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Master Thesis

STAT3 抑制第一型干擾素反應機轉的研究

The suppressive effect of STAT3 on type I interferon-mediated response

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本論文係鄭子珮君（學號 R99449002）在國立臺灣大學醫學院免疫學研究所完成之碩士學位論文，於民國 101 年 07 月 25 日承下列考試委員審查通過及口試及格，特此證明

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Abbreviations

APRF: Acute phase response factor

CBP: CREB-binding protein

CCD: Coiled-coil domain

ChIP: Chromatin Immunoprecipitation

CSF-1: Colony-stimulating factor-1

DBD: DNA binding domain

DNMT: DNA methyltransferase

dsRNA: Double-stranded RNA

EMCV: Encephalomyocarditis virus

GAS: Gamma-IFN-activated sequence

G-CSF: Granulocyte colony-stimulating factor

HDAC: histone deacetylase

IFIT: Interferon-induced protein with tetratricopeptide repeat

IFN: Interferon

iNOS: Inducible nitric oxide synthetase

IRF: Interferon regulatory factor

ISG: Interferon-stimulated gene

ISGF3: Interferon stimulated growth factor 3

ISRE: Interferon stimulated response element

JAK: Janus kinase

KO: Knockout

MDA5: Melanoma differentiation-associated gene 5

MEF: Mouse embryonic fibroblast

MOI: Multiplicity of infection

NTD: N-terminal domain

OAS: Oligoadenylate synthetase

PCR: Polymerase chain reaction

PIAS: Protein inhibitor of activated STAT

PKR: RNA-dependent protein kinase

PTP: Protein tyrosine phosphotase

RIG-I: Retinoic-acid-inducible gene I

RNaseL: Ribonuclease L

SOCS: Suppressors of cytokine signaling

SH2: Src-homology 2

STAT: Signal transducer and activator of transcription

TAD: Transactivation domain

中文摘要

第一型干擾素 (type I IFN) 對於抗病毒扮演非常重要的角色，它可以活化訊號傳導與轉錄子 (STAT) 1, STAT2 和 STAT3。第一型干擾素會藉由 STAT1 及 STAT2 來正向調控抗病毒蛋白產生。而 STAT3 在第一型干擾素訊號傳遞中所扮演的角色還不是很清楚，直到最近發現缺乏 STAT3 的細胞在第一型干擾素刺激下會增加促進干擾素誘導基因 (ISG) 的表現以及增強抗病毒的能力，這顯示 STAT3 會負調控第一型干擾素所引起的抗病毒能力。然而其詳細的機制還需進一步研究。我們利用放回 STAT1, STAT3, 以及 STAT1 和 STAT3 同時放回的 STAT1/STAT3 雙基因剔除鼠胚胎纖維母細胞株 (MEFs) 來研究 STAT3 負調控的機制。我們發現放回的 STAT1 及 STAT3 蛋白的表現量與正常小鼠胚胎纖維母細胞株相似，此外利用反轉錄及時聚合酶鏈式反應 (RT-QPCR) 和基因表現微陣列分析 (microarray) 發現同時放回 STAT1 和 STAT3 與只放回 STAT1 的小鼠胚胎纖維母細胞株相比，STAT3 確實可以抑制第一型干擾素誘導 STAT1 下游基因的產生及抗病毒反應。我們更進一步利用染色質免疫沉澱法 (ChIP assay) 證明在給予第一型干擾素下，STAT3 會阻礙干擾素促進因子 3 複合物 (IFN-stimulated gene factor 3 complex, ISGF3) 被引導到位於 MDA5 及 IFIT1 的干擾素反應片段 (ISRE) 上。由於 STAT3 的 N 端 (胺基酸 1 到 134) 足以造成抑制作用，因此我們進一步釐清 STAT3 中兩個可能被乙醯化位於 49 和 87 的離胺酸 (lysine) 是否參與抑制作用。將 STAT3 上這兩個位置單獨或同時由離胺酸突變為精胺酸 (Arginine) 會阻礙 STAT3 的促進轉錄的能力。然而只將位於 49 的胺基酸由離胺酸換成精胺酸或兩個都換成精胺酸則會抑制 STAT3 負調控第一型干擾素的能力。有趣的是突變為精胺酸的 STAT3 無法被第一型干擾素誘導乙醯化。綜合以上結果顯示 STAT3 會藉由阻斷促進干擾素因子 3 複合物被

引導到促進干擾素因子的啟動子 (promoter) 而影響第一型干擾素的反應。而其詳細之機制則可能與 STAT3 N- 端第 49 及 87 兩個精胺酸的乙醯化有關係。

Abstract

Type I IFNs, activating STAT1, STAT2, and STAT3, are crucial for antiviral response. Stimulation of type I IFN induces the production of antiviral proteins in a STAT1- and STAT2-dependent manner. However, the function of STAT3 in type I IFN response was not completely understood. Recently, STAT3 was reported to negatively regulate type I IFN-mediated antiviral response. Cells lacking STAT3 displayed enhanced ISGs expression and antiviral activity upon IFN stimulation. However, the detailed mechanism remains elusive. To investigate the underlying mechanism, we used STAT1 and STAT3 double knockout (DKO) MEFs that had been restored with STAT1, STAT3, or both molecules. STAT1 or STAT3 was expressed in the DKO MEFs at a level comparable to WT MEFs. Using expression microarray and RT-QPCR, we also found that in STAT1/STAT3-restored MEFs, STAT3 could negatively regulate STAT1-dependent type I IFN-mediated gene induction and antiviral response as compared with STAT1-restored MEFs. Furthermore, using ChIP assay we showed that STAT3 impeded the recruitment of ISGF3 complex to ISRE of MDA5 and IFIT1, two IFN-stimulated genes (ISGs), following type I IFN stimulation. Since NH_2-terminal domain (amino acids 1 to 134) of STAT3 was able to confer suppressive effect, we further dissect the involvement of two potential acetylation sites 49K and 87K in

suppressive activating ofSTAT3. Single or double K to R mutations of these two sites in STAT3 blocked its transactivation ability. However, only K49R or RR mutants inhibited the suppressive effect of STAT3 on type I IFN response. Interestingly, IFNα-dependent acetylation of STAT3 was abolished in RR mutant STAT3. Together, these results indicate that STAT3 may affect type I IFN response by blocking the recruitment of ISGF3 complex to the promoter of ISGs. Acetylation of K49 and K87 in NTD of STAT3 may be involved in the negative regulator of the IFN response.

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Chapter I Introduction

1.1 Interferons and their receptors

Type I interferons (IFNs) were discovered 50 years ago by Isaacs and Lindenmann (Isaacs and Lindenmann, 1957; Isaacs et al., 1957), and has been well recognized as cytokines for inducing cellular resistance to virus infection. Moreover, IFNs also regulate cell growth (Grander et al., 1997) and possess immunomoduatory activities (Biron, 2001). IFNs were classified into three families: type I, type II, and type III IFN. Type I IFN comprises many subtypes of IFN-α and one IFN-β. Furthermore, human IFN-ε, IFN-κ, IFN-ω, and limitins (IFN-δ in pigs and cattle, IFN-τ in ruminants, IFN-ζ in mice) also belong to type I IFNs (Pestka et al., 2004; Vilcek, 2003). All type I IFNs have structural homology and bind to a common receptor, namely type I IFN receptor (consists of two chains IFNAR1 and IFNAR2) (Uze et al., 2007). In contrast, type II IFN has only IFN-γ in this family, and bind to type II IFN receptor (consists of two chains IFNGR1 and IFNGR2) (Bach et al., 1997). Type III IFNs contain IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B), which are recognized by IFN-λ receptor (IFNLR1) and the IL-10Rβ subunit (IL-10Rβ) (Takaoka and Yanai, 2006)

1.2 Type I IFN signaling pathway

Type I IFNs, IFN-α/β, are secreted in most cell types upon viral or other microbial infection, and play an essential role in innate and adaptive immune response. Binding of type I IFNs to IFNAR1 and IFNAR2, expressed in most cell types, initiates several signaling cascades. The intracellular domain of IFNAR1 is constitutively associated with TYK2, one of the Janus Kinases, whereas IFNAR2 with JAK1. Phosphorylation of JAK1 and TYK2 is triggered by the interaction of type I IFN and receptor. Activated JAKs further phosphorylate tyrosine residues in the receptor for recruiting src-homology 2 (SH2) domain-containing proteins including signal transducer and activator of transcription (STAT) 1, STAT2, and STAT3 in most cell types (de Weerd et al., 2007). Other STATs, such as STAT4, STAT5, and STAT6, seem to be strictly activated in limited cell types like endothelial or lymphoid cells. Furthermore, JAKs also phosphorylate STATs on the tyrosine residue. Homodimers (STAT1/1 and STAT3/3) or heterodimers (STAT1/2, STAT1/3, and STAT2/3) of activated STATs translocate into nucleus, bind interferon-γ activated site (GAS) elements ,and drive gene expression. STAT1 and STAT2 interact with IRF9 (p48) to form interferon stimulated gene factor 3 (ISGF3) and target to the promoter containing IFN-stimulated response elements (ISREs).

1.3 Effects of type I IFNs

Type I IFNs regulate several biological responses, such as induction of major histocompatibility complex (MHC) class I expression, activation of natural killer (NK) cell cytotoxicity, maturation of dendritic cells (DCs), and cancer immunoediting. Notably, the most well known effect of type I IFNs is to establish an antiviral state against virus infection by inducing ISG expression (Stark, 2007).

There are more than three hundred ISGs being induced following type I IFN stimulation (Bowie and Unterholzner, 2008; Sadler and Williams, 2008). Some well-characterized ISGs, including protein kinase R (PKR), 2',5'-oligoadenylate-synthetase (2',5'-OAS), RNase L, Viperin (cig5) (Chin and Cresswell, 2001), inducible nitric oxide synthetase (iNOS), and Mx, directly affect virus by suppressing transcription and translocation, interfering RNA stability, blocking protein assembly, or inducing cell apoptosis (Liu et al., 2011).

1.4 STATs

STATs were first discovered in the early 1990s. Mammalian STAT proteins contain seven members, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (Levy and Darnell, 2002). All of STATs have seven conserved domains, such as N-terminal domain (NTD), coiled-coil domain (CCD), DNA binding domain

(DBD), linker domain, SH2 domain, and transactivation domain (TAD). The tyrosine residue that undergoes phosphorylation upon activation, is especially highly conserved in all STATs. Most activated STAT dimers bind to the GAS element (TTCNNNGAA)(Hutchins et al., 2012), a palindromic sequence, whereas STAT1/2 and IRF9 heterotrimers bind to the ISRE ($TTTCN_2TTTC$), a direct tandem repeat (Robertson et al., 2007; Schindler et al., 2007).

1.4.1 STAT1

STAT1 was the first identified in STAT family, which is important in both IFN-α and IFN-γ signaling pathway. STAT1-defecient mice are susceptible to microbial and viral infections and tumor formation because of impaired type I and II IFN-mediated responses (Durbin et al., 1996; Meraz et al., 1996). However, STAT1 also regulates inflammation as well as antagonizes cell proliferation, indicating that STAT1 affects diverse biological responses (Mui, 1999).

There are two isoforms of STAT1, including STAT1α and STAT1β, which are resulting from alternative mRNA splicing. STAT1β lacks 38 amino acids at TAD of STAT1α, but is still efficiently phosphorylate a at Y701 residue, forms dimers with STAT1α isoform, and binds DNA. Nevertheless, overexpression of STAT1β does not activate transcription, suggesting that STAT1β plays a dominant negative role (Lim

and Cao, 2006; Shuai et al., 1993).

1.4.1.1 Post-translational modification of STAT1

Post-translational modification of STAT1 includes the phosphorylation, acetylation, ISGylation (Malakhova et al., 2003), SUMOylation (Rogers et al., 2003), and ubiquitination (Kim and Maniatis, 1996). All the modifications can modulate transcriptional or non-transcriptional activity of STAT1. However, there is still a debate on whether activation of STAT1 is regulated by methylation (Komyod et al., 2005; Meissner et al., 2004).

Recently, it has been reported that activation of STAT1 needs not only phosphorylation but also deacetylation. STAT1 was found to interact with CREB-binding protein (CBP) or p300 (Horvai et al., 1997), and downregulate STAT1-mediated gene expression. Mutation of STAT1 at Lys410 and Lys413, two acetylation sites of STAT1, attenuates the expression of STAT1-downstream gene (Kramer et al., 2009). Besides, cells stimulated with HDAC inhibitor, TSA, suppresses ISG expression, suggesting that deacetylation of STAT1 at Lys410 and Lys413 is required for STAT1-dependent gene expression. The expression of ISGs was suppressed by TSA, a HDAC inhibitor, and histone acetylation of ISRE promoters decrease after IFN treatment (Chang et al., 2004; Nusinzon and Horvath,

2003). In these data, it suggested that promoter of ISGs are unlike other promoters, histone deacetylation is required for its expression. However, these results are controversial and are not consistent with others (Antunes et al., 2011). For example, the interaction of ISGF3 complex with p300 can increase the DNA binding ability, enhance downstream gene expression (Zhang et al., 2005b), and induce histone acetylation (Varinou et al., 2003). Therefore, it is still unclear whether or not acetylation of histone is required for IFN-α-mediated gene expression.

1.4.2 STAT3

STAT3 initially identified as acute phase response factor (APRF) due to its ability to induce acute phase genes in the liver in response to IL-6 (Akira et al., 1994). Biochemical and genetic studies demonstrate that STAT3 plays a crucial role in transducing signal for IL-6 family, IL-10 family, granulocyte colony-stimulating factor (G-CSF), Leptin, IL-21, IL-27, growth factor, oncogenes, and potentially IFNs (Schindler and Plumlee, 2008). Unlike other STATs, ablation of STAT3 leads to embryonic lethality at E6.5-7.5 (Takeda et al., 1997), suggesting that STAT3 is important in development of various organs and cell proliferation.

STAT3, like STAT1, also has two isform, including STAT3α and STAT3β. STAT3β missing the 55 C-terminal amino acids of STAT3α, and has 7 additional

amino acids residues at C terminus (Schaefer et al., 1995).

1.4.2.1 Post-translational modification of STAT3

Like STAT1, the activity of STAT3 is regulated by phosphorylation, acetylation, and SUMOylation (Ohbayashi et al., 2008). Engagement of ligand and receptor activates STAT3 by tyrosine phosphorylation at Y705. In addition, Serine phosphorylation is required for full transactivation ability of STAT3 (Wen et al., 1995). However, unphosphorylated STAT3 can still form dimers and induce transcription (Yang and Stark, 2008). Acetylation of STAT3, unlike STAT1, plays a positive role in gene transcription. Dimerization of STAT3 was inhibited by mutation of STAT3 at K685, an acetylation site by p300, to arginine (Wang et al., 2005; Yuan et al., 2005). STAT3 NTD (amino acids 1 to 130) alone can interact with p300, histone deacetylase (HDAC) 1, and HDAC3 (Hou et al., 2008; Ray et al., 2008). Moreover, in addition to K685, K49 and K87 at NTD of STAT3 can be acetylated by p300 in response to IL-6, and the acetylation affects STAT3 downstream gene expression (Ray et al., 2005). K49R and K87R, two mutations in STAT3, decrease the interaction of STAT3 with p300 and HDAC1 (Hou et al., 2008; Ray et al., 2008). Other than acetylation, STAT3 can be methylated by SET9, when it binds to the promoter (Yang et al., 2010). Moreover, STAT3 can form complex with DNA methyltransferase (DNMT) 1 and

HDAC 1 to silence SHP-1, an tumor suppressor gene in malignant T cells (Zhang et al., 2005a). The acetylation of STAT3 at K685 is important for interaction with DNMT1 to inhibit transcription of tumor suppressors (Lee et al., 2012). These results indicated that post-translational modification of STAT3 may affect the gene expression through the recruitment of histone modifying-enzymes.

1.5 Negative regulators of STAT signaling pathway

The signaling of STATs are not only regulated by post-translational modifications, but also tightly controlled by several negative regulators, such as protein tyrosine phosphotase (PTP), suppressors of cytokine signaling (SOCSs), and protein inhibitor of activated STAT (PIASs) families. SOCS family is induced by activated STATs, resulting in termination of STAT signals. IL-10 can inhibit the activity of pro- inflammatory cytokines like IFN through induced SOCS3. PIAS can interact with STATs, and inhibit STAT-mediated gene induction by a distinct mechanism. For instance, DNA-binding activity of STAT1 and STAT3 is inhibited by PIAS1 and PIAS3 (Chung et al., 1997; Liao et al., 2000). PTPs can also inactivated STATs in either nucleus or cytoplasma by removing phosphate group from activated STATs. It has been identified that TC45 and SHP2 (PTP) can inactivate STAT1 through dephosphorylation in nucleus (Shuai and Liu, 2003). However, a STAT

protein can also suppress other members of the STAT family. For example, activation of STAT1 is prolonged in STAT3-deficient MEFs by IL-6 (Costa-Pereira et al., 2002). STAT3 negatively regulates STAT1-dependent gene expression in IFN treatment (Ho and Ivashkiv, 2006; Wang et al., 2011). Nevertheless, the detailed mechanism remains unclear.

1.6 Rationales

Type I IFN-stimulation leads to activation of STAT1, STAT2, and STAT3, which induces expression of different downstream genes. Besides, STAT3 not only regulates gene expression, but also negatively regulates type I IFN-mediated response and tumor suppressor genes (Ho and Ivashkiv, 2006; Lee et al., 2012; Wang et al., 2011). In this study, we want to investigate the mechanism how STAT3 suppresses type I IFN-mediated response. We have previously shown that STAT3 directly reduce type I IFN-mediated gene expression. Hence, we want to address whether STAT3 may affect phosphorylation, nuclear translocation, or DNA-binding ability of STAT1. Interstingly, NTD of STAT3 is sufficient to suppress IFN-α-mediated gene induction. Acetylation of STAT3 at NTD is critical for its function and interaction with proteins. Furthermore, it has reported that STAT3 could silence gene expression through acetylation-dependent mechanism. From these results, we hypothesize that acetylation at NTD of STAT3 may influence the suppressive effect of type I IFN-mediated response.

Chapter II Materials and methods

2.1 Cells

WT mouse embryonic fibroblast (MEF) and STAT3 knockout (STAT3KO) MEF cell lines were obtained from Dr. Levy's laboratory at New York University. STAT1 and STAT3 double knockout (DKO) MEF cell lines were generated previously by using retroviral transduction of a vector encoding Cre recombinase into $STAT1^{-/-}STAT3^{flox/flox}$ cells. All cells were cultured in DMEM (Gibco) supplemented with 10 % fetal bovine serum (Hyclone), and 10 ng/ml gentamicin (Gibco).

2.2 Plasmids

pLPC-FH_2-mSTAT3 was contructed using the following primers containg BamHI and EcoRI site. Mouse STAT1 was PCR amplified using the following primers. pLPC- FH_2-mSTAT1 was subcloned by XhoI site.

(1) STAT3

Forward: 5'- CGGGATCCGCTCAGTGGAACCAG -3'

Reverse: 5'- GCGAATTCCCATGGGGGAGGTAGC-3'

(2) STAT1

Forward: 5'- CCGCTCGAGATGTCACAGTGGTTCGAG -3'

Reverse: 5'- CCGCTCGAGTACTGTGCTCATCATACTGTC -3'

2.2 Calcium phosphate precipitation transfection

Plasmid DNA was mixing with 250 mM $CaCl_2$, followed by adding 2X BBS drop-wise and then transferred into medium. After incubation for 6 hours at 37 ℃, medium was refreshed.

2.3 Retroviral transduction

The retroviral bicistronic vector pLPC-FH_2 plasmid encoding WT STAT3 or WT STAT1 and puromycin resistant gene, respectively was cotransfected with a helper plasmid (pCL-Eco) into Phoenix A, amphotropic packaging cell line, or HEK 293T cells using calcium phosphate precipitation method. After transfection for two days, the culture supernatant containing pseudo-typed virus was collected. MEFs were incubated with viral supernatant in the presence of 8 μg/ml polybrene and spun at 1100 xg for 45 minutes at room temperature. Two days later, cells were treated with puromycin to select the drug-resistant transfetants .

2.4 RT-QPCR

Total RNA was prepared from MEFs using TRIzol (Invitrogen) or TRIsure (Bioline #Bio-38032) reagent. 1-3 μg of RNA was subjected to reverse transcription (RT) with oligo dT and then cDNA prepared from the reaction was then subjected to QPCR by iCycler IQ (Bio-rad) using the following primer sets.

(1) PKR

Forward: 5'- TGCGCAGACAATGAATGGTA -3'

Reverse: 5'- ATGTGACAACGATAGAGGAT-3'

(2) IP-10

Forward: 5'- TGAGCAGAGATGTCTGAATCCG -3'

Reverse: 5'- TGTCCATCCATCGCAGCA -3'

(3) IRF-7

Forward: 5'- AGCAAGACCGTGTTTACGAC -3'

Reverse: 5'- AGTGCTGAAGTCGAAGATGG -3'

(4) IFIT1

Forward: 5'- AGAGCAGAGAGTCAAGGCAGGT -3'

Reverse: 5'- TGGTCACCATCAGCATTCTCTCCCA -3'

(5) IFIT2

Forward: 5'- ATTGCGAACTACCGTCTG -3'

Reverse: 5'- CTTCAGTGCTAAGAGGAC -3'

(6) Socs3

Forward: 5'- ATGGTCACCCACAGCAAGTTT -3'

Reverse: 5'- TCCAGTAGAATCCGCTCTCCT -3'

(7) JunB

Forward: 5'- TCACGACGACTCTTACGCAG -3'

Reverse: 5'- CCTTGAGACCCCGATAGGGA -3'

(8) β-actin

Forward: 5'-GTGGGGCGCCCCAGGCACCA -3'

Reverse: 5'-CTCCTTACCGTCACGCACGATTT -3'

2.5 Western blot

Whole cell lysates are extracted by lysis buffer at 4 ℃ for 15 minutes. Lysates were cleared of debris by centrifugation at 14,000 rpm (Eppendorf) for 15 minutes. Equal amounts of samples were resolved in 7 % or 10 % sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), followed by transfering to polyvinylidene difluoride membranes (PVDF) and immunoblotting with the indicated antibodies.

2.6 Cytosolic and nuclear extracts

Cells were washed twice with 1XPBS, and then scraped off of the dish and the cell pellets were obtained by centrifugation (300 xg, 5 minutes, 4℃). Cells was resuspended in RSB-G40 buffer and nuclei were centrifugated at 10,000 xg to obtain the cytosolic supernatant. Nuclear extraction was obtained from resuspend nuclei by using nuclear extraction buffer.

2.7 Co-immunopreciptation (CoIP)

Equal amount of whole cell lysetes were immunopreciptated using anti-HA antibodies in cell lysis buffer, which was conjugated with protein G beads (Millipore). Western blotting was performed as described in 2.5 using the indicated antibodies.

2.8 Chromatin immunoprecipitation (ChIP)

MEFs were stimulated with IFN-α4 for 30 minutes, and then fixed in 1.4 % formaldehyde for 15 minutes at room temperature. Cells were lysed with immunoprecipitation buffer prior to sonication (Nelson et al., 2006). Chromatins were sheared by sonication with a Vibra-Cell VCX 130 sonicator (Sonics & Materials). Cells were sonicated using 1 sec on/ 1 sec off pulses for 5 min at 70% power output to shear the DNA to ~200 to 500 base pairs. Protein G-Sepharose beads (Millipore) were

added to cell lysates preincubated with corresponding antibody overnight at 4°C. After extensive washes, bead-bound DNA was reverse-crosslinked by incubation overnight at 67°C. Protein were removed by incubating with 20 μg proteinase K in proteinase K buffer at 55°C for 4 hours. Recovered DNA from ChIP was analyzed by QPCR using primers specific for corresponding ISRE elements in the promoters of the indicated genes. Primer sequences are as follows:

(1) ISRE of IFIT1 promoter

Forward: 5'- GTGGAGAATGCAGTAGGGCAAAC -3'

Reverse: 5'- GTCACACCAACTGGAAGCTCAGG -3'

(2) ISRE of MDA5 promoter

Forward: 5'-ACCAAAGTCCTCACCTAAC-3'

Reverse: 5'-TATTGCCTTCCACCCAC-3'

2.9 In vitro antiviral state assay

MEF cells were pretreated with or without 2-fold serial dilution of IFN-α4 from 240 IU/ml for 24 hours. Cells were infected with EMCV at am MOI of 0.1. After infection for 18 hours, the medium was removed and cells were fixed with 10 % formaldehyde for 20 minutes at RT. After fixation, cells were visualized with crystal violet. The excessive dye was then removed by immersing the plate in water.

2.10 Single primer based site-directed mutagenesis

pLPC-FH2-mSTAT3 was subjected to site-directed mutagenesis using one primer in a single cloning step (Makarova et al., 2000). After PCR, the construct was digested by DpnI twice to remove the parental unmutated strand DNA and then transformed into *Ecoli.*. DNA sequencing was performed to confirm the mutation sites.

The primers used for mutagenesis are as follows

(1) K49Q: GGCATATGCAGCCAGCCAAGAGTCACATGCCAC

Tm= 60 ℃

(2) K87Q: CAACCTTCGAAGAATCCAGCAGTTTCTGCAGAG

Tm= 60 ℃

(3) K49R: GCATATGCAGCCAGCAGAGAGTCACATGCCACG

Tm= 60 ℃

(4) K87R: AACCTTCGAAGAATCAGGCAGTTTCTGCAGAGC

Tm= 58 ℃

(5) K685R: GAGGAGGCATTTGGAAGGTACTGTAGGCCCGAG

Tm= 56 ℃

PCR conditions are as follows

95 ℃, 5 min. 98 ℃, 20 sec. Tm ℃, 20 sec. 72 ℃ 4 min. 72 ℃, 10 min.

2.11 Statistics

A *student's T* test (two-tailed) was performed for statistical analysis.

Chapter III Results

3.1 STAT3 negatively regulates type I IFN-mediated response

Using STAT3KO MEFs, we have previously reported that STAT3 could suppress type I IFN-mediated antiviral response (Wang et al., 2011). Since WT MEFs and STAT3KO MEFs were generated from different mice, it is likely that the epigenetic modifications are different, which may have additional effects independent of STAT3. In this study a different approach was taken, STAT1 and STAT3 double knockout (DKO) MEFs were restored with STAT1, STAT3, or both molecules. In principle, the genetic makeup of different STAT-restored DKO MEFs should be similar. DKO MEFs restored with empty vector (EV), Flag- and HA-tagged STAT1 or STAT3, or both molecular (Fig. 1) with retroviral transduction were stimulated with IFN-α4 for 30 minutes (Fig. 2). STAT1 or STAT3 was expressed in WT, STAT1-restored, STAT3-restored, and STAT1/3-restored DKO MEFs, and all of their STAT1 or STAT3 were phosphorylated in response to IFN-α4. As expected, STAT1 and STAT3 can also be detected by anti-Flag and anti-HA antibodies in the restored cells. More importantly, the phosphorylation of STAT1 and STAT3 in the restored cells was comparable to that in WT MEFs. Interestingly, phosphorylation of STAT2 was increased in cells restored with STAT1. These results suggested that DKO MEFs had

been successfully restored with Flag- and HA-tagged STAT1 and/or STAT3. We next examined the functions of the restored STAT1 and STAT3 in these cells. Expression of STAT1- or STAT3-downstream genes was measured by RT-QPCR. After IFN-α4 stimulation for 1 hour and 2 hours, Socs3 and JunB, two STAT3-dependent genes, were upregulated, respectively, in STAT3- and STAT1/3-restored DKO MEFs (Fig. 3). ISGs, including PKR, IP-10, IRF7, IFIT1, and IFIT2, were induced in STAT1 and STAT1/3-restored DKO MEFs. Consistent with previous studies, the expressions of ISGs were decreased in the STAT1/3-restored DKO MEFs as compared to STAT1-restored DKO MEFs (Fig. 4). Besides, we also perform microarray to do whole gene profiling, and found that there were 137 type I IFN-induced genes, such as STAT2, IRF1, OAS2, and MX1, displaying the same phenotype (Fig. 5). The scatterplot, which showed relationship of gene expression, showed that it a good approach to do the experiment (Fig.6 and Fig. 7A). It revealed that STAT3 could exert suppressive effect in STAT1/3-induced DKO MEFs as compared with STAT1-restored DKO MEFs (Fig. 7B). Furthermore, we used antiviral state assay to confirm negative effect of STAT3 on type I IFN-mediated antiviral response. As shown in Fig. 8 EV and STAT3-restored DKO MEFs were susceptible to EMCV infection, which was due to the absence of STAT1. STAT1- and STAT1/3-restored DKO MEFs were resistant to EMCV infection. Nevertheless, single restoration of STAT1 showed more resistant to

EMCV infection than did STAT1/3 double restoration in DKO MEFs. These results indicate that the phenotype of STAT1- and STAT3-restored DKO MEFs indeed mimicked the phenotypes seen in STAT3KO and WT MEFs, respectively.

3.2 Suppression of type I IFN response by STAT3 is independent of phosphorylation and nuclear translocation of STAT1 or STAT2

Negative regulation of type I IFN by STAT3 may due to competition for receptor occupancy to decrease the phosphorylation of STAT1 or STAT2. To examine this possibility, STAT-restored DKO MEFs were treated with IFN-α4 for different times and activation of STAT1 and STAT2 was assessed. As shown in Fig. 9, phosphorylation of STAT1 was transient and decreased in the time-dependent manner, and the activation of STAT2 was prolonged and remained, at least, for 18 hours. However, STAT1/3-restored DKO MEFs failed to alter IFN-α-induced activation of STAT1 and STAT2 as compared to STAT1-restored DKO MEFs. These results suggested that STAT3 did not affect kinetics of phosphorylation of STAT1 or STAT2, and it also implied that STAT3 does not compete receptor occupancy with STAT1 or STAT2. We next investigate whether STAT3 influences nuclear translocation of STAT1 or STAT2. After IFN-α4 treatment, the level of nuclear STAT1 or STAT2 was comparable in STAT1- and STAT1/3-restored DKO MEFs, suggesting that STAT3 did

not alter nuclear translocation of activated STAT1 and STAT2 following stimulation (Fig. 10).

3.3 STAT3 suppresses type I IFN-mediated response through blocking the recruitment of ISGF3 complex to ISRE in the ISGs promoters

Since STAT3 did alter phosphorylation or nuclear translocation of STAT1 and STAT2, we next examined whether STAT3 influenced the binding of ISGF3 complex binding to ISRE containing promoter using ChIP assay. After IFN-α4 stimulation, STAT1-containing ISGF3 complex was recruited to the ISRE of IFIT1 and MDA5 in STAT1-restored DKO MEFs. However, the binding of STAT1 on ISRE site of IFIT1 and MDA5 was reduced in STAT1/3-restored DKO MEF as compared to STAT1-restored DKO MEFs (Fig. 11), suggesting that STAT3 attenuated ISGF3 complex binding and/or recruitment to the promoter of ISRE of ISGs.

3.4 STAT3 negatively regulates type I IFN-induced gene expression through acetylation-dependent mechanism by HDAC inhibitor

It has been reported that the interaction of STAT1 with p300 can increase the binding ISGF3 complex to ISRE (Zhang et al., 2005b). However, whether STAT1 and histone of ISRE promoter require acetylation is still controversial. We next investigated if acetylation was involved in the suppressive effect of STAT3 by using a

HDAC inhibitor, SAHA. The results showed that ISRE gene expression decreased in the high dose of SAHA treatment, but in 0.1 μM SAHA stimulation, type I IFN-mediated gene induction decreased only in STAT1- but not STAT1/3-restored DKO MEFs (Fig. 12). These results suggested that STAT3 may regulate type I IFN signaling response through acetylation mechanism.

3.5 Lysine 49 and lysine 87 of STAT3 are important for inhibition of type I IFN-mediated gene induction

We have previously shown that NTD alone is sufficient to suppress STAT1-dependent gene expression (Wang et al., 2011). NTD of STAT3 has two lysine residues at positions 49 and 87. Acetylation of Lys 49 and 87 is shown to be critical for STAT3 activation (Ray et al., 2005). K to R mutations in these two positions decreases the interaction of STAT3 with p300 and HDAC1 (Hou et al., 2008; Ray et al., 2008). Therefore, we next examined whether acetylation at NTD of STAT3 is crucial for the suppressive effect of STAT3. Site-directed mutagenesis was performed to generate STAT3 acetylation-deficient K49R or/and K87R ($STAT3^{49R}$, $STAT3^{87R}$, and $STAT3^{RR}$) mutants, and STAT3 acetylation mimics K49Q or/and K87Q ($STAT3^{49Q}$, $STAT3^{87Q}$, and $STAT3^{QQ}$) mutants. EV, WT STAT3, or mutant STAT3s was transfected into STAT3KO MEFs, and the protein expression level was shown to

be comparable (Fig. 13). We further detected acetylation of mutant STAT3, and found RR mutant STAT3 can not be acetylated (Fig. 14). WT STAT3, $STAT3^{49Q}$, $STAT3^{87Q}$, and $STAT3^{QQ}$ could induce Scos3 and JunB expression in response to IFN-α4 stimulation, while the expression was diminished in $STAT3^{49R}$, $STAT3^{87R}$, and $STAT3^{RR}$ mutant-restored cells (Fig. 15). However, both STAT3 acetylation-deficient and STAT3 acetylation mimics mutant failed to negatively regulate type I IFN-triggered gene expression except for $STAT3^{87R}$ mutant (Fig. 16). These results suggested that suppression effect of STAT3 is dependent on posttranslational modification of Lys49 and Lys87.

3.6 Acetylation of STAT3 at Lys 685 also plays a critical role for suppression of type I IFN-induced gene production

K685 of STAT3 blocks acetylation, decreases STAT3 downstream gene induction, and inhibits the interaction with DNMT1 (Lee et al., 2012; Yuan et al., 2005). Hence, we further investigated whether K685 of STAT3 is involved in suppression of type I IFN-mediated gene expression. K685R mutant STAT3 was transfected to STAT3KO MEFs, and revealed that $STAT3^{685R}$ also failed to suppress type I IFN-mediated gene expression as compared to WT STAT3 (Fig. 17), suggesting that K49, K87, and K685 of STAT3 are important to exert suppressive function.

Chapter IV Discussion

Using gain-of-fuction approach by restoring STAT1 and/or STAT3 into DKO MEFs, we have confirmed the regulatory role of STAT3 in type I IFN response. STAT1-restored DKO MEFs induced higher level of ISGs expressions and were more resistant to EMCV infection than STAT1/3-restored DKO MEFs (Fig. 4, Fig. 5, and Fig. 8). The negative effect of STAT3 is not working through activation or nuclear translocation of STAT1 and STAT2 (Fig. 9 and Fig. 10), instead, it affects ISGF3 complex binding to ISRE in the promoter of ISGs (Fig. 11). In addition, acetylation of STAT3 K49 and K87 at NTD and K685 plays a critical role in suppressive effect. Nevertheless, the detailed mechanism remains to be determined.

4.1 Restored DKO MEFs could reduce the epigenetic difference between different cell lines

We generated STAT1- and/or STAT3-restored DKO MEFs to reduced variation of epigenetics in WT and STAT3KO MEFs. Although retroviral transduction may cause some gene overexpression or blockage, and affect cells phenotype, the microarray results suggest the basal gene expression before IFN treatment were comparable between different restored cells, confirming our hypothesis. In addition,

IFN-α-induced gene expression in STAT1- and STAT1/STAT3-restored DKO MEFs were at similar magnetitude, despite a negative effect of STAT3 (Fig. 6). Moreover, most of genes induced by IFN-α4 were known ISGs and the induction was eliminated in EV-restored DKO MEFs (Fig. 7A), suggesting that gain-of-function is better than loss-of-function approach in determing function of genes of interests.

4.3 Acetylated site of STAT3 is critical for the negative regulation

It has been shown that STAT3 could inhibit the expression of tumor suppressor genes through the interaction with DNMT1 (Zhang et al., 2005a). In malignant T lymphocytes, STAT3 was bound to the promoter of SHP-1 with DNMT1 and HDAC1, resulting in DNA methylation and gene silencing. Besides, acetylation of STAT3 at K685 was important for the interaction with DNMT1 (Lee et al., 2012). Acetylated STAT3 was also increased in melanoma tissue as compared to normal skin cells, which enhanced methylation of tumor suppressor genes through DNMT1. Taken together, these data indicated that acetylated STAT3 could interact with DNA modifying-enzymes, leading to suppression of gene expression. In this study, we found that K685 of STAT3 also blocked its suppressive effect. In addition to K685, K49 and K87 at STAT3 NTD are also involved in suppressing type I IFN-mediated responses. For the moment, it is still unclear how K49 and K87 may contribute to the

effect. We propose that acetylation of STAT3 in K49, K87, and K685 is required for interacting with HDAC1 or p300, which facilitates the binding to DNMT1, resulting in methylation of ISRE in the promoters of ISGs and blocking the recruitment of ISGF3.

4.4 STAT3 directly suppressed type I IFN-induced gene expression

Yu's group showed that STAT3 and DNMT1 could bind to promoter of STAT1, which is also an ISG, in cancer cells upon tumor conditioned medium treatment (Lee et al., 2012). Interaction of acetylated STAT3 with DNMT1 resulted in DNA methylaton, and gene silencing. Furthermore, in our microarray data, we found that IFIT2 was decreased in STAT3-restored DKO MEFs after IFN-α4 treatment (Fig. 18A). It implies that STAT3 could directly inhibit type I IFN-mediated gene expression independent of STAT1. In addition to IFIT2, endonuclease domain containing 1 (ENDOD1), which is upregulated upon type I IFN treatment in peripheral blood mononuclear cell (PBMC) (Baechler et al., 2003), was also suppressed by STAT3 in STAT3-restored DKO MEFs (Fig. 18B). These results indicated STAT3 not only affect ISGFs binding to promoter of ISRE and indirectly regulates some ISGs, but it also directly silences gene expression.

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Figures

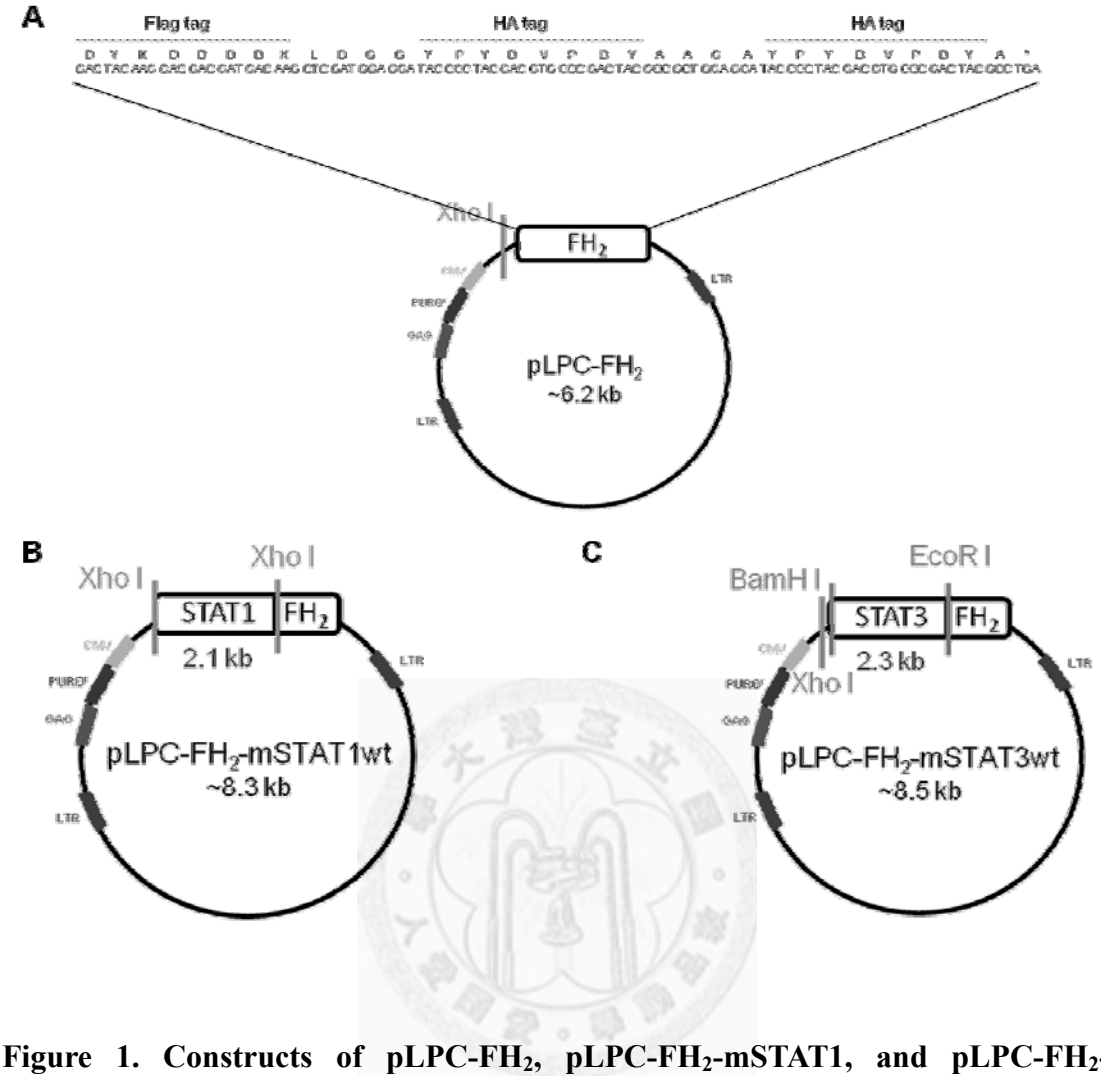


Figure 1. Constructs of pLPC-FH_2, pLPC-FH_2-mSTAT1, and pLPC-FH_2-mSTAT3 (A) The map of the pLPC-FH_2 retroviral vector comprises a Flag and two HA tags (B) mSTAT1 was subcloned into a retroviral vector using XhoI site. (C) mSTAT3 was subcloned into pLPC-FH_2 using BamHI and EcoRI sites. The detailed procedure is described in the Materials and Methods.

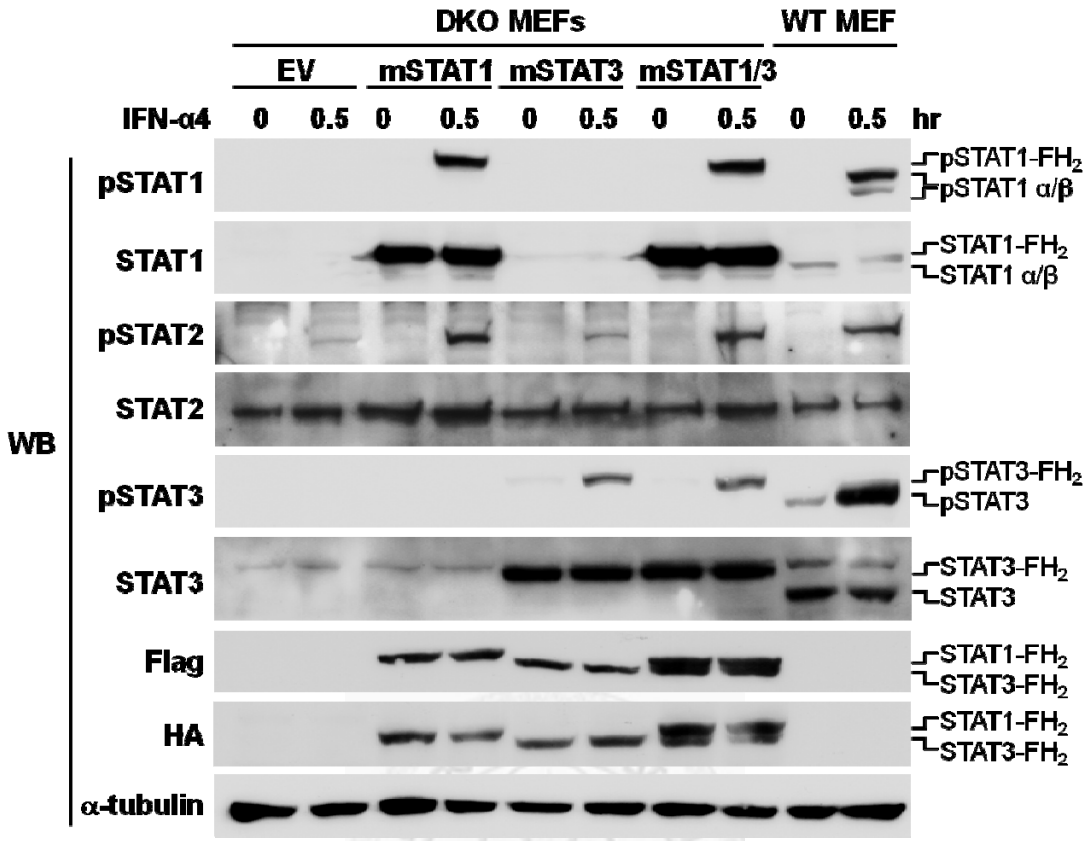


Figure 2. STAT1 and STAT3 are stably expressed and activated in restored DKO MEFs in response to IFN-α4. Empty vector (EV) or vector encoding HA- and Flag-tagged STAT1 or STAT3 stably transduced into DKO MEFs. WT and restored DKO MEFs were treated with IFN-α4 (1000 IU/ml) for 30 minutes. Whole cell lysates were then subjucted to immunoblotting using antibodies to pSTAT1, STAT1, pSTAT2, STAT2, pSTAT3, STAT3, HA, Flag, and α-tubulin.

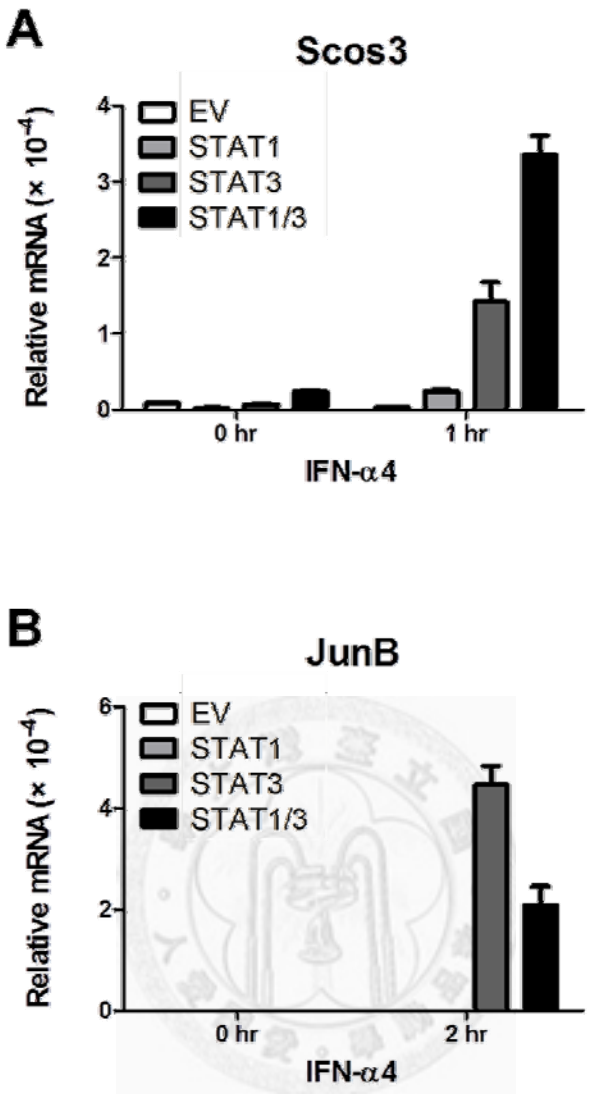
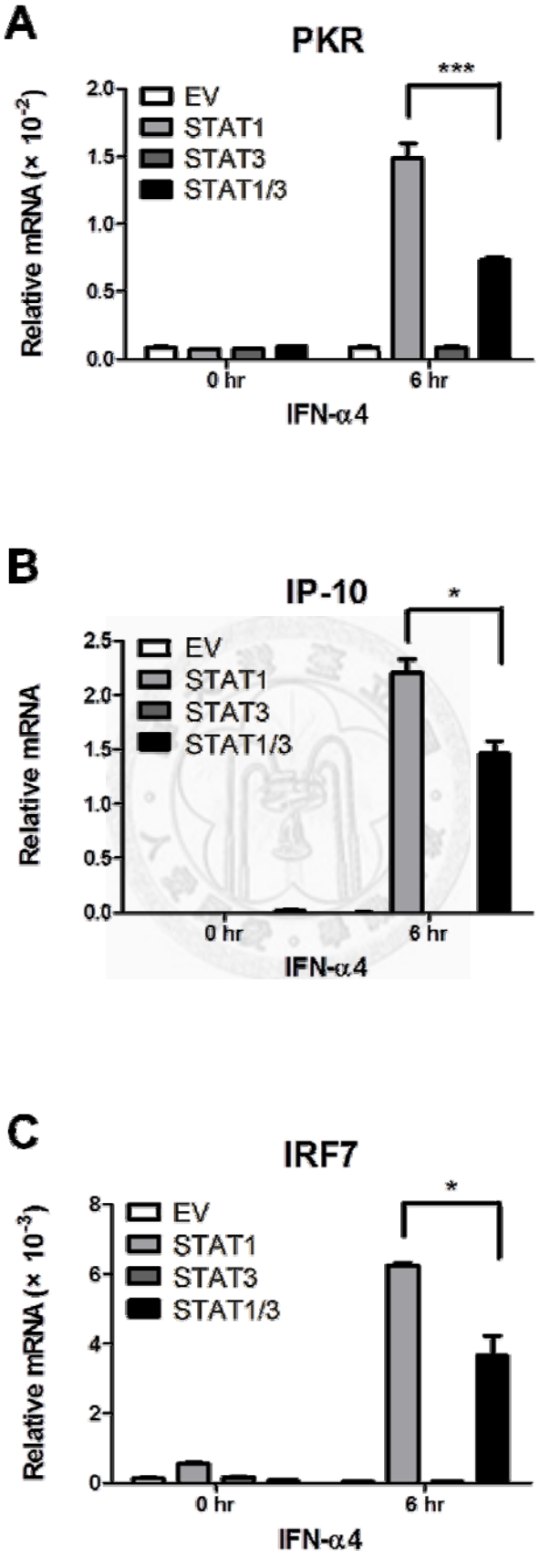


Figure 3. STAT3-dependent genes are induced in STAT3 and STAT1/3 restored DKO MEFs, but not in EV and STAT1 restored DKO MEFs. STAT1- and/or STAT3-restored DKO MEFs were treated with or without IFN-α4 (1000 IU/ml) for 1 or 2 hours, followed by preparing RNA for RT-QPCR using the indicated primers for Socs3 (A) JunB (B) or β-actin. Relative mRNA was calculated by normalizing to β-actin values.


A
PKR
***
EV
STAT1
STAT3
STAT1/3
Relative mRNA (× 10-2)
2.0
1.5
1.0
0.5
0.0
0 hr
6 hr
IFN-α4
B
IP-10
*
EV
STAT1
STAT3
STAT1/3
Relative mRNA
2.5
2.0
1.5
1.0
0.5
0.0
0 hr
6 hr
IFN-α4
C
IRF7
*
EV
STAT1
STAT3
STAT1/3
Relative mRNA (× 10-3)
8
6
4
2
0
0 hr
6 hr
IFN-α4

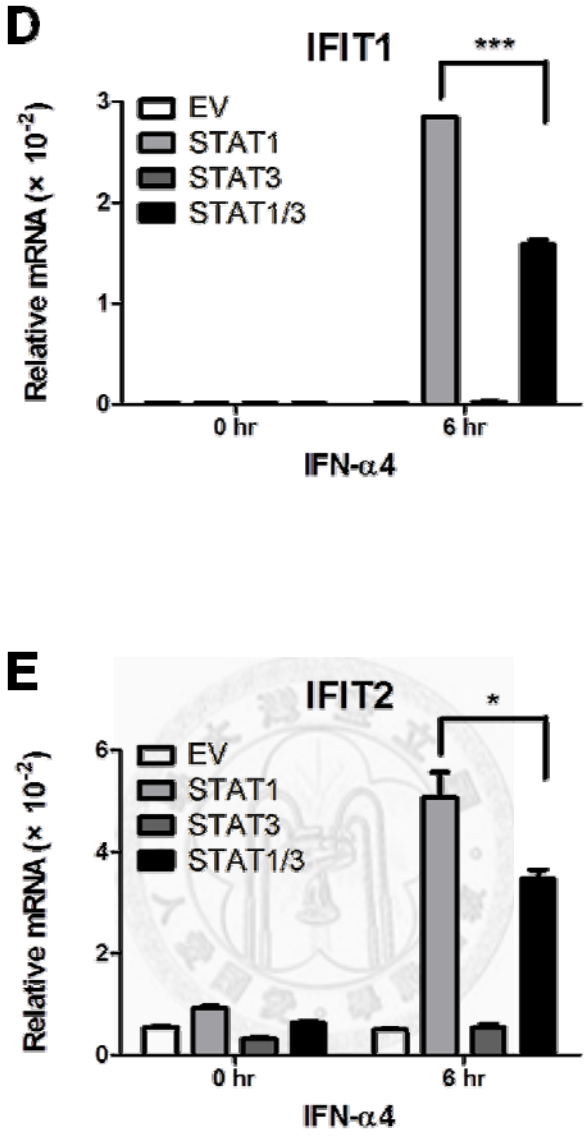
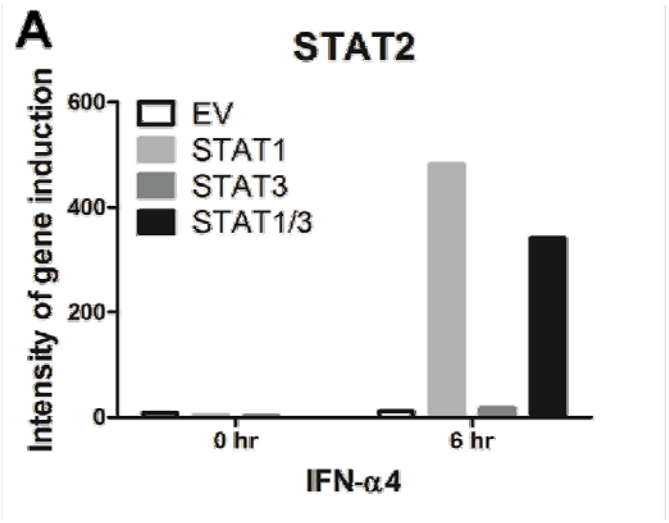
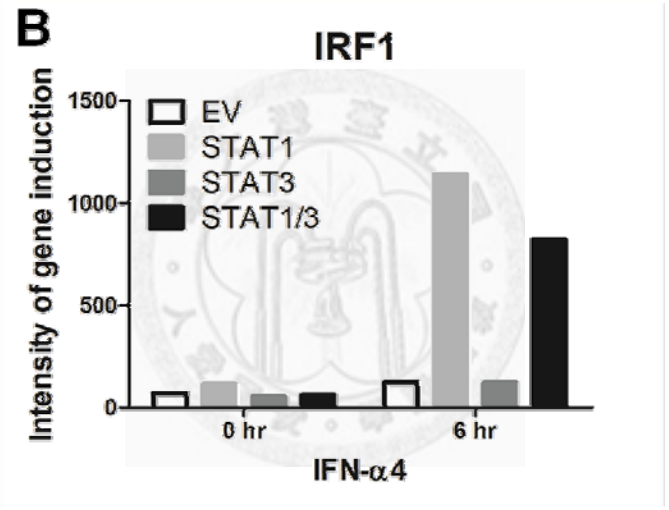
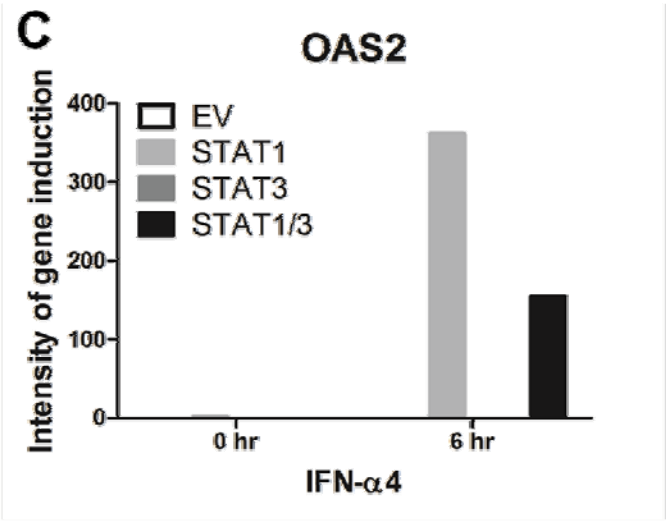


Figure 4. STAT3 negatively regulates STAT1-dependent gene expression in STAT1/3 restored DKO MEFs. STAT1- and/or STAT3-restored DKO MEFs were stimulated with or without IFN-α4 (1000 IU/ml) for 6 hours, followed by preparing RNA for RT-QPCR using primers for PKR (A), IP-10 (B), IRF7 (C), IFIT1 (D), IFIT2 (E) or β-actin. Relative mRNA was calculated by normalizing to β-actin values.


A
STAT2
Intensity of gene induction
600
400
200
0
EV
STAT1
STAT3
STAT1/3
0 hr
6 hr
IFN-α4


B
IRF1
Intensity of gene induction
1500
1000
500
0
EV
STAT1
STAT3
STAT1/3
0 hr
6 hr
IFN-α4


C
OAS2
Intensity of gene induction
400
300
200
100
0
EV
STAT1
STAT3
STAT1/3
0 hr
6 hr
IFN-α4

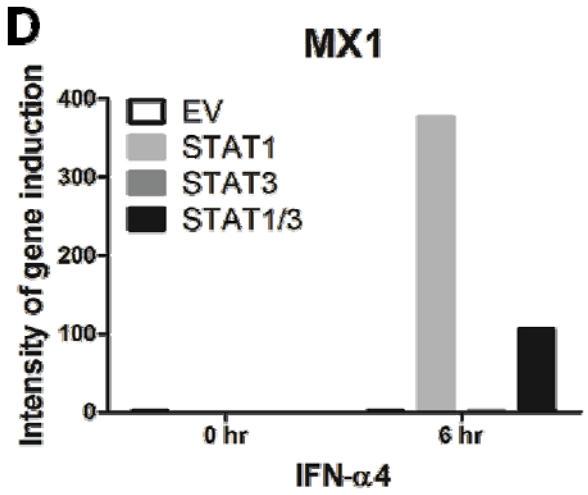


Figure 5. STAT3 suppresses type I IFN-induced gene expression. STAT1- and/or STAT3-restored DKO MEFs were stimulated with or without IFN-α4 for 6 hours, followed by preparing RNA for expression microarray analysis. After normalization, the intensity of OAS2 (A), MX1 (B), STAT2 (C), and IRF1 (D) was showed.

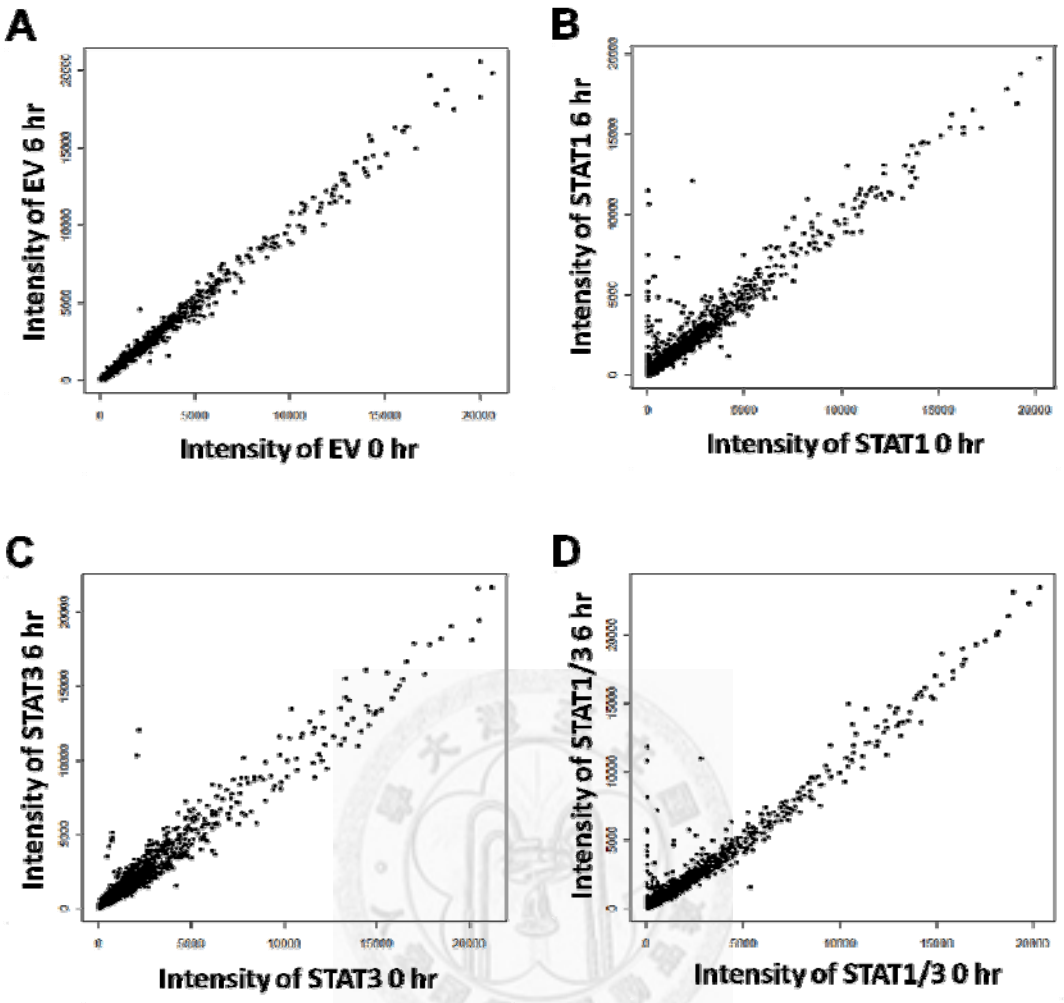
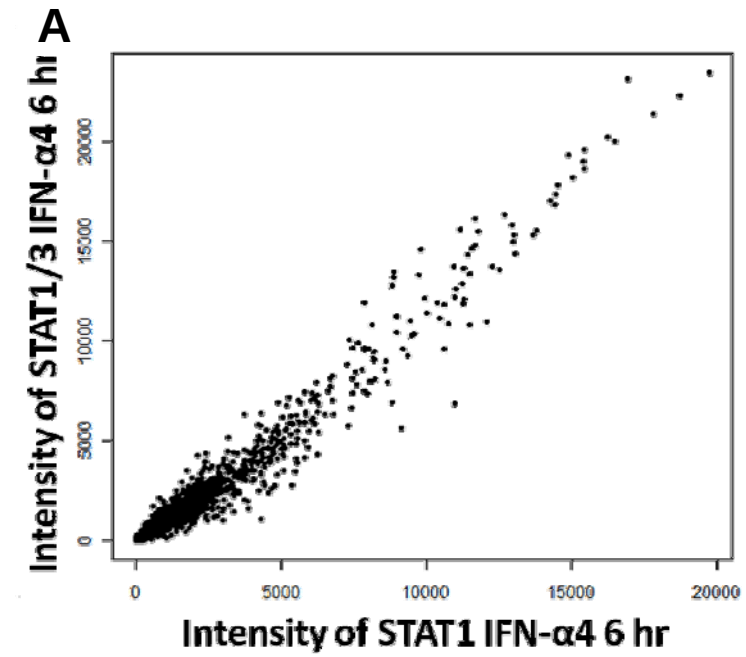


Figure 6. The relationship of gene expression compares between unstimulated and stimulated restored DKO MEFs. Empty vector (EV) (A), STAT1- (B), STAT3- (C), and STAT1/3- (D) restored DKO MEFs were treated with or without IFN-α4 for 6 hours. Total RNA of the treated cells was subjected to expression microarray analysis. The plot was generated by R package.


A
Intensity of STAT1/3 IFN-α4 6 hr
Intensity of STAT1 IFN-α4 6 hr
0
5000
10000
15000
20000

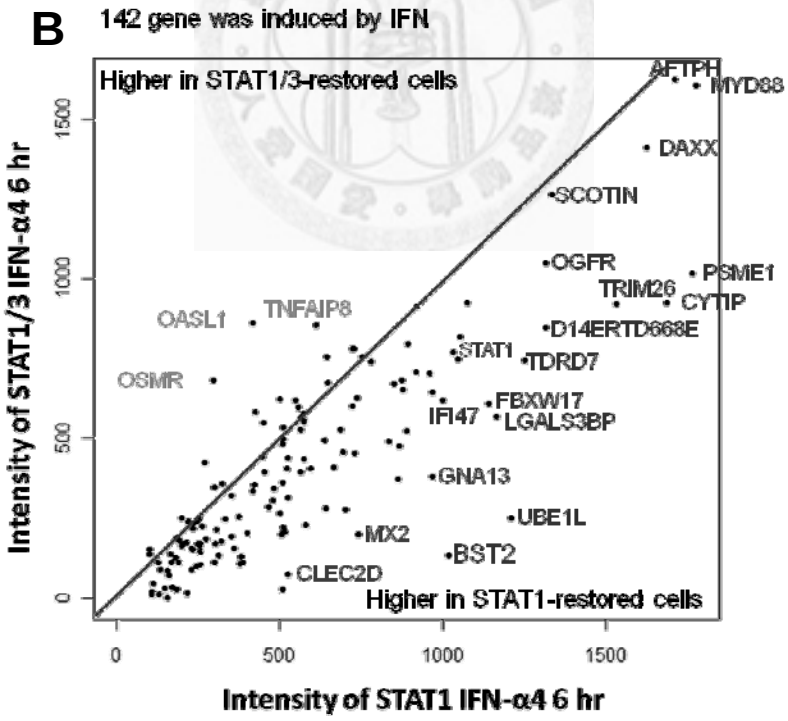

B
142 gene was induced by IFN
Higher in STAT1/3-restored cells
Higher in STAT1-restored cells
Intensity of STAT1/3 IFN-α4 6 hr
Intensity of STAT1 IFN-α4 6 hr
AFTPH
MYD88
DAXX
SCOTIN
OGFR
PSME1
TRIM26
CYTIP
D14ERTD668E
TDRD7
STAT1
FBXW17
LGALS3BP
IFI47
GNA13
UBE1L
MX2
BST2
CLEC2D
OASL1
TNFAIP8
OSMR
0
500
1000
1500

Figure 7. The relationship of gene expression compares STAT1-restored with STAT1/3-restored DKO MEFs. STAT1- and STAT1/3-restored DKO MEFs were treated with IFN-α4 for 6 hours. Total RNA of the treated cells was subjected to microarray. The plot was generated by R package (A). The result was analysis by Excel and the induced genes were indicated (B).

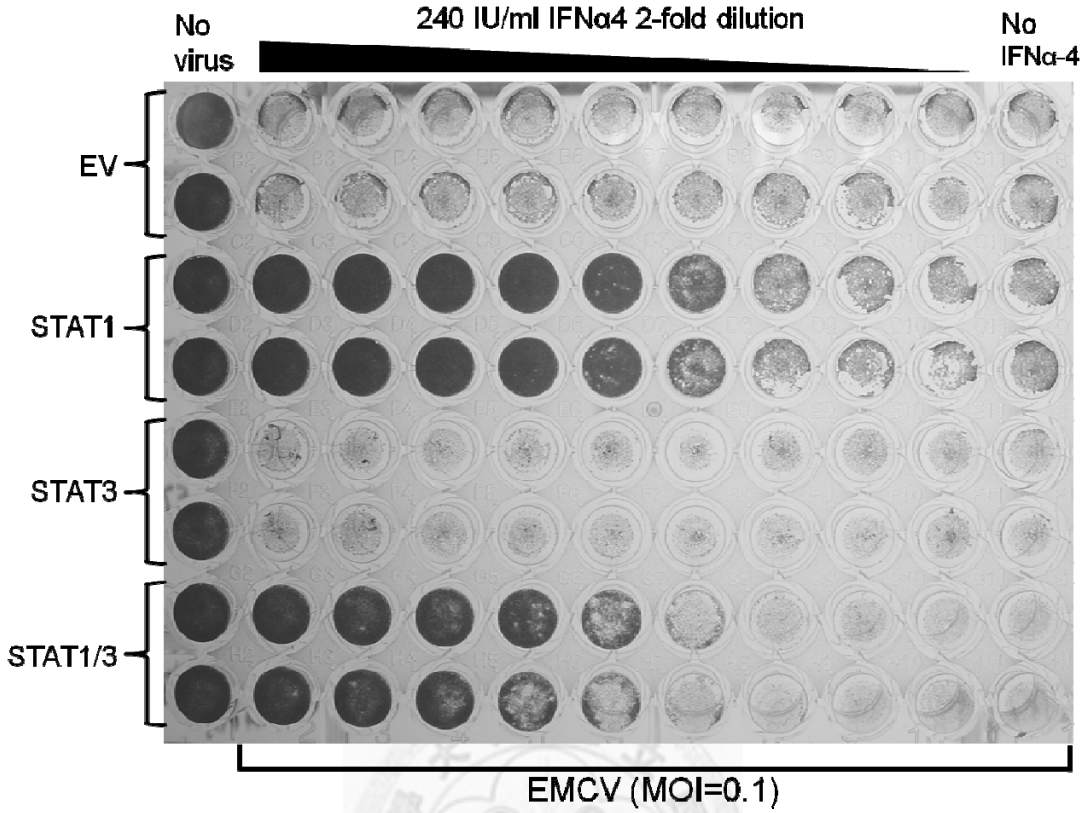


Figure 8. STAT3 suppresses type I IFN-mediated antiviral response in STAT1 and STAT3-restored DKO MEFs. STAT1- and/or STAT3-restored DKO MEFs were stimulated with 2-fold serial dilution of IFN-α4 from 240 IU/ml for 24 hours, followed by infection with EMCV at an MOI of 0.1. Live cells were visualized with crystal violet.

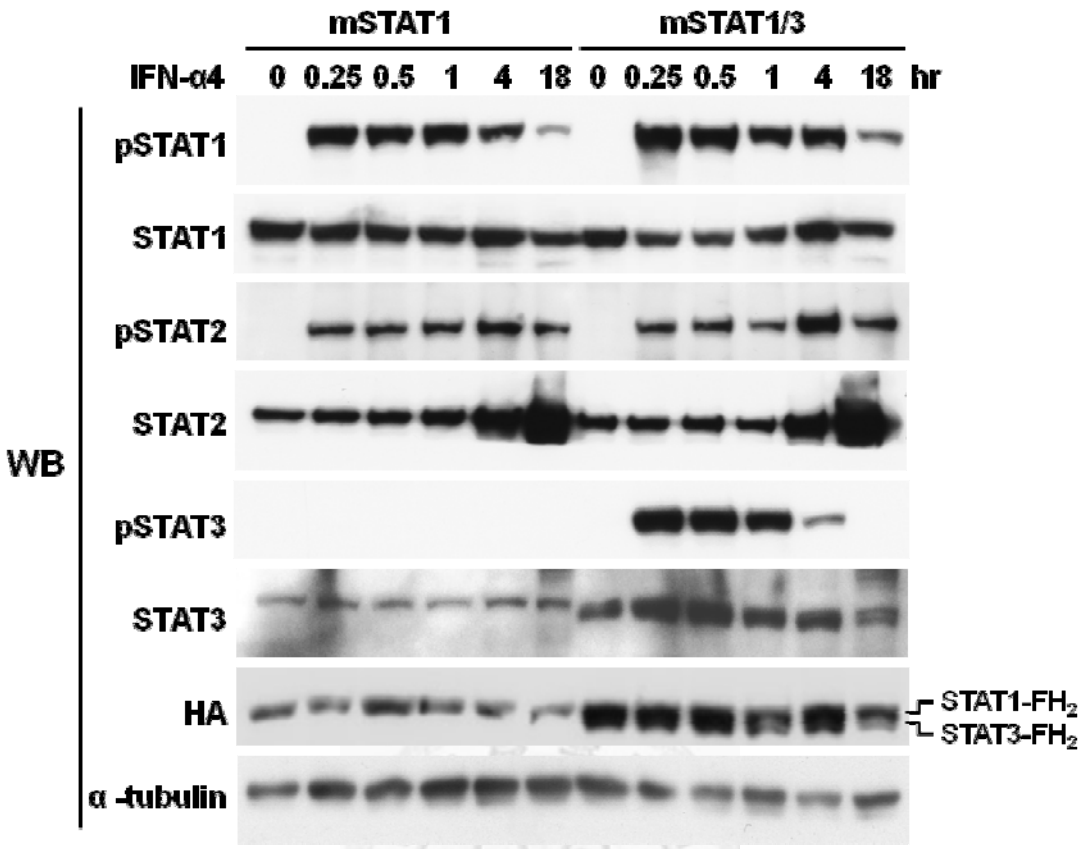


Figure 9. STAT3 does not to affect IFN-α4-activated STAT1 or STAT2. STAT1- and/or STAT3-restored DKO MEFs were treated with or without IFN-α4 (1000 IU/ml) for the indicated durations. Total cell lysates were subjucted to immunoblotting using antibodies to pSTAT1, pSTAT2, pSTAT3, STAT1, STAT2, STAT3, HA, and α-tubulin.

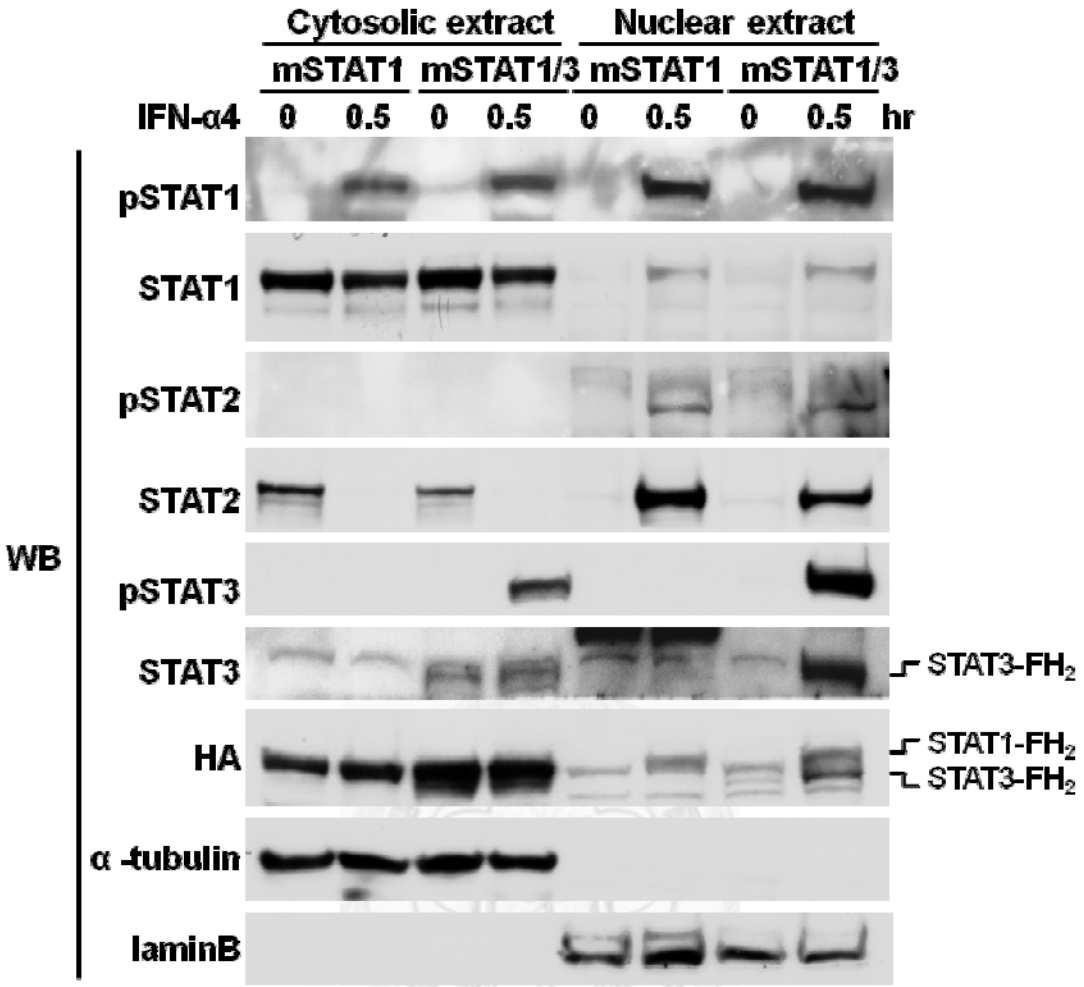


Figure 10. STAT3 does not affect IFN-α4-induced nuclear translocation of STAT1 or STAT2. STAT1- and/or STAT3-restored DKO MEFs were treated with or without IFN-α4 (1000 IU/ml) for 0.5 hours. Cytosolic extracts (left) and nuclear extracts (right) were subjucted to immunoblotting using antibodies to pSTAT1, pSTAT2, pSTAT3, STAT1, STAT2, STAT3, HA, α-tubulin, and laminB.

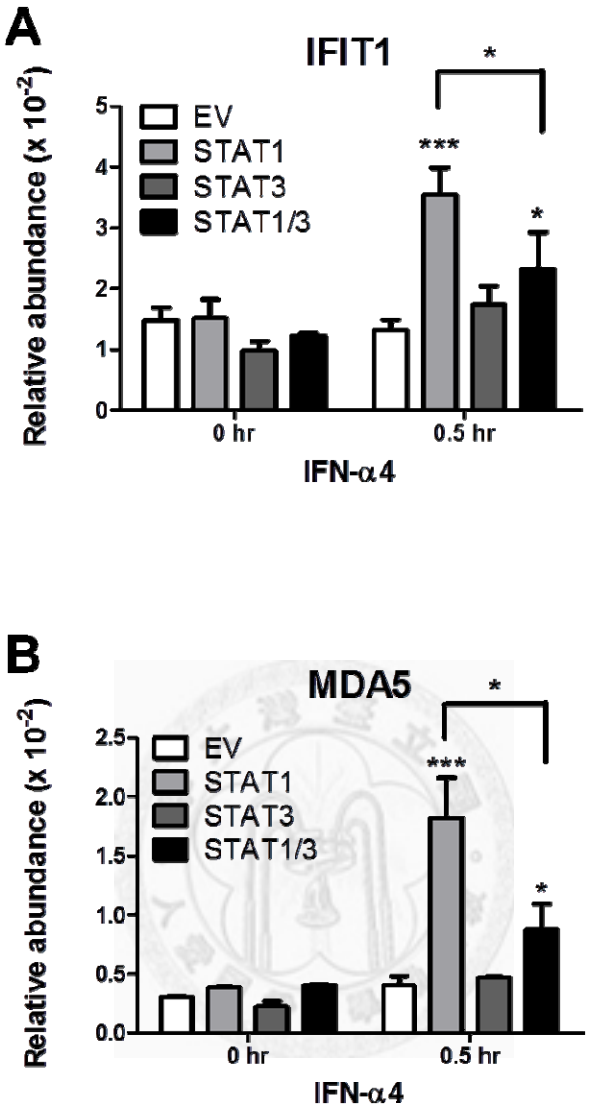
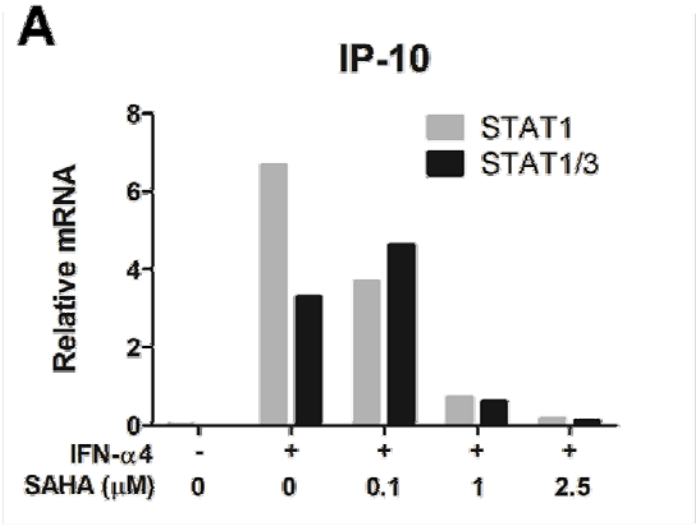
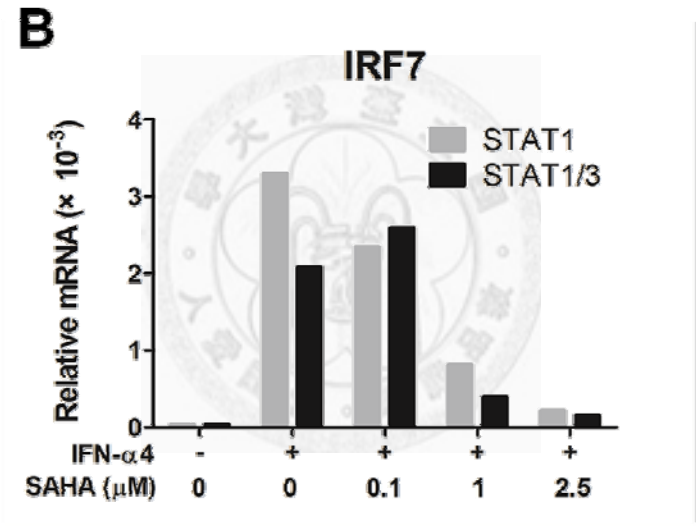


Figure 11. STAT3 decreases the recruitment of ISGF3 complex to the promoter of ISRE. STAT1- and/or STAT3-restored DKO MEFs were treated with or without IFN-α4 for 30 minutes, followed by performing ChIP assay using antibody against STAT1. QPCR analysis was performed using a ChIP-specific primer for the ISRE of MDA5 (A) or IFIT1 (B) promoter. Relative abundance was calculated by normalizing to input control.


A
IP-10
STAT1
STAT1/3
Relative mRNA
8
6
4
2
0
IFN-α4 - + + + +
SAHA (μM) 0 0 0.1 1 2.5


B
IRF7
STAT1
STAT1/3
Relative mRNA (× 10-3)
4
3
2
1
0
IFN-α4 - + + + +
SAHA (μM) 0 0 0.1 1 2.5

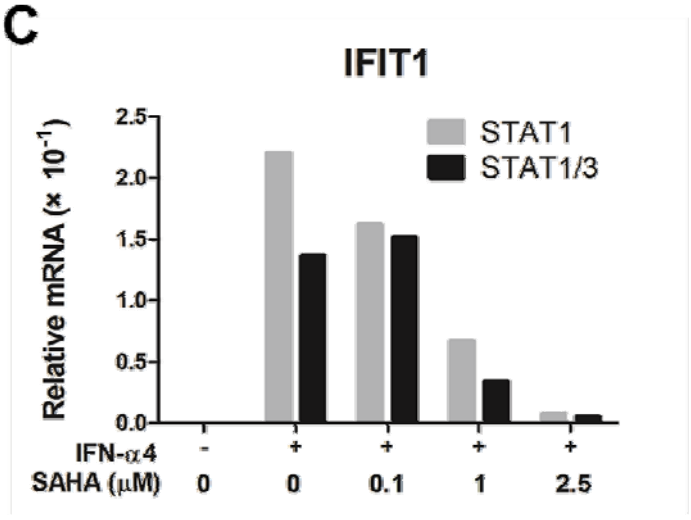

C
IFIT1
STAT1
STAT1/3
Relative mRNA (× 10-1)
2.5
2.0
1.5
1.0
0.5
0.0
IFN-α4 - + + + +
SAHA (μM) 0 0 0.1 1 2.5

Figure 12. HDAC inhibitor blocks suppressive effect of STAT3 on type I IFN-induced gene expression. STAT1- and STAT1/STAT3-restored DKO MEFs were stimulated with or without IFN-α4 alone or simultaneously with the indicated concentration of SAHA for 6 hours. Total RNA of the treated cells was subjected to RT-QPCR using primers for STAT1-mediated genes such as IP-10 (A), IRF7 (B), IFIT1 (C) or β-actin. Relative mRNA was calculated by normalizing to β-actin values.

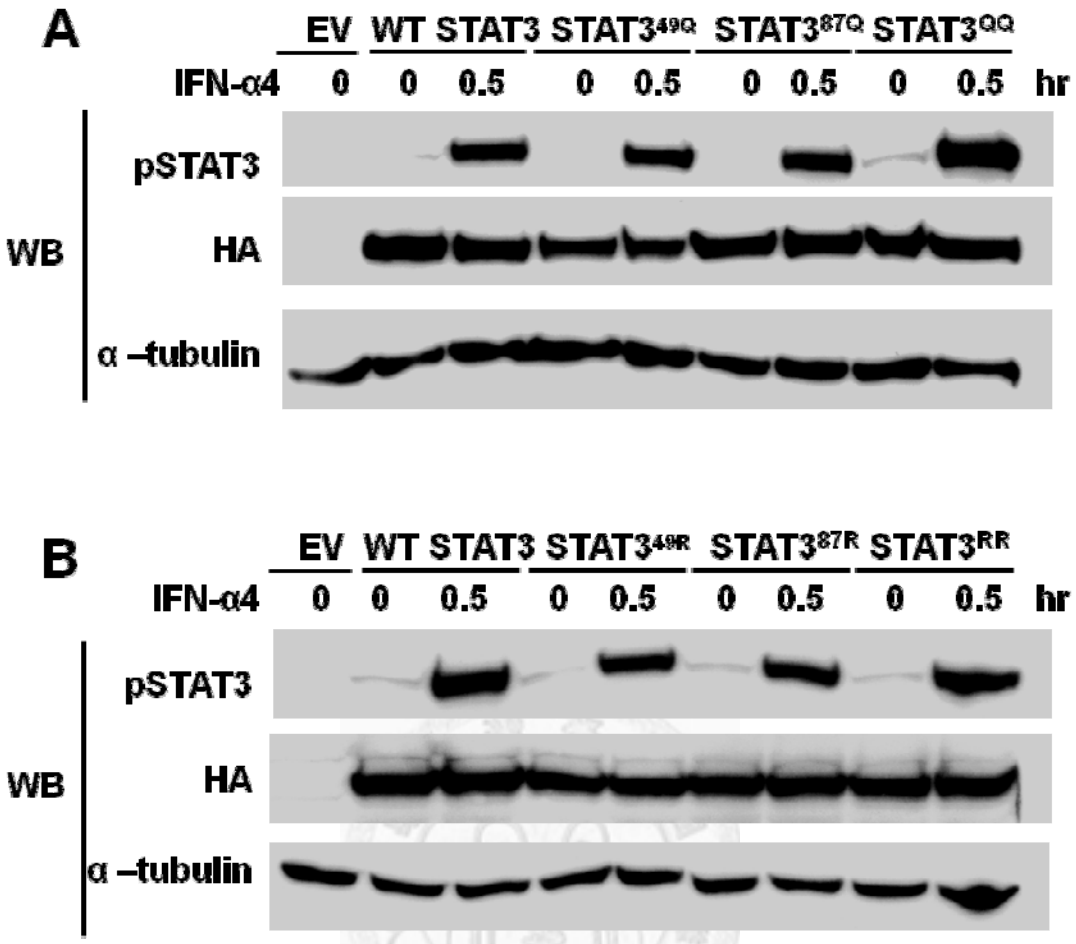


Figure 13. Comparable expression and activation of WT and mutant STAT3s in STAT3KO MEFs. Empty vector (EV), WT STAT3, $STAT3^{49Q}$, $STAT3^{87Q}$, $STAT3^{QQ}$ (A), $STAT3^{49R}$, $STAT3^{87R}$, or $STAT3^{RR}$ (B) was transfected into STAT3KO MEFs for 48 hours. Cells were treated with or without IFN-α4 (1000 IU/ml) for 30 minutes. Whole cell lysates were then subjucted to immunoblotting using antibodies to pSTAT3, HA, and α-tubulin.

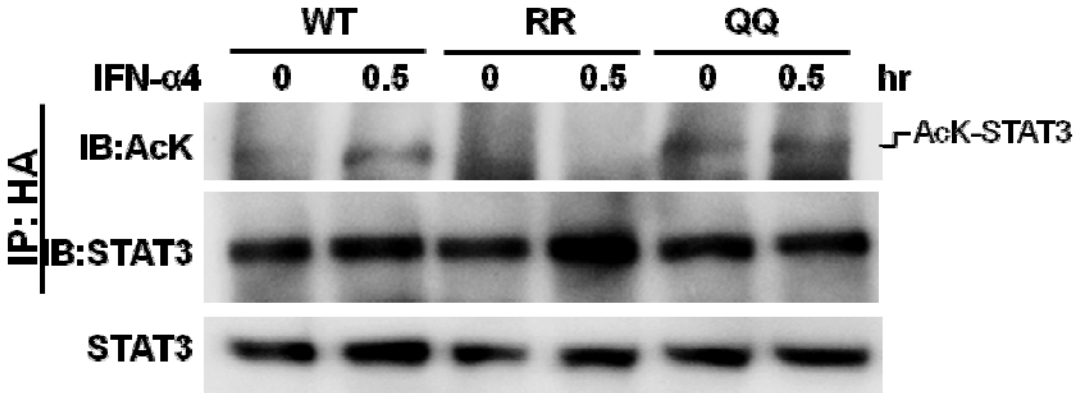


Figure 14. IFNα-dependent acetylation of STAT3 is abolished in RR mutant STAT3. WT STAT3, STAT3^{RR},and STAT3^{QQ} was transfected into STAT3KO MEFs for 48 hours. Cells were treated with or without IFN-α4 (1000 IU/ml) for 30 minutes. Whole cell lysates were then subjucted to immunopreciptation using anti-HA antibodies, and immunoblotting of acetylated-lycine and STAT3. Third raw expressed western blotting for whole cell lysate using anti-STAT3 antibody.

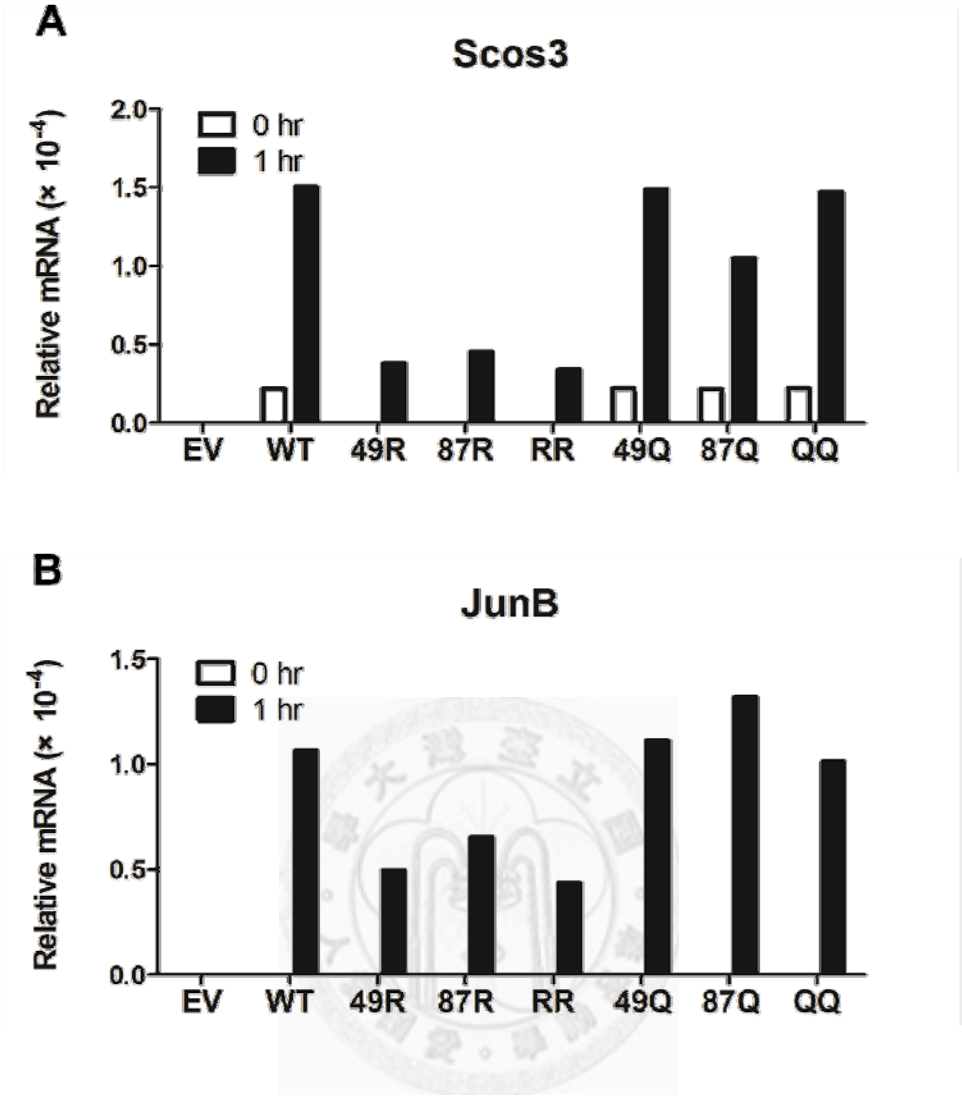


Figure 15. Mutations in Lys49 and Lys87 of STAT3 at NTD affect STAT3 downstream gene induction. Empty vector (EV), WT STAT3, STAT3 acetylation-deficient (49R, 87R, or RR), or STAT3 acetylation mimics (49Q, 87Q, or QQ) was transfected into STAT3KO MEFs. After 48 hours, MEFs were treated with or without IFN-α4 (1000 IU/ml) for 1 hour. Total RNA of the treated cells was subjected to RT-QPCR using primers for Socs3 (A), JunB (B), or β-actin. Relative mRNA was calculated by normalizing to β-actin values.

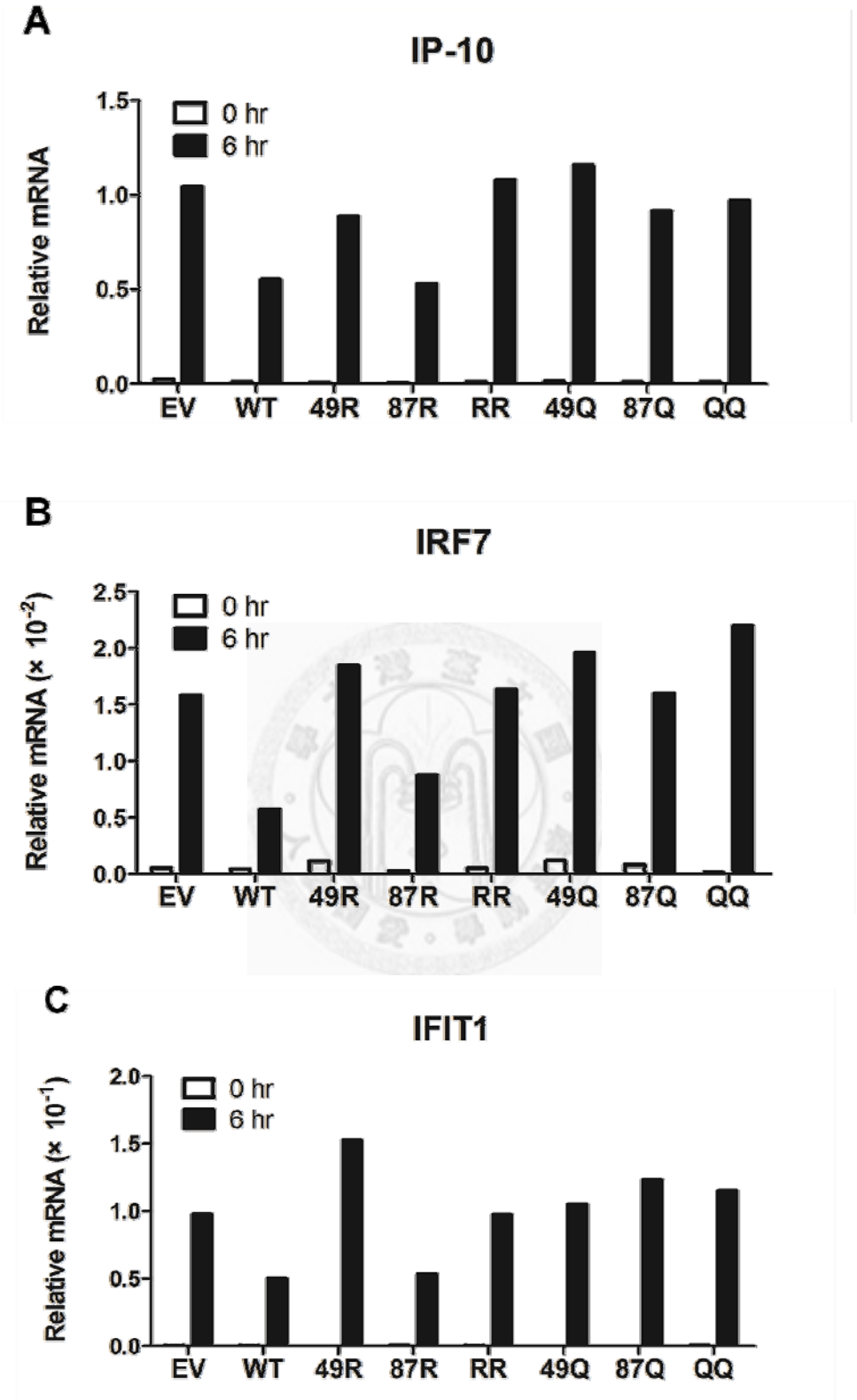
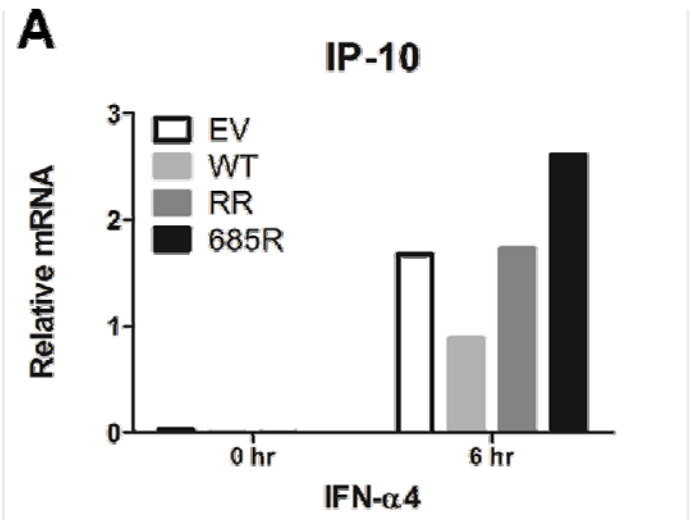
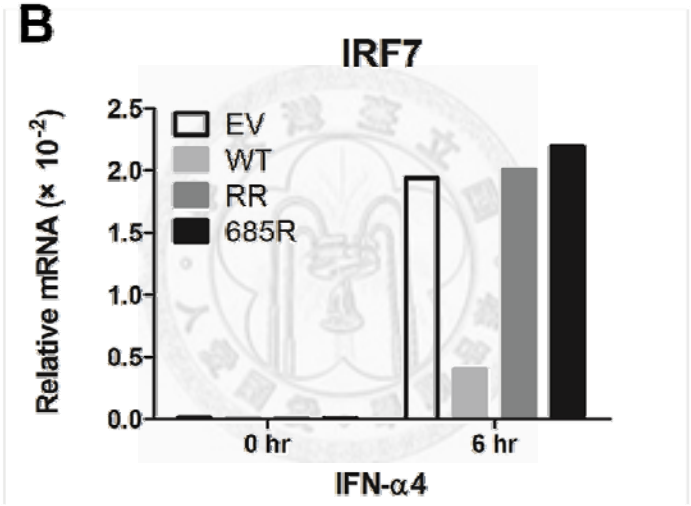

A
IP-10
Relative mRNA
1.5
1.0
0.5
0.0
0 hr
6 hr
EV
WT
49R
87R
RR
49Q
87Q
QQ
B
IRF7
Relative mRNA (× 10-2)
2.5
2.0
1.5
1.0
0.5
0.0
0 hr
6 hr
EV
WT
49R
87R
RR
49Q
87Q
QQ
C
IFIT1
Relative mRNA (× 10-1)
2.0
1.5
1.0
0.5
0.0
0 hr
6 hr
EV
WT
49R
87R
RR
49Q
87Q
QQ

Figure 16. Negative regulation of type I IFN responses by STAT3 is abrogated in Lys49 and/or Lys87 STAT3 mutants. Empty vector (EV), WT STAT3, STAT3 acetylation-deficient, or STAT3 acetylation mimics was transfected into STAT3KO MEFs for 48 hours, followed by treating the cells with or without IFN-α4 (1000 IU/ml) for 1 hour. Expression of IP-10 (A), IRF7 (B), IFIT1 (C), or β-actin was monitored by RT-QPCR. Relative mRNA was normalized with β-actin values.


A
IP-10
Relative mRNA
3
2
1
0
EV
WT
RR
685R
0 hr
6 hr
IFN-α4


B
IRF7
Relative mRNA (× 10-2)
2.5
2.0
1.5
1.0
0.5
0.0
EV
WT
RR
685R
0 hr
6 hr
IFN-α4

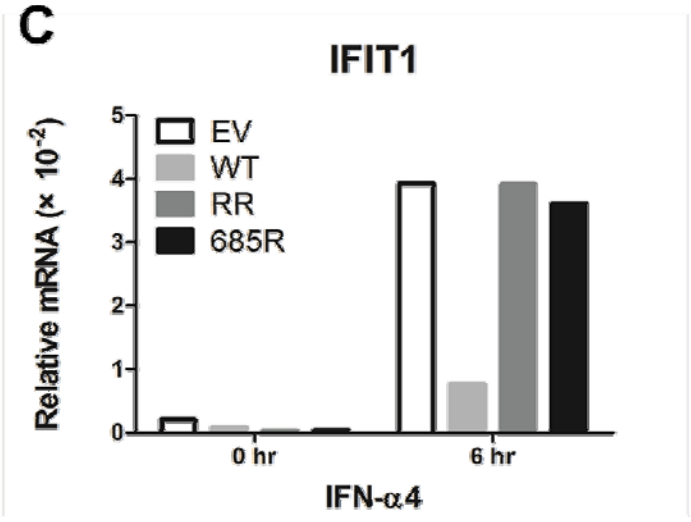

C
IFIT1
Relative mRNA (× 10-2)
5
4
3
2
1
0
EV
WT
RR
685R
0 hr
6 hr
IFN-α4

Figure 17. Acetylation of STAT3 at Lys685 affects negative effect of STAT3 on type I IFN-mediated gene expression. Empty vector (EV), WT STAT3, $STAT3^{RR}$, or $STAT3^{685R}$ was transfected into STAT3KO MEFs. After 48 hours, MEFs were treated with or without IFN-α4 for 6 hour. Total RNA of the treated cells was subjected to RT-QPCR using primers for IP-10 (A), IRF7 (B), IFIT1 (C) or β-actin. Relative mRNA was calculated by normalizing to β-actin values.

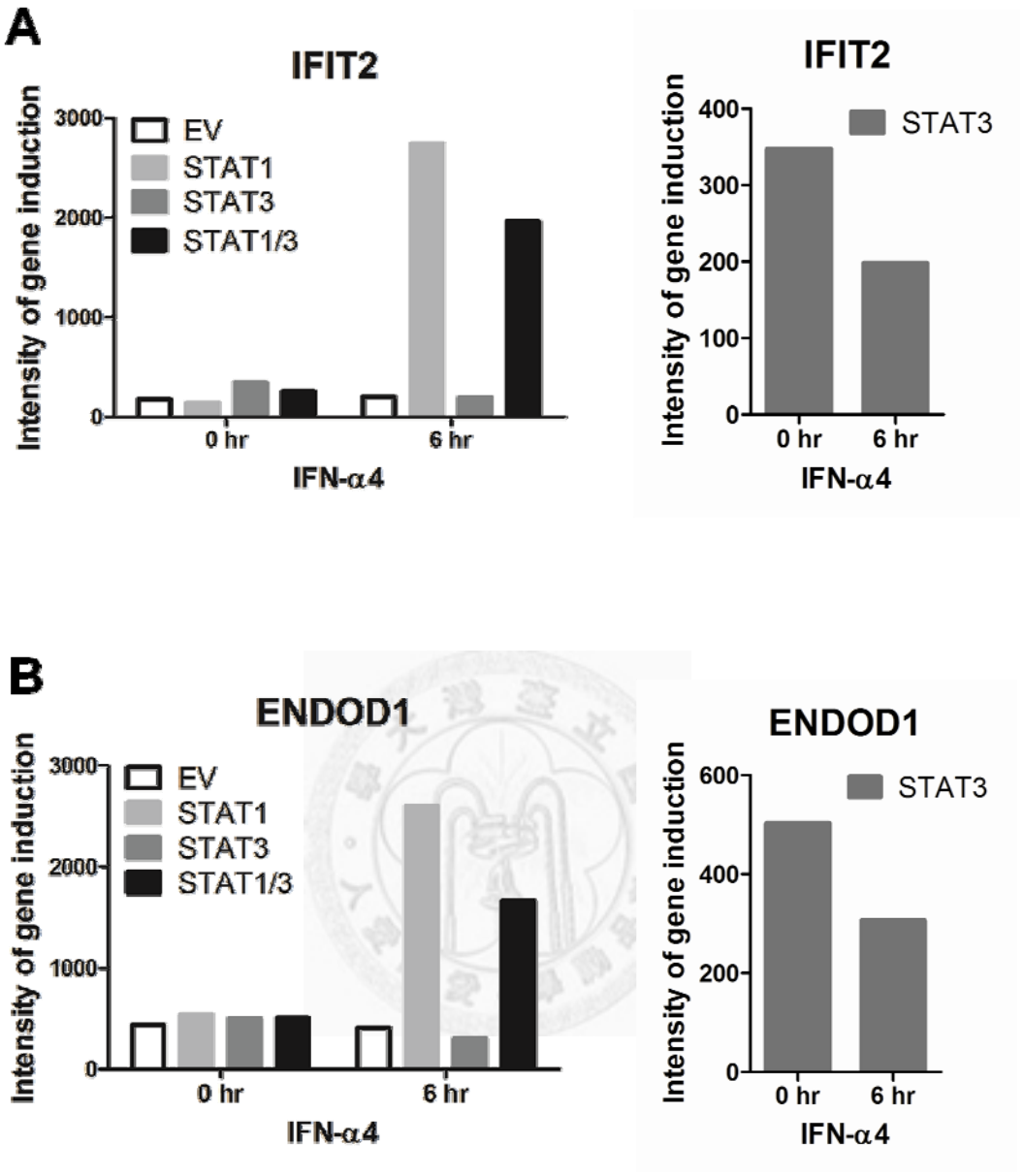


Figure 18. STAT3 directly suppresses gene expression. Restored DKO MEFs were stimulated with or without IFNα4 for 6 hours, followed by preparing RNA for microarray. After analysis, intensity of OAS2 (A), MX1 (B), STAT2 (C), and IRF1 (D) was showed.