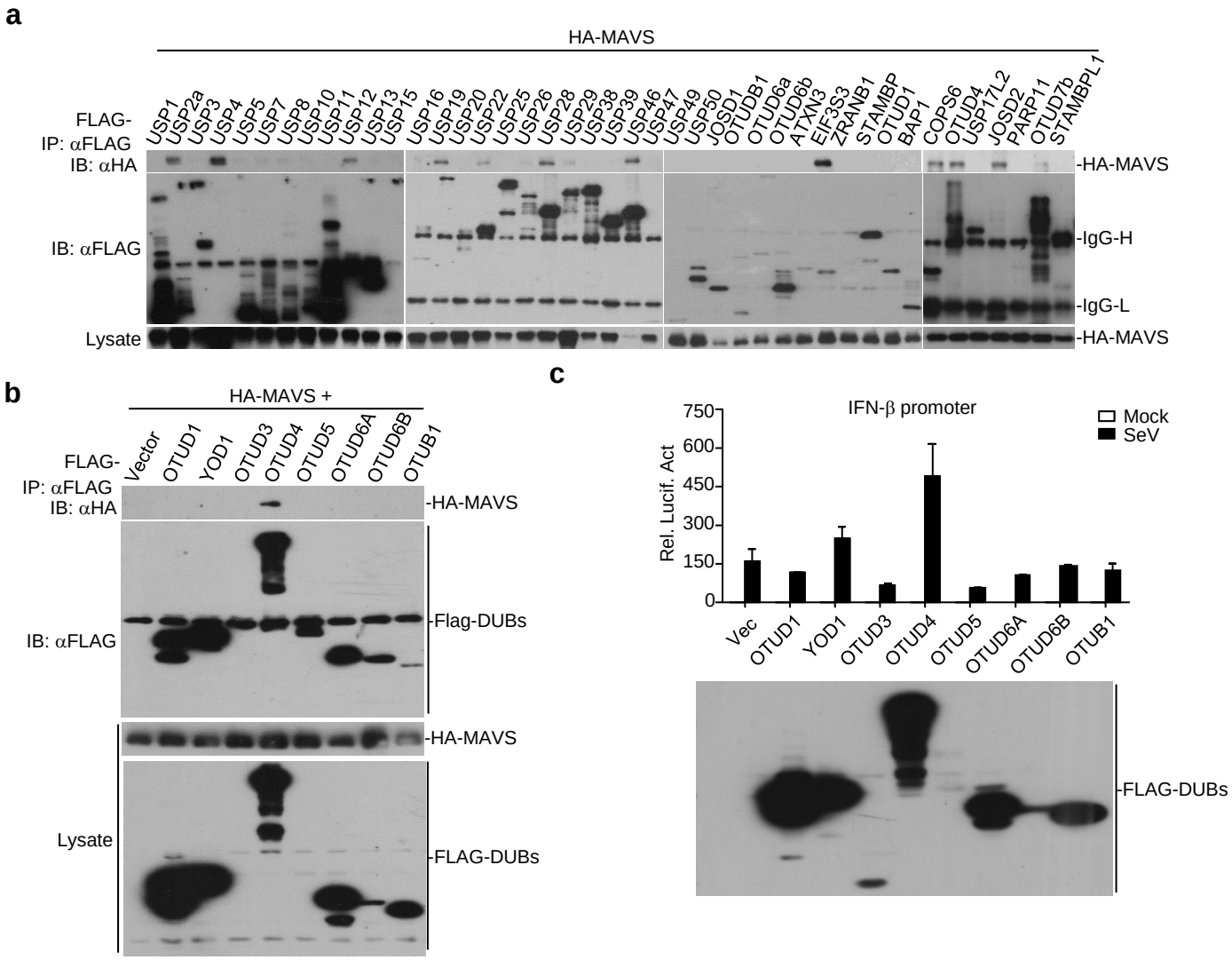
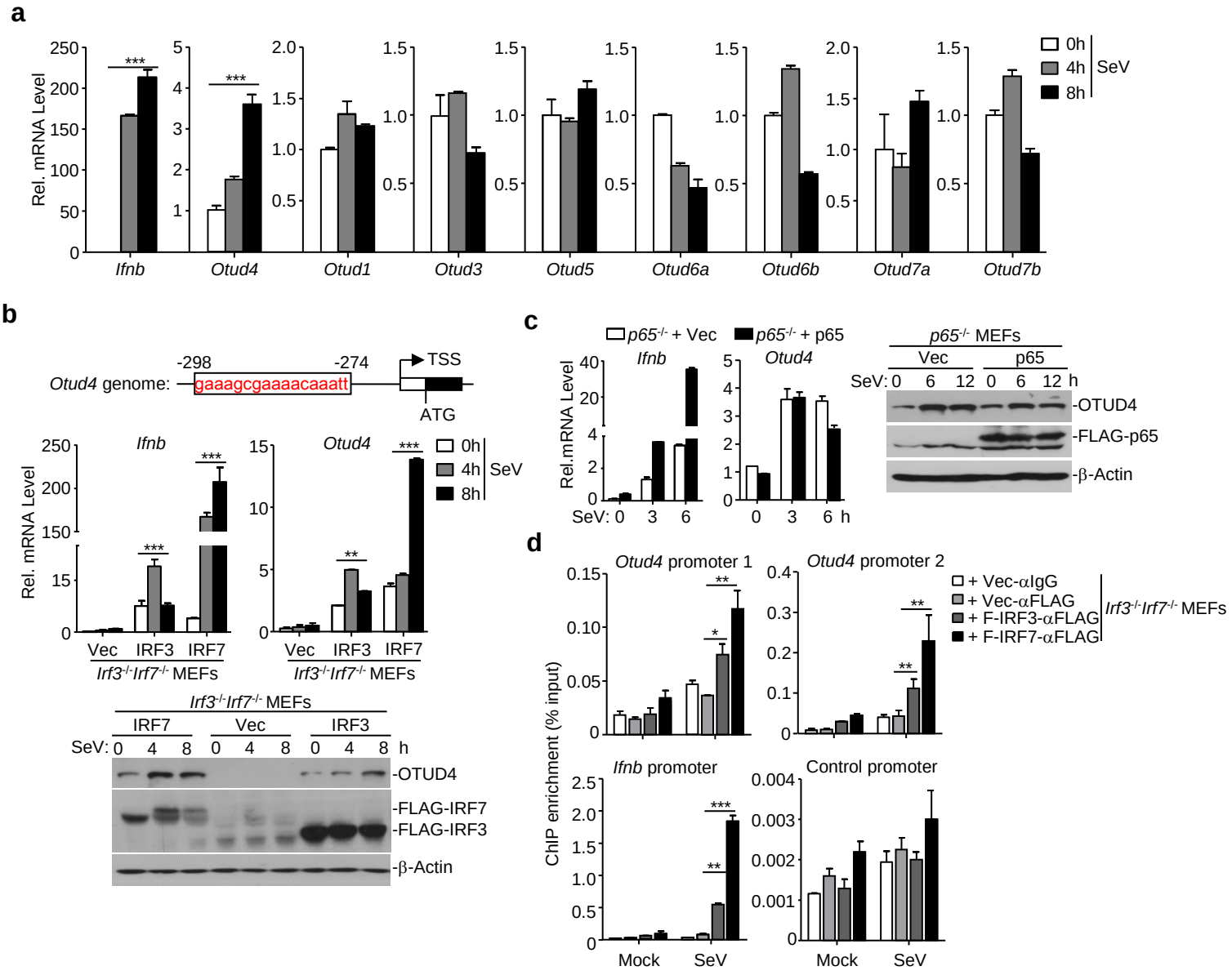
Supplementary information, Figure S1



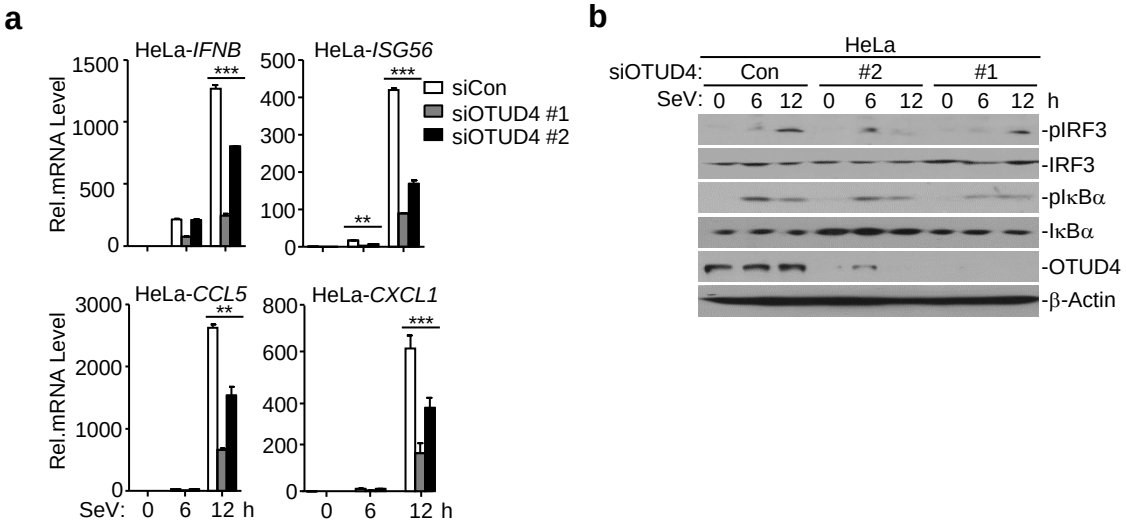
Supplementary Figure S1. Screening of MAVS-interacting DUBs.
(**a-b**) Immunoblot (IB) of HEK293 cells that were transfected to express HA-MAVS and FLAG-tagged OTU family DUBs (OTU domain obtained), lysed and immunoprecipitated (IP) with anti-FLAG. Cell lysate was analyzed by immunoblot with anti-FLAG or anti-HA.
(**c**) Luciferase reporter assays analyzing IFN-β promoter activity in HEK293 cells transfected with OTU family DUBs for 24 hours followed by infection with SeV for 8 hours.
Data are representative of at least three independent experiments (graphs show mean ± S.D. in **c**).

Supplementary information, Figure S2



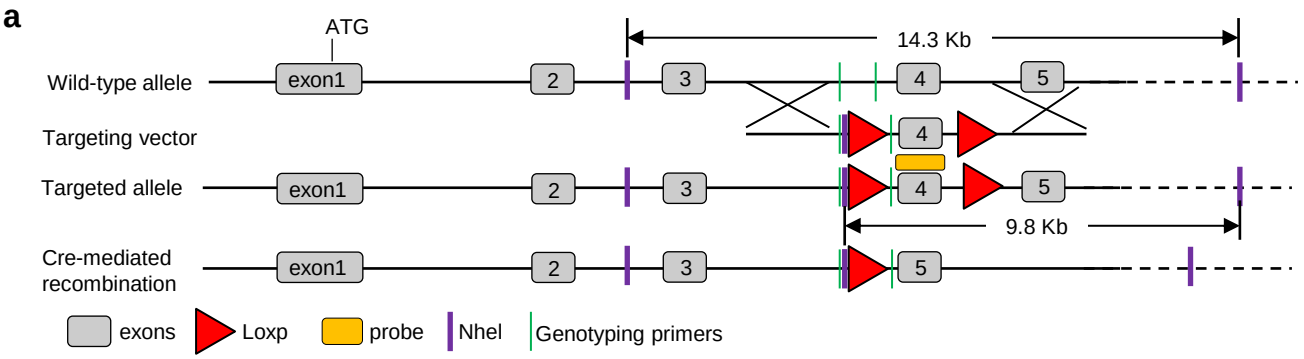
Supplementary Figure S2. Viral infection upregulates the expression of *Otud4* depending on IRF3/7.
(**a**) qRT-PCR analysis of *Ifnb* or OTU family members mRNA in MEFs infected with SeV for 0-8 hours.
(**b-c**) qRT-PCR analysis of *Ifnb* or *Otud4* mRNA or immunoblot analysis (with anti-FLAG, anti-OTUD4 or anti-β-Actin) in *Irf3*$^{-/-}$*Irf7*$^{-/-}$ MEFs reconstituted with empty vector, FLAG-IRF3 or FLAG-IRF7 (**b**) or *p65*$^{-/-}$ MEFs reconstituted with empty vector or FLAG-p65 (**c**) followed by infection with SeV for 0-12 hours.
(**d**) Chromatin immunoprecipitation (ChIP) (with anti-FLAG or IgG as a control) analysis of the binding of IRF3 or IRF7 on the promoter of *Ifnb* or *Otud4* or on a non-specific control promoter in *Irf3*$^{-/-}$*Irf7*$^{-/-}$ MEFs reconstituted with empty vector, FLAG-IRF3 or FLAG-IRF7 followed by SeV infection of 0-4 hours.
*, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ (analysis of two-way ANOVA followed by Bonferroni post-test). Data are representative of at least three independent experiments (graphs show mean ± S.D.).

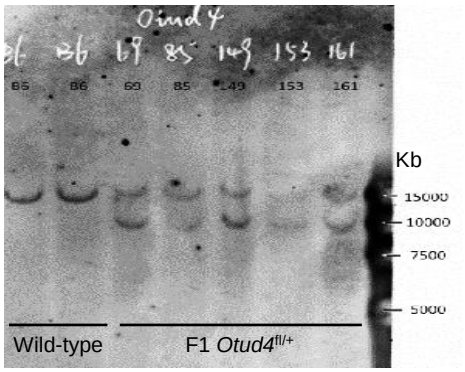
Supplementary information, Figure S3

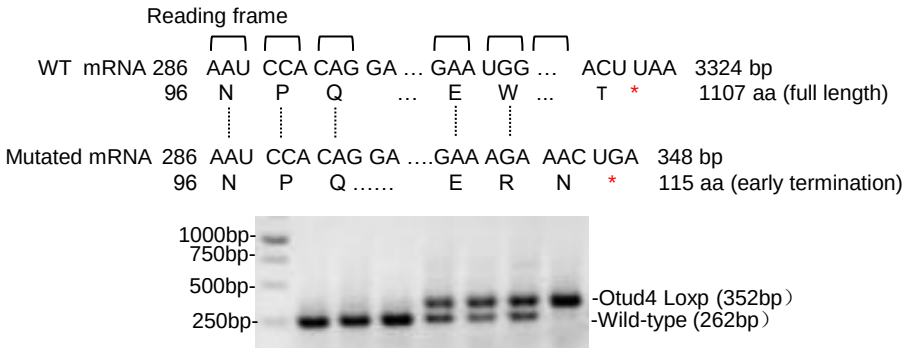


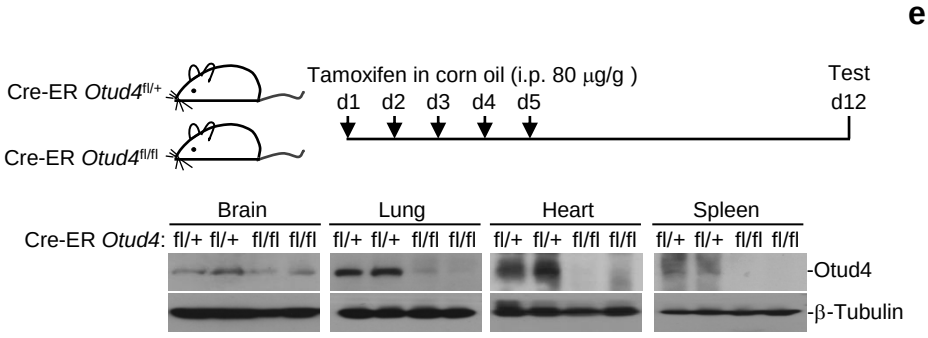
Supplementary Figure S3. Knockdown of OTUD4 impairs RNA virus-induced phosphorylation of IRF3 and IκBα in HeLa cells.
(**a, b**) qRT-PCR analysis of *IFNB*, *ISG56*, *CCL5* and *CXCL1* mRNA (**a**) and immunoblot analysis of total and phosphorylated (p-)IκBα, IRF3, total OTUD4, β-Actin (**b**) in HeLa cells transfected with control siCon or siOTUD4 (#1 or #2) for 36 hours followed by SeV infection for 0-12 hours.
, $P < 0.01$ and ***, $P < 0.001$ (analysis of two-way ANOVA followed by Bonferroni post-test). Data are representative of three independent experiments (mean ± S.D. in **a).

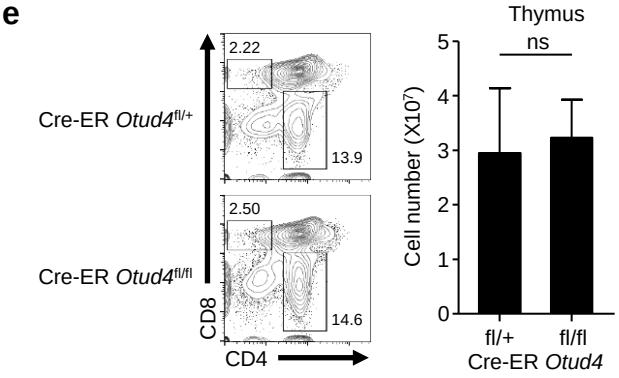
Supplementary information, Figure S4

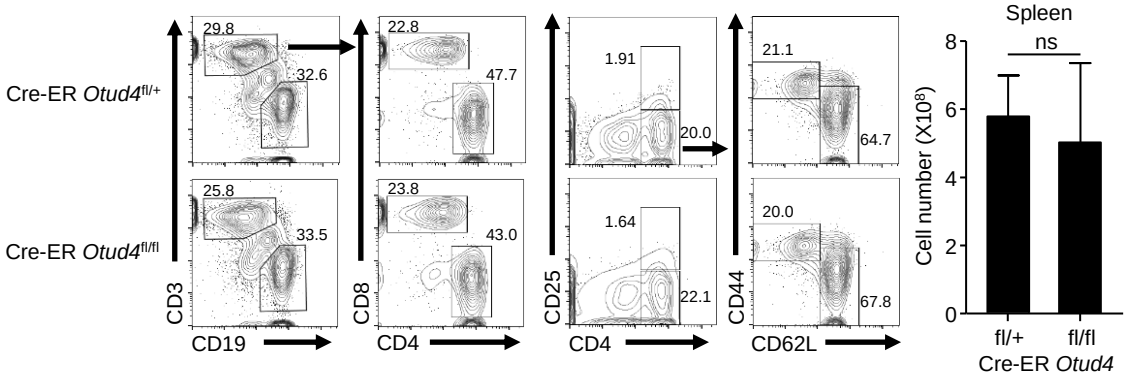


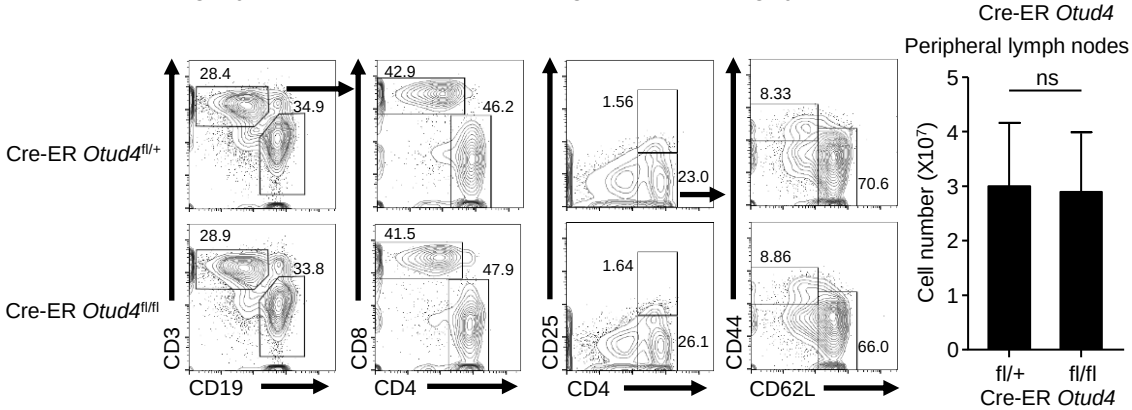


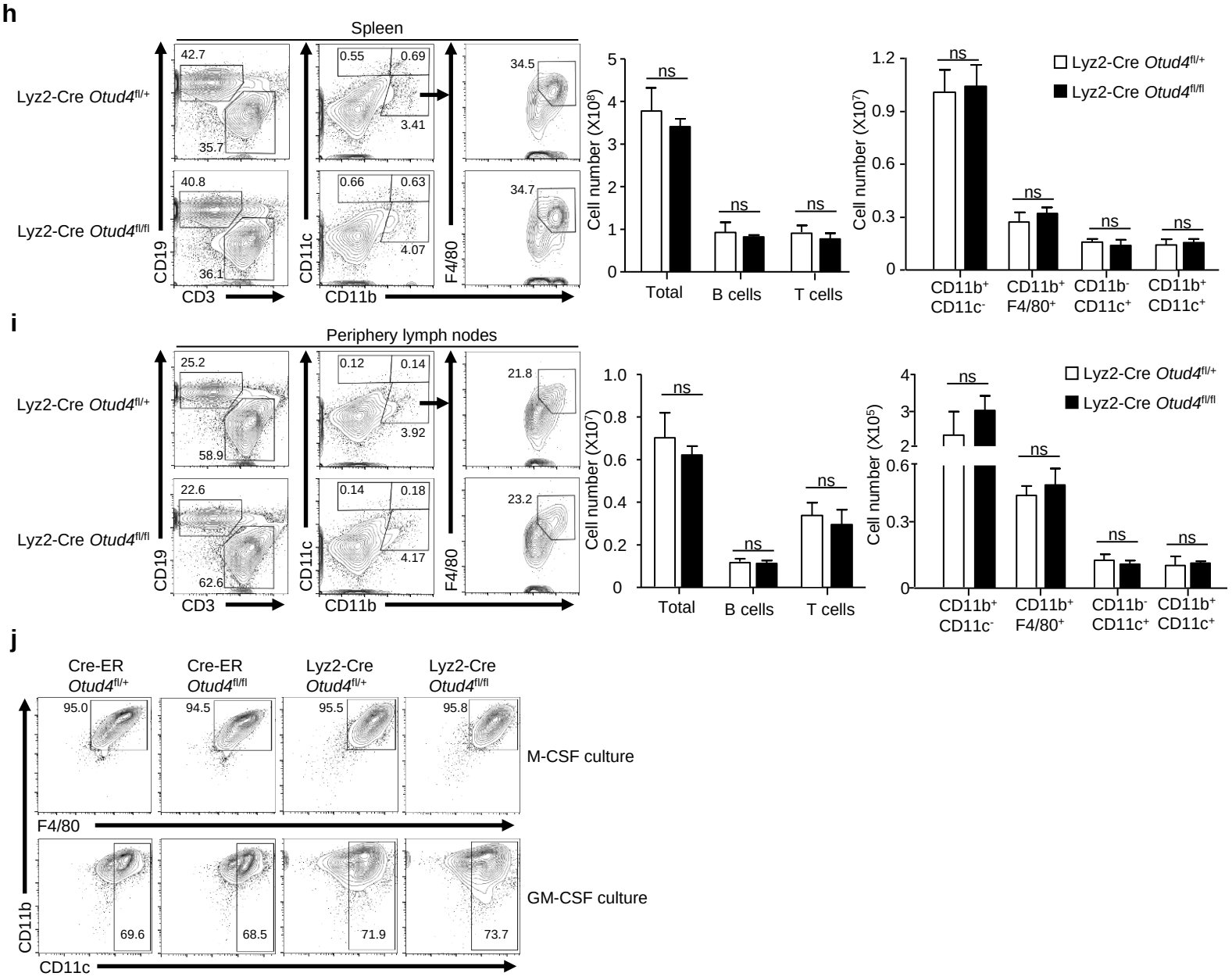












Supplementary Figure S4. Generation of *Otud4*$^{fl/+}$ mice.

(**a**) A scheme for CRIPSR/Cas9-mediated genome editing of the *Otud4* gene locus.

(**b**) Southern blot analysis of the generated F1 *Otud4*$^{fl/+}$ mice.

(**c**) Genotyping analysis of *Otud4*$^{+/+}$, *Otud4*$^{fl/+}$ and *Otud4*$^{fl/fl}$ mice.

(**d**) Immunoblot analysis of OTUD4 and β-Tubulin in brains, lungs, hearts and spleens from Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ mice that were intraperitoneally injected with tamoxifen (80 μg/g in corn oil) for 5 successive days and rested for another 7 days.

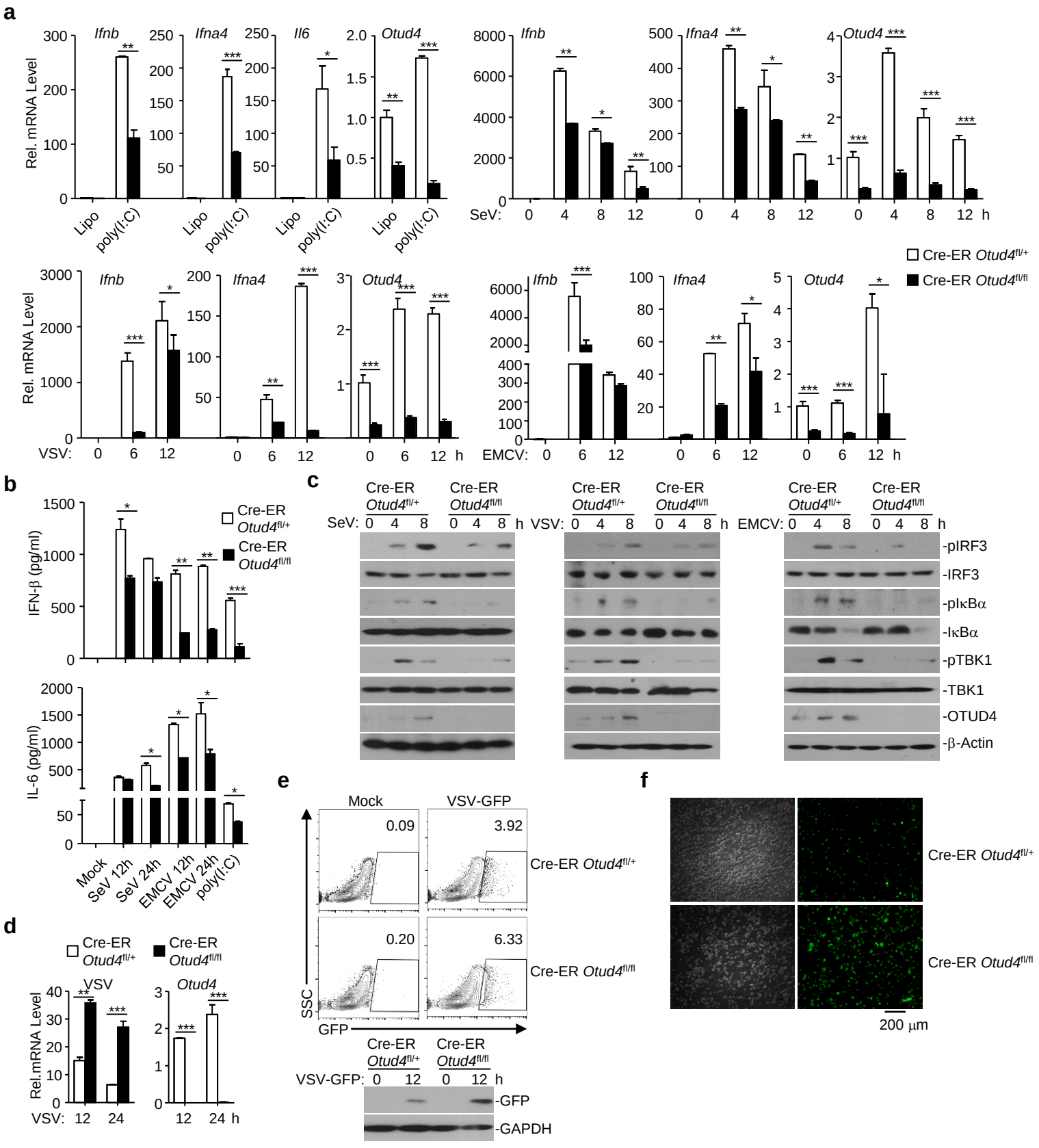
(**e-g**) Flow cytometry analysis of lymphocytes composition in the thymus, spleen or peripheral lymph nodes of Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ mice (n=3) that were intraperitoneally injected with tamoxifen (80 μg/g in corn oil) for 5 successive days and rested for another 7 days.

(**h-i**) Flow cytometry analysis of myeloid or lymphocytes in the spleen or peripheral lymph nodes of Lyz2-Cre *Otud4*$^{fl/+}$ and Lyz2-Cre *Otud4*$^{fl/fl}$ mice (n=3).

(**j**) Flow cytometry analysis of in vitro generated Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ (treated with 4-OH Tam) or Lyz2-Cre *Otud4*$^{fl/+}$ and Lyz2-Cre *Otud4*$^{fl/fl}$ BMDMs or BMDCs .

Data are representative of two independent experiments (**d-j**). Graphs show mean ± S.D. (**e-i**).

Supplementary Figure S5



Supplementary Figure S5. Knockout of OTUD4 inhibits RNA virus-triggered signaling in BMDMs

(**a**) qRT-PCR analysis of *Ifnb*, *Ifna4*, *Il6* and *Otud4* mRNA in Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ BMDMs (4-OH Tam treated) that were transfected with polyIC for 3 hours or infected with SeV, VSV or EMCV for 0-12 hours.

(**b**) ELISA analysis of IFN-β and IL-6 in the supernatants of Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ BMDMs (4-OH Tam treated) that were transfected with polyIC for 12 hours or infected with SeV, VSV or EMCV for 12-24 hours.

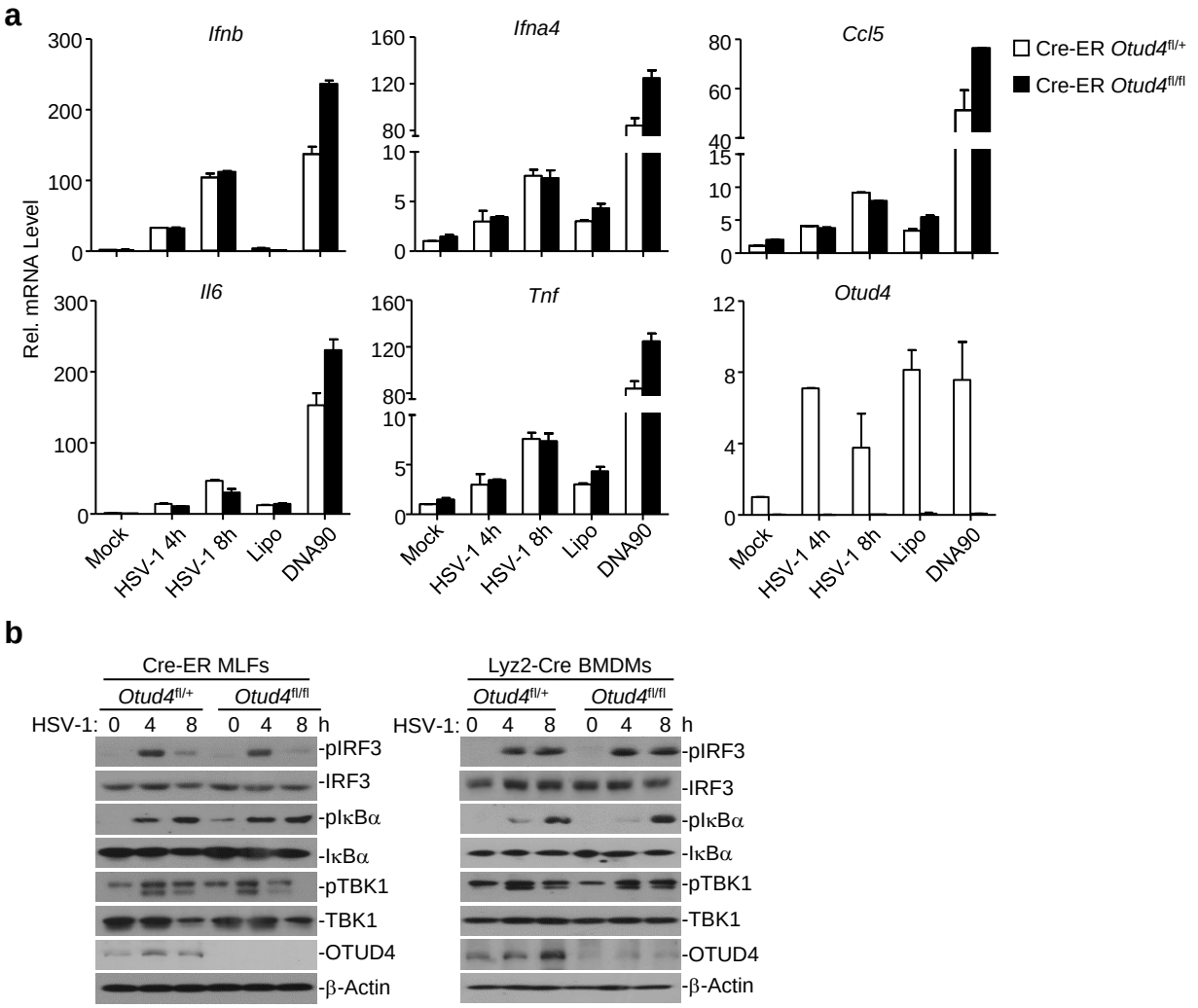
(**c**) Immunoblot analysis of total and phosphorylated (p-)TBK1, IκBα, IRF3, total OTUD4, β-Actin in Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ BMDMs (4-OH Tam treated) that were infected with SeV, VSV or EMCV for 0-8 hours.

(**d**) qRT-PCR analysis of VSV-N mRNA in Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ BMDMs (4-OH Tam treated) that were infected with VSV for 1 hour followed by twice PBS wash and cultured in full medium for 12 or 24 hours.

(**e, f**) Flow cytometry and immunoblot analysis (with anti-GFP and anti-β-Actin) (**e**) and fluorescent microscopy imaging (**f**) of VSV-GFP in Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ BMDMs (4-OH Tam treated) that were uninfected or infected with VSV-GFP (MOI=1). Numbers adjacent to the outlined areas indicate percentages of GFP$^+$ BMDMs.

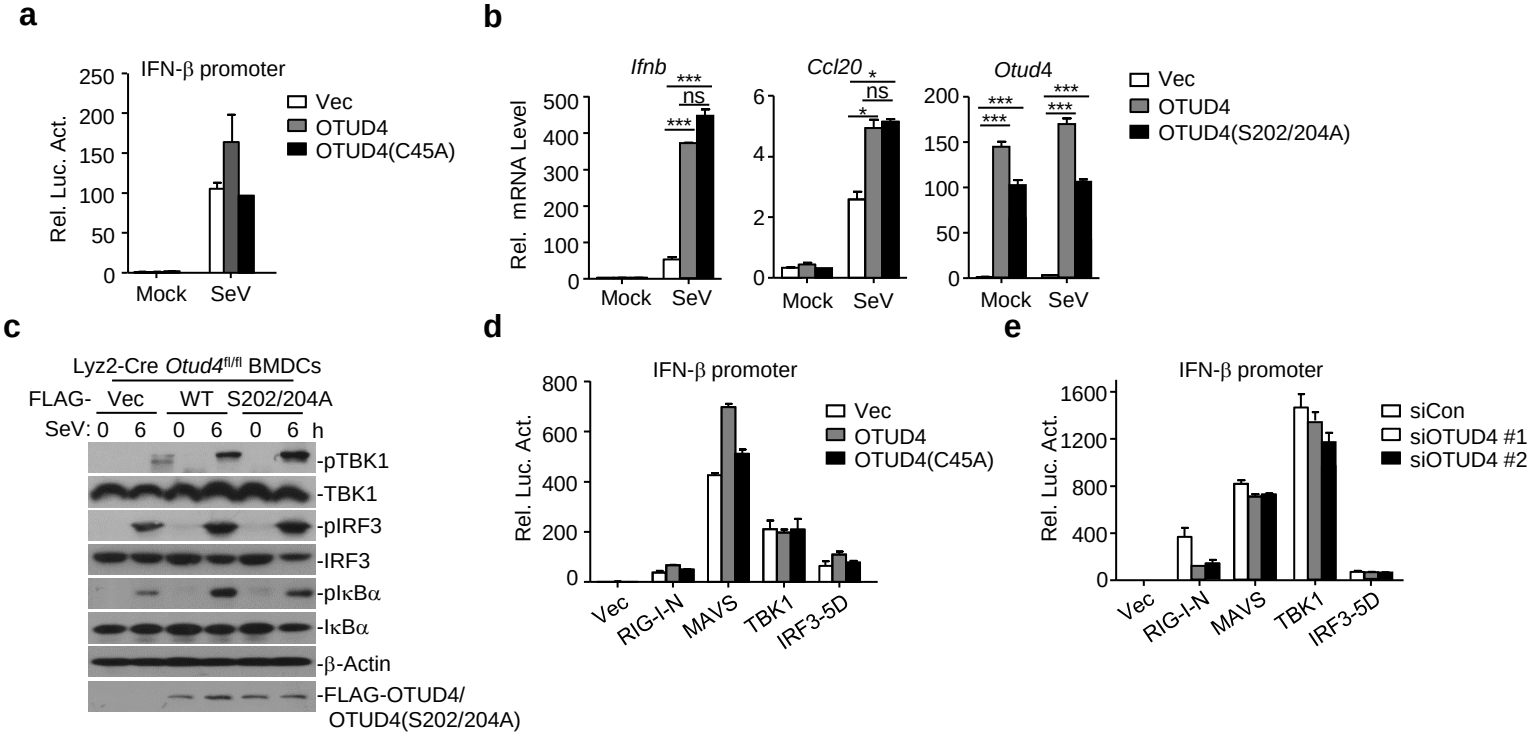
*, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ (analysis of two-way ANOVA followed by Bonferroni post-test). Data are representative of three (**a-c**) or two (**d-f**) independent experiments (mean ± S.D. in **a-b, d**).

Supplementary Figure S6



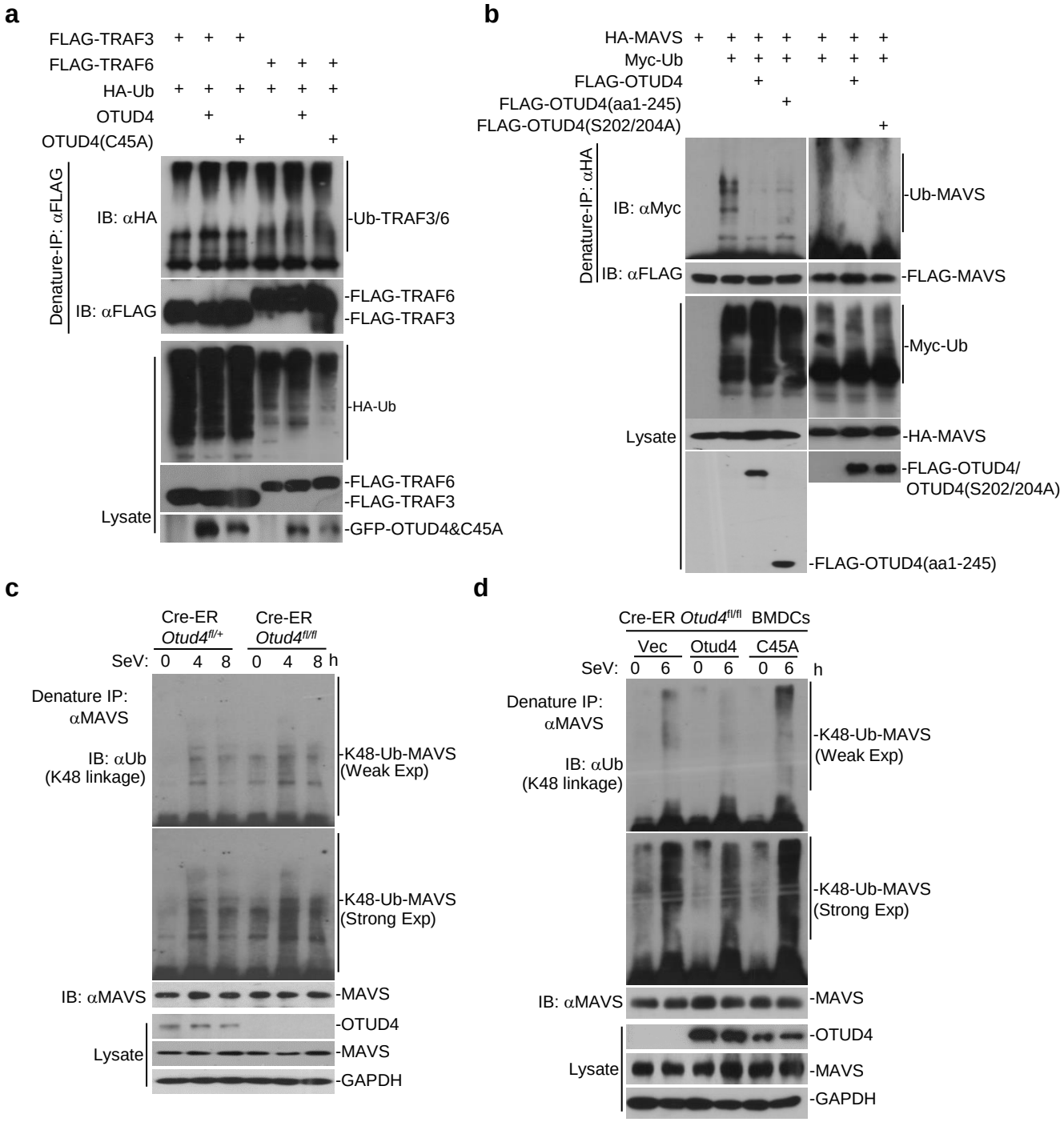
Supplementary Figure S6. Knockout of OTUD4 has little effects on HSV-1-triggered signaling.
(**a**) qRT-PCR analysis of *Ifnb*, *Ifna4*, *Ccl5*, *Tnf*, *Il6* and *Otud4* mRNA in Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ MLFs (4-OH Tam treated) that were infected with HSV-1 for 0-8 hours or transfected with dsDNA for 3 hours.
(**b**) Immunoblot analysis of total and phosphorylated (p-)TBK1, IκBα, IRF3, total OTUD4, β-Actin in Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ MLFs (4-OH Tam treated) or Lyz2-Cre *Otud4*$^{fl/+}$ and Lyz2-Cre *Otud4*$^{fl/fl}$ BMDMs that were infected with HSV-1 for 0-8 hours.
Data are representative of three (**a**) or two (**b**) independent experiments (mean ± S.D. in **a**).

Supplementary information, Figure S7



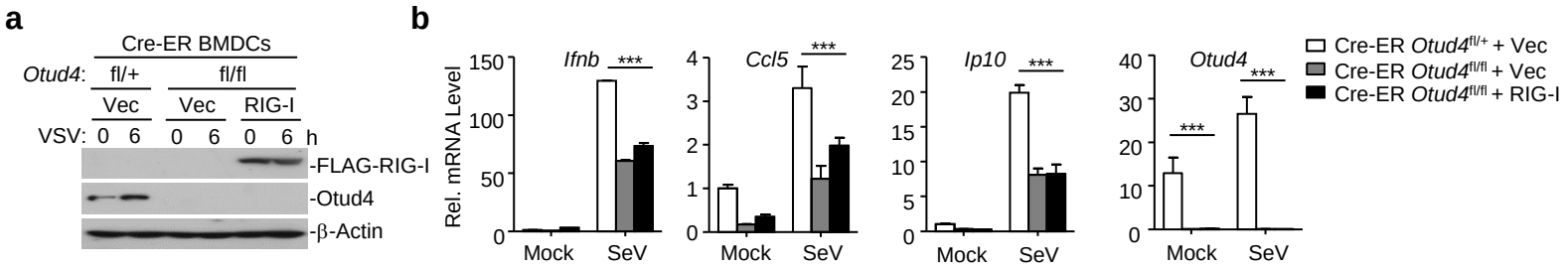
Supplementary Figure S7. OTUD4 functions at the level of MAVS.
(**a**) Luciferase reporter assays analyzing IFN-β promoter activity in HEK293 cells transfected with an empty vector (Vec), OTUD4, or OTUD4(C45A) for 24 hours followed by infection with SeV for 8 hours.
(**b**) qRT-PCR analysis of *Ifnb* and *Otud4* in Lyz2-Cre *Otud4*$^{fl/fl}$ BMDCs reconstituted with an empty vector or FLAG-tagged OTUD4 or OTUD4(S202/204A) followed by SeV infection for 0-6 hours.
(**c**) Immunoblot analysis of total and phosphorylated TBK1, IRF3 and IkBa and β-Actin or FLAG in in Lyz2-Cre *Otud4*$^{fl/fl}$ BMDCs reconstituted with an empty vector or FLAG-tagged OTUD4 or OTUD4(S202/204A) followed by SeV infection for 0-6 hours.
(**d**) Luciferase reporter assays analyzing IFN-β promoter activity in HEK293 cells transfected with plasmids encoding the indicated signaling proteins and Vec, OTUD4 or OTUD4(C45A) for 24 hours.
(**e**) Luciferase reporter assays analyzing IFN-β promoter activity in HEK293 cells transfected with control siCon or siOTUD4 (#1 or #2) for 12 hours followed by transfection of the plasmids encoding the indicated signaling adaptors for 24 hours.
*, $P < 0.05$, **, $P < 0.01$ and ***, $P < 0.001$ (analysis of two-way ANOVA followed by Bonferroni post-test). Data are representative of three independent experiments (mean ± S.D. in **a-b, d-e**).

Supplementary information, Figure S8



Supplementary Figure S8. OTUD4 mediates deubiquitination of MAVS.
(**a**) Denature-immunoprecipitation (Denature-IP) (with anti-FLAG) and immunoblot analysis (IB) (with anti-FLAG, anti-HA or anti-GFP) of HEK293 cells that were transfected to express FLAG-MAVS, HA-Ubiquitin and GFP-OTUD4 or GFP-OTUD4(C45A) for 24 hours.
(**b**) Denature-immunoprecipitation (Denature-IP) (with anti-HA) and immunoblot analysis (IB) (with anti-FLAG, anti-HA or anti-Myc) of HEK293 cells that were transfected to express HA-MAVS, Myc-Ubiquitin and FLAG-OTUD4, FLAG-OTUD4(aa1-245) or FLAG-OTUD4(S202/204A) for 24 hours.
(**c**) Denature-IP (with anti-MAVS) and IB (with anti-K48 linkage polyubiquitination, anti-MAVS, anti-OTUD4, anti-GAPDH) of Cre-ER *Otud4*$^{fl/+}$ and Cre-ER *Otud4*$^{fl/fl}$ BMDCs treated with 4-OH Tam (1 μM) for 3 days followed by by treatment of MG132 for 2 hours prior to SeV infection for 0-8 hours.
(**d**) Denature-IP (with anti-MAVS) and IB (with anti-K48 linkage polyubiquitination, anti-MAVS, anti-OTUD4, anti-GAPDH) of Cre-ER *Otud4*$^{fl/fl}$ BMDCs (4-OH Tam treated) that were reconstituted with Vec, OTUD4 or OTUD4(C45A) followed by treatment of MG132 for 2 hours prior to SeV infection for 0-6 hours.
Data are representative of three independent experiments.

Supplementary information, Figure S9



Supplementary Figure S9. Reconstitution RIG-I into OTUD4 KO cells does not restore SeV-induced expression of downstream genes.
(**a**) Immunoblot analysis of total FLAG-RIG-I, OTUD4 or β-Actin in Cre-ER Otud4$^{fl/+}$ and Cre-ER Otud4$^{fl/fl}$ MLFs (4-OH Tam treated) reconstituted with Vec, or RIG-I followed by infection with SeV for 0-6 hours.
(**b**) qRT-PCR analysis of *Ifnb*, *Ccl5*, *Ip10* and *Otud4* mRNA in cells obtained in (**a**) infected with SeV for 0-6 hours.
***, $P < 0.001$ (analysis of two-way ANOVA followed by Bonferroni post-test). Data are representative of three independent experiments (mean ± S.D. in **b**).

Supplementary information, Table S1 Genotyping primer list

Name	Forward sequence (5‘ to 3')	Reverse sequence (5‘ to 3')
Otud4	GTCTCATTCTTGGCCTCGT	ACATGCTGGCAAAACATTCATC
ER^{-}	AAAGTCGCTCTGAGTTGTTAT	GGAGCGGGAGAAATGGATATG
ER^{+}	AAAGTCGCTCTGAGTTGTTAT	CCTGATCCTGGCAATTTCG
Lyz2-Cre^{-}	CTTGGGCTGCCAGAATTTCTC	TTACAGTCGGCCAGGCTGAC
Lyz2-Cre^{+}	CTTGGGCTGCCAGAATTTCTC	CCCAGAAATGCCAGATTACG

Supplementary information, Table S2 qRT-PCR primer list

Name	Forward sequence (5‘ to 3')	Reverse sequence (5‘ to 3')
m*β-Actin*	ACGGCCAGGTCATCACTATT	TGGCATAGAGGTCTTTACGGA
m*Otud4*	GGAGGTTAGGATGGCCTGTATC	GGCACTTATTTCCACTTGTCCT
m*Ifnb*	TACAACAGATACGCCTGGAT	AGTCCGCCTCTGATGCTTAA
m*Otud1*	GGGTAATGGCACTTTTCACTTT	CCCAAAGCTATCAGGAAACATC
m*Otud3*	TGACATTCCCTTTGAGAAGCAC	AGGGGCATTAAGCTGATGGA
m*Otud5*	GGACGGTGCCTGTCTATTTC	GCAGTTGTTTTTCCGCTTCC
m*Otud6a*	AGGGAGCTACAAGCACACATC	GGCTGTAACCGAATCCACAC
m*Otud6b*	AGACGACTTCCTGCCGTTTCT	TGCCTGTAGTATCTCGATTGGT
m*Otud7a*	GGCAGACACCATGCTAAGAGA	TGGACACTAGGGCTGAAAAGTG
m*Otud7b*	AGGAGAAGTCAAAGCGAGACC	ACTGCTTATTCCAGACCCACTC
m*Ccl5*	CTGCTGCTTTGCCTACCTCT	CTTGAACCCACTTCTTCTCTGG
m*Ccl20*	AAATCTGTGTGCGCTGATCC	TTCAACCCCAGCTGTGATCA
m*Isg15*	GGCCACAGCAACATCTATGA	ACTGGGGCTTTAGGCCATAC
m*Ip10*	GTGAGAATGAGGGCCATAGG	TTTTTGGCTAAACGCTTTCAT
m*Ifna4*	AGGATCACTGTGTACCTGAGA	TCTCCACACTTTGTCTCAGGA
m*Tnf*	ACTGAACTTCGGGGTGATCG	TCTTTGAGATCCATGCCGTTG
m*Il6*	ACAAAGCCAGAGTCCTTCAGA	TCCTTAGCCACTCCTTCTGT
VSV-N	TGATAGTACCGGAGGATTGACGAC	CCTTGCAGTGACATGACTGCTCTT
β-ACTIN	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
IFNB	TTGTTGAGAACCTCCTGGCT	TGACTATGGTCCAGGCACAG
CXCL10	GGTGAGAAGAGATGTCTGAATCC	GTCCATCCTTGGAAGCACTGCA
IL-6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
ISG56	TCATCAGGTCAAGGATAGTC	CCACACTGTATTTGGTGTCTAGG
OTUD4	CTAACTCCTGCGGTGCCTTCTT	GCTGAATCAGGTCCAGTGGTCA