

# Appendix

## **HDAC6 regulates cellular viral RNA sensing by deacetylation of RIG-I**

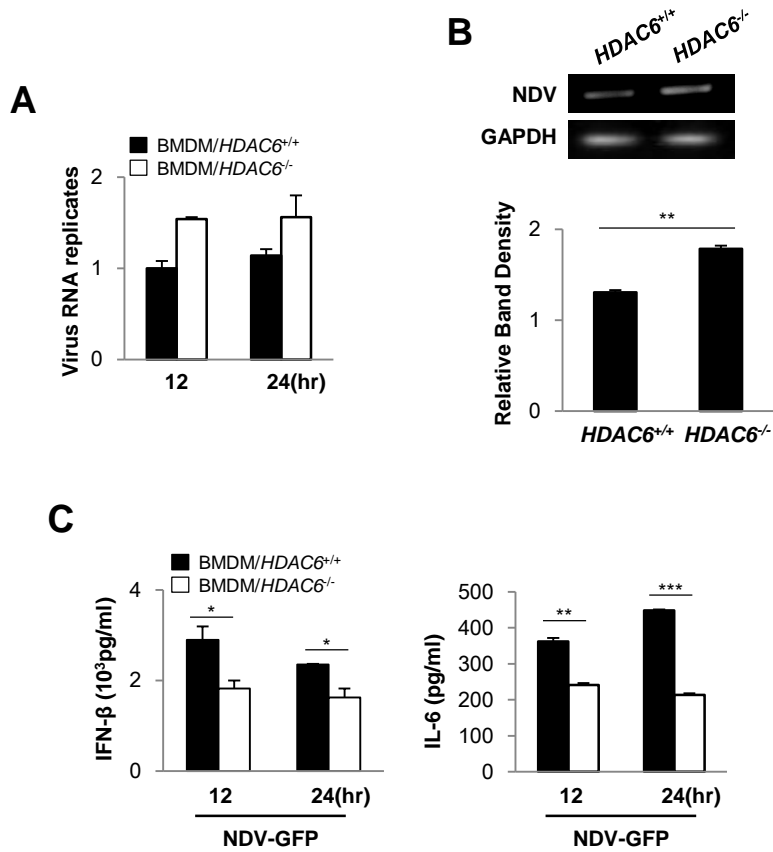
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Appendix Figures S1-S12

Appendix Table S1

Appendix Supplementary Methods

## Appendix Figure S1



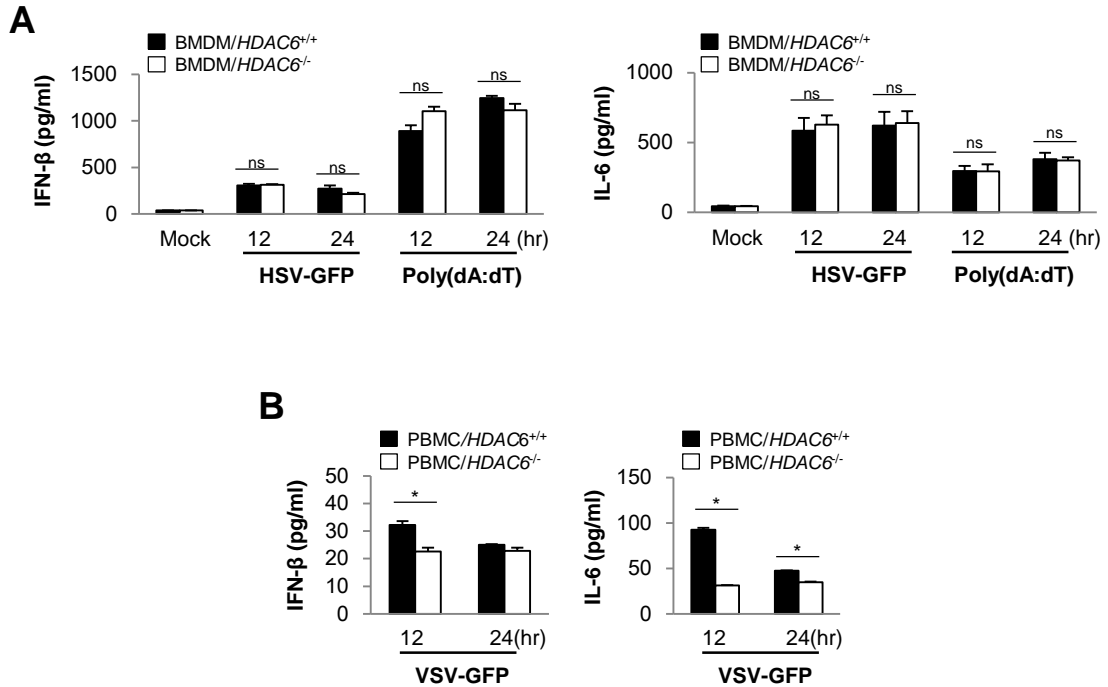
### Appendix Figure S1. HDAC6 inhibits Newcastle disease virus replication in bone marrow derived macrophages.

**A.** *HDAC6*<sup>+/+</sup> and *HDAC6*<sup>-/-</sup> BMDMs were isolated and cells were then infected with NDV-GFP (MOI = 1). Total RNA were extracted from cultured cells and converted into cDNA to measure the amount of viral transcript using quantitative real time PCR.

**B.** RT-PCR was performed with (A) at 24 hpi. Relative band density was measured and normalized to GAPDH.

**C.** Culture supernatant were collected at 12, 24 hpi and used for ELISA bioassay to measure secreted IFN-β (left) and IL-6 (right) levels. Data are representative of at least two independent experiments. Error bars, mean ± SD, \*\**p* < 0.01 (student's t-test).

## Appendix Figure S2



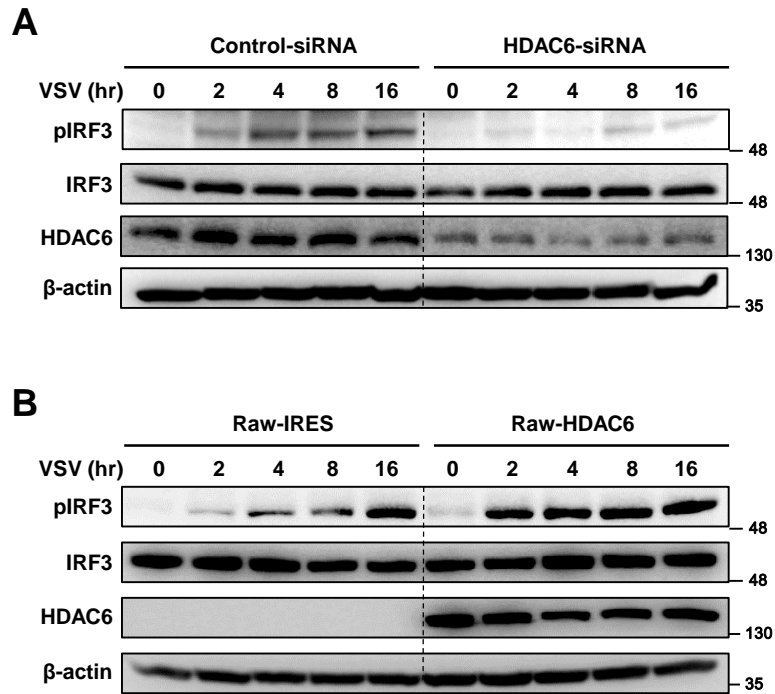
### Appendix Figure S2. Deletion of HDAC6 reduces cytokine secretion in response to RNA virus infection but not in case of DNA virus infection or poly(dA:dT) transfection.

**A.** ELISA of IFN- $\beta$  (left), IL-6 (right) levels in the supernatant of *HDAC6*<sup>+/+</sup> and *HDAC6*<sup>-/-</sup> BMDMs infected with HSV-GFP (MOI = 2) and transfected with poly(dA:dT) (1  $\mu$ g/ml).

**B.** ELISA of IFN- $\beta$  (left), IL-6 (right) levels in the supernatant of *HDAC6*<sup>+/+</sup> and *HDAC6*<sup>-/-</sup> PBMCs infected with VSV-GFP (MOI = 10). Data are representative of at least two independent experiments.

Error bars, mean  $\pm$  SD. \* $p$  < 0.05 (Student's  $t$ -test).

## Appendix Figure S3

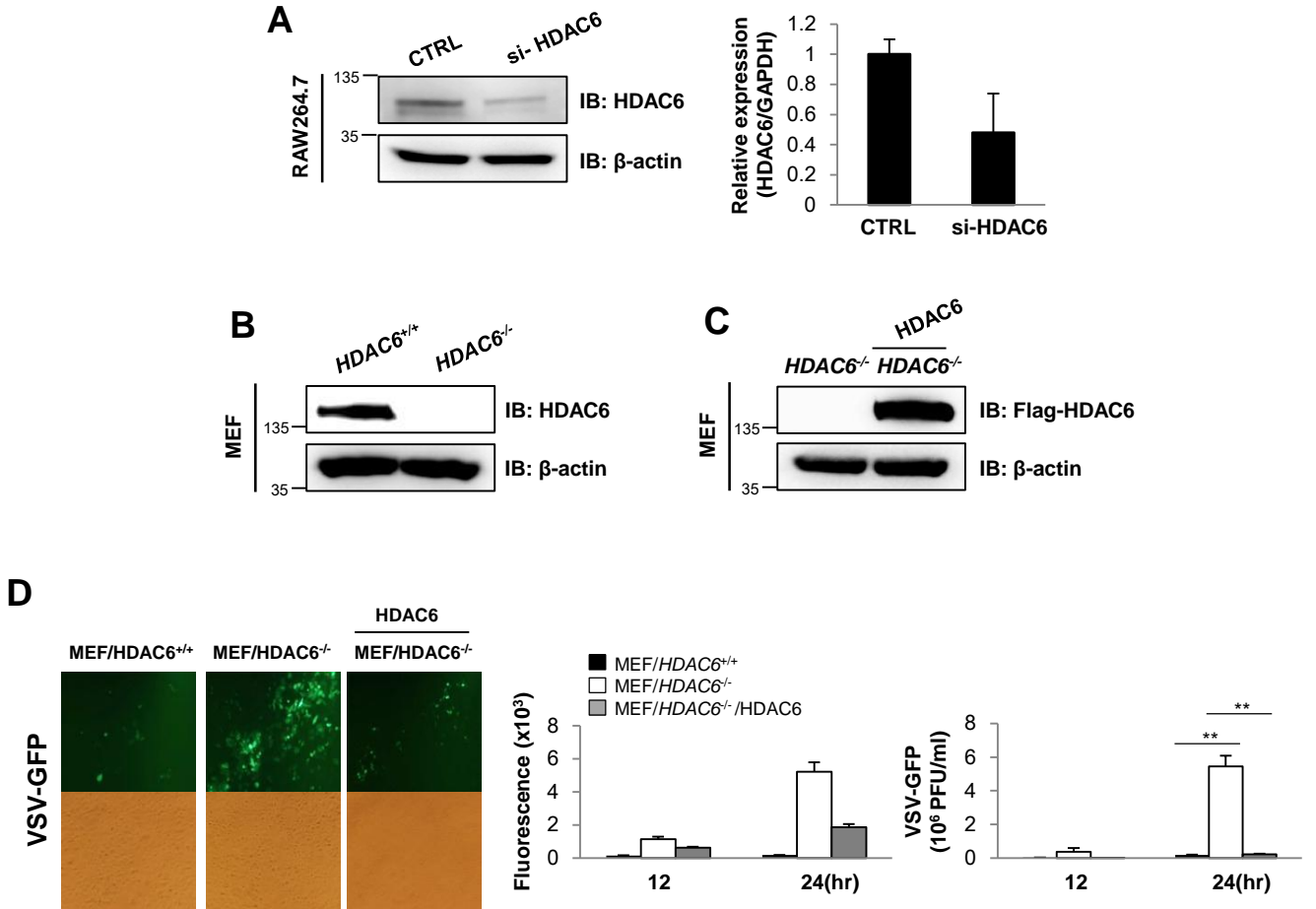


### Appendix Figure S3. HDAC6 is related with phosphorylation of IRF3 in response to VSV-gfp.

**A.** Immunoblot analysis of the phosphorylated and inactive forms of IRF3, HDAC6, and  $\beta$ -actin at the indicated times (0, 2, 4, 8, and 16 h) in control and *HDAC6* knockdown RAW264.7 cells in response to VSV-GFP (MOI = 1) infection.

**B.** Immunoblot analysis of the phosphorylated and inactive forms of IRF3, HDAC6, and  $\beta$ -actin at the indicated times (0, 2, 4, 8, and 16 h) in control and *HDAC6* overexpression RAW264.7 cells in response to VSV-GFP (MOI = 2) infection.

## Appendix Figure S4



### Appendix Figure S4. expression level of HDAC6 in HDAC6 knock-down RAW264.7 cells, MEFs and HDAC6 reconstituted MEF.

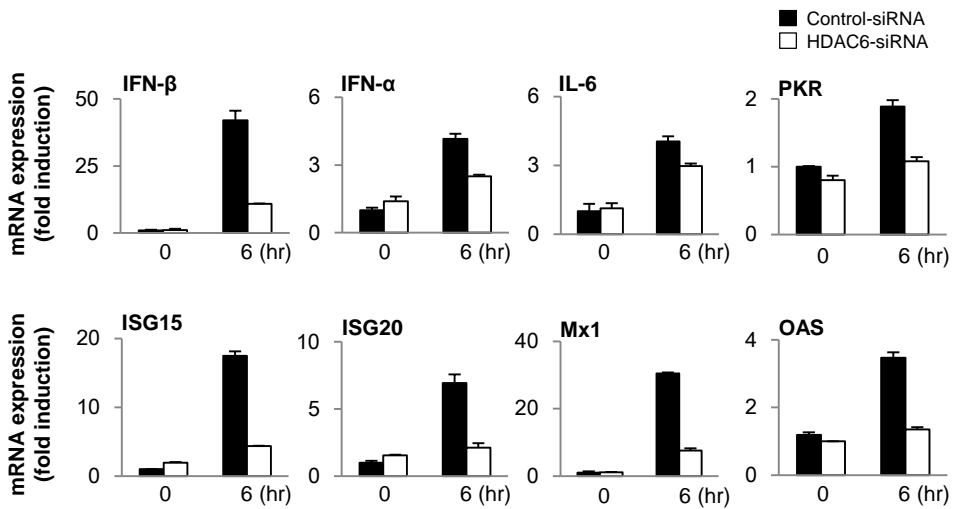
**A.** HDAC6 expression level was decreased in RAW264.7 cell line after siRNA transfection.

**B.** HDAC6 expression in *HDAC6*<sup>+/+</sup> and *HDAC6*<sup>-/-</sup> MEFs. Cells were collected and immune-blotted using anti-HDAC6 antibody to confirm HDAC6 depletion.

**C.** *HDAC6*<sup>-/-</sup> MEF were transfected with HDAC6-IRES-flag to generate stable cell line. Cells were selected using 2  $\mu$ g of puromycin and immunoblotted with anti-Flag Antibody.

**D.** *HDAC6*<sup>+/+</sup>, *HDAC6*<sup>-/-</sup>, and *HDAC6*<sup>-/-</sup> MEFs reconstituted with HDAC6 were infected with VSV-GFP (MOI = 1). VSV-GFP replication was observed by Fluorescence microscopy. Green Fluorescence absorbance was measured at 12, 24 hpi. Virus replication was assessed by standard plaque assay at same time points. Data are representative of three independent experiments. Error bars, mean  $\pm$  SD, \*\**p* < 0.01 (student's t-test).

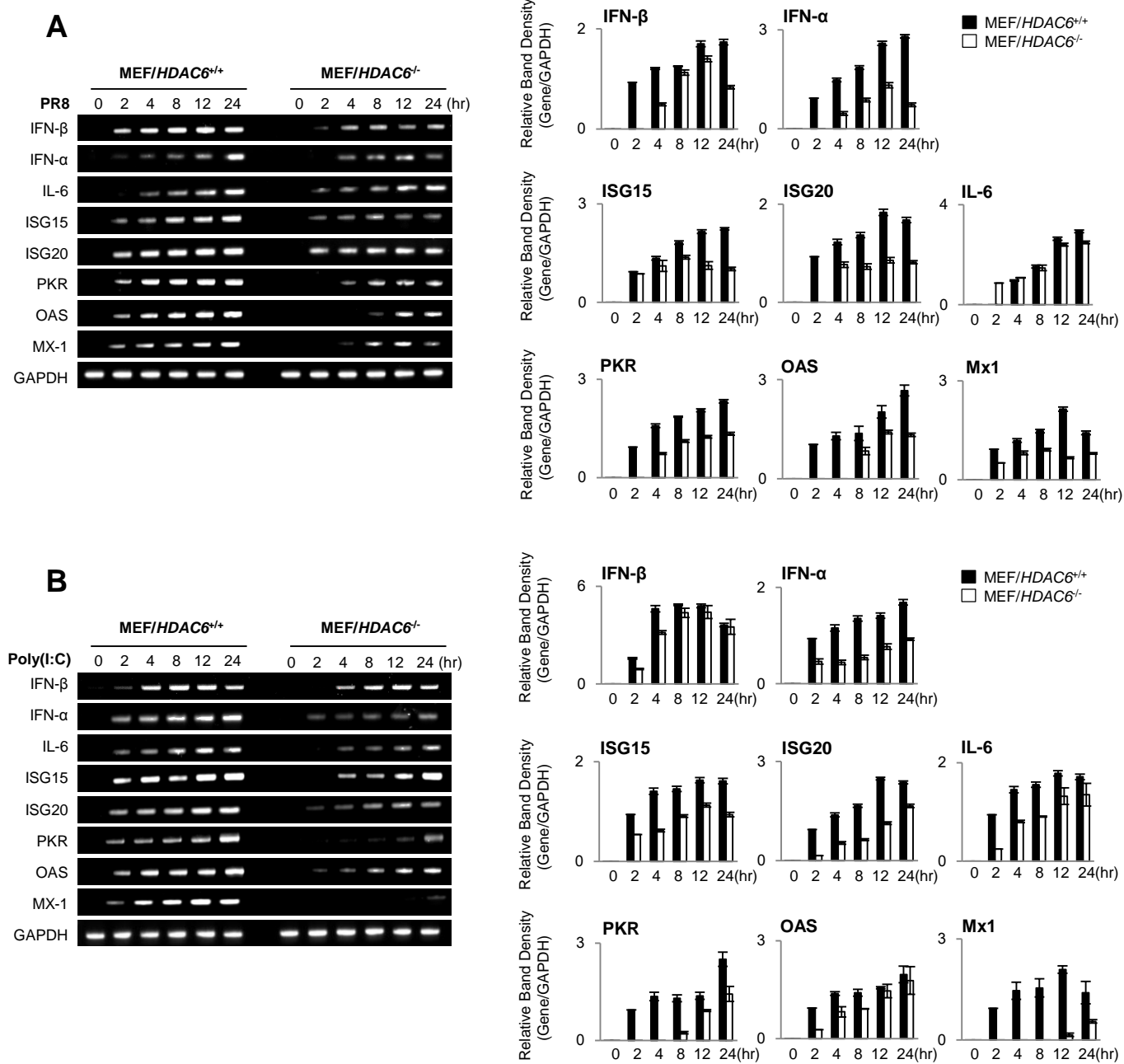
## Appendix Figure S5



### Appendix Figure S5. Knockdown of HDAC6 reduces mRNA induction of type I interferon and related antiviral genes.

Induction of mRNA for type I IFN, IL-6, and other IFN-related antiviral genes was lower in HDAC6 knockdown RAW264.7 cells than in control cells in response to a RIG-I agonist at 6 h. Cells were stimulated with 5'ppp-dsRNA (0.5  $\mu\text{g/ml}$ ) for 6 h.

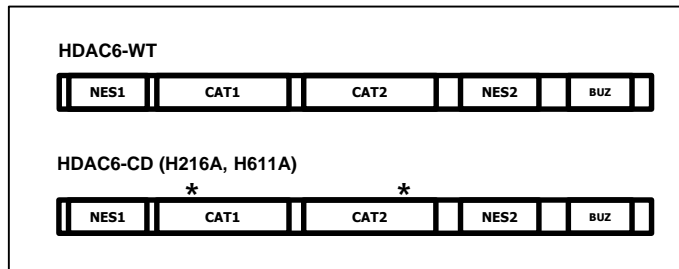
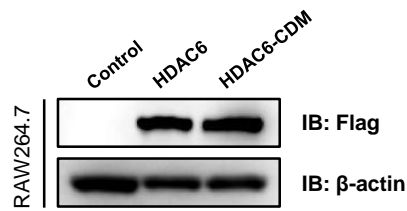
## Appendix Figure S6



### Appendix Figure S6. Induction of mRNA for type I interferon, IL-6, and other interferon-related antiviral genes in *HDAC6*<sup>+/+</sup> and *HDAC6*<sup>-/-</sup> MEFs.

**A, B.** mRNA induction of Type I IFN and other antiviral genes were reduced in *HDAC6*<sup>-/-</sup> MEF than *HDAC6*<sup>+/+</sup> MEF in response to PR8-GFP virus infection (A) or poly(I:C) transfection (B). *HDAC6*<sup>+/+</sup> and *HDAC6*<sup>-/-</sup> MEF were stimulated with PR8-GFP (MOI = 1) or poly(I:C) (1 μg/ml) and harvested at indicated time points to measure mRNA induction of type I IFN and other antiviral genes. Total mRNA was extracted from cultured cells and the RNA was reverse transcribed for cDNA synthesis. Expression levels of indicated genes were determined by end-point RT-PCR. GAPDH was used as a loading control. RT-PCR products were resolved by 2% agarose gel electrophoresis. Data are representative of at least two independent experiments. Error bars, mean ± SD

## Appendix Figure S7

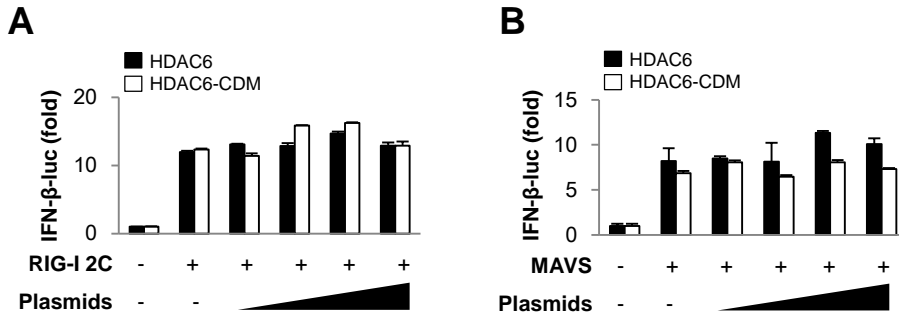


### Appendix Figure S7. Expression of HDAC6, HDAC6-CDM in RAW264.7 cell line.

HDAC6 wild-type and HDAC6-CDM were stably expressed in RAW264.7 cell line. Flag-tagged HDAC6 wild-type, HDAC6-CDM and empty vector plasmids were transfected into RAW264.7 cells for 36 h. Stable cell lines were selected and generated with 2  $\mu$ g of Puromycin treatment as a standard protocol. Cells were then harvested and lysed for immunoblot analysis using anti-Flag Antibody.



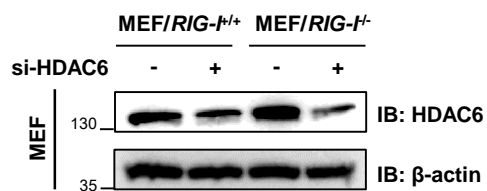
## Appendix Figure S8



### Appendix Figure S8. IFN- $\beta$ induction by CARD of RIG-I (B) and MAVS (C) does not affected by HDAC6 and HDAC6-CDM.

**A, B.** 293T cells were transfected with MAVS or CARD of RIG-I, IFN- $\beta$  luciferase promoter and TK-Renilla together with the expression plasmids HDAC6 or HDAC6-CDM in a dose dependent manner (100, 200, 400, 800 ng). 24 h later, IFN- $\beta$  activity was measured by luciferase reporter assay. Data are representative of at least two independent experiments. Error bars, mean  $\pm$  SD

## Appendix Figure S9



### Appendix Figure S9. Effect of knockdown of HDAC6 in *RIG-I*<sup>+/+</sup> and *RIG-I*<sup>-/-</sup> MEF cell line.

HDAC6 expression level was decreased in *RIG-I*<sup>+/+</sup> and *RIG-I*<sup>-/-</sup> MEF cell line after siRNA transfection.

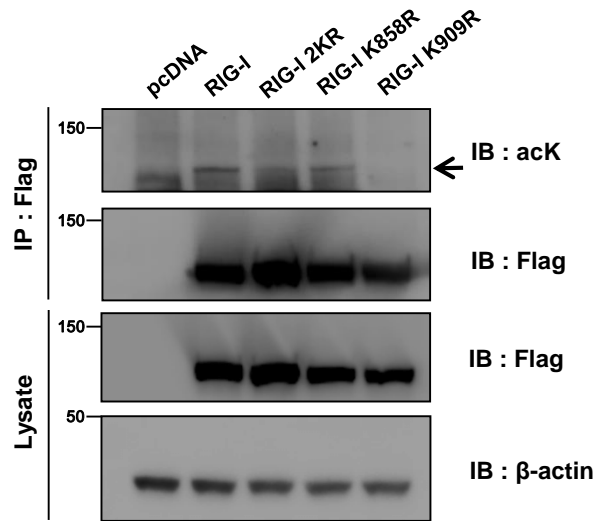
## Appendix Figure S10

H.Sapiens	835	GDAF-KECFVSRPHPKPKQFSSFE <u>K</u> RAKIFCARQNCSDHWGIHVKYKTF-	882
P.Troglodytes	835	GDAF-KECFVSRPHPKPKQFSSFE <u>K</u> RAKIFCARQNCSDHWGIHVKYKTF-	882
M.mulatta	835	GDAF-KECFVRRPHPKPKFSNFD <u>K</u> REKIFCARQNCSDHWGIHMKYKTF-	882
C.lupus	833	GDAF-TKCFVSKLHPKPKSFGHF <u>E</u> RAKIFCARRNCGHDWGIHVKYKTF-	880
B.Taurus	913	RDAF-RECFVTKLHPRPKKFGSFD <u>K</u> KAKIFCARKDCLHDWGIHMKYKTF-	960
M.Musculus	836	GDAF-KERFVCKPHPKPKIYDNFE <u>K</u> KAKIFCAKQNCSDHWGIFVRYKTF-	883
C.Elegans	915	DPGFWSLVRKTRLTDEQQALIKYNA <u>T</u> GSINCRRENCGLKGLQLEIENVTV-	963
X.Tropicalis	870	DKTF-KDRYITKKHKPRTFEGYK <u>K</u> MYKIFCKRPECHEDWGVSGTYQGFQ	918
		858	
H.Sapiens	883	EIPVIKIESFVVEDIATGVQTLYSKW <u>K</u> DF-----HFEKIPFDPAEMSK-	925
P.Troglodytes	883	EIPVIKIESFVVEDIATGVQTLYSKW <u>K</u> DF-----HFEKIPFDPAEMSK-	925
M.Mulatta	883	EIPVIKIESFVVEDIATGVQTLYSKW <u>K</u> DF-----HFEKIPFDPAEMAK-	925
C.Lupus	881	EIPVIKIESFVVEDIATGAQKLYAKW <u>K</u> DF-----PFEKIPFGSPEIPE-	923
B.Taurus	961	EIPVIKIESFVVEDVATGAQTLYAKW <u>K</u> DF-----NFEKIPDAAEMSPW	1004
M.Musculus	884	EIPVIKIESFVVEDIVSGVQNRHSKW <u>K</u> DF-----HFERIQFDPAEMSV-	926
C.Elegans	964	DLPLSALSIVLLVEGTDKRIIVKKW <u>K</u> NI <del>L</del> DKYFTPTAIRQLDVQTMRDA	1013
X.Tropicalis	919	DLPLIKIEQFVIEN-PDGTQEYKDKW <u>V</u> DV-----HFTMKKLSTEEFLSS	961
		909	

### Appendix Figure S10. RIG-I K909 site is well conserved in 7 different species except X. tropicalis.

Sequence alignment of the RIG-I from different species using NCBI-BLAST program. K858 and K909 are marked red and underlines.

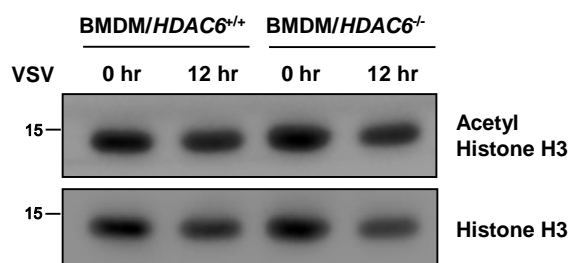
## Appendix Figure S11



### Appendix Figure S11. K909 site of RIG-I is a main acetylation site.

293T cells were transfected with indicated Flag-tagged RIG-I expression plasmid and subjected to immunoprecipitation analysis using anti-Flag antibody. Lysates and IP samples were subjected to immunoblotting with anti-acetyl-lysine and anti-Flag antibodies.  $\beta$ -actin was used as the loading control. Arrow indicates acetyl RIG-I

## Appendix Figure S12



### Appendix Figure S12. HDAC6 has no effects on histone H3 acetylation.

BMDM were isolated from *HDAC6*<sup>+/+</sup> and *HDAC6*<sup>-/-</sup> mice and infected with VSV-GFP (MOI = 5). BMDM cells were harvested at 0 and 12 h after infection and subjected to acid extraction for histone. Samples were analyzed by western blotting with anti-acetyl histone H3 and histone H3 antibody.

**Appendix Table S1 . Primers and siRNA sequences**

	<b>Forward</b>	<b>Reverse</b>
<b>HDAC6</b>	CAGAACTGGTGCTGATCTCAG	TATTTGCGATGGACTTGGATG
<b><math>\beta</math>-Catenin</b>	TGCAGATCTTGGACTGGACA	AAGAACGGTAGCTGGGATCA
<b>IFN-<math>\beta</math></b>	TCCAAGAAAGGACGAACATTCG	TGCGGACATCTCCCAACGTCA
<b>IFN-<math>\alpha</math></b>	CTTGAAGGACAGACATGACTTTGGA	GGATGGTTTCAGCCTTTTGGGA
<b>IL-6</b>	GACAACCTTTGGCATTGTGG	ATGCAGGGATGATGTTCTG
<b>ISG15</b>	CAATGGCCTGGGACCTAAA	CTTCTTCAGTTCTGACACCGTCAT
<b>ISG20</b>	AGAGATCACGGACTACAGAA	TCTGTGGACGTGTCATAGAT
<b>OAS</b>	GAGGCGGTTGGCTGAAGAGG	GAGGAAGGCTGGCTGTGATTGG
<b>PKR</b>	GCCAGATGCACGGAGTAGCC	GAAACTTGGCCAAATCCACC
<b>MX1</b>	ACAAGCACAGGAAACCGTATCAG	AGGCAGTTTGGACCATCTTAGTG
<b>VSV-G</b>	CAAGTCAAATGCCCAAGAGTCACA	TTTCCTTGCATTGTTCTACAGATGG
<b>GAPDH</b>	TGACCACAGTCCATGCCAT	GACGGACACATTGGGGGTAG
	<b>Sense</b>	<b>Antisense</b>
<b>HDAC6<sup>+/+</sup></b>	CTGGTTCGTCTGAAGACA	GTGGACCAGTTAGAAGCC
<b>HDAC6<sup>-/-</sup></b>	CCATGACCGAGATCGGCGAGCA	CGTGAATTCCGATCATATTCAAT
<b>si RNA</b>		
<b>si HDAC6</b>	UCUGCACCGUGAAGGUGCCAACUUU	
<b>si <math>\beta</math>-Catenin</b>	GCGCUUGGCUGAACCAUCAUU	

## Appendix Supplementary Methods

### Histone Purification

BMDMs were isolated from *HDAC6*<sup>+/+</sup> and *HDAC6*<sup>-/-</sup> mice and infected with VSV-GFP (MOI = 5). Cells were harvested at 0 and 12 h after infection and lysed in lysis buffer (10 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, 25 mM KCl, 1% Triton X-100, 8.6% sucrose, pH to 6.5) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma). Supernatants were discarded and pellets were incubated with 0.4 N H<sub>2</sub>SO<sub>4</sub> on the ice for 1 h. After removing supernatant, pellets were analyzed by immunoblotting using anti-acetyl histone H3 (06-599, Millipore) and anti-histone H3 (#3638, Cell signaling) antibodies.

### Modeling of RIG-1-dsRNA complex

The model of the RIG-1/dsRNA complex was constructed based on the reported crystallographical structure (PDB code: 3LRR) using Discovery studio 4.0 (Accelrys Inc. San Diego, USA, <http://accelrys.com/>). Four molecular dynamic (MD) simulation systems were prepared. Each system included one of the following CTD in RIG-I (PDB ID 3LRR, chain A; unmodified CTD, CTD with acetylated K909 and 5'ppp-dsRNA (PDB ID 3LRR, chain C and D). Each system consisted of approximately 2500 atoms in total. MD simulations of the RIG-I/5'ppp-dsRNA complex were performed using the CHARMM program of Discovery studio 4.0, with CHARMM force field as described described below; each model consisting of wild-type or mutant K907R was prepared by mutation module in Discovery studio 4.0. Each system was subjected to full energy minimization using 1000 steps of steepest descent algorithm with a RMS gradient tolerance of 1 (kcal/(mol x Å)) and then followed by a minimization using 2000 steps of conjugated gradient algorithm with RMS gradient tolerance of 0.1 (kcal/(mol x Å)). After the minimization step, each system went through a heating stage with the temperature ranging from 0 to 300 K (27°C) for 10 ps. After heating the system to 300K, the system for the stable state was equilibrated for 10,000 ps with constant pressure of 1 atm. And then, all systems were submitted for unbiased MD runs. Time interval between calculations was set as 1 femtosecond (fs). A 14 Å cutoff was applied to the non-bonded interactions. Long-range electrostatic interactions energy was calculated using the Particle Mesh Ewald method. The coordinates and energies of each atom were stored every 2 picosecond (ps) for further analysis. To avoid locally stable conditions, the process was repeated five times for each model, with different initial velocities for all atoms in the system upon the onset of heating.

## **Luciferase reporter assay**

HEK 293T cells were split into twelve-well plates and transfected with 400 ng of IFN- $\beta$  luciferase reporter construct, 10 ng of Renilla plasmid (pRL-TK), together with gene of interest or empty vector. To stimulate cells, RIG-I (50 ng), CARD of RIG-I (10 ng), MDA-5 (200 ng), MAVS (50 ng) were co-transfected with HDAC6 or HDAC6-CDM simultaneously or were infected with 2 MOI of PR8-GFP or transfected with 1  $\mu$ g of poly(I:C) 24 h after transfection. 24 h to 36 h after transfection, cells were lysed in 20 min at RT and luciferase assay was performed using the Dual-Luciferase Reporter Assay system (Promega) in accordance with the manufacturer's protocols. Firefly Luciferase activity were normalized to Renilla Luciferase activity and represented as a fold change relative to negative control.

## **Plasmid construction**

hHDAC6, hHDAC6-CDM plasmids were gift from Dr. Yao T.P. (Duke University). To generate pIRES-hHDAC6-Flag and pIRES-hHDAC6-V5, hHDAC6 genes were amplified by PCR using ExTaq (TAKARA) with specific primer. Clones were generated using AflII, XbaI, and T4 DNA ligase (TAKARA). Flag-tagged RIG-I K858R, K858Q, K909R, K909Q, K858, 909Q, K858, 909R expression vector was constructed with KOD-plus-mutagenesis kit (TOYOBO).

## **Immunofluorescence**

Cells were grown on coverslips and transfected with 5'-ppp dsRNA for the indicated time. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton in PBS (PBT). Cells were stained with indicated primary antibodies. After overnight incubation at 4°C, coverslips were washed three times in PBT, treated with rhodamine and FITC (Jackson ImmunoResearch), then incubated for 20 min at room temperature. After washing three times in PBT, coverslips were mounted with DAPI. Samples were analyzed using fluorescence microscopy.

## **Interferon and interleukin bioassay**

After cells were infected with viruses, supernatant was collected at indicated time points to measure cytokine levels. Blood from mice were kept at 4°C for 12 h and centrifuged twice at 12000 rpm for 15 min to separate plasma, followed by measurement of cytokines using specific ELISA kit. Murine IL-6 ELISA kit was purchased from BD bioscience, Murine IFN- $\beta$  ELISA kit was from PBL.



## **Immunoblotting, immunoprecipitation, and antibodies**

Cells were lysed in 150 mM NETN lysis buffer (150 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.5% (v/v) NP-40) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma) and 1mM DTT. Whole-cell lysates were subjected to SDS-PAGE and transferred onto a PVDF membrane. Membranes were blocked with 5% skim milk in 0.2% Triton in PBS (PBT) for 1 h and probed with the indicated antibodies.

For immunoprecipitation, cells were transfected with poly(I:C) or 5'-ppp dsRNA or infected with VSV-GFP for indicated amounts of time. Transfected or infected cells were lysed in 100mM NETN lysis buffer (100 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.5% (v/v) NP-40) supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail. Whole cell lysates were incubated with indicated antibodies at 4°C overnight. The following day, protein A or G beads were added and incubated at 4°C for 1 h to precipitate the protein complexes, and samples were washed three times with indicated lysis buffer, and analyzed by immunoblotting. Anti-mouse monoclonal RIG-I D-12, (sc-376845), GAPDH (6c5, sc-32233),  $\beta$ -actin (c4, sc-47778) and anti-rabbit polyclonal HDAC6 (H-300, sc-11420) were purchased from Santa Cruz Biotechnology. MAVS (#4983S, cell signaling), HDAC6 (D21B10, #7612S, cell signaling), pIRF3(#4947, Cell Signaling), IRF3(#4302, Cell signaling), pTBK1(#5483, Cell signaling), TBK1(#3504, Cell signaling), pIKB $\alpha$ (2859, Cell signaling), mouse monoclonal anti-Flag (M2, F1804, Sigma), mouse-monoclonal acetylated lysine (#MA-2021, pierce) and mouse-monoclonal GFP tag (#MA5-15256, pierce) were purchased from indicated suppliers.

Acetyl-specific K909 RIG-I antibodies were generated from rabbit immunized with the specific peptide 905CSKW(ac)KDFHFEEKIPFD919. Peptides were conjugated with keyhole limpet hemocyanin (KLH) and injected with Freund's Adjuvant (complete F5881, incomplete F5506). For antibody purification, affinity resins were prepared with non-acetylated form of the peptide and acetylated form of the peptide separately. Generated antibodies were mixed with PMSF and passed over non-acetylated peptide column to remove any antibodies that have an affinity to non-acetylated peptide. Then unbound solution was passed over acetylated peptide column. Antibodies bound to acetylation peptides were eluted and used for IB.

## **Quantitative real time PCR**

Total RNA was extracted from cells and tissues with RNeasy RNA extraction kit (Qiagen), and cDNA synthesis was performed using ReverTraAce qPCR RT kit (TOYOBO). Quantitative PCR was performed using T-Gradient thermoblock (Biometra) and EmeraldAmp PCR Master Mix (TAKARA). Fluorescence real-time PCR was performed using Rotor-Gene Q instrument (Qiagen) and QuantiTect SYBR green PCR kit (Qiagen). Relative expression of RNA was normalized to GAPDH. Primers used are listed in Table 1.

## **Viruses, infection and replication assay**

VSV and NDV-GFP were amplified on Vero cells and HeLa cells, respectively. PR8-GFP were amplified in SPF chicken eggs. Attached cells were infected with viruses in DMEM supplemented with 1% FBS. 2 h after, each well was replaced with complete media to remove extracellular viruses. Suspended cells were infected with viruses in 1.5 ml tube using RPMI supplemented with 1% FBS. 2 h after, cells were centrifuged at 3000 rpm, 3 min and re-plated into cell culture plates with complete media. After infection, Supernatant and cells were collected at indicated time points and used to measure viral titration and GFP absorbance. Viral titration was performed in Vero cells using standard plaque assay. GFP absorbance of number-matched cells was measured using GloMax Multi Detection system (Promega), Blue optical kit (Ex: 490 nm, Em: 510 - 570 nm).

## **RNA interference**

HDAC6 siRNA,  $\beta$ -catenin siRNA, and nonspecific scrambled siRNA were produced from Bioneer Co. (Refer to Appendix table S1). All siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. At 36h post-transfection, cells were used in experiments.