction of IFN production remain to be elucidated.

Here, we report the crystal structures of the TRAF domain of TRAF5 and that of TRAF3 bound to a peptide from the TIM of Cardif. By comparing the structure of the TRAF3-Cardif complex to that of TRAF5, we found two residues in corresponding structural positions in TRAF3 and TRAF5 that differ and determine the binding abilities of TRAF3

and TRAF5 to Cardif. Mutations of these two residues in TRAF5 to the corresponding TRAF3 residues conferred TRAF3-like antiviral activity on the mutated TRAF5 proteins. Our results have determined a fundamental structure-based mechanism for the selectivity of TRAF3 in promoting the RIG-I-mediated IFN response.

RESULTS

Configuration of the TRAF3-Cardif Complex

First, we sought to identify the TRAF binding site in Cardif that mediates direct interaction with the TRAF domain of TRAF3. We purified the TRAF domains of TRAF3 and TRAF5 as well as glutathione S-transferase (GST)-tagged peptides containing the TRAF binding sites of Cardif residues 138 to 153 (138PSCPKPVQDTQPPESP153, which encompasses T2BM), residues 149 to 163 (149PPESPVENSEQALQT163, which encompasses T6BM1), and residues 425 to 441 (425SEP NHGPEENEYSSFRI441, which encompasses T6BM2). The GST-tagged Cardif peptides were bound to glutathione beads and incubated with the purified TRAF domains of TRAF3 and TRAF5. Only T2BM directly associated with the TRAF domain of TRAF3, and none of the three identified TRAF binding domains in Cardif directly associated with the TRAF domain of TRAF5 (fig. 2.S1). Because T2BM contains a conserved PXQXT sequence, which has been speculated to be a TRAF3 binding motif, our result that T2BM was the relevant TRAF binding domain in Cardif was not surprising.¹ Because Pro153 has been reported to be the first residue in T6BM1, we chose to use a slightly longer Cardif peptide (residues 138 to 158) that included the T2BM and T6BM1 motifs for further cocrystallization studies with TRAF3.

We purified the recombinant TRAF domain of TRAF3 (residues 376 to 565) and synthesized a peptide containing the TIM sequence of Cardif (residues 138 to 158).⁸ Equimolar amounts of the TRAF3 and Cardif peptides were mixed and cocrystallized. Crystals obtained were diffracted to 2.3 Å. The TRAF3-Cardif crystal adopted a P321 space group with one complex in an asymmetric unit; its structure was calculated by

molecular replacement method, with the TRAF domain of TRAF3 as an initial searching model. Table 2.1 gives a summary of crystallographic data and refinement statistics. The Cardif peptide is observed in the $F_o - F_c$ map: The Cardif peptide binds to TRAF3 at the edge of the β -sandwich crevice with a stoichiometry of 1:1 (Fig. 2.1A). After extensive refinement, 10 amino acids from Cardif including the sequence 138PSCPVPVQDT147 were unambiguously built for the backbone and side chains (Fig. 2.1B). Electron density plots for 148QPPE151 had poor resolution, suggesting that the C terminus of the Cardif peptide is flexible. The Gln148 of Cardif has alternative confirmations (fig. 2.S2). However, from the structure, these different conformations of Gln148 do not substantially influence the binding of Cardif peptide with TRAF3. To identify the smallest peptide capable of binding TRAF3, we further truncated the Cardif peptide. Because pulldown assays indicated that the 13-residue peptide observed in the crystal structure (including residues 138 to 150) was sufficient for TRAF3 recruitment, we used this peptide for subsequent in vitro functional assays.

Previous reports have determined that the edge of the β -sandwich TRAF-C domain of TRAF3 is a key docking site for mediating ligand interactions in NF κ B pathways.²¹ Crystal structures of the TRAF domain of TRAF3 indicate that ligand peptides adopt one of two conformations within this crevice.²¹ The TIMs of CD40 and LT β R present with an imbedded “hairpin” configuration; in comparison, the TIMs of BAFFR, TANK, and LMP1 present with a more open “boomerang” configuration. Our structure showed that similar to BAFFR, TANK, and LMP1, the TIM of Cardif adopts an open conformation in the TRAF crevice of TRAF3 (Fig. 2.1C). Although it is not clear why the different ligands adopt different conformations, we suggest that TRAF3 uses the same crevice when

binding to its ligand Cardif in the IFN pathway.

Interactions between the TRAF domain of TRAF3 and the TIM of Cardif

We structurally mapped the TRAF3-Cardif binding interface. The residues 143PVQDT147 in Cardif mediate the interaction with TRAF3; similar to the PXQXT peptides in CD40, TANK, and LMP1, these five residues bind the conserved crevices of the b-sandwich region (Fig. 2.1, C and D).^{15-16, 19} Our structure defined on a molecular level the specific interactions that these Cardif residues form with TRAF3's three hotspot clusters in the crevices. Phe474, Phe511, and Phe520 in TRAF3 form a hydrophobic pocket that binds Pro143 (in the sequence 143PVQDT147) in Cardif. The serine residues Ser517, Ser518, and Ser519 in TRAF3 determine a set of serine fingers that form a network of hydrogen bonds to Gln145 (in the sequence 143PVQDT147) in Cardif. Three additional TRAF3 residues—Arg456, Tyr458, and Asp462—define a hydrophilic region that generates multiple dipole-dipole interactions with Cardif. The guanidinium group of Arg456 and the hydroxyl group of Tyr458 hydrogen-bond to a carboxyl group on Asp146 in Cardif (in the sequence 143PVQDT147); the carboxyl group of Asp462 interacts with the amine and hydroxyl groups of Thr147 in Cardif (143PVQDT147). Our structure provides a means by which the point mutation T147I in Cardif disrupts its interaction with TRAF3: T147I in Cardif disrupts a critical hydrogen bond that forms between Asp462 of TRAF3 and a carbonyl group of Thr147 in wild-type Cardif.⁸

Our structure also located molecular interactions important for TRAF3-Cardif binding that involved the sequence 143PVQDT147 in Cardif but were independent of the

conserved hotspot residues of TRAF3 (Fig. 2.1D). The carboxyl group of Gly532 in TRAF3 hydrogen-bonds to the amino group of Val144 in Cardif (in the sequence 143PVQDT147). Ser531 in TRAF3 provides an amino group that hydrogen-bonds to the carboxyl group of Val144 in Cardif (in the sequence 143PVQDT147). Ala530 in TRAF3 provides a carboxyl group that hydrogen-bonds to the amino group of Asp146 in Cardif (in the sequence 143PVQDT147). Unexpectedly, we observed a disulfide bond between Cys533 in TRAF3 and Cys160 in Cardif in our structure's electron density map (Fig. 2.1D). However, we believe that this covalent bond is an artifact that most likely formed as a result of oxidation that occurred during the crystallization process.

Structure of the TRAF domain of TRAF5

We determined the crystal structure of the TRAF domain of TRAF5 with a molecular replacement method, starting with the C-TRAF domain of TRAF3 as an initial model. To obtain a well-diffracted crystal, we truncated our TRAF5 construct at the N-TRAF domain, so that the construct covered residues 381 through the C-terminal end of TRAF5 (Fig. 2.2A). The crystal adopts a P212121 space group, with three molecules in one asymmetric subunit. Summaries of crystallographic data and refinement statistics are presented in Table 2.1. Our structure showed that TRAF5 forms a homotrimer with the typical mushroom-like structure that is characteristic of TRAF proteins. N-terminal coiled-coil motifs (TRAF5 residues 381 to 399) fold into the alpha helices that form the mushroom's stalk. The mushroom's cap is composed of an eight-stranded β -sandwich fold, consisting of residues 405 to 558. The two layers of an antiparallel β strand, β 1 to β 8, form a hydrophobic core.

The C-terminal 150 amino acids of TRAF3 and TRAF5 share 65% identity (Fig. 2.2B). When crystal structures of the C-terminal TRAF domains of TRAF3 (residues 416 to 567) and TRAF5 (residues 405 to 558) are superimposed, the main chain root mean square deviation (RMSD) between the TRAF domain of TRAF5 and that of TRAF3 is only 0.70 Å, suggesting that these two structures are very similar (Fig. 2.2C). One clear distinction between the two structures is the angle between the stalk and the cap of the mushroom (Fig. 2.2C). We suggest that this is caused by different degrees of flexibility in the hinge regions between the stalk and the cap (residues 400 to 404) of the two molecules.

Our structure confirmed previously predicted hotspot clusters in the TRAF domain of TRAF5, and showed that these hotspots form crevices for binding adaptor proteins. Phe463, Phe500, and Phe509 form a hydrophobic pocket; Arg445, Tyr447, and Asp451 make up a polar pocket; and three sequential serine residues, Ser506, Ser507, and Ser508, form a set of hydrophilic “serine fingers” (Fig. 2.2,B and C). When we compared the crevice residues between the TRAF domain of TRAF3 and the TRAF domain of TRAF5, we found that the hotspot residues from TRAF3 and TRAF5 can be superimposed very well (Fig. 2.2C). Because TRAF5 cannot replace TRAF3 in the RIG-I-mediated type I IFN pathway, we suggest that other critical residues located outside this well-characterized binding crevice are involved in mediating the specific binding of TRAF3 to Cardif.⁸

Structural comparison of the TRAF domains of TRAF3 and TRAF5

To determine on a molecular level why the TIM of Cardif binds the TRAF domain of

TRAF3 but not that of TRAF5, we analyzed the residues in TRAF3 and TRAF5 located near the Cardif binding crevice using sequence and structural alignment. Two residues drew our attention. The corresponding residues in TRAF3 are Phe473 and Tyr440 and in TRAF5 are Tyr462 and Phe429 (Fig. 2.2, B and C). Although both residues are aromatic amino acids and are located on the surface of the b-sandwich domain and not within the crevice, we predicted that these two nonconserved residue positions contributed to the different binding abilities of TRAF3 and TRAF5 for Cardif.

To determine whether Phe473 and Tyr440 in TRAF3 were necessary for the binding of TRAF3 to Cardif, we generated a panel of purified TRAF domain oligopeptides, which included wild-type TRAF3 and TRAF5, TRAF3 F473Y, TRAF3 Y440F, and TRAF3 F473Y/Y440F, and examined the interactions of these peptides with the GST-Cardif TIM fusion proteins. As expected, the Cardif TIM associated with a peptide corresponding to the TRAF domain of wild-type TRAF3 but not wild-type TRAF5 (Fig. 2.3A). In agreement with our structure-based prediction that Phe473 and Tyr440 in TRAF3 enable TRAF3 to bind Cardif, none of the TRAF3 mutant peptides (F473Y, Y440F, or F473Y/Y440F) associated with the TIM of Cardif (Fig. 2.3A).

We reasoned that if sequence differences between these two corresponding residue positions were indeed responsible for the dissimilar Cardif binding abilities of TRAF3 and TRAF5, then mutation of these residues in TRAF5 to the corresponding TRAF3 residues should confer TRAF3-like binding ability for Cardif on TRAF5. To test this hypothesis, we performed the GST-Cardif TIM pulldown assay with the TRAF5 Y462F, F429Y, and F429Y/Y462F TRAF domain peptides, as well as the wild-type TRAF3 and TRAF5 peptides. All three TRAF5 TRAF domain mutants, but not wild-type TRAF5,

associated with the TIM of Cardif (Fig. 2.3A).

We also wanted to confirm that the full-length point-mutant TRAF5 proteins interacted with Cardif. We performed GST pulldowns using a GST-Cardif TIM incubated with lysates from HEK 293T cells expressing wild-type TRAF3 or TRAF5 or full-length TRAF5 Y462F or TRAF5 F429Y. As expected, wild-type TRAF3, but not wild-type TRAF5, associated with the TIM of Cardif. In confirmation of our hypothesis that we could switch TRAF5 toward a more TRAF3-like Cardif-binding phenotype, both TRAF5 Y462F and TRAF5 F429Y interacted with the TIM of Cardif (Fig. 2.3B).

We were surprised that the two residue positions, Phe473 and Tyr440 in TRAF3 and Tyr462 and Phe429 in TRAF5, played such a central role in mediating interactions with Cardif. The TRAF3 mutant Y440A was previously reported to have reduced ability to bind to Cardif.⁸ Unfortunately, our electron density map was unclear for the C-terminal region of the Cardif TIM motif, which is spatially situated to form potential interactions with the TRAF3 residue Tyr440 (fig. 2.S2). In lieu of further structural evidence, we hypothesized that the phenol moiety of Tyr440 forms a hydrogen bond with a carbonyl group on the main chain of Cardif, and that this interaction is destroyed upon replacement of Tyr440 with either a phenylalanine, as in TRAF5, or an alanine, as in the TRAF3 Y440A mutant (Fig. 2.3C). Our in vitro pulldown experimental results confirmed this hypothesis. Cardif peptides with mutations in the C-terminal region, such as P149A, P149S, and P150A, showed reduced binding to TRAF3. Other mutants, such as Cardif 138–148 and P150S, did not bind to TRAF3 (fig. 2.S3). These results show that the TRAF3 binding site in Cardif covers amino acid residues beyond just the minimum 143PVQDT147 motif.

Our TRAF3-Cardif structure indicated that there should be sufficient space around Phe473 in TRAF3 to accommodate substitution of a tyrosine residue, as in either TRAF3 F473Y or the corresponding residue in TRAF5, Tyr462. Although the structure did not show direct interactions between Phe473 in TRAF3 and the Cardif peptide, we wondered whether the phenol moiety of Phe462 played a role in disrupting interactions with Cardif. Our structure showed that in TRAF5, Phe462 lies adjacent to Asp451 (which corresponds to Asp462 in TRAF3). The carboxyl group of Asp462 in TRAF3 interacts with the amino group of Thr147 in Cardif, and disruption of this interaction is sufficient to ablate the formation of the TRAF3-Cardif complex (Fig. 2.3C). Therefore, we hypothesize that Tyr462 in TRAF5 interacts with Asp451, thereby blocking the formation of the TRAF5-Cardif complex by preventing the establishment of hydrogen bonds between Asp451 in TRAF5 and Thr147 in Cardif.

Abolished antiviral function of TRAF3 F473Y and Y440F mutants

Next, we tested whether variants of TRAF3 in which these two residues were mutated to the corresponding TRAF5 residues (TRAF3 F473Y and Y440F) retained antiviral function. Because Cardif is required to recruit TRAF3 upon viral RNA recognition in the TLR-independent IFN secretion pathway, we reasoned that the decreased binding abilities of the TRAF3 F473Y and Y440F mutants for Cardif would attenuate TRAF3-Cardif-dependent antiviral responses.

We reconstituted TRAF3^{-/-} mouse embryo fibroblasts (MEFs) with wild-type TRAF3, wild-type TRAF5, TRAF3 F473Y, TRAF3 Y440F, or TRAF3 F473Y/Y440F (Fig. 2.4A). We assessed the production of type I IFNs by the reconstituted MEFs in response to

infection with a form of vesicular stomatitis virus (VSV) that coexpresses green fluorescent protein (GFP) (Fig. 2.4B). Compared to cells reconstituted with wild-type TRAF3, those reconstituted with TRAF3 F473Y, Y440F, or F473Y/Y440F mutants showed reduced secretion of IFN- β after infection with VSV-GFP. Our results suggest that the TRAF3 F473Y and Y440F mutants abrogate type I IFN production on viral infection.

We next assessed the antiviral state of the reconstituted MEF cell lines after infection with VSV-GFP. We used GFP fluorescence as an indirect measure of viral replication and quantified it as the percentage of VSVGFP– positive cells (reflecting infection across a cell population) multiplied by the geometric mean of the fluorescence intensity (which accounts for the degree of infection within individual cells). As an additional validation, we used viral plaque assay to measure live, infectious virions as a direct measure of viral replication. As expected, cells reconstituted with wild-type TRAF3 were resistant to infection, evidenced by both their low amounts of GFP fluorescence and low viral titers, whereas cells reconstituted with wild-type TRAF5 or vector were more susceptible to infection, as evidenced by both high amounts of GFP fluorescence and high viral titers (Fig. 2.4, C and D). Cells reconstituted with TRAF3 F473Y, Y440F, or F473Y/Y440F had an intermediate phenotype. They showed increased GFP fluorescence compared to wild-type TRAF3-reconstituted cells but less GFP fluorescence compared to cells reconstituted with wild-type TRAF5; similarly, their viral titers were about fivefold higher than those of cells reconstituted with wild-type TRAF3 but were twofold less than those of cells reconstituted with wild-type TRAF5 (Fig. 2.4, C and D).

Acquired antiviral function of TRAF5 F429Y and Y462F

Next, we tested whether mutation of these two TRAF5 residues to the corresponding TRAF3 residue (TRAF5 Y462F and F429Y) endowed TRAF5 with TRAF3-like antiviral function. Because Cardif is required for TRAF3 recruitment upon viral RNA recognition in the TLR-independent IFN secretion pathway, we theorized that the increased binding of the TRAF5 Y462F and F429Y mutants to Cardif could allow them to partially rescue TRAF3-dependent antiviral responses in TRAF3-deficient cells. We reconstituted TRAF3^{-/-} MEFs with wild-type TRAF3, wildtype TRAF5, TRAF5 Y462F, and TRAF5 F429Y (Fig. 2.5A).

We assessed the production of type I IFNs by the reconstituted MEFs in response to infection with both Sendai virus and VSV-GFP (Fig. 2.5, B and C). Compared to cells reconstituted with wild-type TRAF3 and TRAF5, secretion of IFN- α and IFN- β after infection with Sendai virus and VSV-GFP, respectively, was significantly increased in cells reconstituted with the TRAF5 Y462F or F429Y mutants as compared to cells reconstituted with wild-type TRAF5. Similarly, as compared to reconstitution with wildtype TRAF5, cells reconstituted with the TRAF5 Y462F or F429Y mutants showed increased expression of IFN- β mRNA and of IP10 and MX1 mRNAs (both of which are IFN-stimulated genes) at 8 and 12 hours after infection with VSV-GFP. Our results suggested that the TRAF5 Y462F and F429Y mutants restored type I IFN production on viral infection.

We also assessed the antiviral responses of the reconstituted MEFs by quantification of GFP fluorescence and viral titers after infection with VSV-GFP. As expected, cells

reconstituted with wild-type TRAF3 were resistant to infection, evidenced by both their low GFP fluorescence and low viral titers, whereas cells reconstituted with wild-type TRAF5 or vector were more susceptible to infection, as evidenced by both high GFP fluorescence and high viral titers. TRAF5 Y462F– or TRAF5 F429Y–reconstituted cells had an intermediate phenotype: They showed increased GFP fluorescence compared to TRAF3-reconstituted cells but much less GFP fluorescence compared to cells reconstituted with wild-type TRAF5, and their viral titers were about 10-fold lower than those of cells reconstituted with wild-type TRAF5 (Fig. 2.5, D and E).

We also generated single-cell clones for each mutant and wild-type TRAF5 construct. Although cells in a polyclonal population express different amounts of reconstituted protein, individual cells within a clonal cell line will express the same amount of reconstituted protein. Polyclonal populations therefore examine an average phenotype (cells with uneven expression of TRAF3 or TRAF5 can exhibit a corresponding spectrum of phenotype), but our experiments with the clonal cell lines allowed us to examine the effects of TRAF3 and TRAF5 at a given, uniform amount of expression. In agreement with our results using the polyclonal reconstituted cell lines, we found that clonal reconstitution with the TRAF5 Y462F or F429Y mutants, but not wild-type TRAF5, rescued resistance to VSV-GFP infection (Fig. 2.5F). Our findings suggest that, in agreement with our structure-based predictions, the TRAF5 Y462F or F429Y mutants can at least partially rescue resistance to VSV-GFP infection in TRAF3^{-/-} MEFs.

DISCUSSION

TRAF3 plays an important role in activating type I IFN secretion through both TLR-dependent and TLR-independent pathways.⁶ In the TLR independent pathway, cytoplasmic RNA sensors recruit the adaptor Cardif, which in turn associates with and activates TRAF3. The structural basis for the TRAF3-Cardif interaction is still not clear, although three potential TRAF binding sites in Cardif have been reported.^{9, 10} The recruitment of TRAF3 to Cardif is determined through its TRAF domain, and hybrid TRAF5 proteins containing the TRAF domain of TRAF3 gain the ability to bind to Cardif.⁸ Here, we present crystal structures of TRAF5 and the TRAF3-Cardif complex. Analysis of these structures has allowed us to dissect on the molecular level a structural basis for the interaction of TRAF3 with Cardif and its critical role in RIG-I-mediated type I IFN activation. Here, on the basis of crystal structure analysis and coprecipitation experiments, we suggest that TRAF3 can directly associate only with residues 138 to 153 of Cardif (138PSCPVPVQDTQPPESP153, which encompasses T2BM), and not with residues 151 to 165 or 425 to 439 of Cardif (which encompass T6BM1 and T6BM2, respectively).

Superposition of the TRAF3-Cardif and TRAF5 crystal structures allowed us to compare corresponding crevice residues between the TRAF domains of TRAF3 and TRAF5. We identified two residues that are not conserved between TRAF3 and TRAF5: The corresponding residues in TRAF3 are Phe473 and Tyr440 and in TRAF5 are Tyr462 and Phe429. The TRAF3 mutant Y440A was previously reported to have reduced Cardif binding.⁸ We reasoned that if sequence differences between these two corresponding residue positions were indeed responsible for the dissimilar abilities to bind to Cardif

and the RIG-I-mediated antiviral activity of TRAF3 and TRAF5, then point mutations of these residues in TRAF5 to the corresponding TRAF3 residue should switch TRAF5 toward a TRAF3-like function. We showed that two point mutations in the TRAF domain of TRAF5, Y462F and F429Y, give TRAF5 a TRAF3-like function in Cardif binding and in regulating TLR-independent IFN secretion. Considering the different physiological roles of TRAF3 and TRAF5, it is interesting that a single amino acid substitution can switch TRAF5 toward a TRAF3-like specificity.

It will be interesting to investigate how and if these same sites in the TRAF domain of TRAF3 determine binding specificity to other TRAF3 binding partners. Ely et al. have reported that TRAF domain binding crevices undergo subtle structural adjustments when bound to different proteins.²² When human TRAF3 is recruited by TANK, they suggested that the phenyl ring of Tyr377 (corresponding to mouse residue Tyr440) rotates away from an adjacent β strand, thereby disrupting a salt bridge with Arg393 to form a new hydrophobic interaction with Leu193. Given the role of TRAF3 in both TLR-dependent and TLR-independent type I IFN pathways, it will be interesting to determine the interactions between TRAF3 and its adaptors in the TLR-dependent pathway. Ligation of TLRs causes the recruitment of Toll/interleukin-1 receptor (TIR) domain-containing adaptor proteins, including myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adaptor-inducing IFN- β (TRIF), to the signaling complex and triggers activation of downstream transcription factors. Whereas TLR3/4 (TLR3 and TLR4) and TLR7/8/9 trigger IFN secretion through a TRAF3-dependent process, TLR3/4 recruit TRAF3 through TRIF, and TLR7/8/9 recruit TRAF3 through the adaptor protein MyD88.^{6, 23} It will be interesting to determine the residues in TRAF3 that

mediate interactions between TRAF3 and MyD88 or TRIF, and to further characterize the possible dynamic structural adaptation these residues undergo to accommodate interactions with the diverse binding partners of TRAF3. It will also be interesting to learn whether the TRAF5 Y462F and F439Y mutants gain function in TLR-dependent IFN secretion, as well as other TRAF3-regulated signaling pathways including BAFFR and alternative NF- κ B activation.

Our results elucidate a structural basis for the selective role of TRAF3 within the TRAF family in promoting RIG-I-mediated type I IFN secretion. Because type I IFN has a pathogenic role for certain autoimmune disorders including systemic lupus erythematosus, a TRAF3 signaling blockade mediated through the TRIF and MyD88 adaptors could conceivably be a therapeutic target. However, because TRAF3 regulates so many signaling cascades, a complete blockade of TRAF3 signaling would have distinctly decreased utility. Further understanding of the structural specificities of TRAF3 for its various ligands, including Cardif, could allow the development of small-molecule therapeutics that are targeted to the ligand-binding pockets of TRAF3 and designed specifically to inhibit the interaction of TRAF3 with only a subset of ligands. Our results illustrate that by affecting interactions with signaling adaptors, even seemingly minor structural differences between TRAF family members can alter their function. Further analysis of how structural differences in TRAF proteins determine their specific functions will improve efforts to intelligently guide immunological responses to pathogens and autoimmune stimuli.

	TRAF3-Cardif	TRAF5
Data collection		
Detector	SSRF 17U	KEK BL5A
Space group	P321	P2 ₁ 2 ₁ 2 ₁
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	83.491, 83.491, 78.194	71.053, 90.579, 117.216
α , β , γ (°)	90, 90, 120	90, 90, 90
Resolution (Å)	2.20 (2.20- 2.24)*	2.80 (2.80-2.85)*
<i>R</i> _{merge}	0.185 (0.689)	0.169(0.503)
<i>I</i> / σ <i>I</i>	16.84 (2.74)	18.8(5.5)
Completeness (%)	99.9 (100)	93.8(100)
Redundancy	6.9 (5.9)	10.5 (11.0)
Refinement		
Resolution (Å)	2.20-36.8	2.80
No. reflections	15312	16783
<i>R</i> _{work} / <i>R</i> _{free}	0.178/0.223	0.224/0.273
No. atoms		
Protein	1651	4228
Ligand/ion	0	0
Water	151	44
<i>B</i> -factors		
Protein	33.0	50.7
Water	38.5	32.8
R.m.s. deviations		
Bond lengths (Å)	0.007	0.011
Bond angles (°)	1.042	1.156

*Values in parentheses are for the highest-resolution shell.

Table 2.1. Data collection and refinement statistics (molecular replacement).

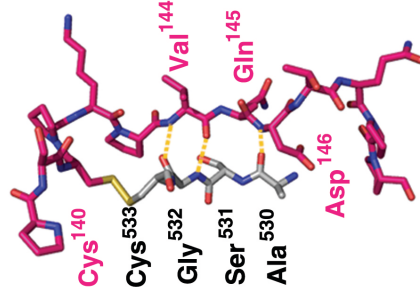
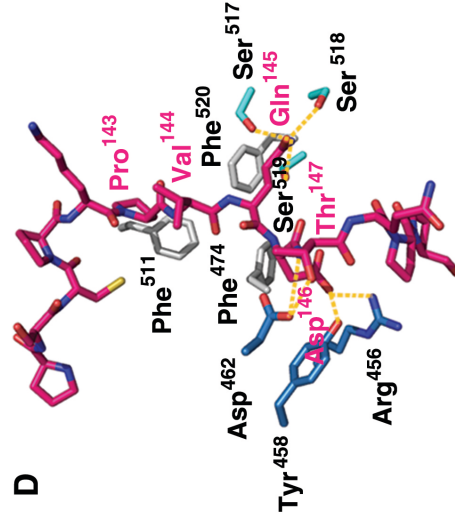
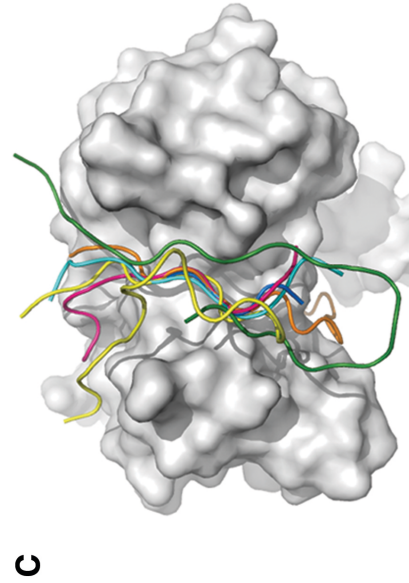
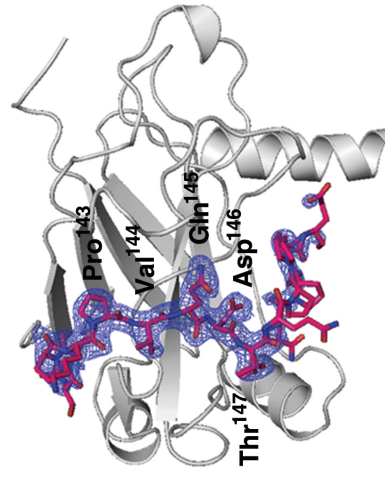
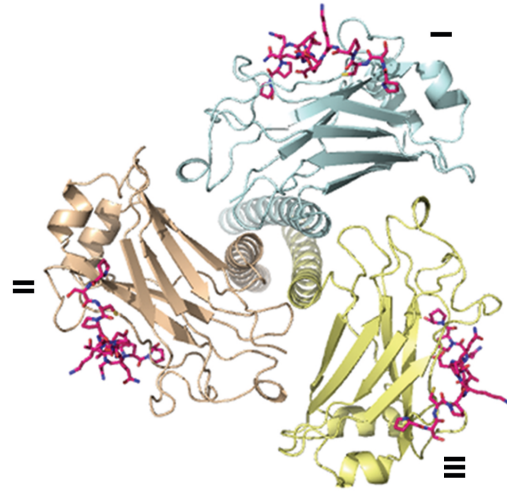
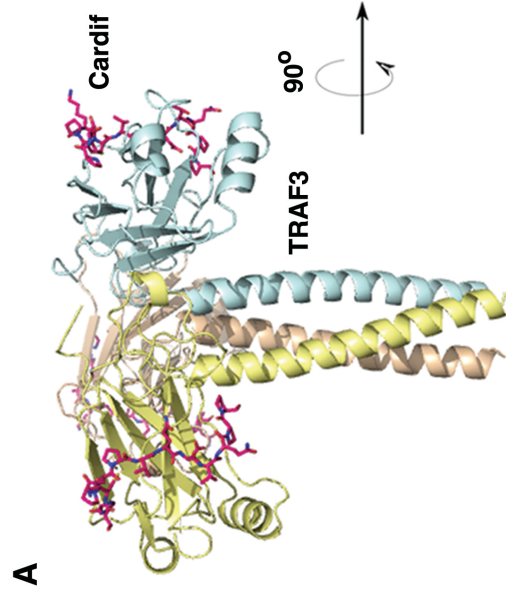
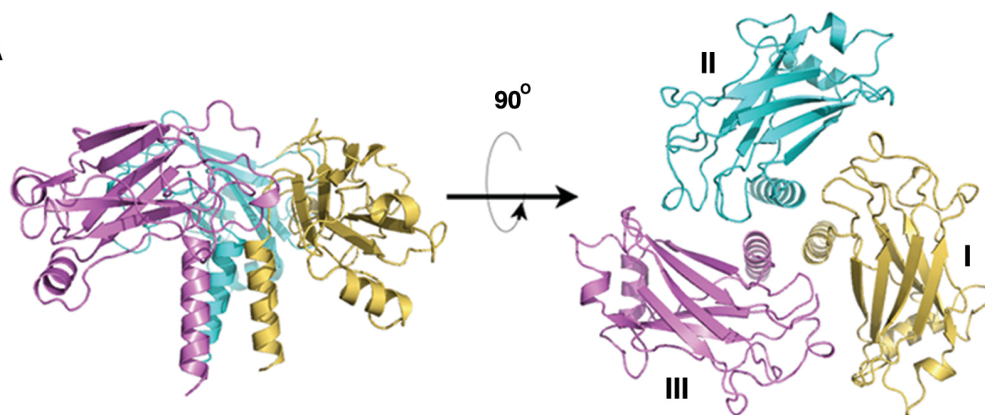
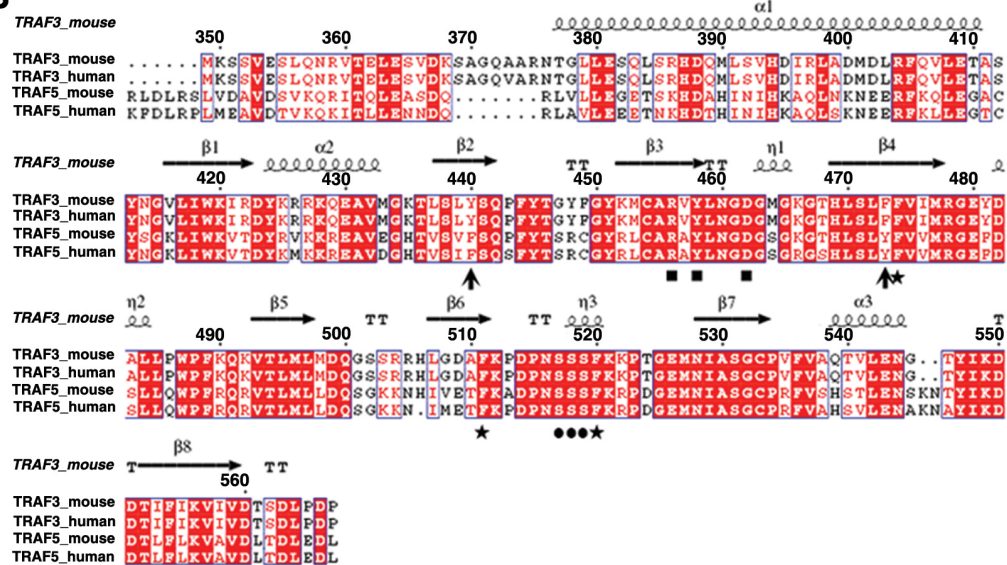


Figure 2.1. Structure of the TRAF3-Cardif complex. A) Representation of TRAF3 homotrimers, shown from both side (left) and top (right) views. The Cardif peptide is in lavender. B) Electron density map of Cardif bound to TRAF3. The blue meshwork represents the $2F_o - F_c$ electron density map contoured at 1.2σ . TRAF3 is gray; the Cardif peptide backbone is lavender. Cardif residues ¹⁴³PVQDT¹⁴⁷ are labeled. C) Different peptides binding to TRAF3. The surface of TRAF3 is gray; peptides shown are from Cardif (red), CD40 (green), TANK (cyan), LT β R (yellow), LMP1 (blue) and BAFF-R (orange). D) Molecular TRAF3-Cardif interactions. Key TRAF3-Cardif interactions are shown by yellow dotted lines. Conserved ‘hot spot’ residues - the hydrophobic patch, the serine fingers and the polar residues – are in grey, cyan and blue, respectively. For clarity, only one of the alternative conformations of Cardif is shown in panels A and D.

A



B



C

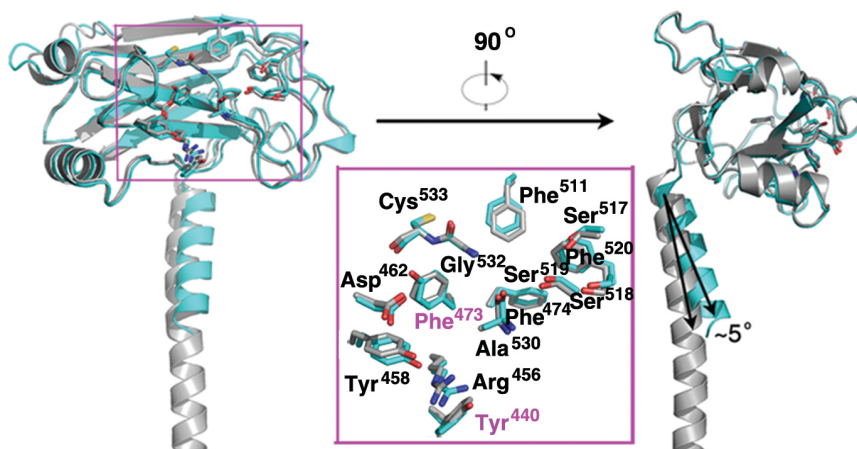


Figure 2.2. Structural comparison of TRAF5 and TRAF3. A) Representation of TRAF5 homotrimers. B) Sequence alignment of the TRAF domains of TRAF3 and TRAF5. The 'hotspot' residues in the hydrophobic patch, the serine fingers and the polar residues are labeled as stars, dots and squares, respectively. The amino acids that play crucial roles in "switching" TRAF5 to TRAF3 are highlighted by black arrows. C) Superposition of TRAF3 and TRAF5 structures. TRAF3 is grey and TRAF5 is cyan. Conserved 'hotspot' residues, Phe⁴⁷³ and Tyr⁴⁴⁰ in TRAF3, and Tyr⁴⁶² and Phe⁴²⁹ in TRAF5 are shown within the magnified insert.

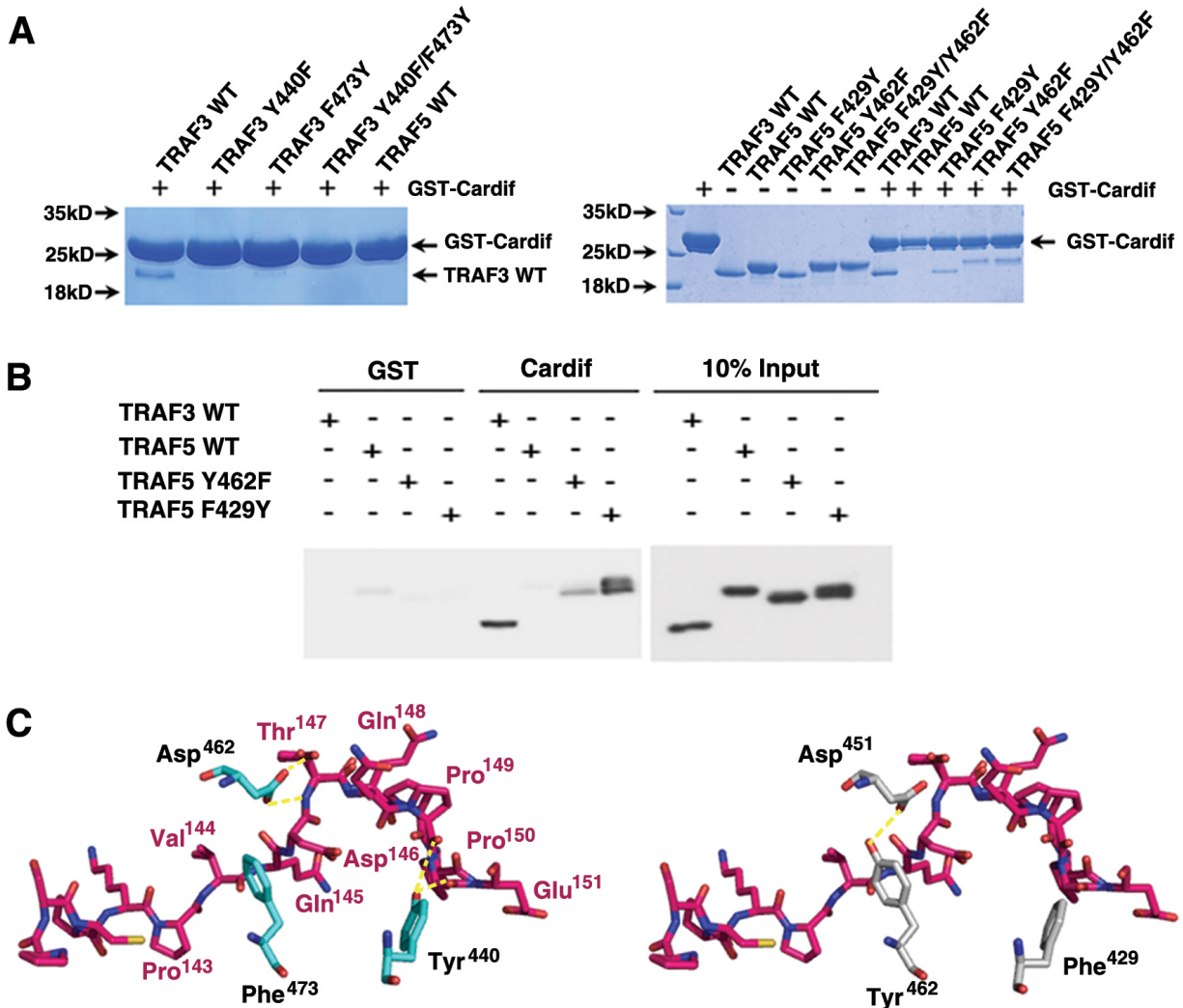


Figure 2.3. Tyr⁴⁴⁰ and Phe⁴⁷³ in TRAF3 are necessary for interaction with Cardif. A) Immunoblot analysis of interactions between a 13 residue Cardif peptide (residues 138-150) and the TRAF domains of wild-type TRAF3, wild-type TRAF5, TRAF3 mutants (left-panel), and TRAF5 mutants (right-panel). B). Lysates from cells expressing Flag-tagged wild-type TRAF3, wild-type TRAF5, TRAF5 Y462F and TRAF5 F429Y constructs were immunoprecipitated with GST-Cardif TIM and assayed by immunoblot. C) The presumed interactions (orange dotted line) between TRAF3 (left panel) and Cardif are absent in TRAF5 (right panel). Results in panel A and panel B are confirmed by 3 independent experiments.

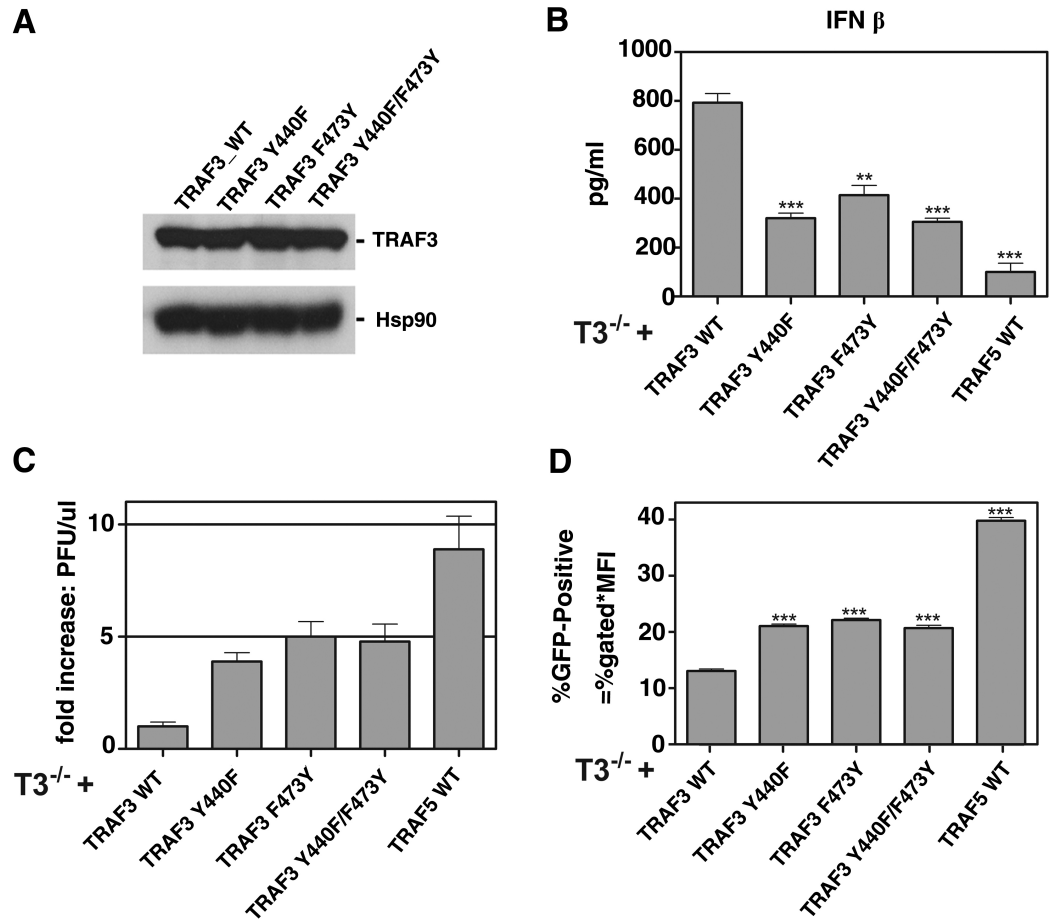
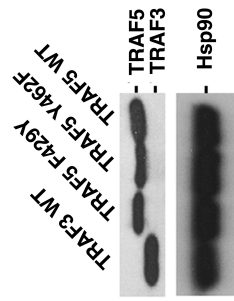
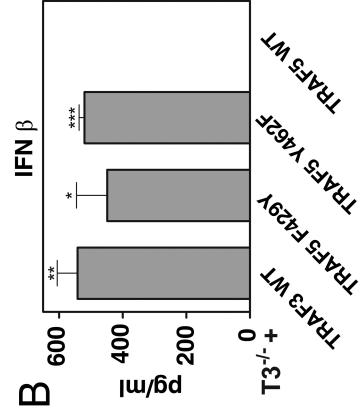


Figure 2.4. TRAF3 Y440F and F473Y lose antiviral function. A) Immunoblot of *Traf3*^{-/-} MEFs reconstituted with wild-type TRAF3, wild-type TRAF5, TRAF3 F440Y, TRAF3 Y473F, or TRAF3 F440Y/Y473F. B) IFN- β production was assessed in reconstituted *Traf3*^{-/-} MEFs infected with VSV-GFP. (C) Viral titer was assessed in reconstituted *Traf3*^{-/-} MEFs infected with VSV-GFP (D). VSV-GFP fluorescence was quantified and was calculated by the product of the percent GFP-positive cells and the geometric mean fluorescence intensity (MFI) of GFP. Results represent an average of 3 independently-analyzed biological replicates and were confirmed by 3 separate experiments. Error bars indicate the SD. Unpaired *t* test, two-tailed, *n*=3. *: *p*<0.05; **: *p*<0.01.

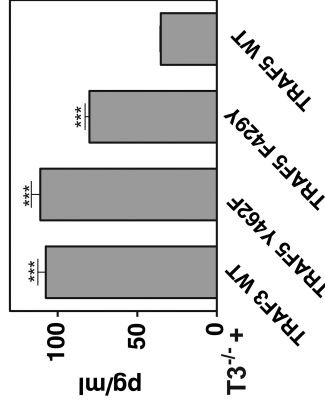
A



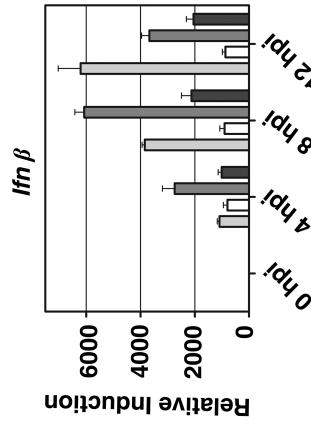
B



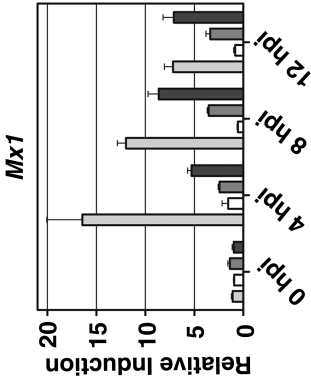
IFN α



C



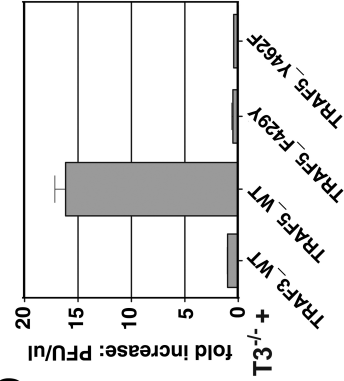
Relative Induction



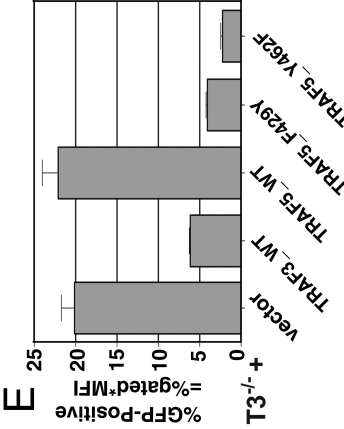
Relative Induction

Legend: TRAF3 WT (white), TRAF5 WT (light gray), TRAF5 F429Y (dark gray), TRAF5 Y462F (black)

D



E



F

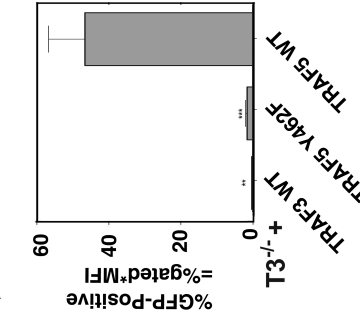
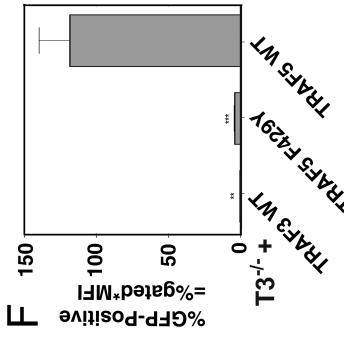


Figure 2.5. The TRAF5 Y462F and F429Y mutants gain TRAF3-like antiviral function.

A) Immunoblot of *Traf3*^{-/-} MEFs that were reconstituted with wild-type TRAF3, wild-type TRAF5, TRAF5 F429Y, TRAF5 Y462F, or vector. B) IFN- α production by Sendai virus-infected reconstituted *Traf3*^{-/-} MEFs and IFN- β production by VSV-GFP-infected reconstituted *Traf3*^{-/-} MEFs. C) mRNA was collected at 0, 4, 8, and 12 hours after infection with VSV-GFP and assayed by Q-PCR for induction of *IFN- β* , *IP10* and *MX1*. D-E) Viral titer was assessed in reconstituted *Traf3*^{-/-} MEFs infected with VSV-GFP (D). VSV-GFP fluorescence was quantified (E). F) Single-cell clones were generated from stably-reconstituted *Traf3*^{-/-} MEFs. GFP fluorescence was determined in cells were infected with VSV-GFP. Single-cell clones from cells reconstituted with TRAF5 F429Y (left-panel) and Y462F (right-panel) were tested in separate experiments. Plots represent average GFP fluorescence of three single-cell clones per construct. All results represent an average of 3 biological replicates and were confirmed by 3 separate experiments. Error bars indicate the SD. Unpaired *t* test, two-tailed, n=3. *: p<0.05; **: p<0.01.

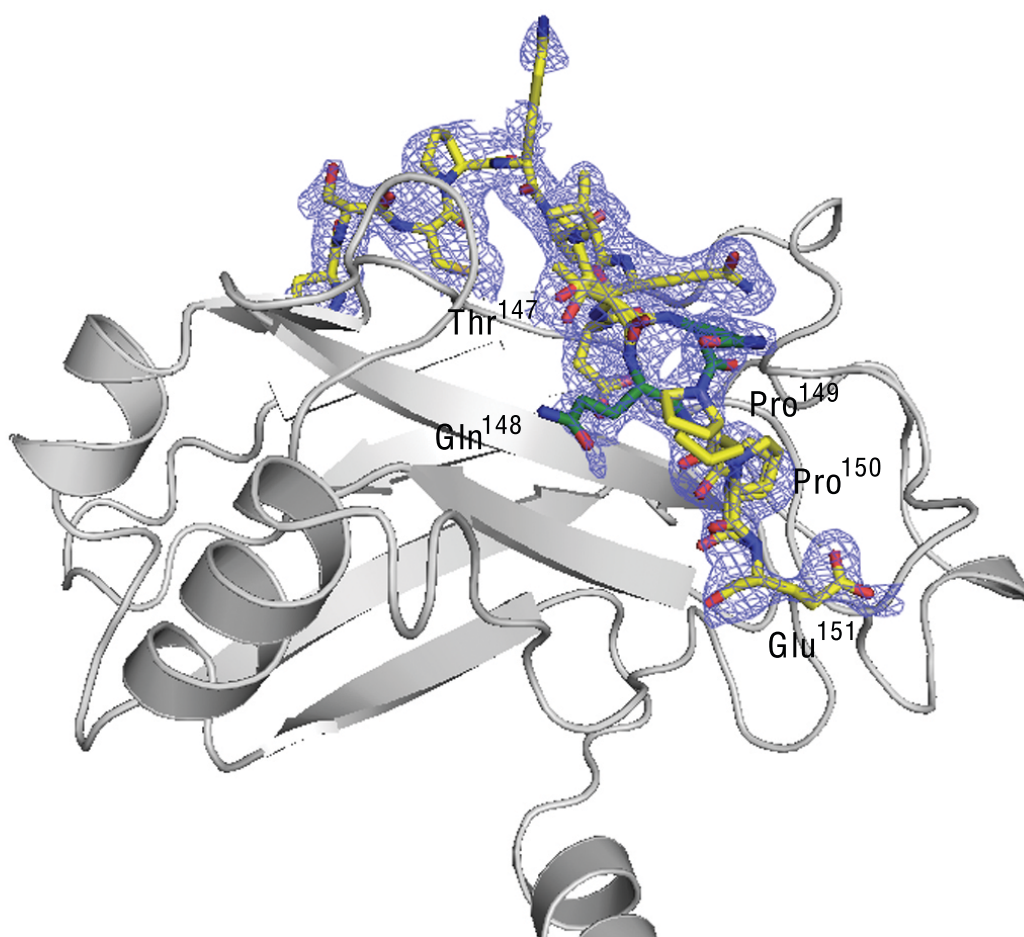


Figure 2.S2. Alternative confirmation of the C-terminal peptide of Cardif bound to TRAF3. The blue meshwork represents the 2F_o-F_c electron density map contoured at 0.7 σ. TRAF3 is in gray; the Cardif peptide is in yellow. Gln¹⁴⁸ is highlighted in green. The C-terminal residues of Cardif are labeled.

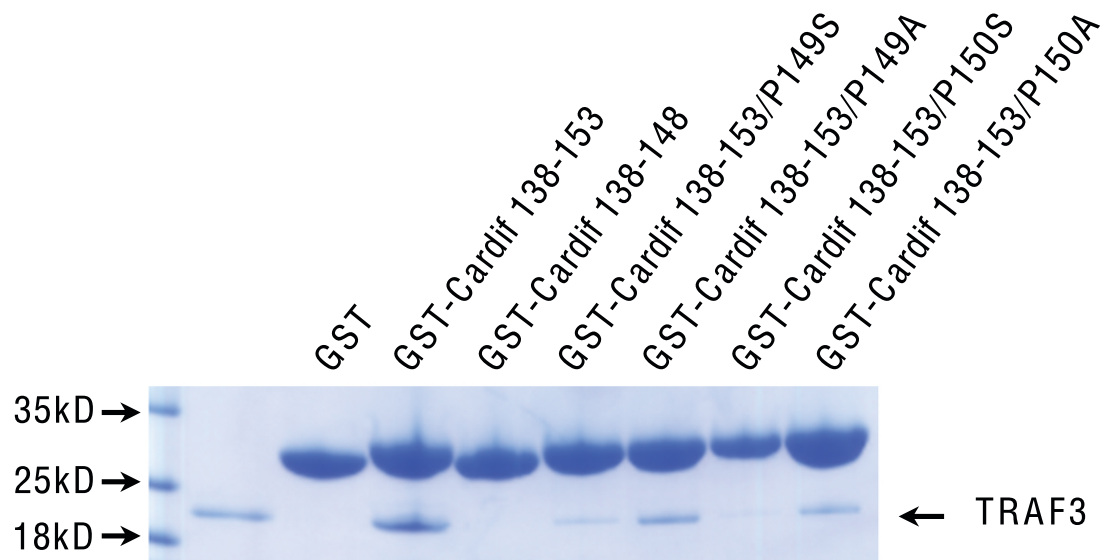


Figure 2.S3. The C-terminal region of the Cardif peptide is necessary for the interaction with TRAF3. The N-terminally GST-tagged Cardif peptides (residues 138-153, 138-148, P149A, P149S, P150A, P150S) were bound to glutathione beads. After incubation with TRAF3, the glutathione beads were washed and analyzed by SDS-PAGE for TRAF3-Cardif binding. These results were confirmed using different batches of proteins (N=3 independent experiments).

MATERIALS AND METHODS

Constructs and protein expression and purification

The TRAF domain of mouse TRAF3 (residues 376 through the C terminus) was cloned into a pET21b vector (Invitrogen). The plasmid was transformed into *Escherichia coli* strain BL21(DE3) cells. Bacteria were cultured in LB medium at 310 K with ampicillin (100 mg/liter). When the absorbance at 600 nm (A_{600}) reached 0.6 to 0.8, the culture was induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG); bacteria were harvested 4 hours after induction by centrifugation at 5000 rpm for 10 min. The pellet was resuspended with denaturing lysis buffer [6 M urea, 25 mM tris (pH 7.5), 5 mM imidazole, 5 mM β -mercaptoethanol] and lysed by sonication. The lysate was separated by centrifugation at 30,700g for 30 min. The supernatant was bound to Ni-NTA (nickel-nitrilotriacetic acid) beads (Qiagen Inc.) and washed several times with denaturing lysis buffer. TRAF3 was refolded in urea gradient buffer (4 to 0 M) with 25 mM tris at pH 7.5 and 5 mM β -mercaptoethanol. To produce a shortened TRAF3 for crystallization, TRAF3 was digested by trypsin (1:1000 molecular ratio) for 4 hours at 277 K. The reaction was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride (final concentration). The shortened TRAF3 was further purified by gel filtration with a Superdex 75 column (GE Healthcare Inc.) on fast-flow protein purification system.

The TRAF domain of TRAF5 (residues 365 through C terminus) was cloned into pGEX-6p-1. The plasmids were transformed to BL21(DE3) cells grown in LB-ampicillin medium at 37°C. When culture density reached 1.5 (A_{600}), the bacteria were induced with 0.1 mM IPTG at 16°C for 24 hours. The bacteria were harvested by centrifugation at 7000g for 10 min and lysed under 15,000 psi in 20 mM tris (pH 7.5), 150 mM NaCl, 5

mM EDTA, 1 mM dithiothreitol (DTT). GST tags were cleaved by PreScission Protease at 4°C for 16 hours. The TRAF domain of TRAF5 was separated by gel filtration with Superdex 200 (GE Healthcare Inc.).

The Cardif peptide (138PSCP KP VQDTQPPE SPVENSE158) used for cocrystallization with TRAF3 was purchased from GL Biochem Ltd. and was 99% pure.

Crystallization

For cocrystallization of Cardif with TRAF3, the Cardif peptide was dissolved in filtered water at 10 mg/ml and added to purified TRAF3. The ratio of Cardif peptides to TRAF3 was 1:1, and the final concentration was 3 to 4 mg/ml as confirmed by Coomassie blue staining of SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Crystals were observed in multiple conditions as screened with the Crystal Screen and Index reservoir solutions (Hampton Research Inc.) with hanging drops at 289 K. The crystals grew to a hexagonal shape with usable size in the conditions of 0.1 M Hepes (pH 7.5), 10% polyethylene glycol 6000 (PEG6000), and 5% (v/v) 2-methyl-2,4-pentanediol.

The TRAF5 TRAF domain [crystallization buffer: 20 mM tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM DTT] was crystallized with 0.1 M ammonium acetate, 0.1 M bis-tris (pH 6.5), 15% (w/v) PEG10000 in a hanging-drop diffusion system at 16°C.

Crystallographic analysis

Diffraction data were collected at the Photon Factory, KEK (Japan) and Shanghai Synchrotron Radiation Facilities (SSRF, China). Crystals were flash-frozen before data collection with 10% glycerol. Data were processed by HKL2000 (24). The structure of

TRAF5 was solved by Phaser (25) with a molecular replacement method. The native truncated TRAF3 atomic coordinates 2GKW were used as an initial model. The TRAF3/Cardif complex was refined with the native TRAF3 atomic coordinates. An $F_o - F_c$ difference map was used to build the Cardif peptide. Structure refinement and model rebuilding were done iteratively with Phenix (26) and Coot (27). The Ramachandran plots were generated with Procheck (28); 97.44 and 2.56% of the amino acids in TRAF3/Cardif and 95.98 and 4.02% of the amino acids in TRAF5 were in the most favorable and additional allowed regions, respectively. No amino acids were in the disallowed region. The atomic coordinates and diffraction data have been deposited in the Protein Data Bank (4GHU for TRAF3/Cardif and 4GJH for TRAF5). All figures were prepared by Coot and Pymol (<http://www.pymol.org/>). The angle between the N-terminal α helices in TRAF3 and TRAF5 in Fig. 2C was calculated with Pymol. The superposition is performed with Coot with the C-TRAF domains (TRAF3 416–559 and TRAF5 353–498).

Reagents

Anti-FLAG (M2) antibodies were obtained from Sigma. Anti-Hsp90 (H-114) antibodies were obtained from Santa Cruz Biotechnology. FuGENE 6 reagent was purchased from Roche. VSV-GFP was a gift from G. Barber.

Constructs

GST-Cardif (TIM) was constructed as previously described.²⁹ Murine TRAF3, TRAF5, and mutant constructs were generated as previously described in either pBABE-

puromycin or pBABE-puro-TAP retroviral vectors with N-terminal FLAG tags.⁸ Single point mutants of TRAF3 and TRAF5 were constructed with a QuikChange kit (Stratagene) and the pBABE-puro-TAP-TRAF3 vector as template.

Cell culture and reconstitution

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). We performed transient transfection of HEK 293T cells using standard calcium phosphate methods. To reconstitute TRAF3^{-/-} cells, we transfected HEK 293T cells with a Moloney murine leukemia virus-Ψ_A helper construct plus either pBABE-puro alone or the indicated pBABE-puro construct using FuGENE 6. TRAF3^{-/-} cells were then infected with the filtered HEK 293T cell supernatants and then selected with puromycin (5.0 mg/ml).

Immunoblotting

Cells were lysed in modified radioimmunoprecipitation assay buffer containing 0.5% (v/v) NP40 and 0.1% (w/v) sodium deoxycholate supplemented with protease inhibitor tablets (Roche). Equal amounts of whole-cell lysates were resolved by 8% SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

GST pulldown assays

GST pulldown assays were performed as previously described.⁸ Glutathione beads (Sigma-Aldrich) were incubated with E. coli-expressed GST alone or GST-TIM

for 12 hours. Glutathione beads were then washed and incubated for 12 hours with lysates of HEK 293T cells expressing the indicated TRAF3 or TRAF5 constructs. For Fig. 2.3A and figs. 2.S1 and 2.S3, the TRAF3 or TRAF5 proteins were purified as described above. After being washed, proteins were eluted from the beads, resolved by 8% SDS-PAGE, and analyzed by Western blotting with the indicated antibodies, as described previously.⁸ All these results were confirmed by three independent experiments.

Viral inhibition assays

Viral inhibition experiments were performed as described previously.⁶ Reconstituted TRAF3^{-/-} MEFs were infected with either VSV-GFP or Sendai virus; cell supernatants were collected 12 hours after infection for analysis by enzyme-linked immunosorbent assay, plaque assay, and measurement of GFP fluorescence.

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CHAPTER 3

Optineurin is a Positive Regulator of Non-Canonical NF κ B Signaling

ABSTRACT

Optineurin (optic neuropathy inducing) facilitates the assembly of large, multimolecular complexes that mediate diverse cellular functions. Optineurin is known to interact with TRAF3 (a protein that limits non-canonical NF κ B signaling), and so we explored whether Optineurin functions to regulate non-canonical NF κ B through this association with TRAF3. In our system, overexpression of optineurin enhances, and conversely knockdown of optineurin inhibits, activation of non-canonical NF κ B. We suggest that upon stimulation of the non-canonical NF κ B pathway, optineurin is recruited to the TRAF-clAP-NIK complex, wherein it acts to promote TRAF3 degradation and support NIK accumulation. In light of our lab's data showing that TRAF3 functions to inhibit the cytosolic IFN β response to DNA via an inhibition of the NIK-TBK1 axis, we hypothesize that optineurin should function to promote IFN β induction in the cytosolic DNA pathway.

INTRODUCTION

Optineurin (optic neuropathy inducing) is a widely-expressed 67-kDa protein.¹ It is a cytosolic protein that is not secreted, and it is ubiquitously expressed in wide range of tissues, including heart, brain, skeletal muscle, kidney, pancreas, adrenal cortex, liver, and the eye.² Mutations in Optineurin have been linked with normal-tension glaucoma (a subtype of primary open angle glaucoma, or POAG), both familial and sporadic amyotrophic lateral sclerosis (ALS), and Paget's disease of bone.³⁻⁶ Optineurin is most closely related to NEMO (human Optineurin shares 53% amino acid homology), and is a member of the ubiquitin-binding domain in ABINs (A20-binding inhibitors of NFκB) and NEMO (UBAN) family.² The protein consists of a NEMO-like domain, leucine zipper motif, multiple coiled-coil motifs, an ubiquitin binding domain (UBD, or UBAN), a microtubule associated protein 1 light chain 3 (LC3)-interacting motif, and a carboxyl (C)-terminal zinc finger.¹

Optineurin interacts with a wide array of proteins, acting as a platform to facilitate the assembly of large, multimolecular complexes that mediate various cellular functions.

Optineurin is involved in regulating such diverse processes as protein trafficking, regulation of exocytosis and vesicle traffic from the Golgi to the plasma membrane, maintenance of the Golgi apparatus, and glutamate receptor signaling.⁷⁻¹⁴ Optineurin also functions in innate immunity: it is an autophagy receptor that links the TBK1 signaling pathway to the autophagic elimination of cytosolic bacteria.¹⁵⁻¹⁷

Optineurin likely has additional (though still controversial) functions in regulating NFκB and antiviral IFN pathways. Initial work suggested that Optineurin was a negative

regulator of the canonical NF κ B pathway (induced by TNF α); the suggested mechanism for this negative regulation was competition of Optineurin with NEMO for binding to polyubiquitinated RIP.¹⁸ Separate work also suggested that Optineurin was a negative regulator of IFN β production in response to RNA virus infection; the suggested mechanism for this negative regulation involved interaction of Optineurin with a TBK1/TRAF3 complex.¹⁹ However, two separate studies have since proposed a conflicting hypothesis that Optineurin does not influence NF κ B in innate immune cells, and that Optineurin actually positively regulates the TBK1/IRF3 pathway in the antiviral immune response.^{20, 21} Additional work is needed to clarify what role, if any, Optineurin plays in regulation of NF κ B and IFN β production.

RESULTS

Optineurin overexpression enhances activation of non-canonical NF κ B

Optineurin is known to interact with TRAF3 and TBK1. Our own lab's work suggests that TRAF3's negative regulation of the non-canonical NF κ B pathway limits association of NIK with TBK1; this NIK-TBK1 axis promotes IFN β production in response to cytosolic DNA. We therefore considered Optineurin a candidate molecule, first, for regulation of non-canonical NF κ B, and second, for regulation of the IFN β response to cytosolic DNA.

First we examined an overexpression system. We transfected wild type (wt) murine embryonic fibroblast (MEF) cells with either vector control or GFP-tagged Optineurin. 24 hours after transfection we stimulated the MEF cells with an agonist antibody against LT β R (α LT β R), and collected whole-cell lysate (WCL) for immunoblot (IB). As shown in figure 3.1, there is enhanced accumulation of NIK in MEF cells transfected with Optineurin versus vector alone. The enhanced NIK accumulation suggests that overexpression of Optineurin promotes activation of non-canonical NF κ B, at least in response to stimulation with α LT β R. In accordance with the enhanced accumulation of NIK, we see in figure 3.1 that cells transfected with Optineurin versus vector show increased processing of p100 to p52. This suggests that the enhanced accumulation of NIK observed on overexpression of Optineurin translates downstream to increased activation of IKK α kinase activity, targeting p100 for partial degradation to p52. This provides further support that overexpression of Optineurin promotes activation of non-canonical NF κ B in response to stimulation with α LT β R. Additional blots in figure 3.1

confirm equivalent loading (IB:Hsp90) and expression of the GFP-tagged Optineurin construct (IB:GFP).

Stable knockdown of Optineurin decreases non-canonical NF κ B

We next examined a knockdown system. We infected wt MEFs with lentiviral particles containing a mixture of shRNA constructs, against either control vector or against endogenous Optineurin. After infection, we used puromycin selection to enrich for infected cells expressing the shRNA constructs. This allowed us to establish cell lines with either stable knockdown of control vector (shCTRL) or Optineurin (shOPTN). As demonstrated in figure 3.2, we were able to achieve partial knockdown of Optineurin by our methodology.

We next used these stable knockdown cells to examine activation of the non-canonical NF κ B pathway. First, we tested the cells using α LT β R, as demonstrated in figure 3.3. When we knockdown Optineurin, we see less NIK accumulation, and we see less processing of p100 to p52. Additional blots confirm equivalent loading (IB:Hsp90). These results suggest that cells expressing less Optineurin have decreased activation of non-canonical NF κ B after stimulation with α LT β R. Next, we directly examined the activation status of the non-canonical NF κ B pathway by probing for p52:RelB nuclear translocation (again, after stimulation with α LT β R). These results are shown in figure 3.4. In the shOPTN cells, we see decreased translocation of p52 to the nucleus, and decreased translocation of RelB to the nucleus. These results suggest that shOPTN cells have decreased translocation of the p52:RelB dimer to the nucleus and supports

the hypothesis that shOPTN cells show diminished activation of the non-canonical NFκB pathway.

We also tested activation of the non-canonical NFκB pathway in response to stimulation with TNF-like weak inhibitor of apoptosis (TWEAK); TWEAK signals through Fn14 to activate non-canonical NFκB. The results are shown in figure 3.5: as expected, the stable-Optineurin knockdown cells show reduced accumulation of NIK after stimulation with TWEAK. This suggests that cells expressing less Optineurin have decreased activation of non-canonical NFκB after stimulation with TWEAK.

We also tested activation of the non-canonical NFκB pathway in response to stimulation with SMAC mimetic (SM). SMAC mimetic is a small molecule inhibitor that mimics the pro-apoptotic Smac protein to antagonize cIAP function, promoting degradation of cIAP1/2. Therefore, on treatment with SMAC mimetic, NIK is allowed to accumulate by virtue of disintegration of the TRAF-cIAP ubiquitin ligase complex; this leads to activation of non-canonical NFκB. Figure 3.6 shows results of SM-treatment in shCTRL and shOPTN cells. The stable-Optineurin knockdown cells show reduced accumulation of NIK after stimulation with SM. This suggests that cells expressing less Optineurin have decreased activation of non-canonical NFκB after treatment with SM. Additional blots in figure 3.6 show equivalent loading (IB:Hsp90).

We demonstrated that stable-knockdown of Optineurin leads to decreased activation of non-canonical NFκB. We confirmed this for multiple non-canonical agonists, including

α LT β R, TWEAK, and SM. In combination with the overexpression results – in which cells expressing increased Optineurin demonstrated increased NIK accumulation and p100/52 processing – our results suggest that Optineurin functions as a positive regulator to promote activation of non-canonical NF κ B.

Optineurin associates with TRAF3 and NIK in overexpression system

Our results to this point have not addressed the mechanism of how Optineurin can promote non-canonical NF κ B signaling. The literature regarding Optineurin's role in RNA-viral infections, though, suggested a possible route. Optineurin is already known to associate with TRAF3, and TRAF3 obviously associates with NIK. We wondered if Optineurin associated with TRAF3 and NIK in the context of non-canonical NF κ B signaling. Perhaps with appropriate stimuli, Optineurin is recruited to the TRAF-cIAP-NIK complex and functions somehow to promote NIK accumulation.

We began by screening for interactions of Optineurin with relevant proteins of the non-canonical NF κ B signaling pathway. In HEK 293T cells, we coexpressed GFP-tagged Optineurin with an array of FLAG-tagged potential binding partners. We immunoprecipitated WCL with anti-FLAG antibody and then immunoblotted for GFP. The results are shown in figure 3.7. As expected, Optineurin interacts with TRAF3. Interestingly, Optineurin also interacts with NIK. However, Optineurin does not appear to interact with IKK α , p100, or cIAP1/2. These results suggest that the mechanism of how Optineurin functions to promote non-canonical NF κ B signaling is at the NIK-TRAF3 level.

Optineurin associates with TRAF3 endogenously after stimulation with α LT β R

We also wanted to examine if Optineurin interacts with TRAF3/NIK in an endogenous system. These results are shown in figure 3.8. We stimulated MEF cells with α LT β R, and immunoprecipitated WCL with anti-Optineurin antibody and then immunoblotted for TRAF3. We found that there is an inducible association between Optineurin and TRAF3: at baseline, they do not interact, but after stimulation with α LT β R, they associate to form a complex. Unfortunately, the antibody for NIK is very weak, and we were unable to demonstrate endogenous Optineurin-NIK interactions. However, we feel this is most likely a technical difficulty arising from the NIK antibody.

Optineurin promotes degradation of transfected TRAF3

To this point, our results suggest that Optineurin promotes activation of non-canonical NF κ B, and that Optineurin associates with TRAF3 (and possibly NIK) endogenously in a ligand-dependent fashion. We hypothesized that through its association with TRAF3, Optineurin is able to promote TRAF3 degradation, thereby promoting non-canonical NF κ B activation through promotion of TRAF3 degradation.

We began by checking if Optineurin could promote TRAF3 degradation in an overexpression system. Using HEK 293Ts, we cotransfected FLAG-tagged TRAF3 with increasing amounts of GFP-tagged Optineurin. Control vector was added to maintain the total amount of transfected DNA constant. We immunoblotted with anti-FLAG antibody to probe specifically for transfected TRAF3. Results are shown in figure 3.9.

There is a clear decrease in TRAF3 moving from lanes 2 to 6: lane 2 has the greatest amount of TRAF3 and lane 6 the least. This correlates inversely with the addition of increasing amounts of GFP-tagged Optineurin moving from lanes 2 to 6: lane 2 has no Optineurin transfected, and lane 6 has the largest amount of Optineurin transfected. Additional blots in figure 3.9 show equivalent loading (IB:Hsp90).

Our results show that transfection of increasing amounts of Optineurin decreases detection of FLAG-tagged TRAF3. The transfection of increasing amounts of Optineurin is either promoting TRAF3 degradation in accordance with our hypothesis, or it is somehow preventing TRAF3 expression and is an artifact unrelated to our hypothesis. In order to better distinguish between these two interpretations, we repeated the same experiment using FLAG-tagged TRAF2 instead of TRAF3. Because TRAF2 shares significant homology with TRAF3, any artifact interfering with TRAF3 expression should similarly interfere with TRAF2 expression. Results are shown in figure 3.9. In contrast to results obtained with TRAF3, we see no change in TRAF2 levels as the amount of Optineurin transfected increases (moving from lanes 2 to 6). In combination, these results suggest that transfection of increasing amounts of Optineurin does not prevent expression of either TRAF3 or TRAF2. It appears that transfection of increasing amounts of Optineurin promotes increasing degradation of transfected TRAF3, but does not affect degradation of transfected TRAF2. These results support the hypothesis that Optineurin is able to promote TRAF3 degradation.

Optineurin promotes degradation of endogenous TRAF3

We next examined if Optineurin could promote degradation of endogenous TRAF3, again using an overexpression system. In HEK 293Ts, we transfected increasing amounts of GFP-tagged Optineurin; control vector was added to maintain the total amount of transfected DNA constant. Results are shown in figure 3.10. Immunoblotting for endogenous TRAF3, we see moving from lane 1 to 5 a decrease in TRAF3. This correlates inversely with the amount of Optineurin transfected: lane 1 has no Optineurin transfected, and lane 5 has the largest amount of Optineurin transfected. Similarly to the case with FLAG-tagged TRAF3, our results show that transfection of increasing amounts of Optineurin decreases detection of endogenous TRAF3. In accordance with our hypothesis, this suggests that transfection of increasing amounts of Optineurin is promoting degradation of endogenous TRAF3.

Similarly to the case for FLAG-TRAF2, this appears to be a TRAF3-specific phenomenon. In contrast to results obtained for endogenous TRAF3, we see no change in levels of endogenous TRAF6 moving from lane 1 to 5. In a similar but separate experiment, we see no change in levels of endogenous cIAP1 or TRAF2 as the amount of Optineurin transfected increases. Additional blots in figure 3.10 show equivalent loading (IB:Hsp90) and confirm expression of transfected GFP-tagged Optineurin (IB:GFP). These results support our hypothesis that Optineurin is specifically able to promote TRAF3 degradation.

DISCUSSION

The data presented here suggests a role for Optineurin as a positive regulator of non-canonical NF κ B. In our system, overexpression of Optineurin enhances, and conversely knockdown of Optineurin inhibits, activation of non-canonical NF κ B. Based on interaction studies, we suggest that upon stimulation of the non-canonical NF κ B pathway, Optineurin is recruited to the TRAF-cIAP-NIK complex, wherein it acts to promote TRAF3 degradation and support NIK accumulation.

In light of our lab's data showing that TRAF3 functions to inhibit the cytosolic IFN β response to DNA via an inhibition of the NIK-TBK1 axis, we hypothesize that Optineurin should function to promote IFN β induction in the cytosolic DNA pathway. Our results suggest that Optineurin promotes accumulation of NIK, which our lab has shown interacts with TBK1 to promote IFN β induction in the cytosolic DNA pathway. It is possible that Optineurin is part of the NIK-TBK1 complex, even – Optineurin is already known to interact with TBK1, and we show here that Optineurin associates with NIK, at least in an overexpression system. It will be interesting to know if Optineurin does actually promote IFN β production in the cytosolic DNA pathway, especially considering the current controversy regarding whether Optineurin functions to promote or inhibit IFN β production in the RLR pathway. Potentially, Optineurin plays opposite roles in promoting IFN β in response to cytosolic DNA and inhibiting IFN β in response to cytosolic RNA. As our lab has shown with TRAF3, such opposite functions for regulation of the cytosolic DNA versus RNA IFN β responses do occur.

A collaborator's group has established knockout Optineurin mice, from which they have derived complete knockout MEF cells. In contrast to our results, they do not see a role for Optineurin in regulation of the non-canonical NF κ B pathway. Results are shown in figure 10. On stimulation with α LT β R, our competitors observe no difference in cytoplasmic p100 degradation or nuclear p52/RelB accumulation between the wt and knockout cells. These results would suggest that Optineurin does not play a role in regulation of the non-canonical NF κ B pathway. These results are opposite to ours, and confusing to make sense of. The situation is reminiscent of the protein TANK – initial knockdown and overexpression studies had suggested TANK was necessary for the IFN β response to RLR signaling, but only with characterization of the knockout mouse was it realized that TANK was not involved in the RLR IFN β response. Additional work needs to be done to clarify Optineurin's roles, if any, regulating the non-canonical NF κ B and IFN β responses. Fortunately, availability of the knockout mouse will greatly aid these studies and allow conclusive answers.

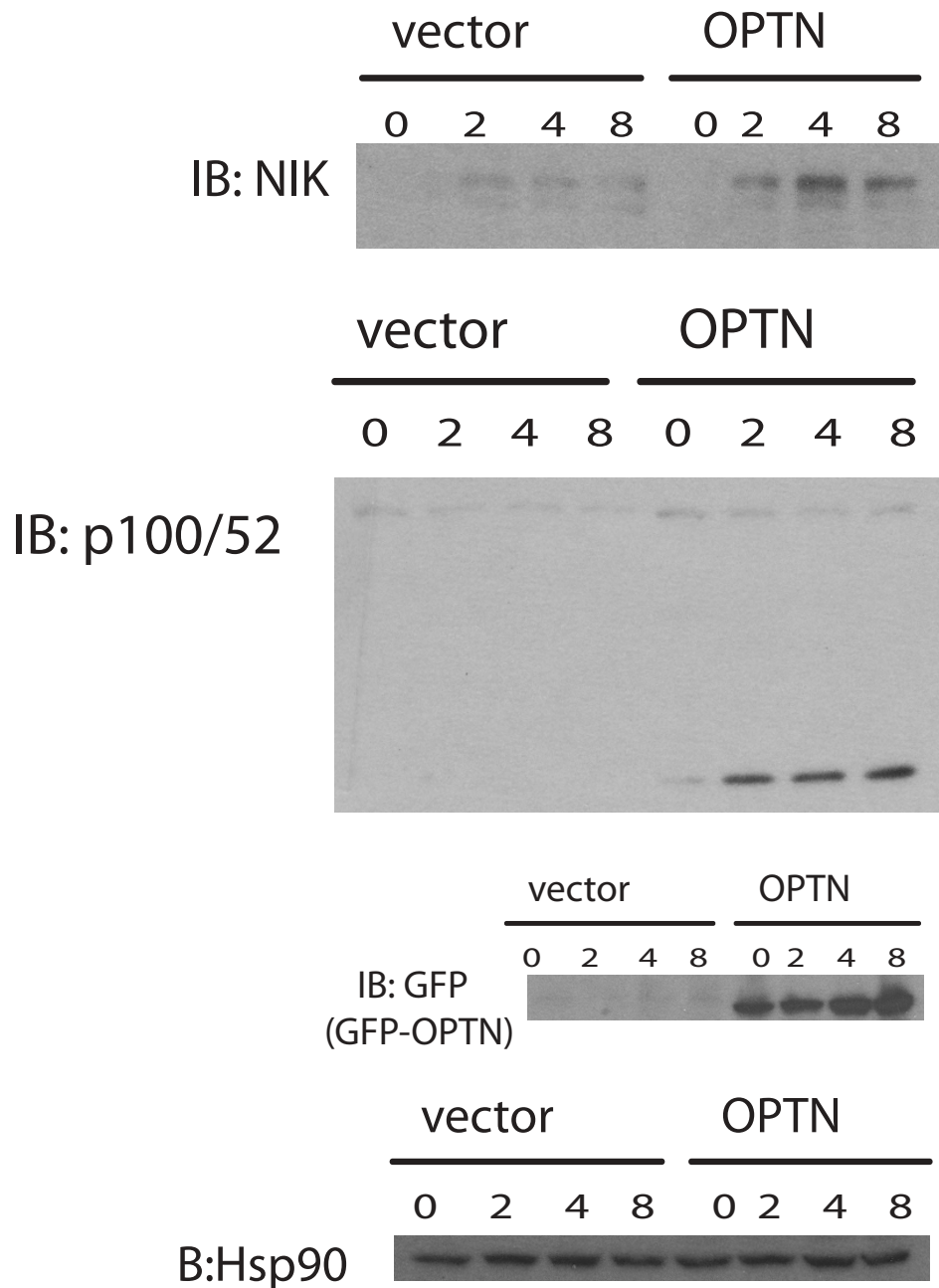


Figure 3.1. Optineurin overexpression enhances activation of non-canonical NF κ B. WT MEFs were transfected with either control or GFP-tagged Optineurin. 24 hours after transfection, MEF cells were stimulated with agonist α LT β R. WCL was collected at 0, 2, 4, and 8 hours post-stimulation, and assayed for protein expression of NIK, p100/52, GFP, and Hsp90.

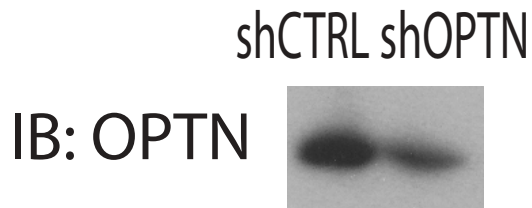


Figure 3.2. Stable knockdown of Optineurin. We infected WT MEFs with lentiviral particles containing a mixture of shRNA constructs, against either control vector or against endogenous Optineurin. After infection, we used puromycin selection to enrich for infected cells expressing the shRNA constructs. We collected WCL and assayed for expression of Optineurin.

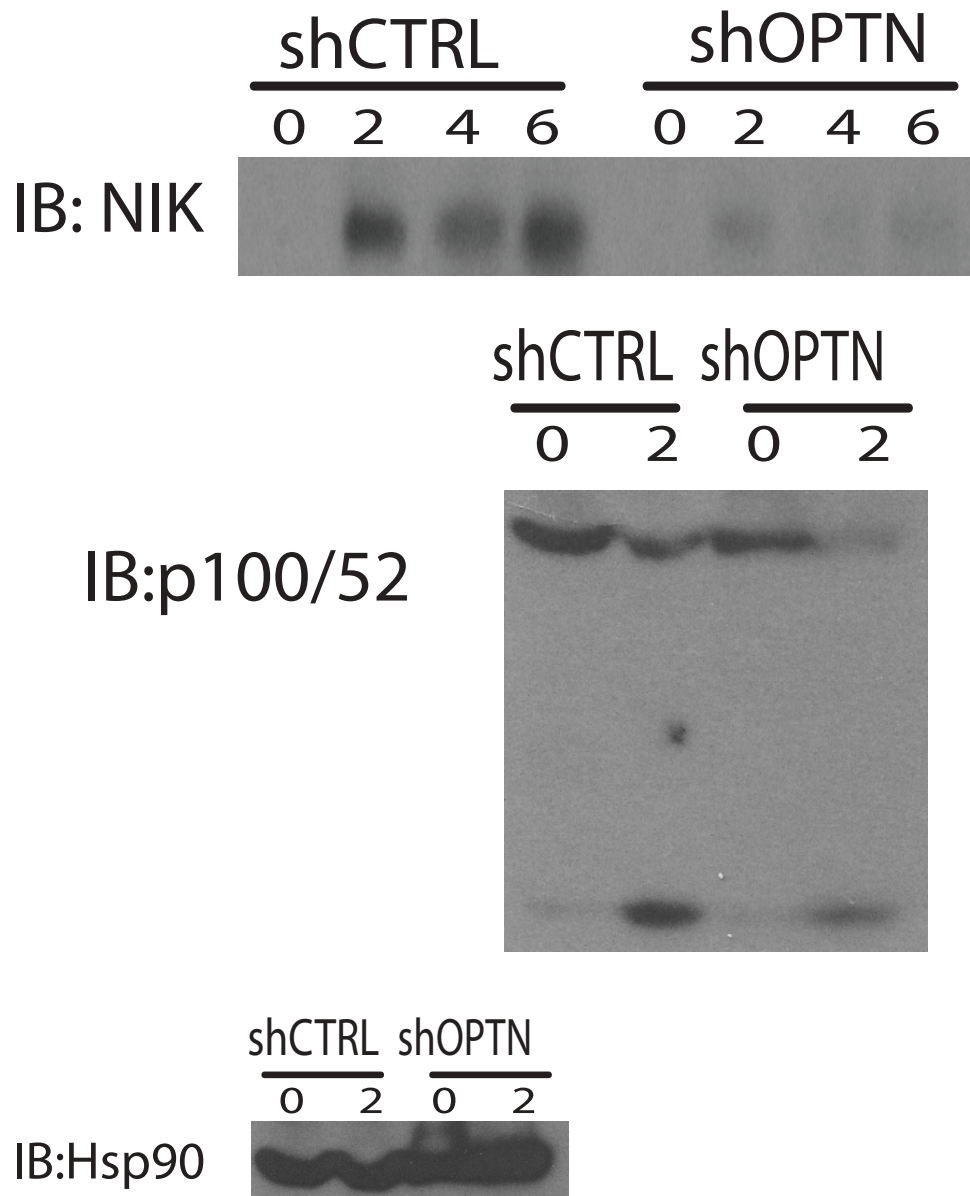


Figure 3.3. Stable Optineurin knockdown decreases non-canonical NF κ B on stimulation with agonist α LT β R. Stable shCTRL and shOPTN cells were stimulated with agonist α LT β R. WCL was collected at 0 and 2 hours post-stimulation, and assayed for protein expression of p100/52 and Hsp90.

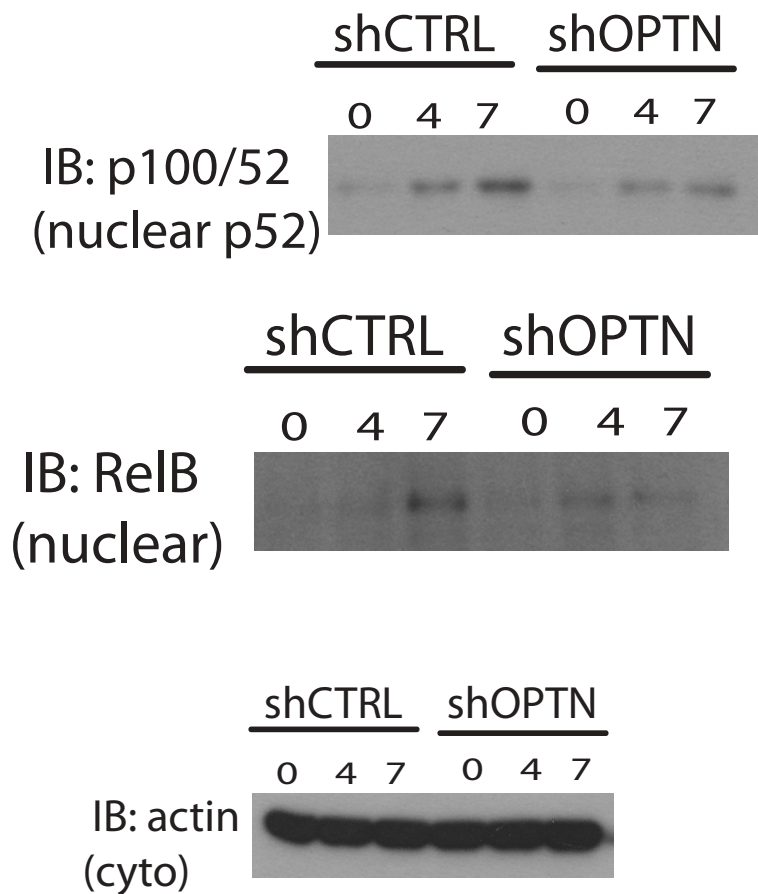


Figure 3.4. Stable Optineurin knockdown decreases nuclear translocation of p52:RelB on stimulation with agonist α LT β R. Stable shCTRL and shOPTN cells were stimulated with agonist α LT β R. Nuclear and cytoplasmic fractions were collected at 0, 4, and 7 hours post-stimulation and assayed for expression of p52, RelB, and actin.

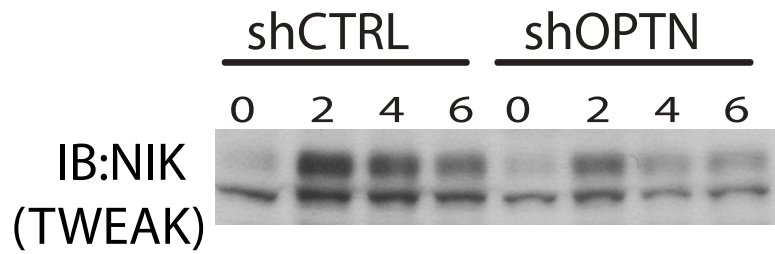


Figure 3.5. Stable Optineurin knockdown decreases non-canonical NF κ B activation on stimulation with TWEAK. Stable shCTRL and shOPTN cells were stimulated with TWEAK. WCL was collected at 0, 2, 4, and 6 hours post-stimulation and assayed for NIK.

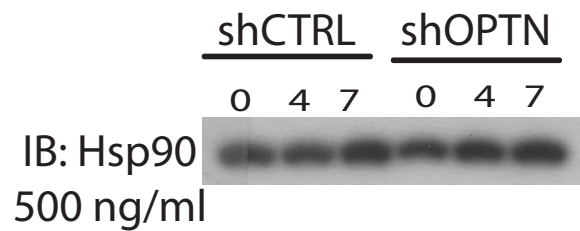
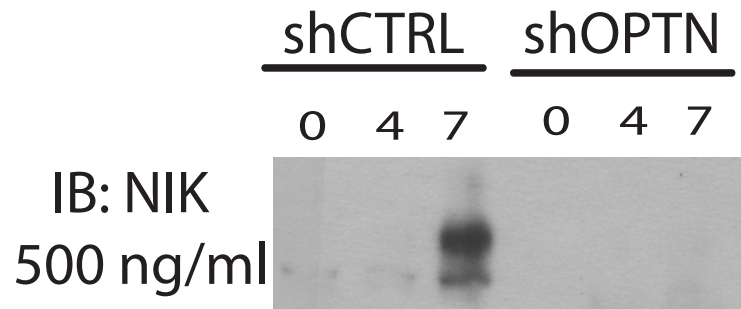


Figure 3.6. Stable Optineurin knockdown decreases activation of non-canonical NFκB on stimulation with SMAC mimetic. Stable shCTRL and shOPTN cells were stimulated with SMAC mimetic at 500 ng/ml. WCL was collected at 0, 4, and 7 hours post-stimulation and assayed for expression of NIK and Hsp90.

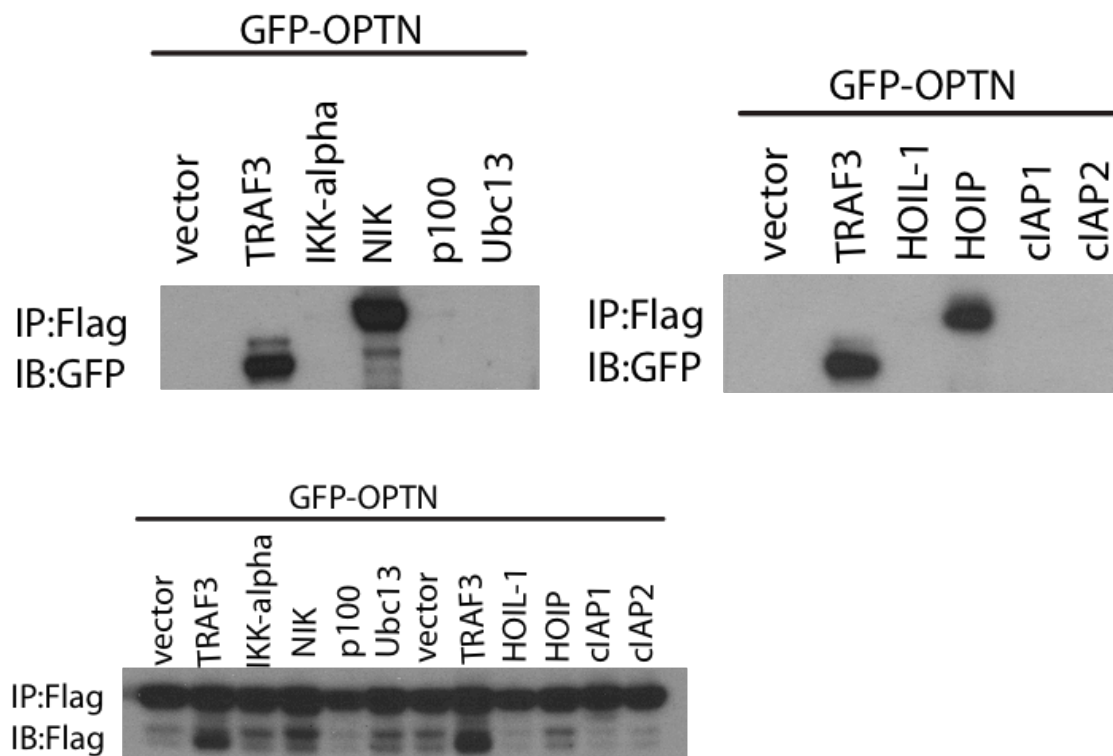


Figure 3.7. Optineurin associates with TRAF3 and NIK in overexpression system.

HEK 293Ts were cotransfected with GFP-Optineurin and an array of Flag-tagged bait proteins. 36 hours post-transfection, WCL was collected, immunoprecipitated with anti-Flag antibody, and assayed for expression of GFP. An initial aliquot of input WCL was saved and assayed for expression of Flag.

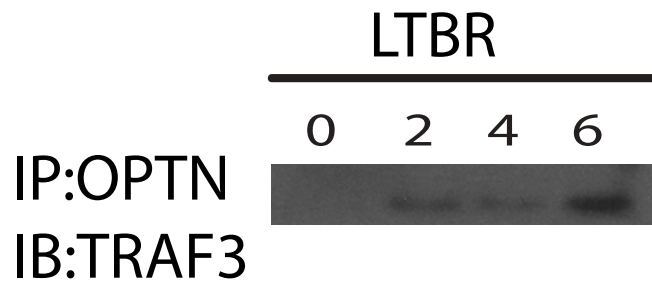


Figure 3.8. Optineurin associates with TRAF3 after stimulation with agonist

α LT β R in endogenous setting. WT MEF cells were stimulated with agonist α LT β R.

WCL was collected 0, 2, 4, and 6 hours post-stimulation, immunoprecipitated with anti-Optineurin antibody, and assayed for expression of TRAF3.

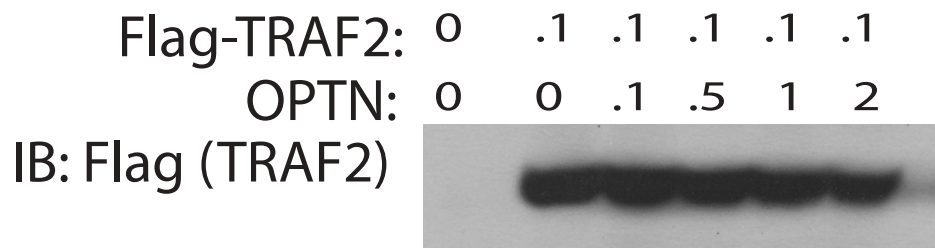
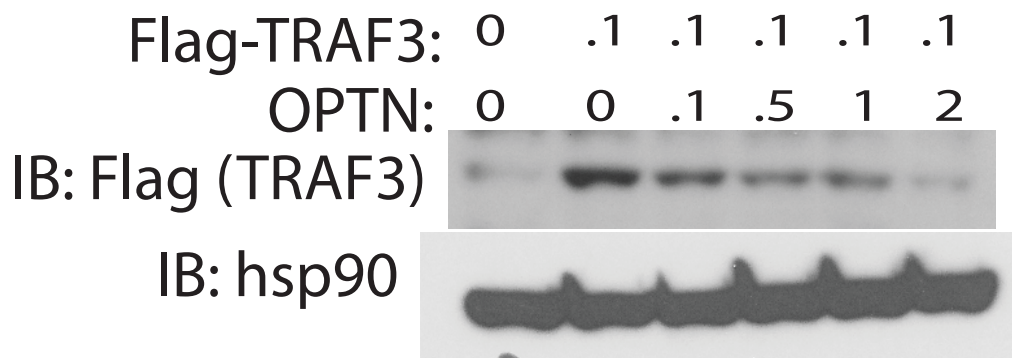


Figure 3.9. Optineurin promotes degradation of transfected TRAF3. HEK 293Ts were cotransfected with Flag-tagged TRAF3 and increasing amounts of GFP-tagged Optineurin. 36 hours post-transfection, WCL was collected and assayed for expression of TRAF3, TRAF2, and Hsp90.

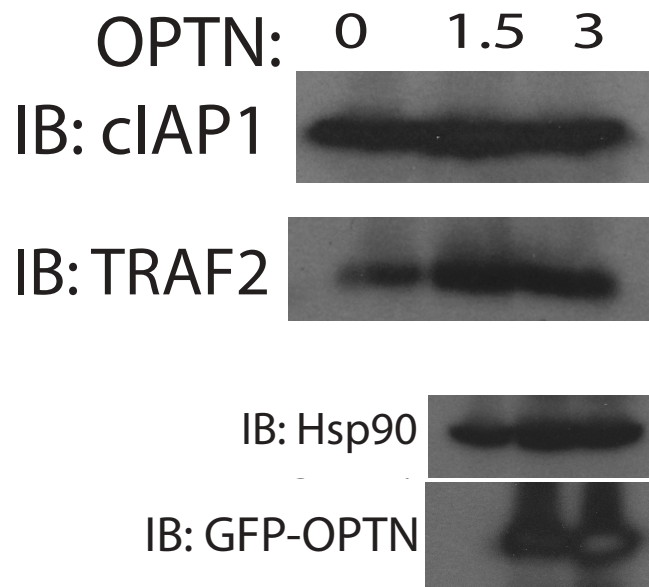
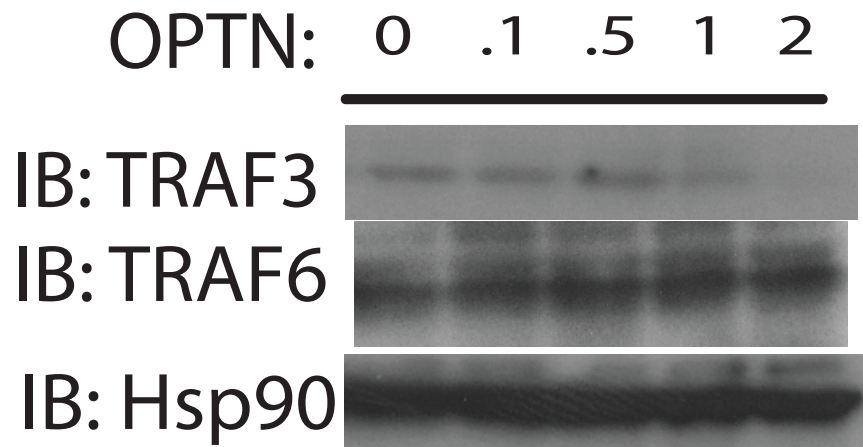


Figure 3.10. Optineurin promotes degradation of endogenous TRAF3. HEK 293Ts were cotransfected with increasing amounts of GFP-tagged Optineurin. 36 hours post-transfection, WCL was collected and assayed for expression of TRAF3, TRAF6, TRAF2, cIAP1, and Hsp90.

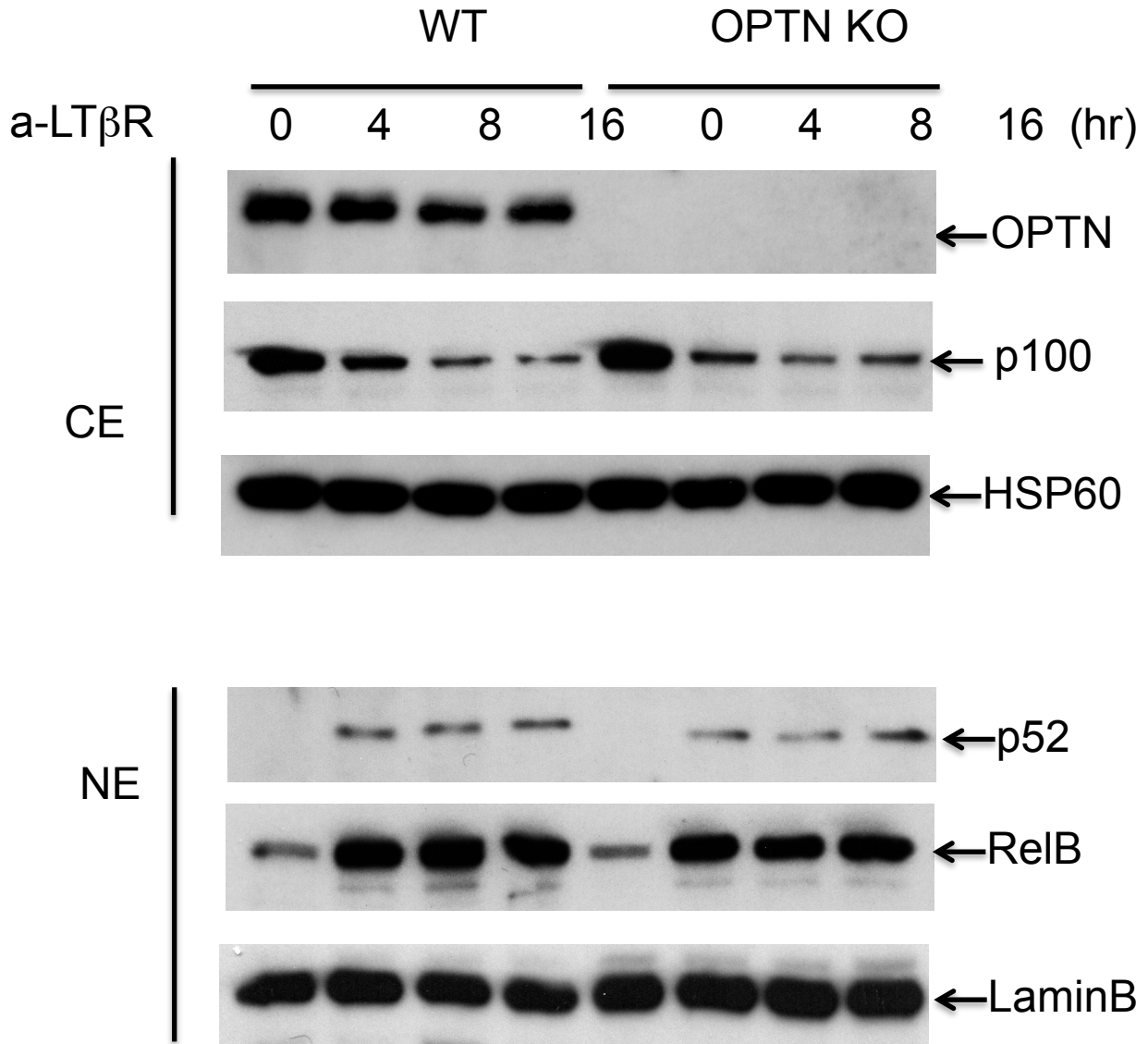


Figure 3.11. Optineurin KO cells show no defect in non-canonical NFκB activation. Another group generated Optineurin KO mice, which they used to generate Optineurin KO MEFs. WT and KO cells were stimulated with agonist αLTβR. Nuclear and cytoplasmic fractions were collected at 0, 4, 8, and 16 hours post-stimulation and assayed for expression of p100/p52 and RelB. Additional blots confirm for LaminB and Hsp60 confirm equivalent loading; a blot for Optineurin confirms protein knockout.

MATERIALS AND METHODS

Cell Culture:

Generation of WT MEFS was previously described. Human embryonic kidney (HEK) 293T cells and MEFs were cultured in DMEM (Gibco) supplemented with 10% FBS and 1% Pen/Strep.

Reagents:

Fugene 6 (Roche) was used for all transfections. SMAC mimetic was generously provided by Xiaodong Wang (University of Texas Southwestern Medical Center). Antibodies were obtained from the following companies: FLAG (Sigma); TRAF3, Optineurin (Abcam); TRAF2, TRAF6, RelB, GFP, Hsp90 (Santa Cruz Biotechnology); cIAP1, NIK, p100/52, actin (Cell Signaling).

Immunoblotting:

For western blotting, cells were homogenized for 15 minutes at 4°C in a modified radioimmune precipitation (mRIPA) buffer, containing 0.5% (vol/vol) NP-40, 0.1% (wt/vol) Na-Deoxycholate, and no SDS. Protease inhibitor cocktail (Sigma) was included in all lysates. Equal amounts of whole-cell lysates were resolved by SDS-PAGE and analyzed by Western blotting with the indicated antibodies.

Generation of Stable Cell Lines:

WT MEF cells were plated overnight for 60% confluency. Media was replaced with fresh, complete medium supplemented with Polybrene (10 ug/ml), and cells were incubated with shRNA particles (Santa Cruz Biotechnology) for 16 hours. Media was

replaced with fresh, complete medium without Polybrene. 24 hours later, cells were selected with Puromycin (5 mg/ml). Cells were expanded, and assayed for Optineurin expression.

Immunoprecipitation

For immunoprecipitation assays, cells were lysed as described above. Cell lysates were incubated with primary antibody for 2 hours, before adding Protein A/G agarose.

Lysates were incubated overnight at 4° on a shaker, before washing the agarose and eluting protein. The immunoprecipitated complexes were then separated by SDS-PAGE and blotted with the indicated antibodies.

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CHAPTER FOUR

LATS1 Promotes IFN β Activation via the RLR and DNA-Sensor Pathways

ABSTRACT

LATS1 is a serine/threonine kinase best known for its involvement in regulation of Hippo pathway signaling. LATS1 has not been directly linked to antiviral activity; but in a screen of 575 kinases, we found that LATS1 overexpression activates both IFN β and ISRE reporter elements. Using overexpression, transient knockdown, knockout and reconstitution assays, we show that LATS1 promotes IFN β activation in response to activation of the RLR and cytosolic DNA sensor pathways. We show that LATS1 forms part of a ligand-dependent complex of TBK1 and IRF3, and that LATS1 is able to enhance phosphorylation of TBK1 and IRF3 as part of that complex. Additionally, we show that LATS1 kinase activity is required for its role promoting IFN β induction. We propose that LATS1 functions as a type of adaptor protein that enhances formation of the TBK1/IRF3 complex, thereby promoting TBK1/IRF3 phosphorylation and downstream induction of IFN β . From our results, we suggest that LATS1 might be a signaling bridge connecting viral infection and Hippo signaling pathways.

INTRODUCTION

Our current understanding of viral detection is by no means comprehensive, and elucidating in closer molecular detail the mechanisms of type I IFN induction via the RLR and DNA-sensor pathways remains an active research area. Towards this end, we set out to identify novel kinases involved in regulation of type I IFNs; from an initial screen, we identified the protein Large Tumor Suppressor 1 (LATS1) as a candidate.

LATS1, along with its close homologue LATS2, belongs to the AGC family of serine/threonine kinases.¹⁻⁴ LATS1/2 recognize the substrate consensus sequence HxRxxS.⁴⁻⁶ Activation of the LATS1/2 kinases requires phosphorylation of two key residues: Ser909/872 on the activation segment, and Thr1079/1041 on the C-terminal hydrophobic motif.^{4, 7, 8} Both sites are essential for kinase activity: mutation of either one is sufficient to abrogate kinase function.⁴ In addition to phosphorylation, LATS1/2 are also regulated by E3 ubiquitin ligase-mediated degradation. Both the NEDD4 and ITCH E3 ubiquitin ligases interact with, and facilitate LATS1/2 degradation.^{9, 10}

LATS1/2 are best implicated for their involvement in the Hippo signaling pathway. The Hippo pathway is a highly conserved signaling node involved in regulation and coordination of the processes of cell growth, cell proliferation and apoptosis, and stem cell identity.^{1, 11-15} Activation of Hippo signaling turns on expression of genes that promote cell death and inhibit cell proliferation.¹⁶ Proper Hippo signaling is essential for wound repair and tissue regeneration; dysregulation of Hippo signaling is linked to multiple human cancers.¹⁷⁻¹⁹ The core Hippo pathway comprises a highly-conserved

kinase cascade: the mammalian STE20-like protein kinases 1/2 (MST1/2) phosphorylate and activate LATS1/2; LATS1/2 in turn phosphorylate and inactivate the transcriptional co-activators Yes-associated protein (YAP) and Taz (TAZ).⁵³ The identity and signaling mechanisms of upstream inputs to this core kinase cascade, however, are poorly understood. It appears that the Hippo-YAP pathway integrates multiple signals, including spatial readouts of cellular architecture and cytoskeletal dynamics, mechanosensing of tissue structure and tension, GPCR-mediated detection of extracellular ligands and hormones, and even metabolic signals from the intracellular department.²⁰⁻²⁹

Neither LATS1/2 has directly been linked to antiviral activity. However, both LATS1/2 have been directly linked to molecules that are established in induction of antiviral response. The same E3 ubiquitin ligases responsible for regulation of LATS1/2 protein levels - NEDD4 and ITCH – have also been implicated for potential roles in antiviral signaling.^{30, 10, 9, 31} ISG15 inhibits activity of NEDD4 E3 ubiquitin ligase, so enhancing the antiviral response. ITCH degrades Cardif, thereby inhibiting RLR-mediated IFN β activation. More recently, LATS2 was described to associate with TBK1 in a screen of the Hippo pathway interactome; TBK1 is well-established as a central regulator of both RLR and cytosolic DNA-sensor pathways.¹ Additionally, it appears that at least one virus targets LATS1/2. Kaposi sarcoma-associated herpesvirus (KSHV) encodes a viral G-protein-coupled receptor (vGPCR) that inhibits LATS1/2, so promoting activation of YAP/TAZ in order to facilitate vGPCR-mediated cell proliferation and tumorigenesis.³² Considering the role of Hippo pathway in integrating multiple readouts of a cell's

environment, it would not be surprising if viral infection, though any effects on extracellular environment and intracellular homeostasis, were able to alter Hippo signaling. However, greater understanding of input signaling to the Hippo pathway will be required to more clearly elucidate any potential role or association with viral infection.

RESULTS

Screening for Kinases Involved in IFN β Production

We set out for the current study with the goal of identifying novel protein kinases involved in regulation of the type I IFN antiviral response. We screened 575 kinases using a luciferase overexpression approach. Kinase expression plasmids and luciferase reporter plasmids were co-transfected into HEK 293T cells; we screened four separate luciferase reporter constructs for each kinase: IFN β , ISRE, 2XkB, and AP1. The IFN β and ISRE reporter constructs measured activation of the type I IFN response: specifically, the IFN β promoter and interferon stimulated response elements (ISRE) promoter elements, respectively. The 2XkB measured activation of the inflammatory NF κ B response, and the AP1 reporter measured activation of the activator protein 1 (AP1) mitogen-activated protein kinase (MAPK) pathway. 36 hours post transfection, luciferase levels were measured in order to quantify activation of the relevant signaling pathway. We included as a negative control the co-transfection of an empty plasmid with the various luciferase reporter plasmids; we also included TBK1 as a positive control since TBK1 is already well-established as a central regulator of IFN β induction. We classified the kinases by color according to induction levels: low-levels of activation (<10 fold) were denoted by the color black; medium levels (10-50 fold) by the color yellow; and finally, high levels (>50) by the color red. Results are shown in figure 4.1. As expected, empty vector did not activate any of the reporter constructs. TBK1 had high levels of induction for the IFN β , ISRE, and 2XkB reporters, and low levels for AP1. According to our screen, co-transfection of LATS1 promoted moderate induction of the IFN β , ISRE, and 2XkB reporter constructs, and only low induction for AP1 reporter

construct. In our screen, LATS1 was one of just ten kinases, besides TBK1, that could activate both IFN β and ISRE reporter elements. Because LATS1 has been linked to multiple molecules involved in regulation of the type I IFN response, we proceeded with our studies to determine if LATS1 functions to regulate IFN β production.

Overexpression of LATS1 promotes IFN β Induction

We proceeded to verify the kinase screen results that showed that LATS1 overexpression induces IFN β activation. Along with IFN β firefly luciferase and Renilla control luciferase, we overexpressed either empty vector or LATS1 into HEK 293T cells; we measured luciferase at 24 hours post-transfection. Results are shown in figure 4.2. In agreement with the kinase-screen, we see that overexpression of LATS1 is sufficient to induce activation of IFN β luciferase. It should be noted that we see only 2-3 fold induction of IFN β luciferase with LATS1 overexpression, which is less than what we saw in the kinase screen. We hypothesize that this is due to differences in conditions between the two assays.

We also proceeded to verify if LATS1 overexpression could synergize with pIC transfection to activate the type I IFN pathway. Results are shown in figure 4.3. Again, we used HEK 293T cells. We transfected empty vector and LATS1, with and without pIC. We cotransfected control Renilla luciferase, along with IFN β , ISRE, or 5xkB firefly luciferase; luciferase values were quantified 24 hours post-transfection. We normalized luciferase value separately for empty vector and LATS1-transfected samples. As expected, transfection of pIC promotes activation of IFN β , ISRE, and 5xkB luciferase

reporters; this occurs through via pIC-induced activation of the RLR pathway (which activates both type I IFN and NF κ B signaling). Co-transfection of LATS1 with pIC enhances type I IFN signaling – we see increased activation of the IFN β and ISRE luciferase reporters when LATS1 is transfected. This supports our hypothesis that LATS1 enhances activation of RLR-induced type I IFN signaling.

Surprisingly, we did not see any enhancement of the 5x κ B luciferase reporter upon LATS1 co-transfection. This was unexpected because in our kinase-screen results, LATS1 overexpression activated 2x κ B luciferase activity. Based on this screen result, we would have predicted that LATS1 would enhance pIC-mediated activation of 5x κ B luciferase activity. It is possible that the low activation of 5x κ B – we were only able to achieve 2-fold activation – is responsible. Again, however, due to different conditions between the luciferase experiments, we would expect some differences. In our hands, though, overexpression of LATS1 does not enhance pIC-mediated NF κ B luciferase activation.

We also proceeded to verify if LATS1 overexpression could synergize with BDNA transfection to activate the type I IFN pathway. Results are shown in figure 4.4. Again, we used HEK 293T cells. We transfected empty vector and LATS1, with and without BDNA. We cotransfected control Renilla luciferase, along with IFN β , ISRE, or 5x κ B firefly luciferase; luciferase values were quantified 24 hours post-transfection. We normalized luciferase value separately for empty vector and LATS1-transfected samples. As expected, transfection of BDNA promotes activation of IFN β , ISRE, and

5xkB luciferase reporters. Co-transfection of LATS1 with BDNA enhances type I IFN signaling – we see increased activation of the IFN β and ISRE luciferase reporters when LATS1 is transfected. This supports our hypothesis that LATS1 enhances activation of RLR-induced type I IFN signaling. Again, similar to our results with pIC, we did not see any enhancement of the 5xkB luciferase reporter upon LATS1 co-transfection with BDNA. It is worth mentioning that there is disagreement over whether HEK 293T cells express STING, and if they are a relevant system to examine activation of cytosolic DNA-sensors. In our hands at least, though, LATS1 overexpression can enhance BDNA-mediated type I IFN activity in HEK 293T cells.

Knockdown of LATS1 Inhibits IFN β Induction

We next proceeded to examine the effects of transient siRNA-mediated knockdown of LATS1 expression in HEK 293T cells upon type I IFN signaling. First, we used luciferase assays to examine activation of type I IFNs within this system. We transfected HEK 293T cells with either control siRNA, or siLATS1. 24 hours post-transfection, we transfected the same cells with IFN β firefly and Renilla control luciferase reporters, with and without pIC. Luciferase values were then quantified 24 hours after the second transfection; results are shown in figure 4.5. We see that knockdown of LATS1 inhibits pIC-mediated activation of the IFN β luciferase reporter. Next, we used infection with the RNA virus VSV-GFP to activate the RLR pathway, instead of pIC transfection. Again, we transfected HEK 293T cells with either control siRNA or siLATS1; we also included siTBK1 as a positive control. Results are shown in figure 4.5. 24 hours later, we transfected the same cells with IFN β firefly and Renilla control luciferase reporters, we

then infected with VSV-GFP at MOI=0.1, and determined luciferase values 12 hours later. Similar to the results seen with pIC-transfection, we see a defective activation of the IFN β luciferase reporter upon knockdown of either LATS1 or TBK1. These knockdown results suggests that LATS1 is a positive inducer of the RLR-mediated type I IFN response, and are consistent with our results showing that overexpression of LATS1 enhances RLR-mediated type I IFN activation.

We proceeded to use QPCR to examine more directly the effects that transient siRNA-mediated LATS1 knockdown exerts upon IFN β induction in the RLR pathway. Parallel to our luciferase assays, we transfected HEK 293Ts with either control siRNA or siLATS1. 24 hours post-transfection, we transfected the same cells with and without pIC. 12 hours after pIC transfection, we collected cells for RNA extraction. Results are shown in figure 4.6. Similar to our IFN β luciferase reporter results, we see that knockdown of LATS1 inhibits pIC-mediated activation of IFN β transcription. Next, as before, we used infection with the RNA-virus VSV-GFP to activate the RLR pathway. We transfected HEK 293T cells with either control siRNA or siLATS1; 24 hours later, we infected the same cells with VSV-GFP at an MOI=0.1. We collected cells at 12 hpi; results are shown in figure 4.6. As expected, we see defective induction of IFN β transcription in response to VSV-GFP infection upon knockdown of LATS1. These QPCR results are in agreement with our luciferase results that suggested transient knockdown of LATS1 decreased activation of type I IFNs through the RLR-pathway.

Having determined that LATS1 knockdown inhibits activation of type I IFN through the RLR-pathway, we proceeded to investigate the effects of LATS1 knockdown on activation of type I IFN through the cytosolic DNA pathway. Again, we began by using luciferase assays to examine activation of type I IFNs. We transfected HEK 293T cells with either control siRNA or siLATS1; 24 hours post-transfection, we transfected the same cells with IFN β firefly and Renilla control luciferase reporters, with and without BDNA. Luciferase values were then quantified 24 hours after the second transfection; results are shown in figure 4.7. We see that knockdown of LATS1 inhibits BDNA-mediated activation of the IFN β luciferase reporter. Additionally, we used QPCR to examine more directly the effects that transient LATS1 knockdown exerts on IFN β induction in the cytosolic DNA pathway. Parallel to the luciferase assays, we transfected HEK 293Ts with either control siRNA or siLATS1; 24 hours post-transfection, we transfected the same cells with or without BDNA. 6 hours after BDNA transfection, we collected cells for RNA extraction; results are shown in figure 4.7. Similar to our IFN β luciferase reporter results, we see that knockdown of LATS1 inhibits BDNA-mediated activation of IFN β transcription. Again, though, it is worth mentioning that there is disagreement over whether HEK 293T cells express STING, and if they are a relevant system to examine activation of cytosolic DNA-sensors. In our hands at least, though, LATS1 knockdown inhibits BDNA-mediated type I IFN activity in HEK 293T cells.

LATS1 KO MEFs have reduced IFN β Induction

We next proceeded to examine directly LATS1's involvement in IFN β induction in wt and LATS1 KO MEF cells. We began by comparing RLR-mediated IFN β induction.

First, we transfected pIC into wt and KO cells, and we used QPCR to monitor gene induction. Results are shown in figure 4.8. In parallel with our knockdown results, we see that the LATS1 KO MEF cells exhibit defective IFN β induction on pIC transfection. We also see that LATS1 KO MEF cells exhibit defective induction of MX1 and IL6 – both are ISGs stimulated by induction of IFN β . These results show that LATS1 KO MEF cells exhibit decreased activation of the RLR pathway by pIC transfection.

Next, we used infection with the RNA virus VSV-GFP in order to activate the RLR pathway, instead of pIC transfection. Again, we used QPCR to monitor gene induction in wt and KO MEF cells; results are shown in figure 4.9. In agreement with our results from pIC transfection, the LATS1 KO MEF cells exhibit defective IFN β induction on infection with VSV-GFP. We also see that LATS1 KO MEF cells exhibit defective IP10 induction on infection with VSV-GFP – IP10 is an ISG stimulated by induction of IFN β . In addition to monitoring gene induction by QPCR, we also used flow cytometry to monitor viral replication in wt and KO MEF cells. Results are shown in figure 4.10. As expected, LATS1 KO MEF cells exhibit increased GFP fluorescence after infection with VSV-GFP, as compared to WT cells. The increased GFP fluorescence in LATS1 KO cells is indicative of greater viral replication, and decreased cellular antiviral-response – ie, decreased induction of the IFN β in response to detection of the VSV-GFP virus. Again, both the QPCR and flow cytometry results suggest that LATS1 KO MEF cells exhibit decreased activation of the RLR pathway by VSV-GFP infection. This phenotype is not specific to infection with VSV-GFP. We also tested infection with Sendai virus, another RNA virus that activates the RLR pathway. Again, we used QPCR to monitor

gene induction in wt and KO MEF cells; results are shown in figure 4.11. In parallel to the results from infection with VSV-GFP, we see that LATS1 KO MEF cells exhibit defective induction of IFN β on Sendai viral infection.

Having determined that LATS1 KO MEF cells exhibit impaired activation of the IFN β response via the RLR pathway, we proceeded to compare the IFN β response of wt and LATS1 KO MEF cells after cytosolic DNA activation. We began by transfecting BDNA into wt and KO MEF cells, and using QPCR to monitor gene induction. Results are shown in figure 4.12. In parallel with the knockdown results (obtained with HEK 293T cells), we see that LATS1 KO MEF cells exhibit defective IFN β induction upon BDNA transfection. We also see that LATS1 KO MEF cells exhibit defective induction of MX1 and IL6 (both ISGs) on BDNA transfection. These results show that LATS1 KO MEF cells exhibit decreased activation of IFN β upon activation of the cytosolic DNA pathway by BDNA transfection.

Next, we used infection with the DNA virus HSV1 in order to activate the cytosolic DNA pathway, versus BDNA transfection. Again, we used QPCR to monitor gene induction in wt and KO MEF cells; results are shown in figure 4.13. In agreement with our results from BDNA transfection, the LATS1 KO MEF cells exhibit defective IFN β induction on infection with HSV1. We also see that LATS1 KO MEF cells exhibit defective induction of IL6 on infection with HSV1 (IL6 is an ISG). These results are in agreement with the BDNA-transfection results. Both results suggest that LATS1 KO MEF cells exhibit decreased activation of IFN β upon activation of the cytosolic DNA pathway.

Reconstitution of LATS1 KO MEFs rescues IFN β Production

Having determined a phenotype for LATS1 KO cells relating to induction of IFN β in response to activation of either the RLR or the cytosolic DNA pathways, we next proceeded to determine if we could rescue this phenotype in LATS1 KO MEF cells by restoring LATS1 expression. First, we attempted to rescue the LATS1 KO phenotype via transient transfection. We transfected LATS1 KO MEFs with either empty vector or with LATS1. Because MEF cells are difficult to transfect, we used western blot to confirm that the 'rescue transfection' does restore LATS1 expression to LATS1 KO cells; the blot is shown in figure 4.14. 24 hours after this 'rescue transfection,' we stimulated the RLR pathway by pIC transfection. We used QPCR to monitor gene induction; results are shown in figure 4.15. In agreement with our hypothesis that LATS1 facilitates IFN β activation through the RLR pathway, we see that cells transfected with LATS1 have greater IFN β induction than cells transfected with just empty vector. We also see that cells transfected with LATS1 have greater induction of various downstream ISGs, including MX1, RANTES, IL6, and IP10. These results are shown in figure 4.16. They suggest that transient transfection of LATS1 into LATS1 KO cells is able to rescue IFN β induction in response to pIC transfection. Next, we used infection with the RNA virus VSV-GFP to activate the RLR pathway, instead of pIC. Again, we used QPCR to monitor gene induction; results are shown in figure 4.17. In agreement with our results from pIC transfection, we see that cells transfected with LATS1 have greater IFN β induction than cells transfected with just empty vector. We also see that cells transfected with LATS1 have greater induction of the downstream

ISGs IP10 and IL6 (results shown in figure 4.18). Again, these results suggest that even transient transfection of LATS1 into LATS1 KO cells is sufficient rescue IFN β induction in response to activation of the RLR pathway.

Because the transient 'rescue transfection' was able to restore IFN β induction upon RLR pathway activation to LATS1 KO cells, we proceeded to generate the corresponding, stable cell lines. We reconstituted the LATS1 KO cells with either empty vector or with LATS1. To reconstitute the cells, we transfected HEK 293T cells with a Moloney murine leukemia virus Ψ A helper construct, plus either empty pBABE-puro vector or pBABE-puro-LATS1 vector. We then infected LATS1 KO MEFS with the filtered HEK 293T cell supernatants, and selected with puromycin. For a control, we used western blot to confirm that the cells reconstituted with LATS1 indeed expressed LATS1. The western blot is shown in figure 4.19: WT cells and cells reconstituted with LATS1 express LATS1 (lanes 1 and 3); LATS1 KO and cells reconstituted with vector do not express LATS1 (lanes 2 and 4). Having established stable cell lines reconstituted with either vector or LATS1, we tested the IFN β response of these cells to pIC transfection. Again, we used QPCR to monitor gene induction; results are shown in figure 4.20. In agreement with our results using just transient transfection, we see that cells reconstituted with LATS1 have increased IFN β induction after pIC transfection compared to cells reconstituted with empty vector. We also see that cells reconstituted with LATS1 versus vector have increased induction of various downstream ISGs, including IP10, MX1, and IL6 (shown in figure 4.21). These results suggest that the stable reconstitution of LATS1 into LATS1 KO cells is able to restore IFN β induction in

response to pIC transfection. We also compared the IFN β response to pIC transfection of WT, LATS1 KO, and the stable reconstituted cell lines all together; results are shown in figure 4.22. In parallel with previous results, we see that LATS1 KO cells, in comparison to WT cells, have greatly reduced induction of IFN β after pIC transfection. We see that cells reconstituted with vector show similar induction of IFN β as LATS1 KO cells. Finally, we see that cells reconstituted with LATS1 show intermediate induction of IFN β – showing that reconstitution is able to at least partially restore IFN β induction after pIC transfection.

Next, we used infection with the RNA virus VSV-GFP to activate the RLR pathway in the stably reconstituted cell lines. We infected the stably reconstituted cell lines with MOIs of 0, 1.5, 3.0, and 6.0; 6 hours after infection, we collected cells for RNA extraction. Results are shown in figure 4.23; again, we used QPCR induction to monitor gene induction. In agreement with the results from pIC transfection, we see that cells reconstituted with LATS1 versus vector show increased induction of IFN β to VSV-GFP infection. This supports our hypothesis that LATS1 can promote activation IFN β through the RLR pathway. In addition to monitoring gene induction by QPCR, we also used flow cytometry to monitor viral replication in the stably reconstituted cells. Results are shown in figure 4.24. As expected, cells reconstituted with just vector exhibit increased GFP fluorescence after infection with VSV-GFP, as compared to cells reconstituted with LATS1. The increased GFP fluorescence is indicative of greater viral replication, and decreased cellular antiviral-response – ie, decreased induction of the IFN β in response to detection of the VSV-GFP virus. Again, both the QPCR and flow cytometry results

suggest that reconstitution of LATS1 into LATS1 KO cells restores IFN β induction via the RLR pathway.

Having determined that restoring LATS1 expression to LATS1 KO cells could restore IFN β induction through the RLR pathway, we proceeded to determine if it could similarly restore IFN β induction through the cytosolic DNA pathway. As before, we began by attempting to rescue the LATS1 KO phenotype via transient transfection. Again, we transfected LATS1 KO MEFs with either empty vector or with LATS1; 24 hours after this 'rescue transfection,' we stimulated the cytosolic DNA pathway via BDNA transfection. We used QPCR to monitor gene induction; results are shown in figure 4.25. In agreement with our hypothesis that LATS1 facilitates IFN β activation through the cytosolic DNA pathway, we see that cells transfected with LATS1 have increased IFN β induction then cells transfected with just empty vector. These results suggest that even just transient transfection of LATS1 into LATS1 KO cells is able to at least partially rescue IFN β induction in response to BDNA transfection.

As with the RLR pathway, we proceeded next to determine if stable reconstitution of LATS1 into LATS1 KO cells could rescue IFN β induction through the cytosolic DNA pathway. We began by testing the response of cells reconstituted with either vector or LATS1 to BDNA transfection. We used QPCR to monitor gene induction; results are shown in figure 4.26. In parallel with our transient 'rescue transfection' results, we see that cells reconstituted with LATS1 versus vector have increased IFN β induction after BDNA transfection. We also see that cells reconstituted with LATS1 versus vector have

increased induction of MX1, a downstream ISG, after BDNA transfection (results in figure 4.27). Both results suggest that stable reconstitution of LATS1 into LATS1 KO cells is able to rescue IFN β induction in response to BDNA transfection. We also compared the IFN β response to BDNA transfection of WT, LATS1 KO, and the stable reconstituted cell lines all together; results are shown in figure 4.28. In parallel with previous results, we see that LATS1 KO cells, in comparison to WT cells, have greatly reduced induction of IFN β after BDNA transfection. We see that cells reconstituted with vector show similar induction of IFN β as LATS1 KO cells. Finally, we see that cells reconstituted with LATS1 show intermediate induction of IFN β – showing that reconstitution is able to at least partially restore IFN β induction after BDNA transfection.

Next, we used infection with the DNA virus HSV1 in order to activate the cytosolic DNA pathway in the stably reconstituted cells. Again, we used QPCR to monitor gene induction; results are shown in figure 4.29. In agreement with our results from BDNA transfection, cells reconstituted with just vector exhibit defective IFN β induction on infection with HSV1; in contrast, cells reconstituted with LATS1 exhibit increased IFN β induction on HSV1 infection. These results are in agreement with the BDNA-transfection results. Both results suggest that reconstitution of LATS1 expression into LATS1 KO cells restores IFN β induction on activation of the cytosolic DNA pathway.

LATS1 operates at TBK1/IRF3 level to modulate IFN β

Our data indicate that LATS1 functions to promote induction of IFN β upon activation of either the RLR or cytosolic DNA pathways. Having verified a functional role for LATS1,

we now directed our investigations towards determining the mechanism through which LATS1 exerts its promotion of IFN β induction. First, we sought to determine the epistatic relationship between LATS1 and various key signaling molecules that are already well-established in pathways mediating IFN β induction. We used luciferase assays in combination with siRNA-mediated knockdown of LATS1, and overexpression of proteins known to induce the IFN β promoter. We transfected HEK 293T cells with either control siRNA or siLATS1. 24 hours post-transfection, we transfected the same cells with IFN β firefly and Renilla control luciferase reporters, along with vector, RIGI, Cardif, TBK1, IRF3, STING, or TRIF. Luciferase values were then quantified 24 hours after the second transfection; results are shown in figures 4.30 through 4.32. First, we looked at RIGI and Cardif – both of which are involved in activating IFN β through the RLR pathway. As illustrated in figure 4.30, knockdown of LATS1 impedes IFN β induction in response to overexpression of both RIGI and Cardif. This suggests that LATS1 functions downstream of RIGI and Cardif in the RLR pathway. In contrast, we see that knockdown of LATS1 does not impair IFN β induction in response to overexpression of either TBK1 or IRF3 (figure 4.31). This suggests that LATS1 functions at the level of TBK1/IRF3 in its activation of the RLR pathway.

We also considered STING- and TRIF-induced IFN β induction; results again are shown in figure 4.32. STING is a crucial mediator for IFN β induction via the cytosolic DNA pathway; TRIF is a key adaptor that functions to promote IFN β induction via LPS stimulation. As illustrated, knockdown of LATS1 impedes IFN β induction in response to overexpression of both STING and TRIF. In combination with the previous results

showing that LATS1 knockdown does not impair IFN β induction in response to TBK1 or IRF3 overexpression, these results allow us to pinpoint where LATS1 operates in the cytosolic DNA- and LPS-mediated IFN β pathways. In both cases, LATS1 appears to function downstream of STING/TRIF, and to operate at the level of TBK1/IRF3.

It is not surprising that LATS1 functions at the level of TBK1/IRF3 in the RLR and cytosolic DNA pathways. Both pathways converge at the TBK1/IRF3 level. Since our data suggests that LATS1 promotes IFN β induction through both the RLR and cytosolic DNA pathways, we would expect it to operate at or downstream of these two pathways' convergence. Similarly, it is not surprising that LATS1 operates downstream of TRIF-mediated IFN β induction. Given that LATS1 operates at the TBK1/IRF3 level, because the TRIF-mediated pathway connects to TBK1/IRF3, we would expect LATS1 to also effect TRIF-mediated IFN β induction via its actions at the TBK1/IRF3 level.

LATS1 associates with TBK1/IRF3 complex

In light of our data showing that LATS1 operates at the TBK1/IRF3 level in the RLR and cytosolic DNA pathways, we wondered if LATS1 can associate with either protein. First, we checked for LATS1 interactions with TBK1/IRF3 in an overexpression system. We co-transfected HEK 293T cells with LATS1 and either vector, Flag-TBK1, or Flag-IRF3. Next, we immunoprecipitated with either control IgG or Flag antibody (to pull down the Flag-TBK1 and Flag-IRF3). After washing, we then used western blotting to probe for LATS1. As shown in figure 4.33, LATS1 is pulled down by both Flag-TBK1 and Flag-IRF3. We do not see LATS1 pulldown for either the vector or IgG controls, suggesting

that the LATS1 interaction with Flag-TBK1 and Flag-IRF3 is specific and real. While LATS1 has not been reported to interact with either protein thus far, it is noteworthy that LATS2 recently was reported to interact with TBK1. In light of the overlapping homology between LATS1 and LATS2, our results showing a LATS1-TBK1 interaction are not surprising.

It is known that overexpression systems are prone to artifacts, especially for ‘false-positives’ between potentially interacting protein partners. Therefore, we examined if LATS1 can associate with TBK1/IRF3 in the endogenous setting. We transfected WT MEF cells with pIC to activate the RLR-pathway. We then immunoprecipitated the whole-cell lysate with either IgG control or anti-TBK1 antibody. After washing, we used western blotting to probe for LATS1 and IRF3; results are shown in figure 4.34 (IB:LATS1) and 4.35 (IB:IRF3). We do not see association of LATS1 or IRF3 with TBK1 at baseline. However, after pIC stimulation, we see that both LATS1 and IRF3 are pulled down by the anti-TBK1 antibody. We do not see pulldown of LATS1/IRF3 for the IgG controls, suggesting that the pIC-induced interactions of LATS1/IRF3 with TBK1 are specific. We also performed a parallel immunoprecipitation. We transfected WT MEF cells with pIC to activate the RLR pathway; we then immunoprecipitated the whole-cell lysate with either IgG control or anti-LATS1 antibody. After washing, we probed for TBK1; results are shown in figure 4.34. Again, we do not see any interaction of TBK1 with LATS1 at baseline. However, after pIC transfection, we see induction of TBK1 interaction with LATS1. Again, we do not see pulldown of TBK1 for the IgG controls, suggesting that the pIC-induced association of TBK1 with LATS1 is specific. Combined,

these results suggest that on activation of the RLR pathway, LATS1 is recruited to the TBK1/IRF3 complex.

We also examined LATS1 association with TBK1/IRF3 in the context of cytosolic DNA activation. We transfected WT MEF cells with BDNA to activate the cytosolic DNA pathway. We then immunoprecipitated the whole-cell lysate with either IgG control or with anti-TBK1 antibody. After washing, we used western blotting to probe for LATS1 and IRF3; results are shown in figure 4.35 (IB:IRF3) and 4.36 (IB:LATS1). Again, we do not see association of LATS1 or IRF3 with TBK1 at baseline. As we saw with pIC stimulation though, we see that both LATS1 and IRF3 are pulled down by the anti-TBK1 antibody after BDNA transfection. Because we do not see pulldown of LATS1/IRF3 for the IgG controls, the BDNA-induced interactions of LATS1/IRF3 with TBK1 are specific. These results suggest that on activation of the cytosolic DNA pathway, LATS1 is recruited to the TBK1/IRF3 complex.

Our results provide strong evidence that LATS1 associates with the TBK1/IRF3 complex. We see association of LATS1 with both TBK1 and IRF3 individually in an overexpression system. In the endogenous setting, we also see interaction of LATS1 with the TBK1/IRF3 complex after activation of either the RLR or cytosolic DNA pathways. Our results show that LATS1 exerts its promotion of IFN β induction as part of the TBK1/IRF3 complex.

These results raise the possibility that LATS1 could function as an adaptor protein, capable of interacting with both TBK1 and IRF3 and by doing so, enhancing their association with each other. Perhaps by enhancing association of TBK1/IRF3, LATS1 is able to promote IFN β induction. In a transient knockdown system, we did see that knockdown of LATS1 disrupted TBK1/IRF3 interaction. Using HEK 293T cells, we transfected cells with either siCTRL or siLATS1. For control, we used western blotting to confirm knockdown of LATS1; results are shown in figure 4.37. 24 hours post-transfection, we transfected the cells with TBK1, and either vector or Flag-tagged IRF3. 24 hours after the second transfection, we immunoprecipitated the whole cell lysate with anti-Flag, pulling down IRF3. After washing, we used western blotting to probe for TBK1 association with IRF3. Results are shown in figure 4.37. Lanes 1 and 3 do not express Flag-IRF3, and as expected, TBK1 is not pulled down. However, in lanes 2 and 4, we do see association of TBK1 with IRF3. Comparing between lanes 2 (transfected with siCTRL) and 4 (transfected with siLATS1), we see reduced levels of TBK1 in lane 4. We used densitometric analysis to quantify the TBK1 bands in lanes 2 and 4: the results are plotted in figure 4.37. As evidenced, siLATS1-transfected cells show decreased pulldown of TBK1 compared to siCTRL-transfected cells. These results support the hypothesis that LATS1 functions as a type of adaptor protein that enhances formation of the TBK1/IRF3 complex.

However, we have not confirmed this in the endogenous setting yet; and until we do so, our hypothesis that LATS1 promotes the association of TBK1/IRF3 remains unproved.

LATS1 enhances TBK1/IRF3 phosphorylation

It has been established that phosphorylation of TBK1 and IRF3 is a necessary step towards downstream induction of IFN β . Based on our results showing that LATS1 forms part of a ligand-dependent complex of TBK1 and IRF3, we wondered if LATS1 were able to enhance phosphorylation of TBK1 and IRF3 as part of that complex. We began by examining if overexpression of LATS1 could enhance TBK1/IRF3 phosphorylation. We transfected either vector or LATS1 into HEK 293T cells; we also co-transfected vector, WT TBK1, or a kinase-dead TBK1 construct into the same cells. We used the K38A TBK1 construct; this construct is known to lack kinase activity. 24 hours post transfection, we collected the cells; and we used western blotting to probe for phosphorylation of TBK1 and IRF3. Results are shown in figure 4.38; additional blots show total TBK1, total IRF3, and actin loading controls. Comparing lanes 2 (transfected with vector and WT TBK1) and 5 (transfected with LATS1 and WT TBK1), we see that transfection of LATS1 in lane 5 promotes increased phosphorylation of TBK1 compared to transfection of vector alone. This suggests that LATS1 does act to promote TBK1 phosphorylation. It should be noted, though, that LATS1 is acting here to promote phosphorylation of transfected, overexpressed TBK1. It is interesting to notice that LATS1 expression is not able to promote phosphorylation of kinase-dead TBK1 – we see no pTBK1 in either lanes 3 (transfected with vector and KD TBK1) or 6 (transfected with LATS1 and KD TBK1). This would suggest that LATS1 is not able to directly phosphorylate TBK1 on its own. Additionally, we see that overexpression of LATS1 promotes phosphorylation of IRF3. Lanes 4-6 (all transfected with LATS1) show significantly increased pIRF3 compared to lanes 1-3 (all transfected with vector). It is

interesting to note that, in this case, LATS1 is able to enhance phosphorylation of endogenous IRF3 protein.

With evidence that overexpression of LATS1 can promote phosphorylation of TBK1 and IRF3, we next sought to corroborate this function in a more physiological setting. As before, we wanted to confirm that the overexpression results were not simply an artifact. We took advantage of our WT and LATS1 KO MEF cells to examine phosphorylation patterns of IRF3 after activation of the RLR and cytoplasmic DNA pathways. We transfected pIC and BDNA into WT and LATS1 KO MEF cells; we collected cell lysate four hours after transfection. We again used western blotting to probe for phosphorylated IRF3. Results are shown in figure 4.39; additional blots show total IRF3 and Hsp90 loading controls. As expected, we do not see any phosphorylated IRF3 in the unstimulated state for either WT (lane 1) or LATS1 KO cells (lane 3). Still, the WT MEFs exhibit clear, strong accumulation of phosphorylated IRF3 on transfection of either pIC (lane 2) or BDNA (lane 3). We do see some accumulation of phosphorylated IRF3 in the LATS1 KO MEFs after transfection of either pIC (lane 5) or BDNA (lane 6). However, the accumulation of phosphorylated IRF3 in LATS1 KO cells is substantially decreased compared to WT cells (compare lanes 2 and 5, and lanes 3 and 6). These results show that LATS1 KO cells exhibit reduced phosphorylation of IRF3 on transfection of pIC and BDNA.

We have shown that overexpression of LATS1 can enhance phosphorylation of both TBK1 and IRF3. We also show that LATS1 KO MEFs have reduced phosphorylation of

IRF3 after transfection of plC and BDNA. Both results support the hypothesis that upon activation of either the RLR or cytosolic DNA pathway, LATS1 is recruited to the TBK1/IRF3 complex, where it acts to enhance phosphorylation of TBK1 and IRF3. We suggest that this is the means through which LATS1 acts to promote IFN β induction on activation of either the RLR or cytosolic DNA pathways. Because phosphorylation of TBK1 and IRF3 is required for downstream IFN β induction, by enhancing TBK1/IRF3 phosphorylation, LATS1 would so act to promote IFN β induction.

LATS1 kinase function is required

We began our investigation of LATS1 as part of a kinase screen, so it was natural to ask whether LATS1 kinase function is required for its role promoting IFN β induction in response to RLR and cytosolic DNA pathways. For our studies, we used the LATS1D846A construct; this point mutant is already characterized to lack kinase activity.⁸ First, we examined whether kinase-dead LATS1 is able to enhance phosphorylation of TBK1/IRF3. We transfected vector, WT LATS1, or KD LATS1 into HEK 293T cells. **In order to activate the RLR pathway**, we co-transfected into the same cells either vector or a WT TBK1 construct. 24 hours post transfection, we collected cells and used western blotting to probe for TBK1 phosphorylation. Results are shown in figure 4.40; additional blots show total TBK1 and actin loading controls. Comparing lanes 4 (transfected with vector and WT TBK1) and 5 (transfected with WT LATS1 and WT TBK1), we see that overexpression of LATS1 enhances phosphorylation of overexpressed TBK1. This is in agreement with earlier results. Considering, though, lane 6 (transfected with KD LATS1 and WT TBK1), we see that overexpression of

kinase-dead LATS1 does not enhance TBK1 phosphorylation, as compared to vector overexpression. These results show that in contrast to overexpression of WT LATS1, overexpression of KD LATS1 does not enhance TBK1 phosphorylation. These results suggest that LATS1 kinase activity is required for its function to enhance phosphorylation of TBK1/IRF3.

Next, taking a more direct approach to determine if LATS1 kinase function is required to promote IFN β induction, we tested if reconstitution of kinase-dead LATS1 could rescue the LATS1 KO phenotype on activation of the RLR and cytosolic DNA pathways. In parallel with previous experiments, we used a transient transfection approach. We transfected LATS1 KO MEFs with empty vector, WT LATS1, or KD LATS1. 24 hours after this 'rescue transfection,' we stimulated either the RLR pathway by pIC transfection, or the cytosolic DNA pathway by BDNA transfection. We used QPCR to monitor gene induction; results are shown in figure 6. Looking first at the pIC transfection results, we see, in agreement with previous results, that cells transfected with WT LATS1 have greater IFN β induction than cells transfected with just empty vector. In contrast, though, cells transfected with KD LATS1 do not show any enhancement of IFN β induction. In fact, cells transfected with KD LATS1 show similar levels of IFN β induction as do cells transfected with vector. These results show that while transient transfection of WT LATS1 into LATS1 KO cells can rescue IFN β induction in response to pIC transfection, transient transfection of KD LATS1 does not rescue IFN β induction. These results suggest that LATS1 kinase activity is required for its function to promote IFN β induction on RLR pathway activation. Looking next at the

BDNA transfection results, we see a similar pattern to what we saw with pIC transfection. Again, in agreement with previous results, we see that cells transfected with WT LATS1 have greater IFN β induction than cells transfected with just empty vector. In contrast, though, cells transfected with KD LATS1 do not show any enhancement of IFN β induction – cells transfected with KD LATS1 show similar levels of IFN β induction compared to cells transfected with vector alone. These results show that while transient transfection of WT LATS1 into LATS1 KO cells can rescue IFN β induction in response to BDNA transfection, transient transfection of KD LATS1 does not rescue IFN β induction. Similar to the case for RLR pathway activation, these results suggest that LATS1 kinase activity is required for its function to promote IFN β induction on activation of the cytosolic DNA pathway.

We are able to conclude that LATS1 kinase activity is indeed required for its role promoting IFN β induction in response to RLR and cytosolic DNA pathways. Our results show that LATS1 kinase activity is required for its function to enhance phosphorylation of TBK1/IRF3. Our results also show that LATS1 kinase activity is required to rescue the LATS1 KO phenotype on activation of the RLR and cytosolic DNA pathways. At this point, the identity of the molecular target for LATS1 kinase activity regarding IFN β induction is unknown. Further studies are necessary to further elucidate in detail this mechanism.

DISCUSSION

We set out for the current study with the goal of identifying novel protein kinases involved in regulation of the type I IFN antiviral response. After screening 575 kinases, we focused our investigation to the kinase LATS1. LATS1 is a serine/threonine kinase best implicated for involvement in the Hippo signaling pathway. While LATS1 has not been directly linked to antiviral activity, it has been linked to molecules established in induction of antiviral response, including the E3 ubiquitin ligases NEDD4 and ITCH. Our screen showed that LATS1 overexpression activates both IFN β and ISRE reporter elements. In light of our screen results, and the fact that LATS1 has been linked to multiple molecules involved in regulation of the type I IFN response, we chose to proceed with our studies to determine if, indeed, LATS1 does regulate IFN β production.

We began by verifying the kinase screen results that showed LATS1 overexpression induces IFN β activation. We further showed that LATS1 overexpression synergizes with pIC and BDNA transfection to activate type I IFNs. Our results show that overexpression of LATS1 enhances activation of RLR-induced and BDNA-mediated type I IFN signaling. Next, we used transient siRNA-mediated knockdown of LATS1. By luciferase and QPCR assays, we showed that LATS1 knockdown decreases activation of type I IFNs through the RLR-pathway (mediated by pIC transfection or VSV infection), and through the cytosolic DNA pathway (mediated by BDNA transfection). Consistent with our overexpression results, our knockdown results suggest that LATS1 is a positive inducer of the RLR-mediated and cytosolic DNA-mediated type I IFN responses.

We proceeded to compare IFN β induction in wt and LATS1 KO MEF cells. We showed that LATS1 KO MEF cells exhibit defective IFN β and downstream ISG induction on RLR-pathway activation (by pIC transfection, VSV-GFP infection, and Sendai virus infection). We also showed that LATS1 KO MEF cells have defective IFN β and ISG induction in response to cytosolic DNA pathway activation (by BDNA transfection, and HSV1 infection). Crucially, we showed that we could rescue this IFN β phenotype in LATS1 KO MEF cells by restoring LATS1 expression. Using both transient transfection and retroviral-generation of stable cell lines, we restored IFN β induction levels in response to RLR-pathway and cytosolic DNA pathway activation. Our data indicate that LATS1 functions to promote induction of IFN β upon activation of either the RLR or cytosolic DNA pathways.

Having verified a functional role for LATS1, we now directed our investigations towards determining the mechanism through which LATS1 promotes IFN β induction. First, we sought to determine the epistatic relationship between LATS1 and various key signaling molecules established in mediating IFN β induction. Using luciferase assays, we showed that LATS1 functions at the level of TBK1/IRF3 in the RLR and cytosolic DNA pathways. Since our data suggests that LATS1 promotes IFN β induction through both the RLR and cytosolic DNA pathways, we were not surprised to discover that LATS1 operates at the convergence point of these two pathways'. In light of our data showing that LATS1 operates at the TBK1/IRF3 level in the RLR and cytosolic DNA pathways, we next wondered if LATS1 is able to associate with either protein. Indeed, our results provide

strong evidence that LATS1 associates with the TBK1/IRF3 complex. We see association of LATS1 with both TBK1 and IRF3 individually in an overexpression system. In the endogenous setting, we also see interaction of LATS1 with the TBK1/IRF3 complex after activation of either the RLR or cytosolic DNA pathways. Our results show that LATS1 exerts its promotion of IFN β induction as part of the TBK1/IRF3 complex.

Based on our results showing that LATS1 forms part of a ligand-dependent complex of TBK1 and IRF3, we wondered if LATS1 were able to enhance phosphorylation of TBK1 and IRF3 as part of that complex. Indeed, we show that overexpression of LATS1 can enhance phosphorylation of both TBK1 and IRF3. We also show that LATS1 KO MEFs have reduced phosphorylation of IRF3 after transfection of pIC and BDNA. Both results support the hypothesis that upon activation of either the RLR or cytosolic DNA pathway, LATS1 is recruited to the TBK1/IRF3 complex, where it acts to enhance phosphorylation of TBK1 and IRF3. We suggest that this is the means through which LATS1 acts to promote IFN β induction on activation of either the RLR or cytosolic DNA pathways. Because phosphorylation of TBK1 and IRF3 is required for downstream IFN β induction, by enhancing TBK1/IRF3 phosphorylation, LATS1 would so act to promote IFN β induction. We also show that LATS1 kinase activity is required for its role promoting IFN β induction in response to RLR and cytosolic DNA pathways. Our results show that LATS1 kinase activity is required for its function to enhance phosphorylation of TBK1/IRF3. Our results also show that LATS1 kinase activity is required to rescue the LATS1 KO phenotype on activation of the RLR and cytosolic DNA pathways.

Our current study of LATS1 role in regulation of viral detection is by no means comprehensive. Further work is needed to elucidate in closer molecular detail the mechanisms of LATS1-mediated type I IFN induction via the RLR and DNA-sensor pathways. It is still unclear if LATS1 functions as a type of adaptor protein that enhances formation of the TBK1/IRF3 complex. The identity of the molecular target for LATS1 kinase activity regarding IFN β induction is unknown. Further work is needed to characterize LATS1 role in regulation of TRIF-mediated IFN β induction. Perhaps the most intriguing question, though, is whether there is interplay between LATS1 role in promoting IFN β induction and its role in the Hippo signaling pathway. Hippo signaling has not yet been linked to viral infection. However, considering the role of Hippo pathway in integrating multiple readouts of a cell's environment, it would not be surprising if viral infection, through any effects on extracellular environment and intracellular homeostasis, were able to alter Hippo signaling. Potentially LATS1 could serve as a bridge between viral infection and Hippo signaling. Potentially even, therapeutics developed to target Hippo signaling could effect antiviral signaling via LATS1. Regardless, our studies show that LATS1 promotes IFN β activation. We hope that the mechanisms of LATS1-induced type I IFN induction via the RLR and DNA-sensor pathways remains an active research area.

Gene	Reporter Construct				Key (Fold Induction)				
	IFN- β	ISRE	2XKB	AP1		IFN- β	ISRE	2XKB	AP1
Vector Alone					Low	<10	<10	<10	<10
RIPK2					Moderate	10-50	10-50	10-50	10-50
RIOK3					High	>50	>50	>50	>50
EPHA4									
ARK5									
PRKCM									
LATS1									
PRKG2									
CDC2L5									
LCK									
MAP3K4									
KIAA0781									
SLK									
CAMK2D									
RIPK3									
AAK1									
MAPK10									
PRKACB									
IKBKB									
IRAK4									
STK19									
TRIO									
TBK1									

Figure 4: Activation of Interferon- β promoter transcription factors.

36 hours after Co-transfection of the kinase expression plasmids and the different luciferase plasmids, luciferase levels were quantitated. To facilitate classification, levels of induction were color coded as low (black: little induction), moderate (yellow: 10 to 50 times induction) or High (red: high, greater than 50 times induction).

As a negative control, the levels of empty plamid induction of the luciferase plasmids is shown as well as a positive control of tranfection with TBK1, a known kinase in interferon induction.

Figure 4.1. Screening for kinases involved in IFN β production. We screened 575 kinases by a luciferase overexpression approach. Kinase expression plasmids and luciferase reporter plasmids were co-transfected into HEK 293T cells; we screened four separate luciferase reporter constructs for each kinase (IFN β , ISRE, 2XkB, and AP1). 36 hours post transfection, luciferase levels were measured. We classified the kinases by color according to induction levels: low-levels of activation (<10 fold) were denoted by the color black; medium levels (10-50 fold) by the color yellow; and finally, high levels (>50) by the color red.

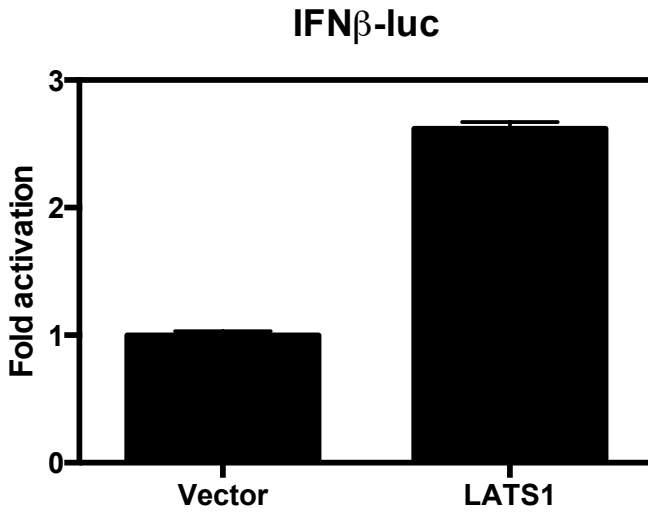


Figure 4.2. Overexpression of LATS1 promotes IFN β induction. HEK 293T cells were co-transfected with an IFN β luciferase reporter, along with either empty vector or LATS1. Cells were harvested 24 hours post-transfection, and assayed for luciferase activity using a luminometer. Renilla was used as a transfection control, and relative luciferase units were normalized to vector activation.

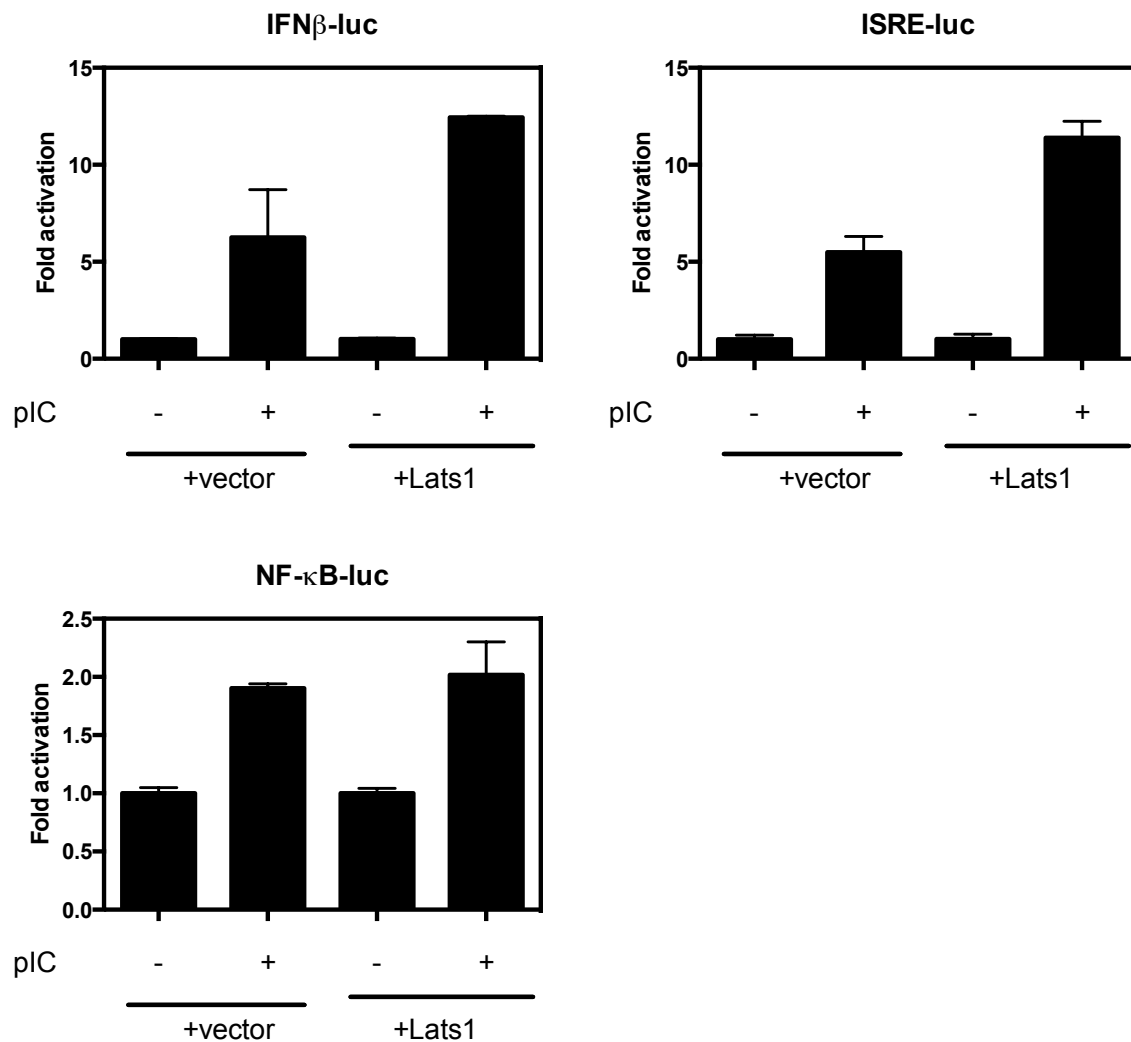


Figure 4.3. Overexpression of LATS1 synergizes with pIC transfection to promote IFN β induction. HEK 293T cells were co-transfected with empty vector and LATS1, with and without pIC. Cells were transfected with either IFN β , ISRE, or 5xkB luciferase reporters. Renilla was used as a transfection control, and relative luciferase units were normalized to activation without pIC transfection.

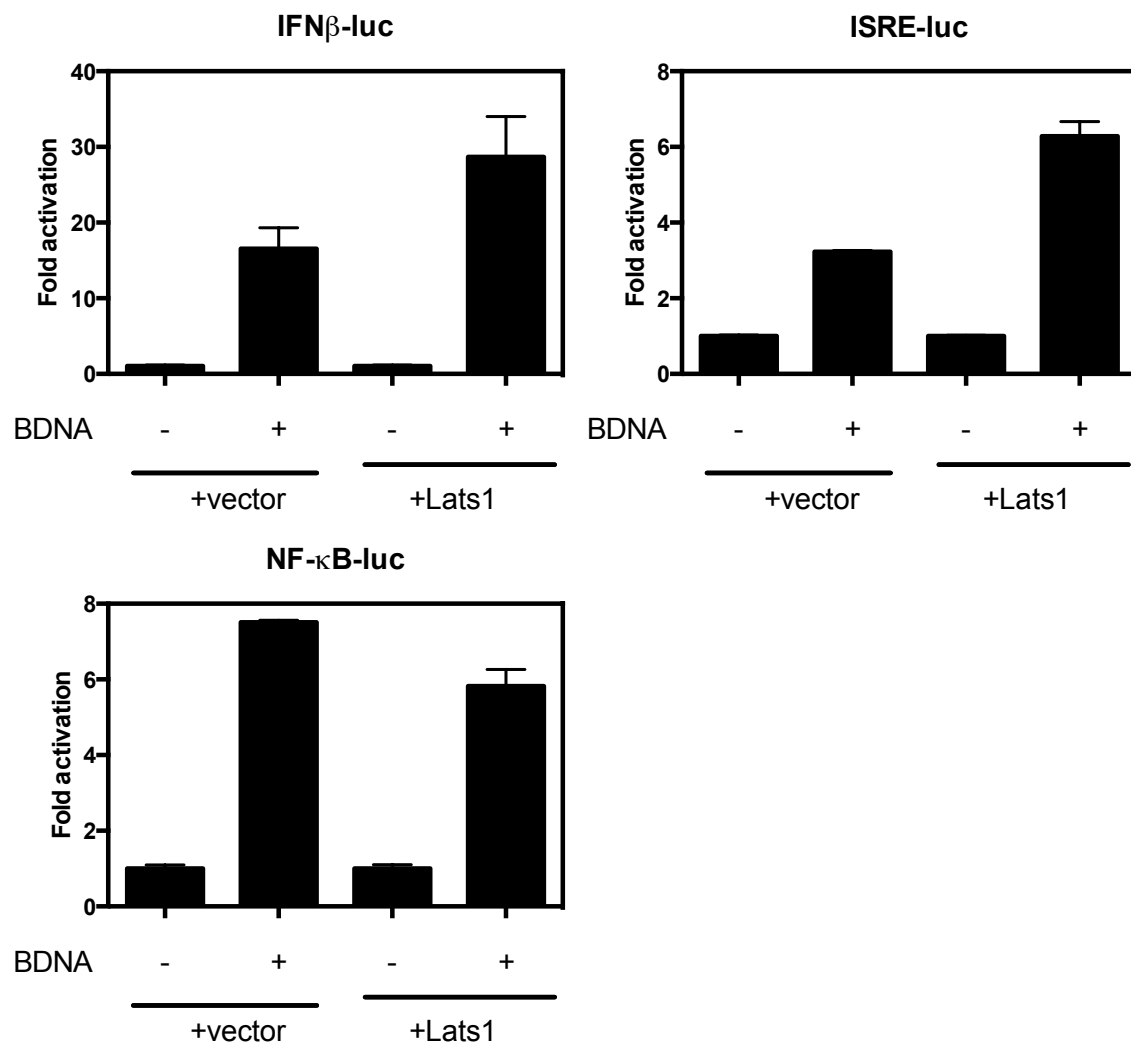


Figure 4.4. Overexpression of LATS1 synergizes with BDNA transfection to promote IFN β induction. HEK 293T cells were co-transfected with empty vector and LATS1, with and without BDNA. Cells were transfected with either IFN β , ISRE, or 5xkB luciferase reporters. Renilla was used as a transfection control, and relative luciferase units were normalized to activation without BDNA transfection.

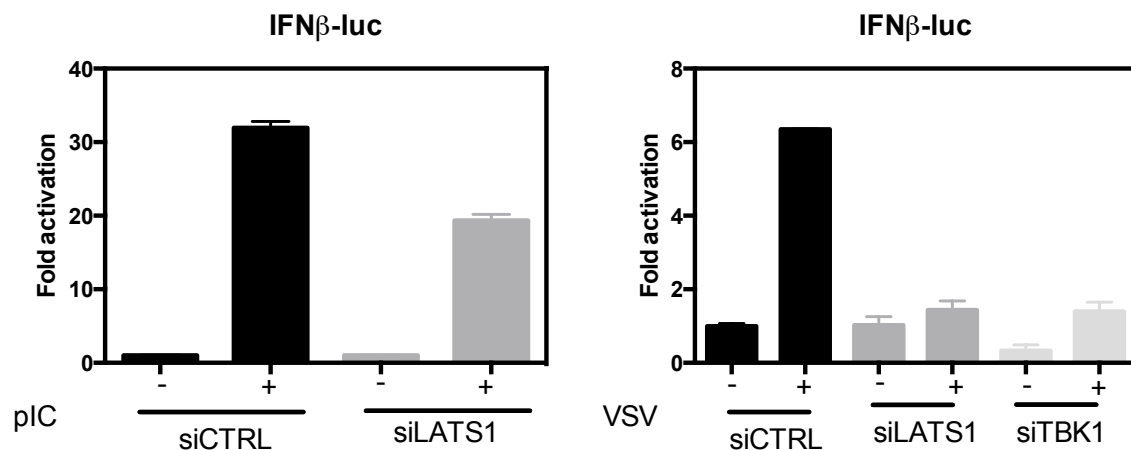


Figure 4.5. Knockdown of LATS1 inhibits IFN β response to pIC transfection and VSV infection, as measured by luciferase. We transfected HEK 293T cells with siRNA. 24 hours post transfection of siRNA, we transfected the same cells with IFN β firefly and Renilla control luciferase reporters. Luciferase values were then quantified 16 hours after the luciferase reporter transfection. At the time of luciferase reporter transfection, we activated the RLR pathway, via either a pIC transfection (on the left) or VSV-GFP infection (on the right; MOI=0.01).

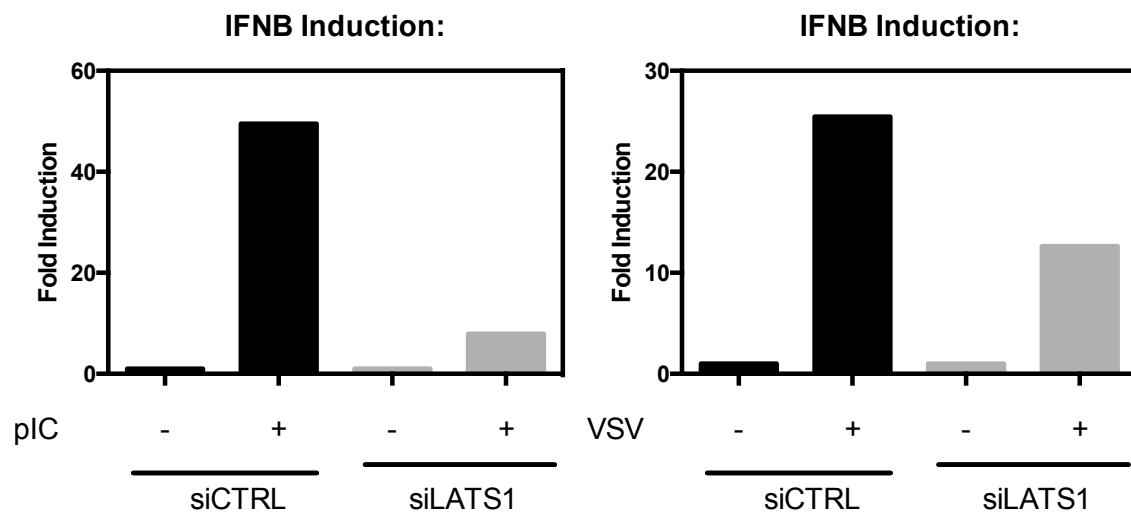


Figure 4.6. Knockdown of LATS1 inhibits IFN β response to pIC transfection and VSV infection, as measured by QPCR. We transfected HEK 293T cells with siRNA. 24 hours after siRNA-transfection, we activated the RLR pathway, via either a pIC transfection (on the left) or VSV-GFP infection (on the right; MOI=0.01). We collected cells for RNA extraction 16 hours after pIC transfection/VSV-GFP infection.

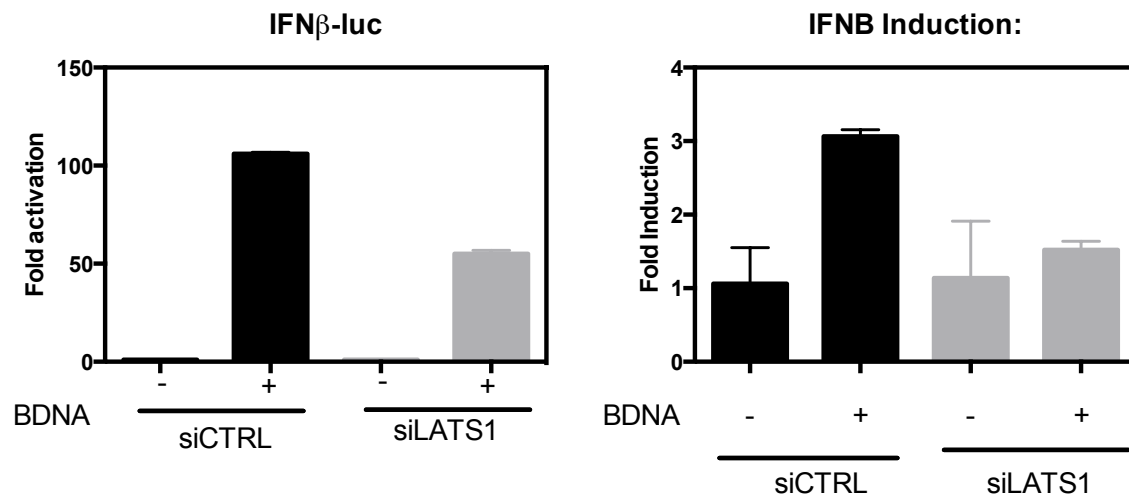


Figure 4.7. Knockdown of LATS1 inhibits IFN β response to BDNA transfection.

The left-hand shows activation of an IFN β -luciferase construct. We transfected HEK 293T cells with siRNA. 24 hours post transfection of siRNA, we transfected the same cells with IFN β firefly and Renilla control luciferase reporters, with or without BDNA transfection. Luciferase values were then quantified 16 hours after the luciferase reporter transfection. The right-hand graph plots induction of IFN β mRNA levels. We transfected HEK 293T cells with siRNA. 24 hours after siRNA-transfection, we transfected the same cells with and without BDNA. We collected cells for RNA extraction 16 hours after BDNA transfection.

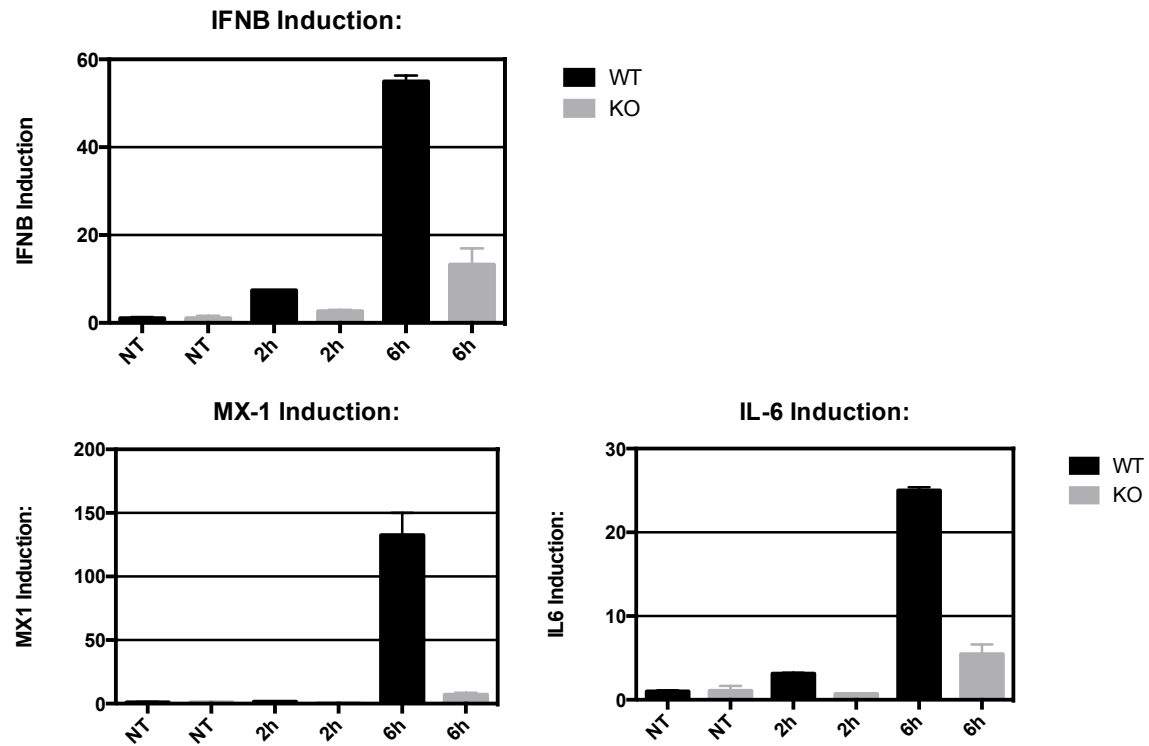


Figure 4.8. LATS1 KO MEFs have reduced IFN β response to pIC transfection. We transfected pIC into wt and KO cells, and we used QPCR to monitor gene induction.

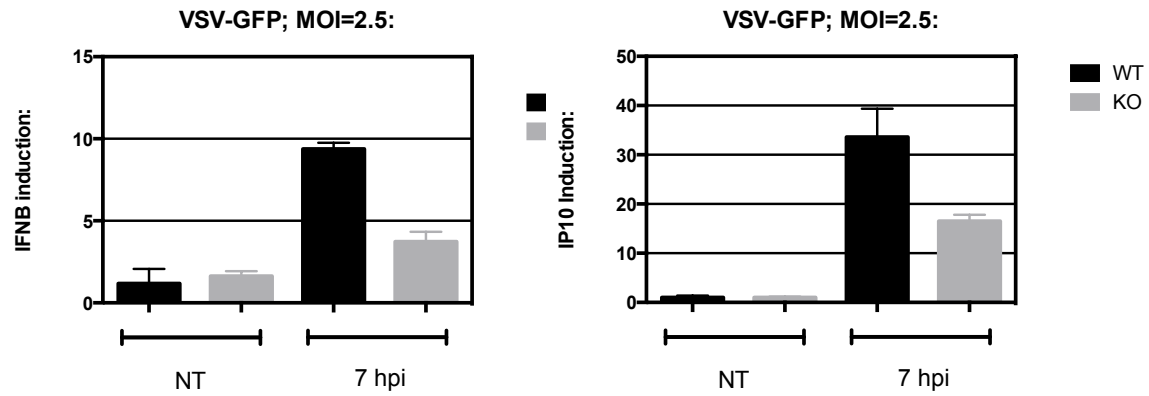


Figure 4.9. LATS1 KO MEFs have reduced IFN β response to VSV-GFP infection.

We infected wt and KO cells with VSV-GFP at MOI=2.5, and we used QPCR to monitor gene induction.

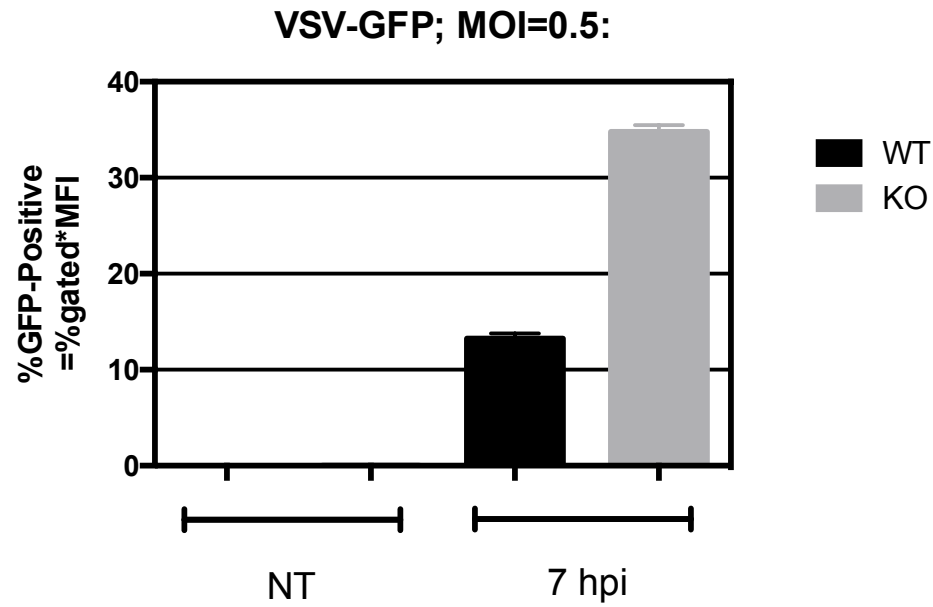


Figure 4.10. LATS1 KO MEFs show increased viral replication on infection with VSV-GFP. We infected wt and KO cells with VSV-GFP at MOI=0.5, and we used flow cytometry to measure GFP fluorescence.

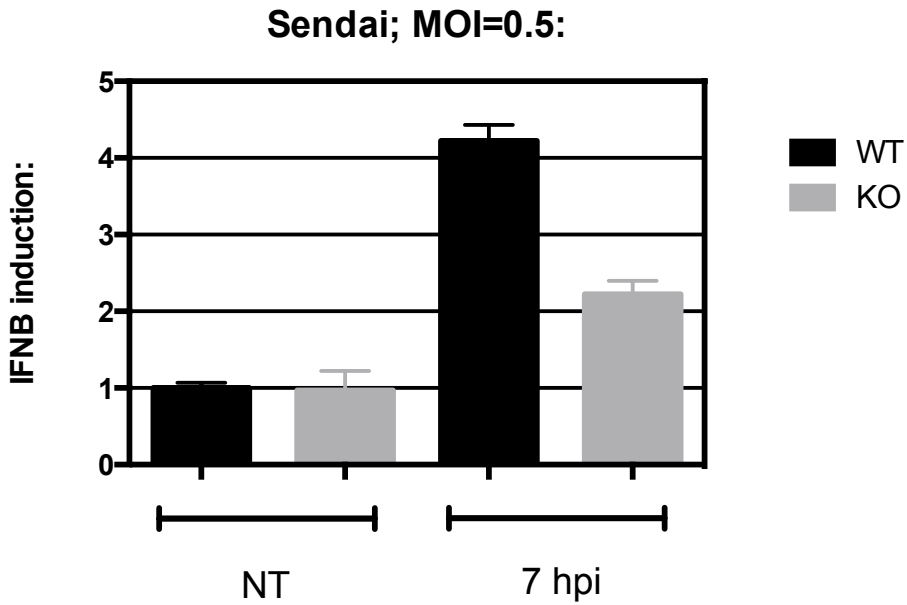


Figure 4.11. LATS1 KO MEFs have reduced IFN β response to Sendai viral infection. We infected wt and KO cells with Sendai virus at MOI=0.5, and we used QPCR to monitor gene induction.

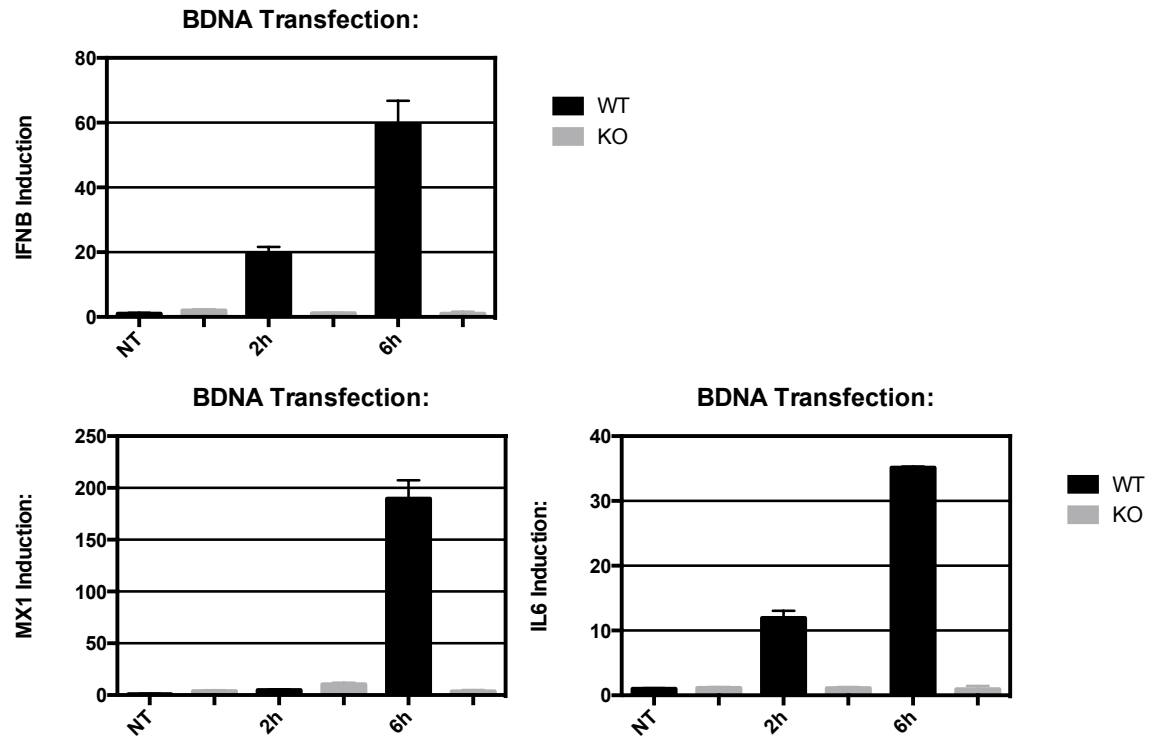


Figure 4.12. LATS1 KO MEFs have reduced IFN β response to BDNA transfection.

We transfected BDNA into wt and KO cells, and we used QPCR to monitor gene induction.

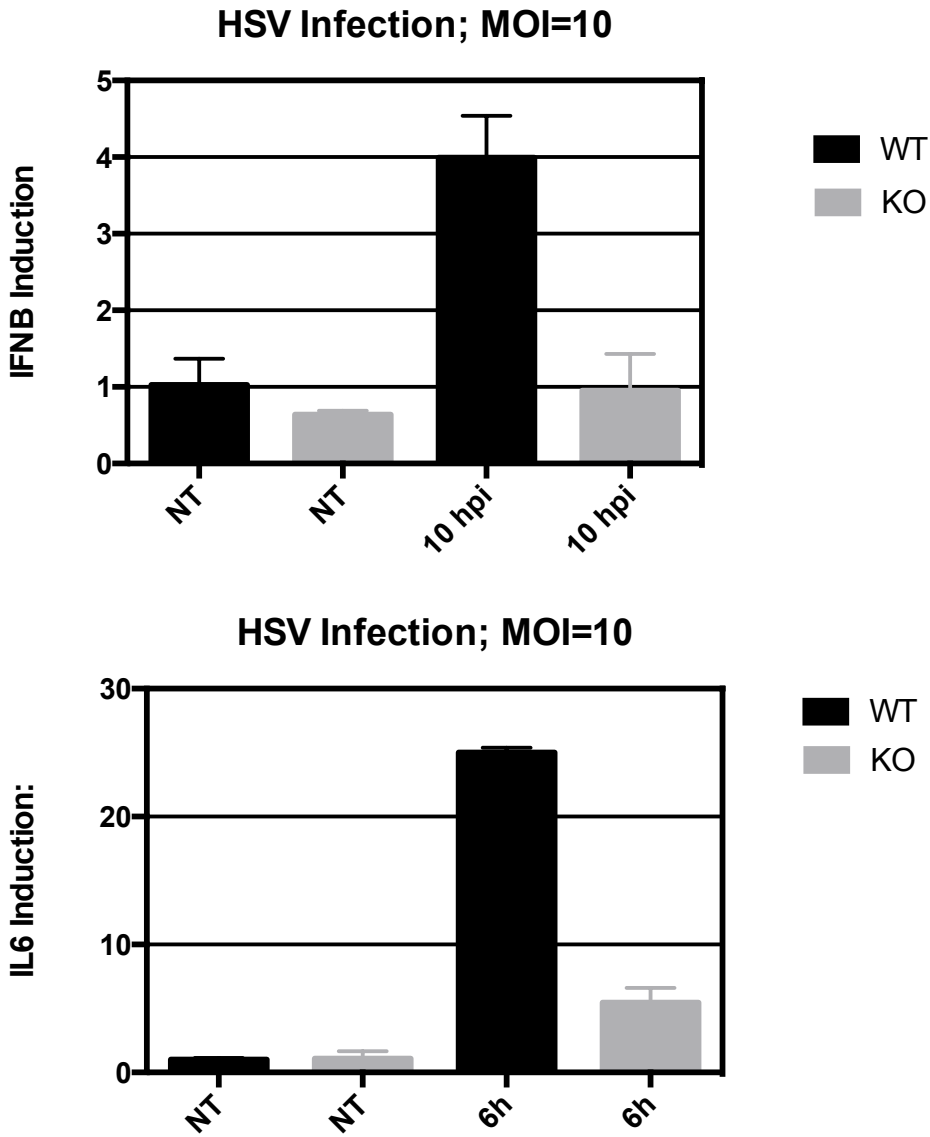


Figure 4.13. LATS1 KO MEFs show reduced IFN β response to HSV1 infection. We infected wt and KO cells with HSV1 at MOI=10, and we used QPCR to monitor gene induction.

- | | | |
|-----------|---|---|
| 1. Vector | + | - |
| 2. LATS1 | - | + |

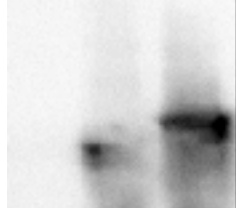


Figure 4.14. Transient transfection restores LATS1 expression to LATS1 KO MEFs. We transfected LATS1 KO MEFs with either empty vector or with LATS1. 24 hours later we collected WCL and assayed for LATS1 protein expression by immunoblot.

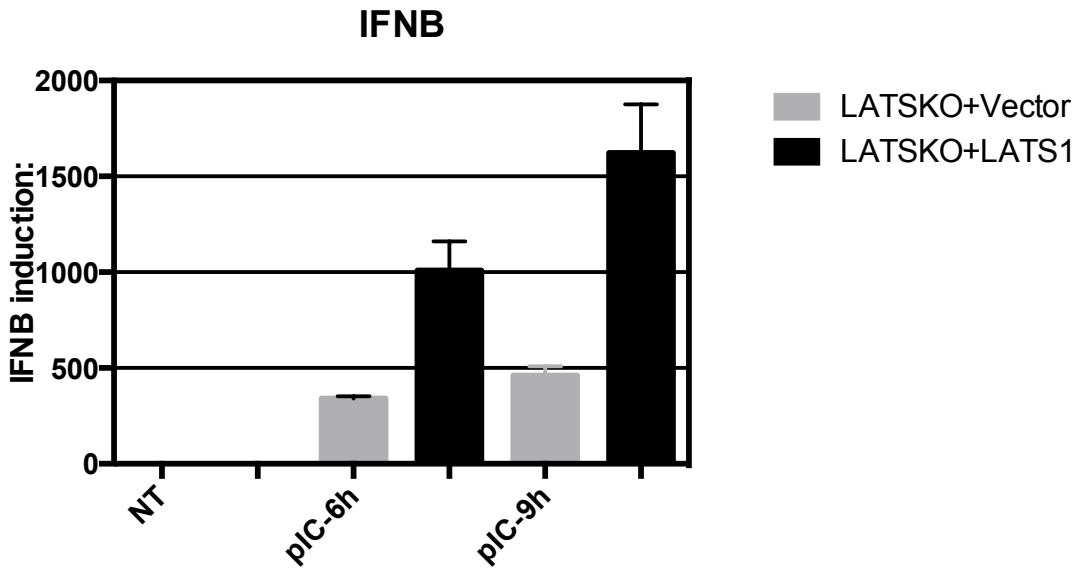


Figure 4.15. Transient transfection of LATS1 plasmid into LATS1 KO MEFs rescues induction of IFN β in response to pIC transfection. We transfected LATS1 KO MEFs with either empty vector or with LATS1. 24 hours later we transfected the same cells with pIC, and we used QPCR to monitor gene induction.

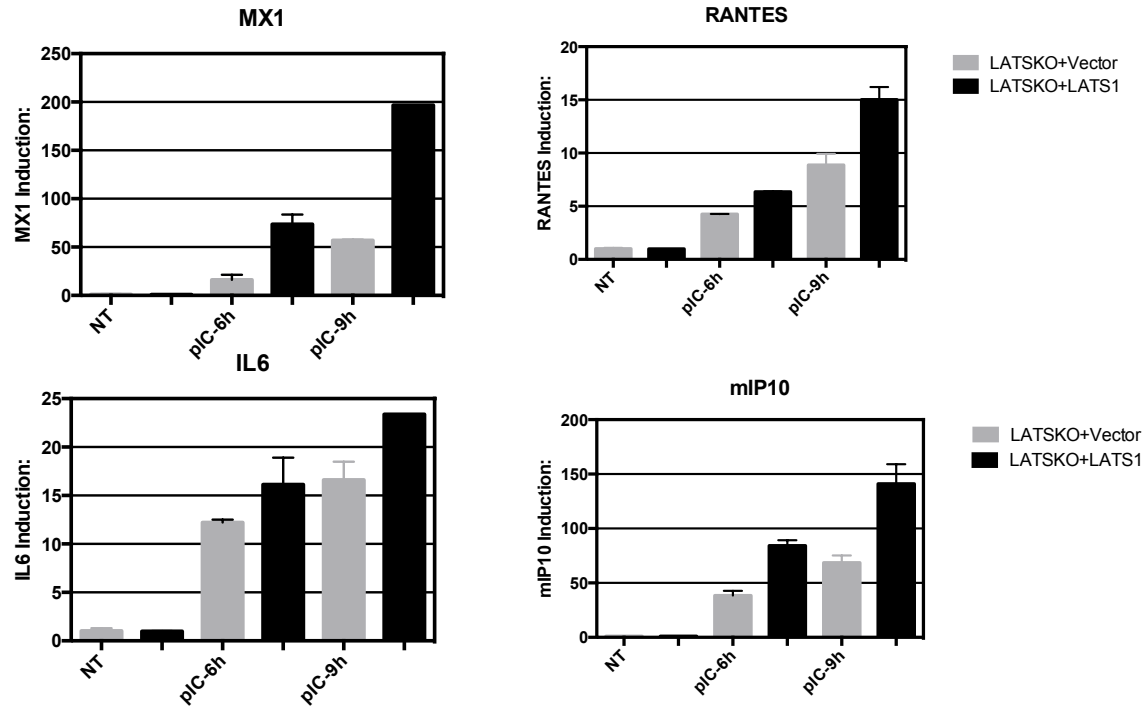


Figure 4.16. Transient transfection of LATS1 plasmid into LATS1 KO MEFs rescues IFN β response to pIC transfection. We transfected LATS1 KO MEFs with either empty vector or with LATS1. 24 hours later we transfected the same cells with pIC, and we used QPCR to monitor gene induction.

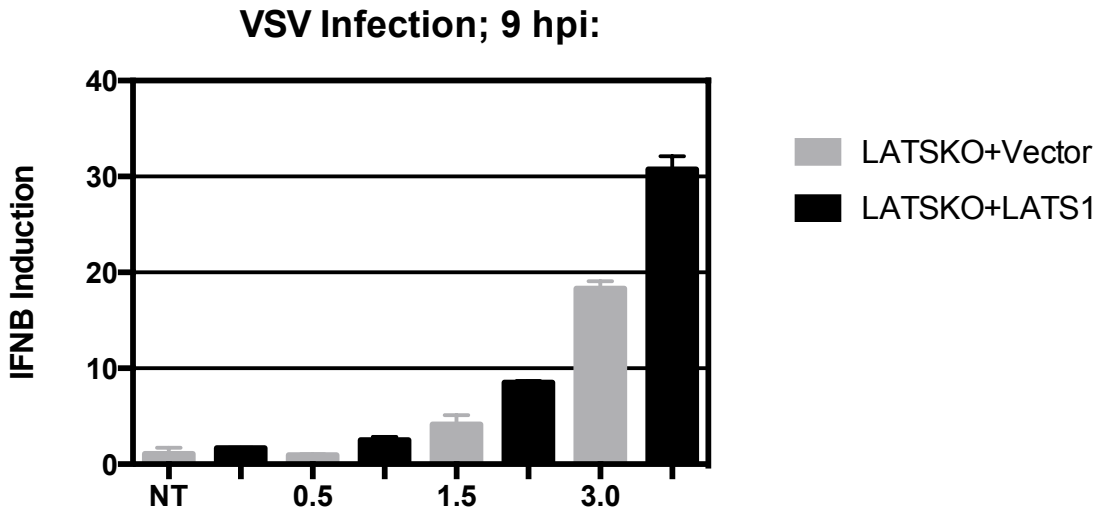


Figure 4.17. Transient transfection of LATS1 plasmid into LATS1 KO MEFs rescues induction of IFN β in response to infection with VSV-GFP. We transfected LATS1 KO MEFs with either empty vector or with LATS1. 24 hours later we infected the same cells with VSV-GFP at MOI=2.5, and we used QPCR to monitor gene induction.

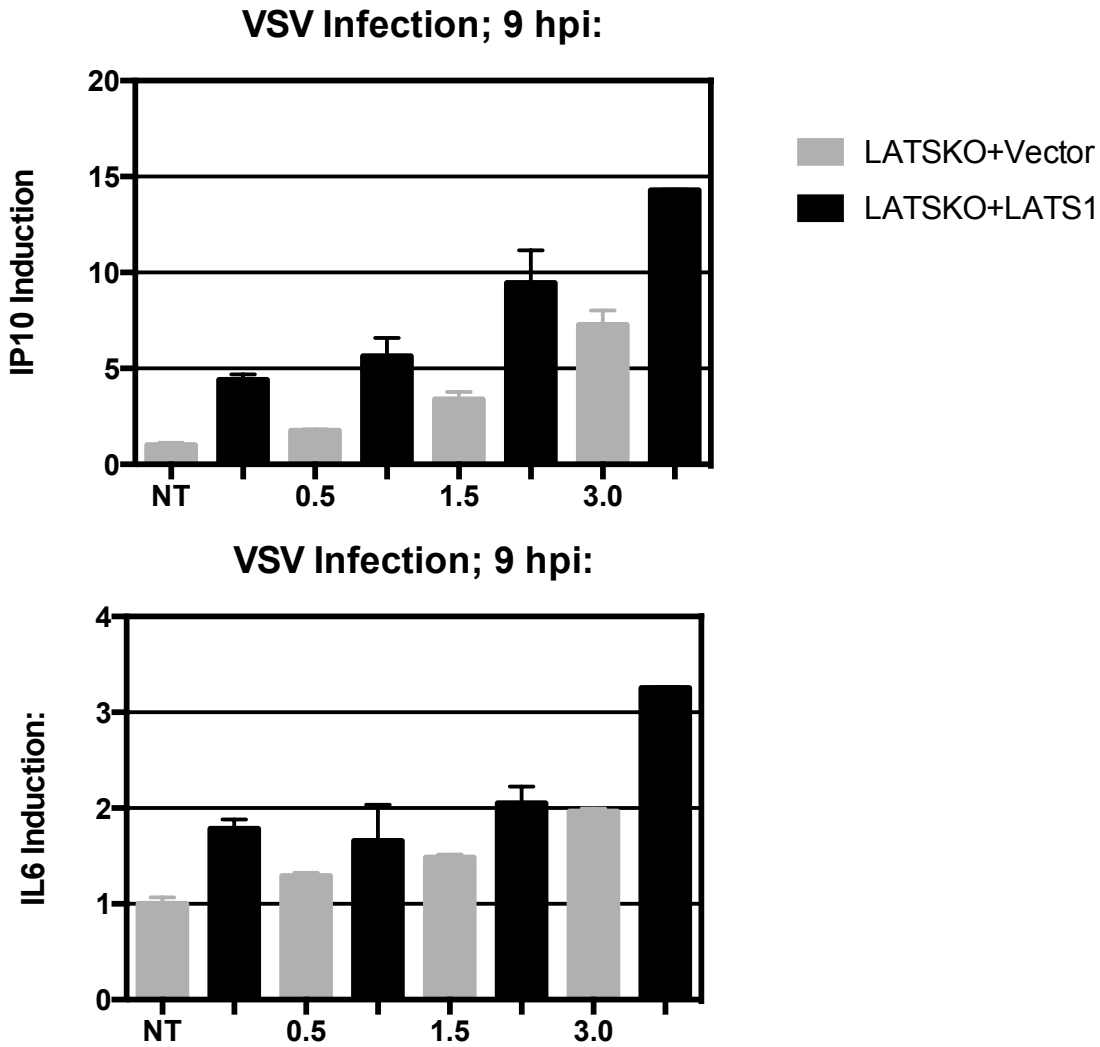


Figure 4.18. Transient transfection of LATS1 plasmid into LATS1 KO MEFs

rescues IFN β response to VSV-GFP infection. We transfected LATS1 KO MEFs with either empty vector or with LATS1. 24 hours later we infected the same cells with VSV-GFP at MOI=2.5, and we used QPCR to monitor gene induction.

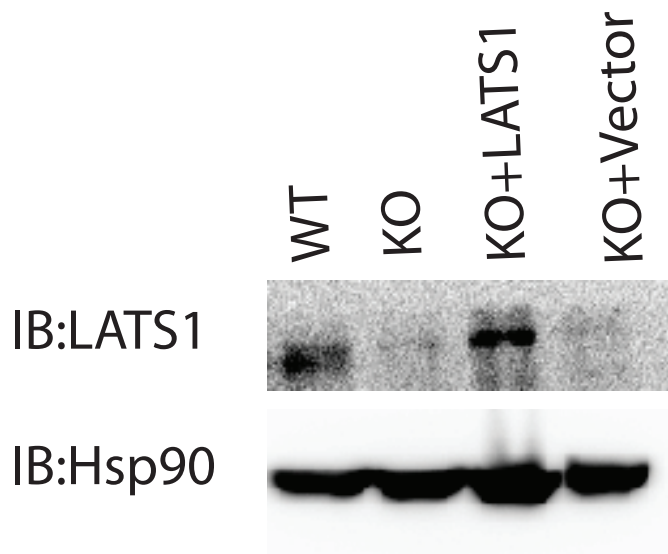


Figure 4.19. Expression of LATS1 in stably-reconstituted cells. We used a Moloney murine leukemia virus Ψ A helper construct to generate stably-reconstituted cells. We transfected HEK 293T cells with a Moloney murine leukemia virus Ψ A helper construct, plus either empty pBABE-puro vector or pBABE-puro-LATS1 vector. We then infected LATS1 KO MEFS with the filtered HEK 293T cell supernatants, and selected with puromycin. After selection, we collected WCL and assayed for LATS1 protein expression by immunoblot.

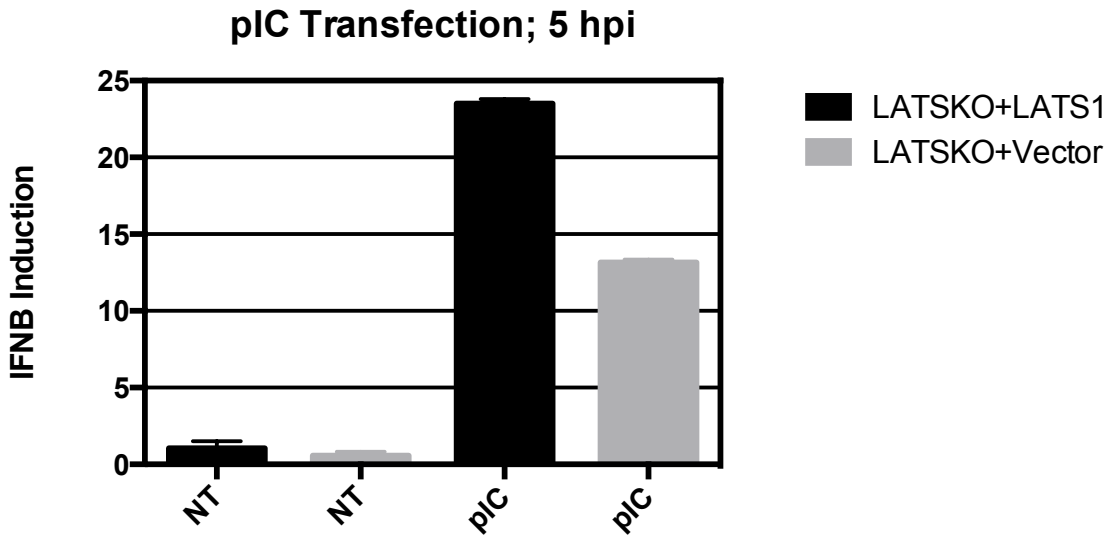


Figure 4.20. Stable reconstitution of LATS1 into LATS1 KO MEFs rescues induction of IFN β in response to pIC transfection. Having established stable cell lines reconstituted with either vector or LATS1, we transfected these same cells with pIC, and we used QPCR to monitor gene induction.

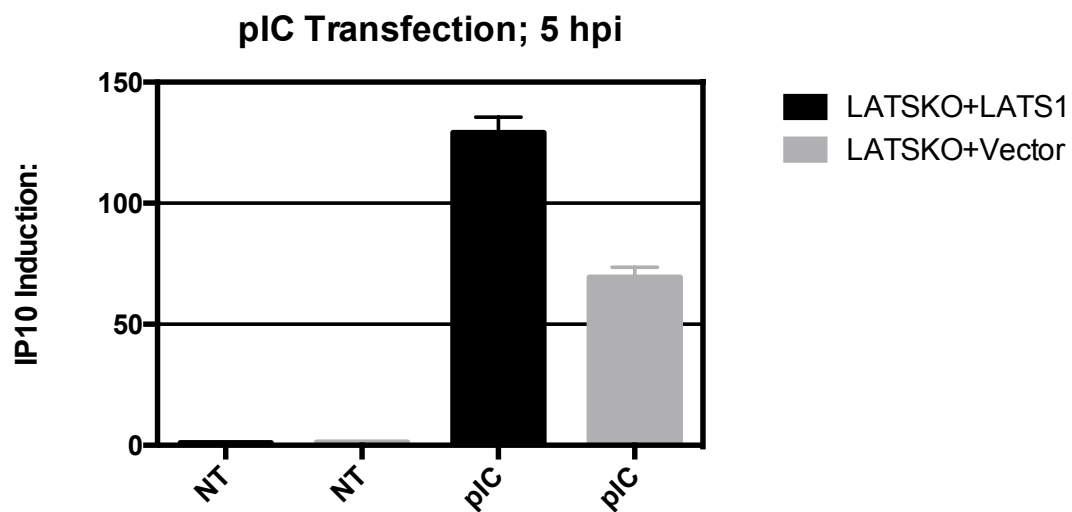
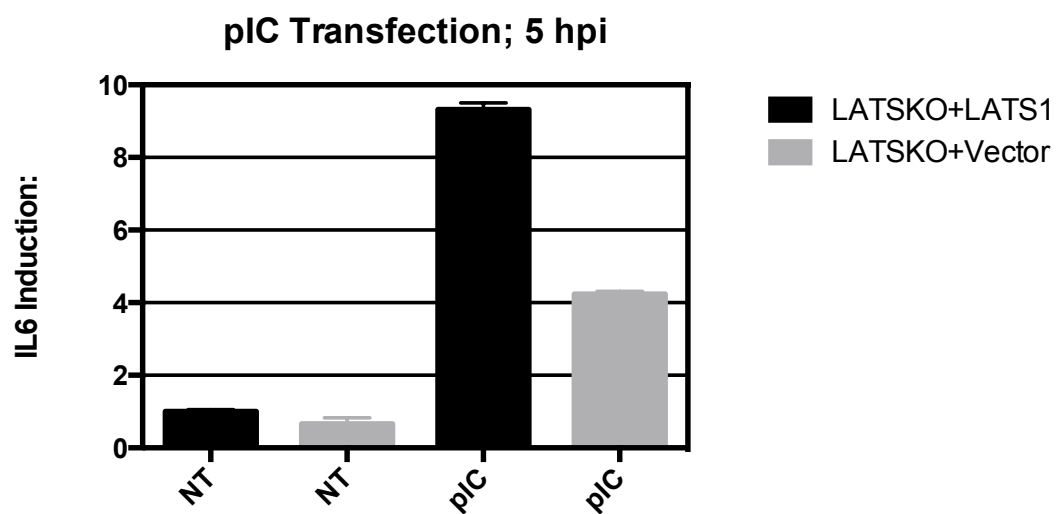
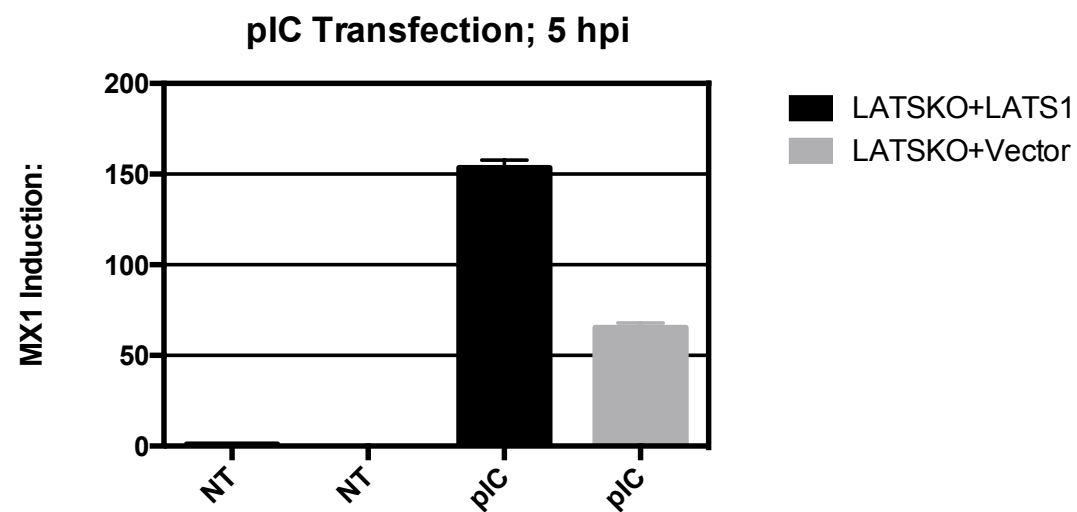


Figure 4.21. Stable reconstitution of LATS1 into LATS1 KO MEFs rescues IFN β response to pIC transfection. Having established stable cell lines reconstituted with either vector or LATS1, we transfected these same cells with pIC, and we used QPCR to monitor gene induction.

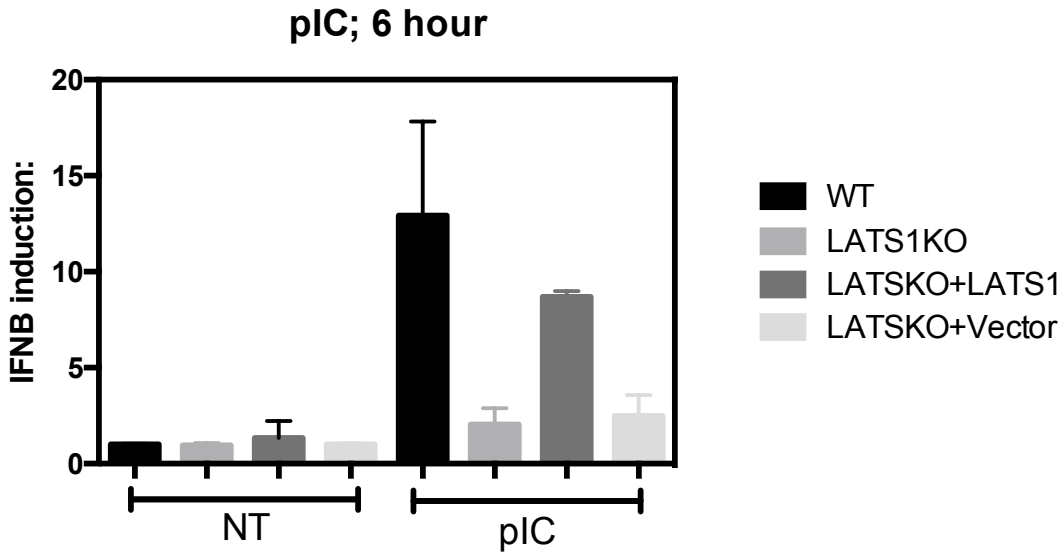


Figure 4.22. Comparison of IFN β induction levels after pIC transfection between cell lines. In parallel with WT and KO cells, we transfected the stably-reconstituted cells with pIC, and we used QPCR to monitor gene induction.

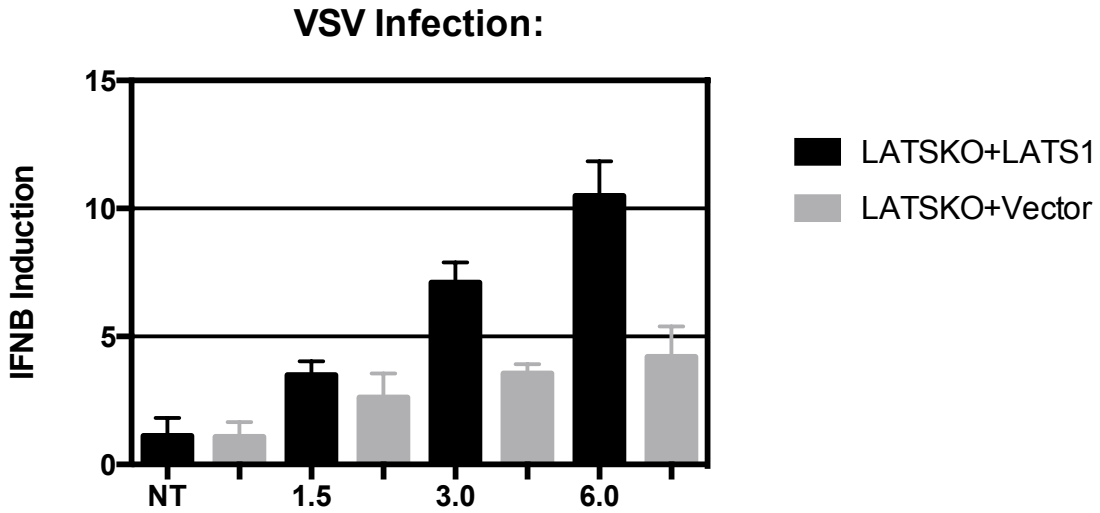


Figure 4.23. Stable reconstitution of LATS1 into LATS1 KO MEFs rescues induction of IFN β in response to VSV-GFP infection. Having established stable cell lines reconstituted with either vector or LATS1, we infected these same cells with VSV-GFP at MOI=0, 1.5, 3.0 or 6.0, and we used QPCR to monitor gene induction at 6 hpi.

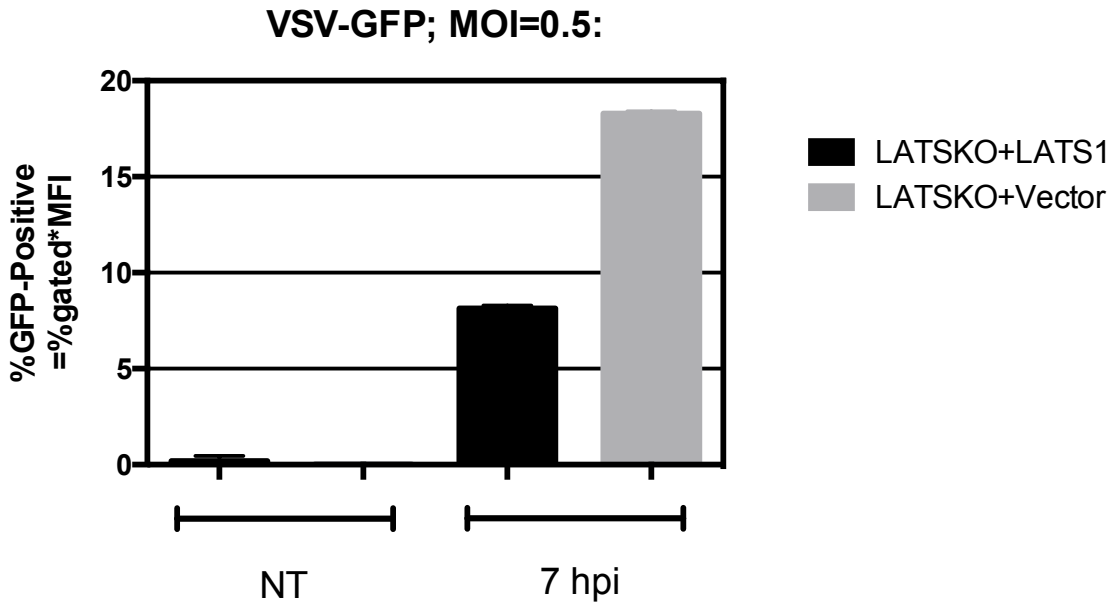


Figure 4.24. Stable reconstitution of LATS1 into LATS1 KO MEFs reduces viral replication on infection with VSV-GFP. Having established stable cell lines reconstituted with either vector or LATS1, we infected these same cells with VSV-GFP at MOI=0.5, and we used flow cytometry to measure GFP fluorescence at 7 hpi.

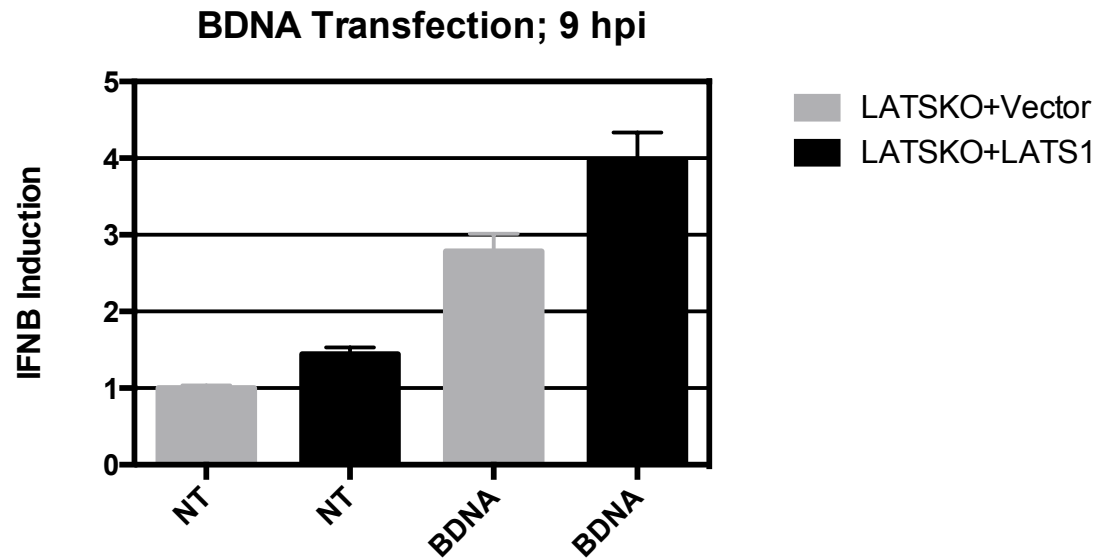


Figure 4.25. Transient transfection of LATS1 plasmid into LATS1 KO MEFs rescues induction of IFN β in response to BDNA transfection. We transfected LATS1 KO MEFs with either empty vector or with LATS1. 24 hours later we transfected the same cells with BDNA, and we used QPCR to monitor gene induction.

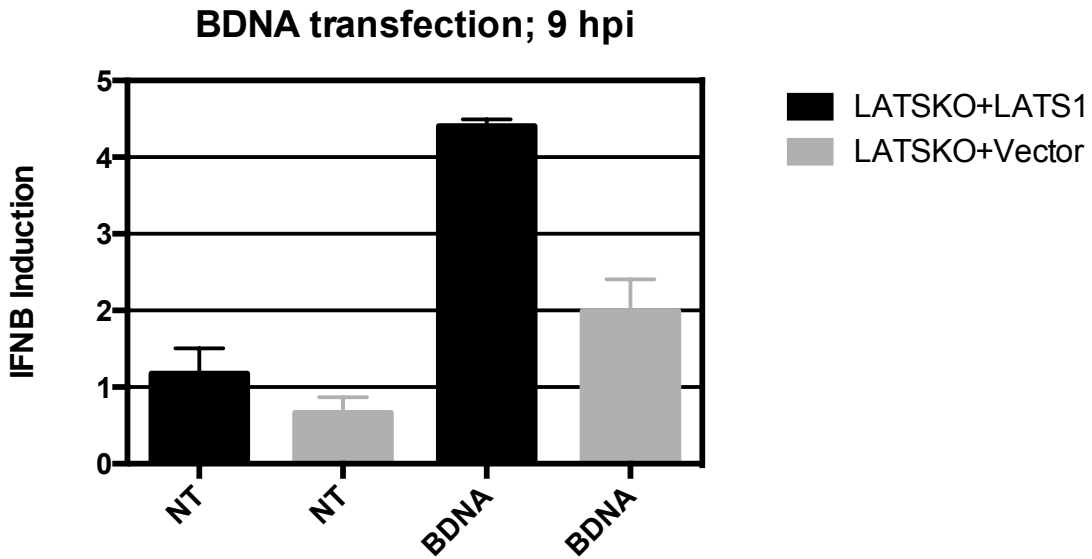


Figure 4.26. Stable reconstitution of LATS1 into LATS1 KO MEFs rescues induction of IFN β in response to BDNA transfection. Having established stable cell lines reconstituted with either vector or LATS1, we transfected these same cells with BDNA, and we used QPCR to monitor gene induction.

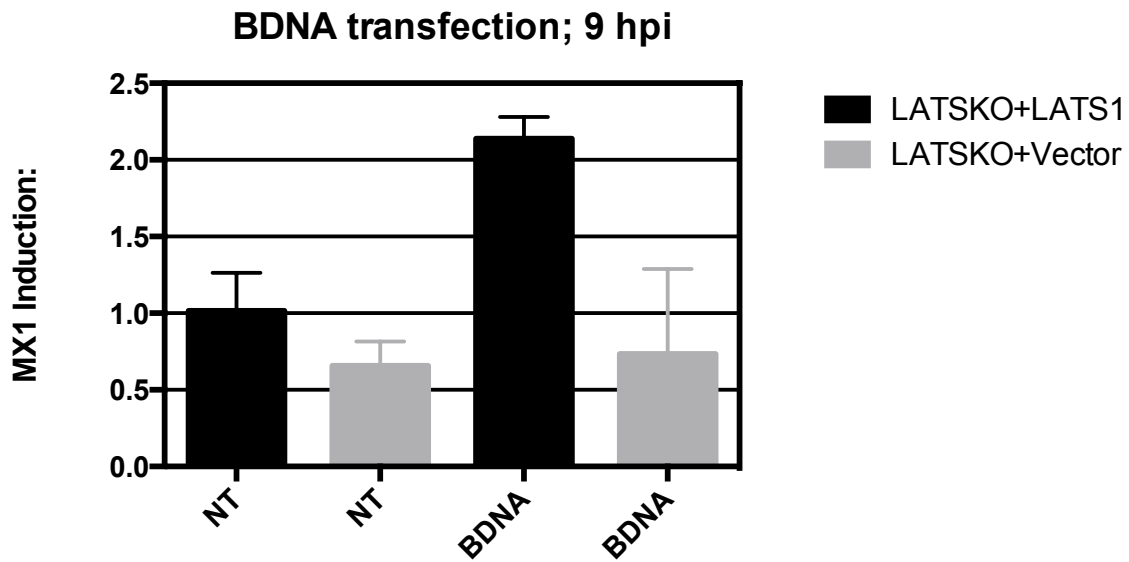


Figure 4.27. Stable reconstitution of LATS1 into LATS1 KO MEFs rescues IFN β response after BDNA transfection. Having established stable cell lines reconstituted with either vector or LATS1, we transfected these same cells with BDNA, and we used QPCR to monitor gene induction.

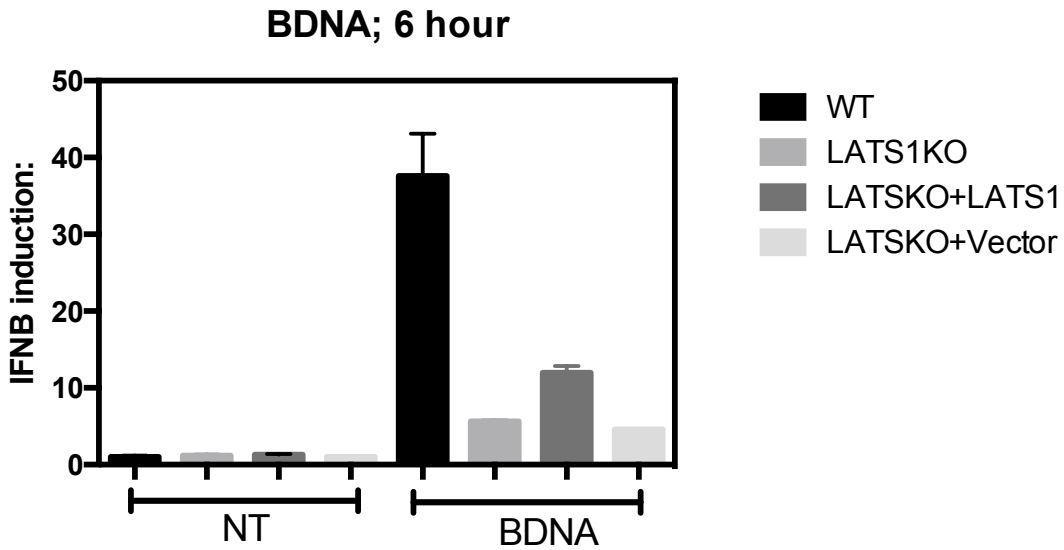


Figure 4.28. Comparison of IFN β induction levels after BDNA transfection between cell lines. In parallel with WT and KO cells, we transfected the stably-reconstituted cells with BDNA, and we used QPCR to monitor gene induction.

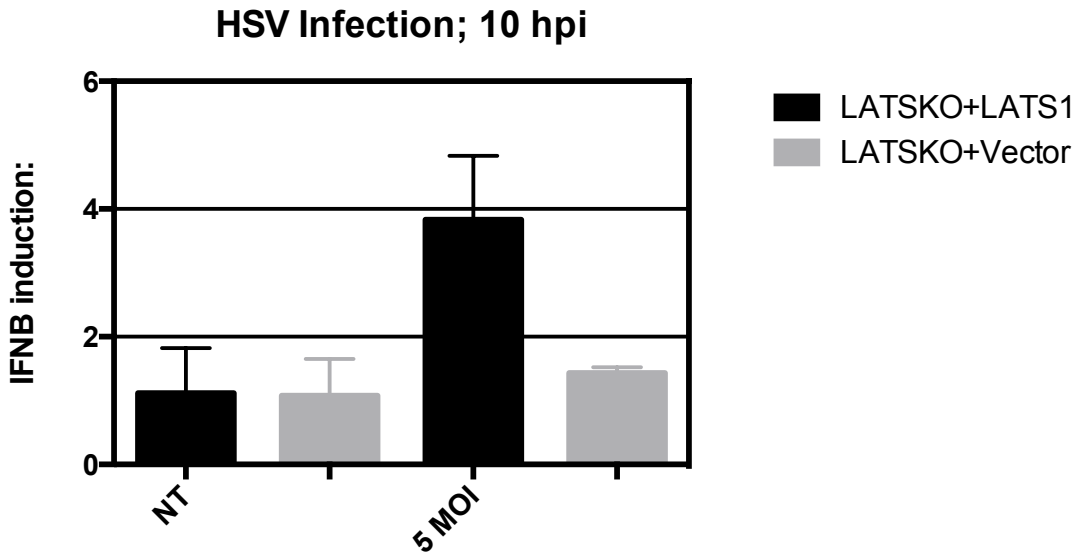


Figure 4.29. Stable reconstitution of LATS1 into LATS1 KO MEFs rescues induction of IFN β in response to HSV1 infection. Having established stable cell lines reconstituted with either vector or LATS1, we infected these same cells with HSV1 at MOI=5, and we used QPCR to monitor gene induction at 10 hpi.

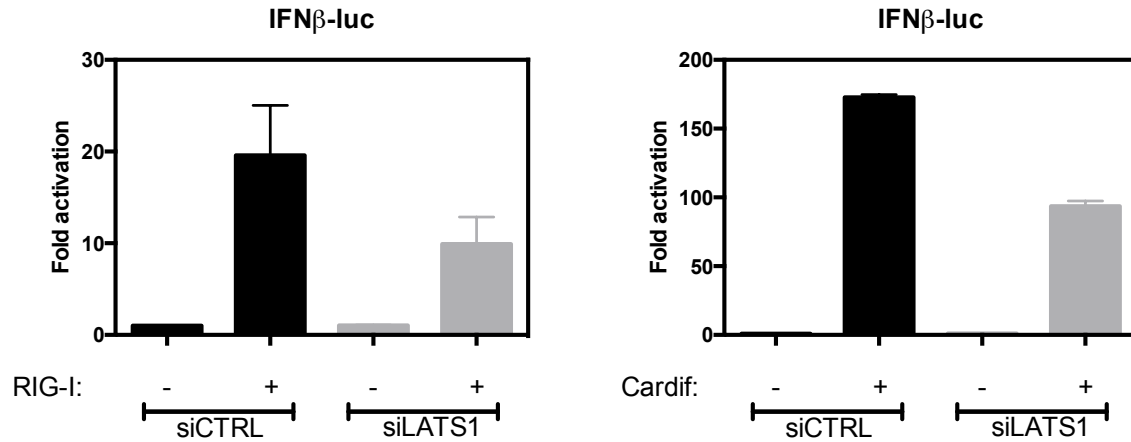


Figure 4.30. LATS1 operates downstream of RIGI/Cardif to modulate IFN β . We transfected HEK 293T cells with either control siRNA or siLATS1. 24 hours post-transfection, we transfected the same cells with IFN β firefly and Renilla control luciferase reporters, along with vector, RIGI, or Cardif. Luciferase values were then quantified 24 hours after the second transfection. Renilla was used as a transfection control, and relative luciferase units were normalized to activation with just vector transfection.

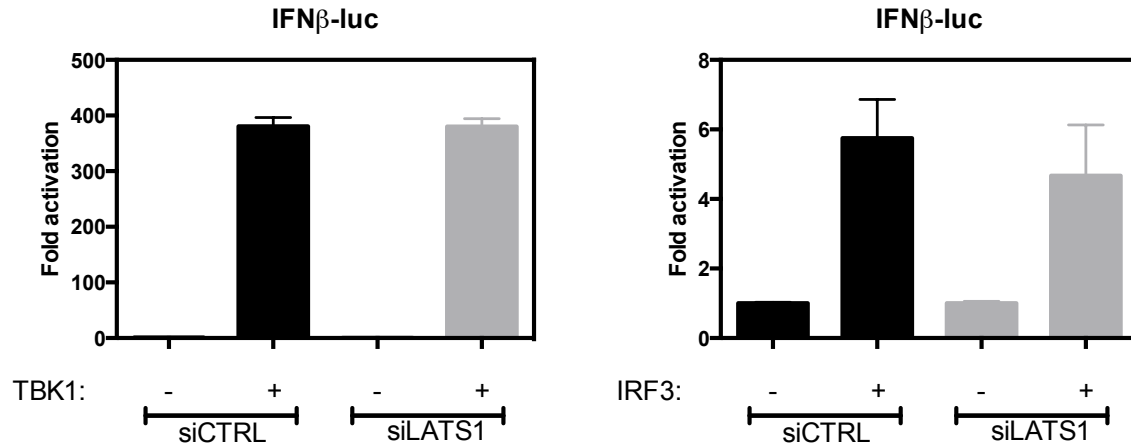


Figure 4.31. LATS1 operates at TBK1/IRF3 level to modulate IFN β . We transfected HEK 293T cells with either control siRNA or siLATS1. 24 hours post-transfection, we transfected the same cells with IFN β firefly and Renilla control luciferase reporters, along with vector, TBK1, or IRF3. Luciferase values were then quantified 24 hours after the second transfection. Renilla was used as a transfection control, and relative luciferase units were normalized to activation with just vector transfection.

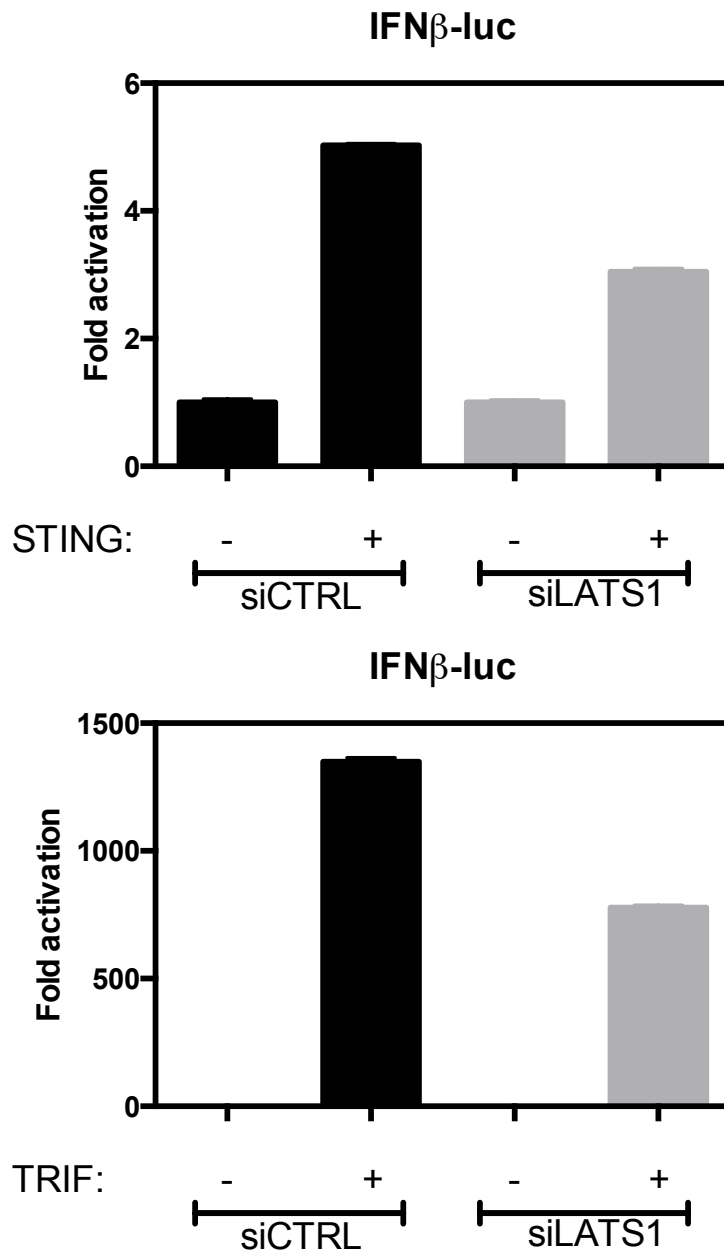


Figure 4.32. LATS1 operates downstream of STING and TRIF to modulate IFN β .

We transfected HEK 293T cells with either control siRNA or siLATS1. 24 hours post-transfection, we transfected the same cells with IFN β firefly and Renilla control luciferase reporters, along with vector, STING, or TRIF. Luciferase values were quantified 24 hours after the second transfection. Renilla was used as a transfection control, and relative luciferase units were normalized to activation with just vector transfection.

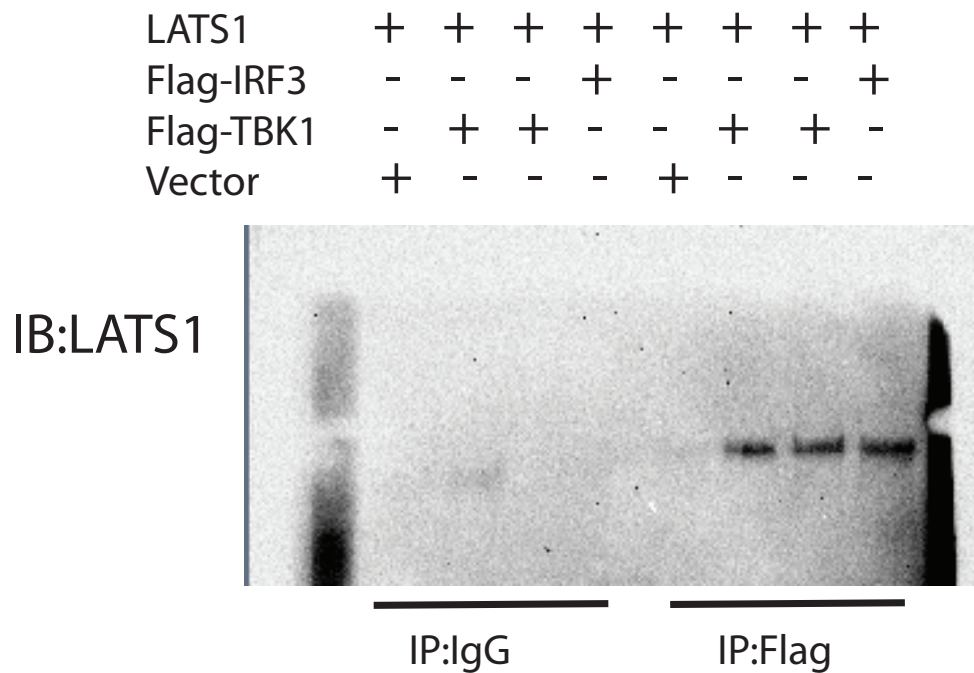


Figure 4.33. LATS1 associates with TBK1 and IRF3 in overexpression system. We co-transfected HEK 293T cells with LATS1 and either vector, Flag-TBK1, or Flag-IRF3. We immunoprecipitated with either control IgG or Flag antibody, and after washing, we used western blotting to probe for LATS1.

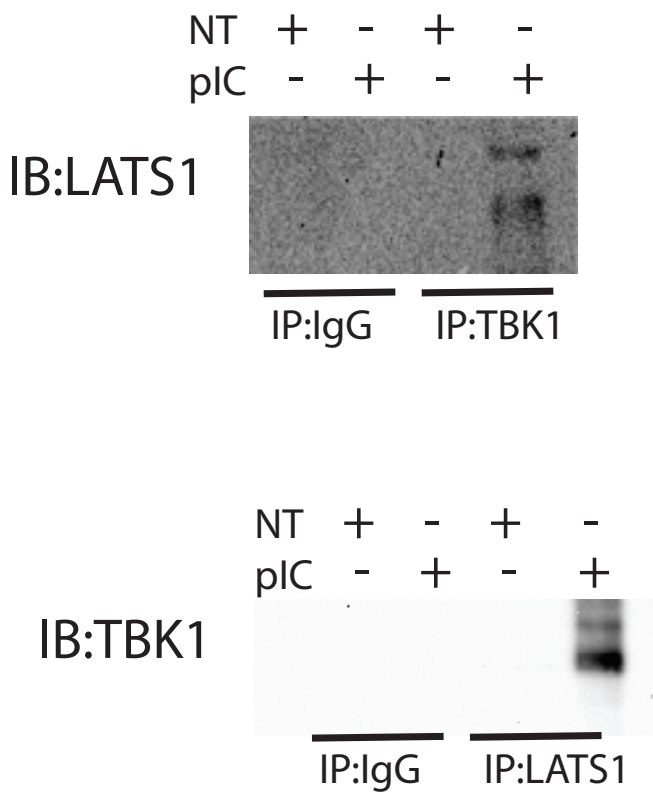
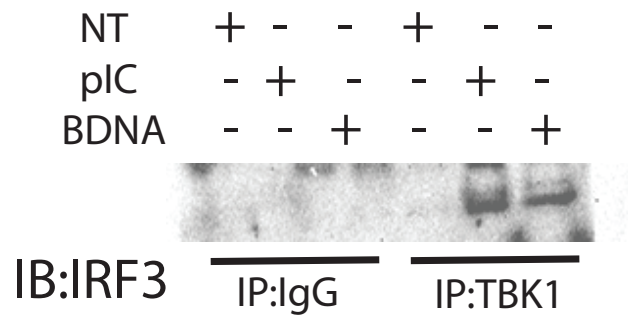


Figure 4.34. LATS1 interacts with TBK1 after pIC transfection. For the top blot, we transfected WT MEF cells with pIC and immunoprecipitated the WCL with either IgG control or anti-TBK1 antibody. After washing, we used western blotting to probe for LATS1. For the bottom blot, we transfected WT MEF cells with pIC and immunoprecipitated the WCL with either IgG control or anti-LATS1 antibody. After washing, we used western blotting to probe for TBK1.



4.35. LATS1 interacts with IRF3 after transfection of pIC or BDNA. We transfected WT MEF cells with either pIC or BDNA, and immunoprecipitated the WCL with either IgG control or anti-TBK1 antibody. After washing, we used western blotting to probe for IRF3.

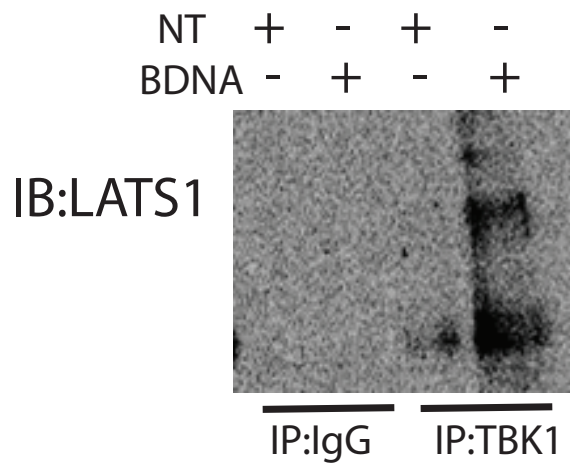


Figure 4.36. LATS1 interacts with TBK1 after BDNA transfection. We transfected WT MEF cells with BDNA and immunoprecipitated the WCL with either IgG control or anti-TBK1 antibody. After washing, we used western blotting to probe for LATS1.

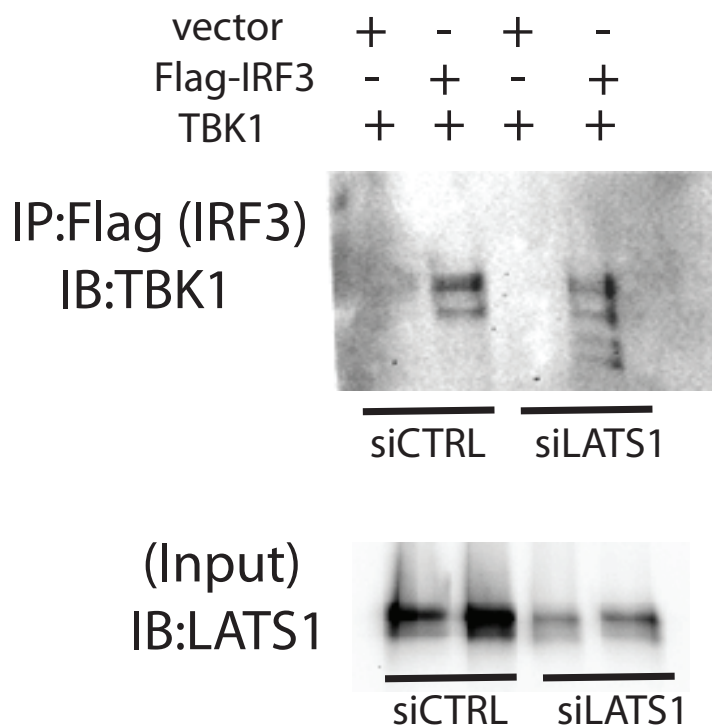


Figure 4.37. LATS1 knockdown disrupts TBK1/IRF3 interaction. We transfected HEK 293T cells with either siCTRL or siLATS1. After 24 hours, we transfected the same cells with TBK1, and either vector or Flag-tagged IRF3. 24 hours after the second transfection, we immunoprecipitated the WCL with anti-Flag antibody; after washing, we used western blotting to probe for TBK1.

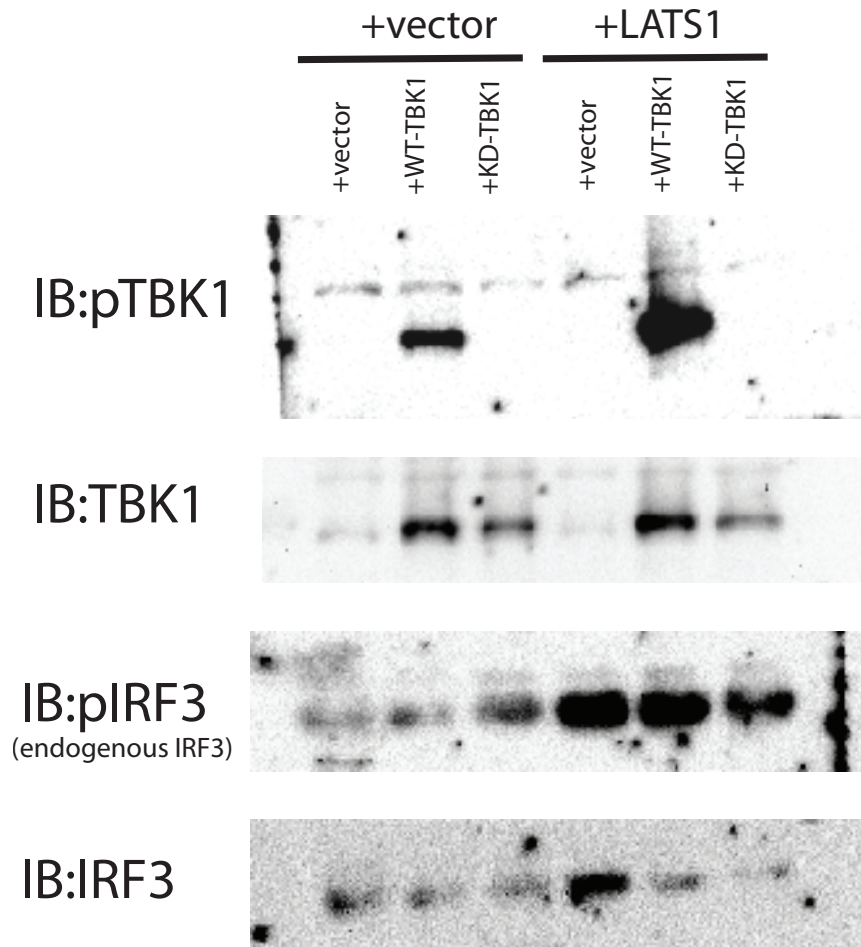


Figure 4.38. Overexpression of LATS1 promotes phosphorylation of TBK1 and IRF3. We transfected either vector or LATS1 into HEK 293T cells; we also co-transfected vector, WT TBK1, or a KD TBK1 construct into the same cells. After 24 hours, we collected the WCL and used western blotting to probe for TBK1 and IRF3 phosphorylation.

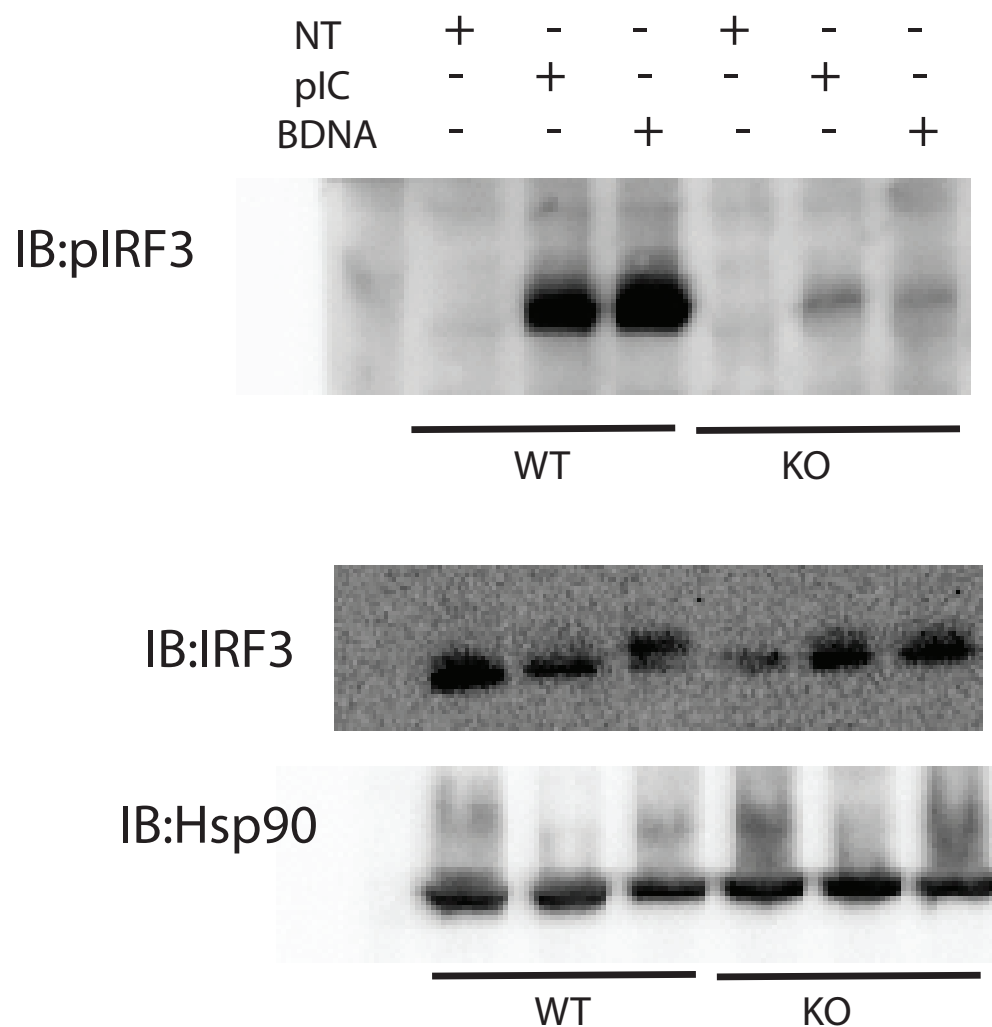


Figure 4.39. LATS1 KO cells exhibit reduced phosphorylation of IRF3 on transfection of pIC and BDNA. We transfected either pIC or BDNA into WT and LATS1 KO MEFs. Four hours after transfection, we collected WCL and used western blotting to probe for IRF3 phosphorylation.

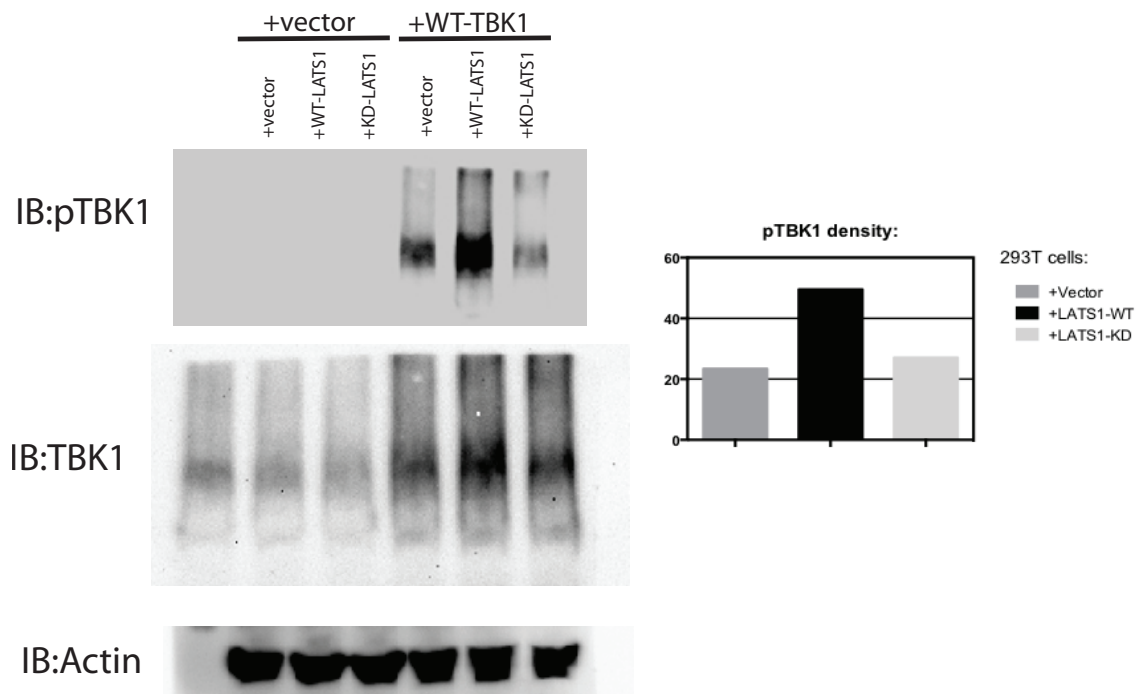


Figure 4.40. LATS1 kinase activity is required to enhance TBK1 phosphorylation.

We transfected vector, WT LATS1, or KD LATS1 into HEK 293T cells, together with either vector or a WT TBK1 construct. 24 hours after transfection, we collected WCL and used western blotting to probe for TBK1 phosphorylation. We used densitometric analysis to quantify band intensity.

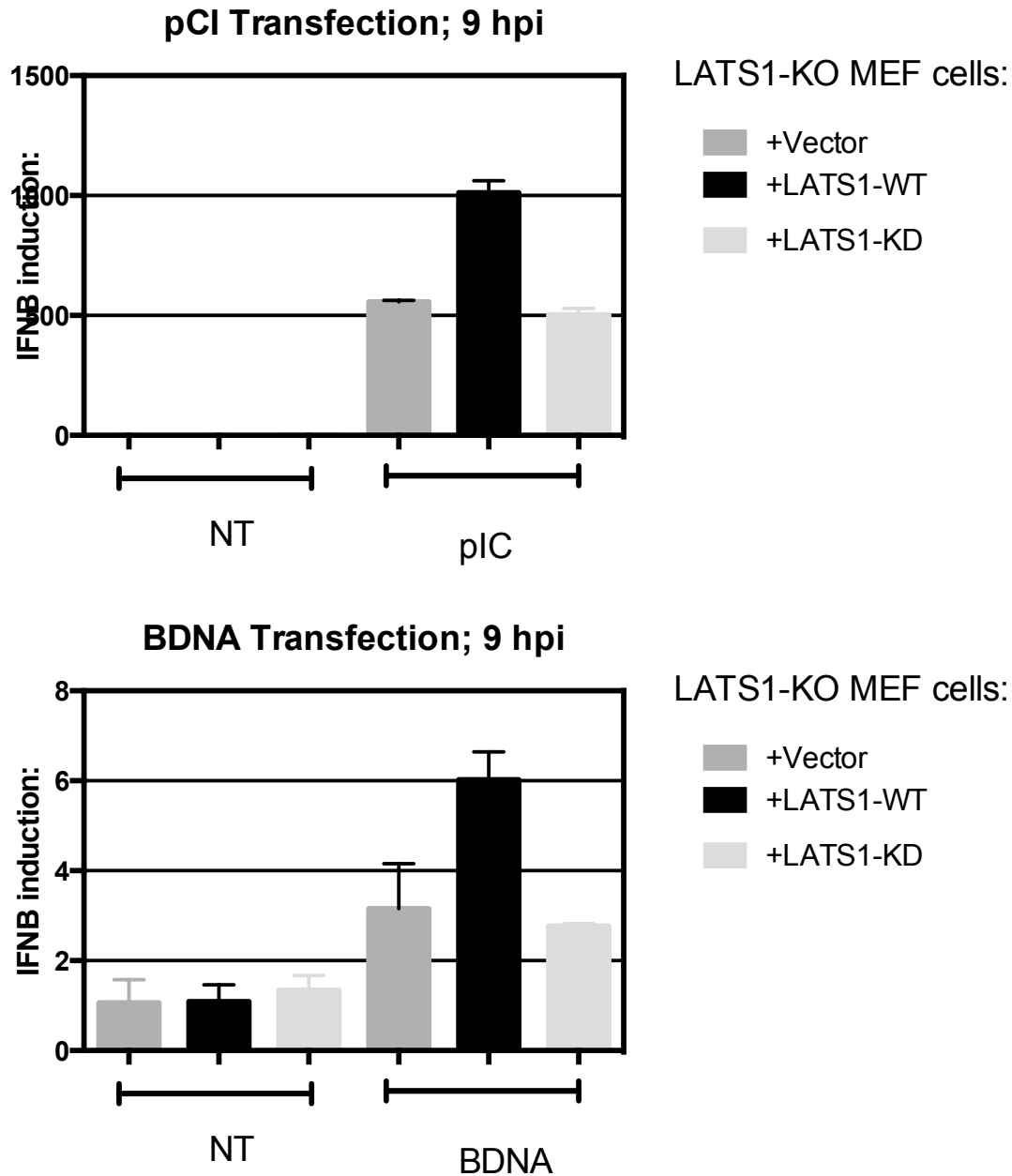


Figure 4.41. LATS1 kinase activity is required to promote IFN β induction in response to RLR and cytosolic DNA pathways. We transfected LATS1 KO MEFs with either empty vector, with WT LATS1, or with KD LATS1. 24 hours later we transfected the same cells with either pCI (top graph) or BDNA (bottom graph), and we used QPCR to monitor gene induction.

MATERIALS AND METHODS

Cell Culture:

LATS1 KO MEFS were generously provided by Dr. Xiaolong Yang. Generation of MEFS was previously described. Human embryonic kidney (HEK) 293T cells and MEFs were cultured in DMEM (Gibco) supplemented with 10% FBS and 1% Pen/Strep.

Reagents:

Fugene 6 (Roche) was used for all transfections. Antibodies were obtained from the following companies: FLAG, HA (Sigma); LATS1 (Abcam); IRF3, Hsp90 (Santa Cruz Biotechnology); TBK1, pTBK1, pIRF3, Actin (Cell Signaling). LATS1 expression vectors were generously provided by Dr. Xiaolong Yang, and used to generate pBABE-LATS1-WT vectors by previously-described technique. Poly(I:C) and poly(dA:dT)poly(dA:dT) were purchased from Invivogen and Sigma, respectively, and were transfected into MEFs using Lipfectamine 2000 (Invitrogen).

Immunoblotting:

For western blotting, cells were homogenized for 15 minutes at 4°C in a modified radioimmune precipitation (mRIPA) buffer, containing 0.5% (vol/vol) NP-40, 0.1% (wt/vol) Na-Deoxycholate, and no SDS. Protease inhibitor cocktail (Sigma) was included in all lysates. Equal amounts of whole-cell lysates were resolved by SDS-PAGE and analyzed by Western blotting with the indicated antibodies. Quantitative analysis of band intensity was performed by Quantity one software.

Generation of Stable Cell Lines:

HEK 293T cells were transfected with a Moloney murine leukemia virus- Ψ_A helper construct plus either pBABE-puro alone or the indicated pBABE-puro construct using FuGENE 6. These HEK 293T cell supernatants were collected 48 hours post-transfection, filtered, supplemented with Polybrene (10 ug/ml), and used to infect LATS1 KO MEF cells (plated overnight for 60% confluency). After 16 hours, media was replaced with fresh, complete medium without Polybrene. 24 hours later, cells were selected with Puromycin (5 mg/ml). Cells were expanded, and assayed for LATS1 expression.

Immunoprecipitation

For immunoprecipitation assays, cells were lysed as described above. Cell lysates were incubated with primary antibody for 2 hours, before adding Protein A/G agarose. Lysates were incubated overnight at 4° on a shaker, before washing the agarose and eluting protein. The immunoprecipitated complexes were then separated by SDS-PAGE and blotted with the indicated antibodies.

Virus Infections and Assays

VSV-GFP was a gift from Dr. Glen Barber. HSV-1 was a gift from Dr. William Halford. Sendai virus (Cantell strain, VR-907) was purchased from ATCC. Virally infected GFP-positive cells were fixed with 2% paraformaldehyde and examined by flow cytometry. Data was analyzed by FlowJo.

Quantitative PCR

Cells were collected in trizol and RNA was isolated by standard isopropanol precipitation. RNA was quantitated and 1 ug of RNA was reverse transcribed to cDNA using IScript (BioRad) according to the manufacturer's instructions. QPCR analysis was done using the iCycler thermocycler (BioRad). Primer sequences for IFN β , IP10, MX1, IL6, RANTES, L32, and h36B4 were previously published. Amplification conditions were: 95°C (3 min), 40 cycles of 95°C (20 s), 55°C (30 s), 72°C (20 s). Expression values were normalized to either L32 control (MEF cells) or h36B4 control (HEK 293T cells), and fold-induction was normalized to untreated control.

Luciferase Assays:

Cells were transfected with the specified constructs, along with a Firefly luciferase pGL2-basic reporter plasmid and *Renilla* luciferase for normalization. Cells were lysed and luciferase values were quantified on a luminometer using a dual luciferase assay system (Promega).

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CHAPTER FIVE

Conclusions and Future Perspectives

This series of studies sought to elucidate in closer molecular detail the mechanisms of type I IFN induction via the RLR and DNA sensor pathways. Beginning in chapter two, we determined the crystal structures of the TRAF domains of TRAF5 and of TRAF3 bound to a peptide from the TIM of Cardif. By comparing the structure of the TRAF3-Cardif complex to that of TRAF5, we identified two residues near the Cardif binding pocket in TRAF3 that allow TRAF3 to bind to Cardif. We showed that mutation of these two residues in TRAF5 to the corresponding TRAF3 residues conferred TRAF3-like antiviral activity on the mutated TRAF5 proteins. Our results determined a structure-based mechanism for the selectivity of TRAF3 in promoting the RIGI-mediated IFN response. Our results illustrate that a single amino acid substitution can switch TRAF5 towards a TRAF3-like specificity. It is our hope that further understanding of the structural specificities of TRAF3 for its various ligands, including Cardif, could allow for the development of small-molecule therapeutics that are targeted to the ligand-binding pockets of TRAF3 and designed specifically to inhibit the interaction of TRAF3 with only a subset of ligands.

Continuing to chapter three, we presented data suggesting a role for Optineurin as a positive regulator of non-canonical NF κ B. We showed that overexpression of Optineurin enhances, and conversely knockdown of Optineurin inhibits, activation of non-canonical NF κ B. Based on interaction studies, we proposed a model such that on stimulation of the non-canonical NF κ B pathway, Optineurin is recruited to the TRAF-cIAP-NIK complex, wherein it acts to promote TRAF3 degradation and support NIK accumulation. In light of our lab's data showing that TRAF3 functions to inhibit the cytosolic IFN β

response to DNA via an inhibition of the NIK-TBK1 axis, we hypothesize that Optineurin should function to promote IFN β induction in the cytosolic DNA pathway. We propose that Optineurin could potentially play opposite roles in promoting IFN β induction in response to cytosolic DNA and inhibiting IFN β in response to cytosolic RNA. However, we also present data from another group that shows no difference between wt and complete Optineurin KO cells in p100/52 degradation/accumulation on stimulation with α LT β R. Additional work needs to be done to clarify Optineurin's roles, if any, regulating the non-canonical NF κ B and IFN β responses.

Advancing to chapter four, we screened 575 kinases towards the goal of identifying novel protein kinases involved in regulation of the type I IFN antiviral response. We finally focused our studies on the kinase LATS1, which we propose functions to promote IFN β activation in response to cytosolic RNA and DNA pathways. We show that LATS1 overexpression promotes, and LATS1 knockdown inhibits, IFN β activation in response to both pIC and BDNA-transfection. We show that LATS1 KO MEF cells exhibit defective IFN β induction in response to both cytosolic RNA and DNA, and we show that we can rescue this IFN β phenotype by restoring LATS1 expression. Mechanistically, we show that LATS1 associates with the TBK1/IRF3 complex after activation of either cytosolic RNA or DNA pathways, and that LATS1 acts to promote phosphorylation of TBK1 and IRF3, thereby so enhancing downstream IFN β induction. Further work is needed to elucidate in closer molecular detail the mechanisms of LATS1-mediated IFN β induction via the RLR and DNA sensor pathways. Additional work is also needed to examine if there is interplay between LATS1 role in promoting IFN β induction and its

role in the Hippo signaling pathway. It remains to be determined if viral infection, through any effects on extracellular environment and intracellular homeostasis, is able to alter Hippo signaling, and if LATS1 serves as a bridge between viral infection and Hippo signaling.

Cells are equipped with a comprehensive set of sensors that permits rapid detection of viral infection and initiation of an antiviral immune response. Currently, three primary groups of PRRs have been identified as responsible for detection of viral nucleic acids: these include endosomal TLRs, RLRs, and the cytosolic DNA sensors. Several of the major proteins that are key players in these pathways have been identified; however, our current understanding of viral detection is by no means comprehensive. We still have much to discover about IFN PRR signaling pathways, and the fine-tuning of this regulation is a major area of research. We suggest that microtubule-based trafficking of signaling components and regulation of polyubiquitin-mediated signaling are two areas that are particularly interesting for future studies. Hopefully, a more intricate knowledge of the subtleties of PRR-signaling will aid development of better-targeted therapeutics.