Supplementary Information

The E3 ubiquitin ligase ARIH1 promotes antiviral immunity and autoimmunity by inducing mono-ISGylation and oligomerization of cGAS

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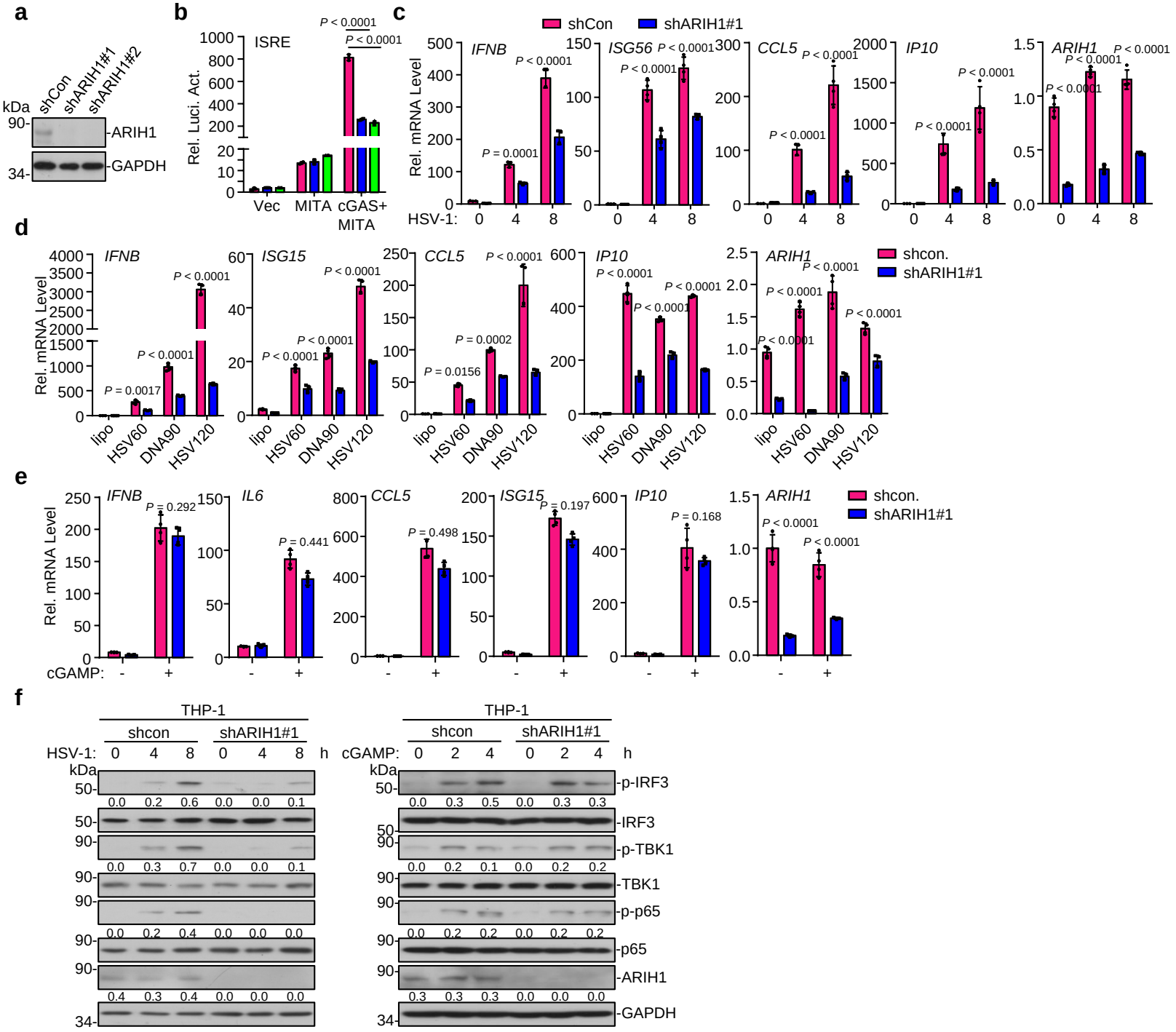
Supplementary Figure 1



Supplementary Figure 1 Knockdown of ARIH1 inhibits HSV-1-induced expression of downstream genes.

(**a**) Immunoblot analysis of ARIH1 and GAPDH in HEK293 cells that were transfected with shCon, shARIH1#1 or shARIH1#2 for 36 h.

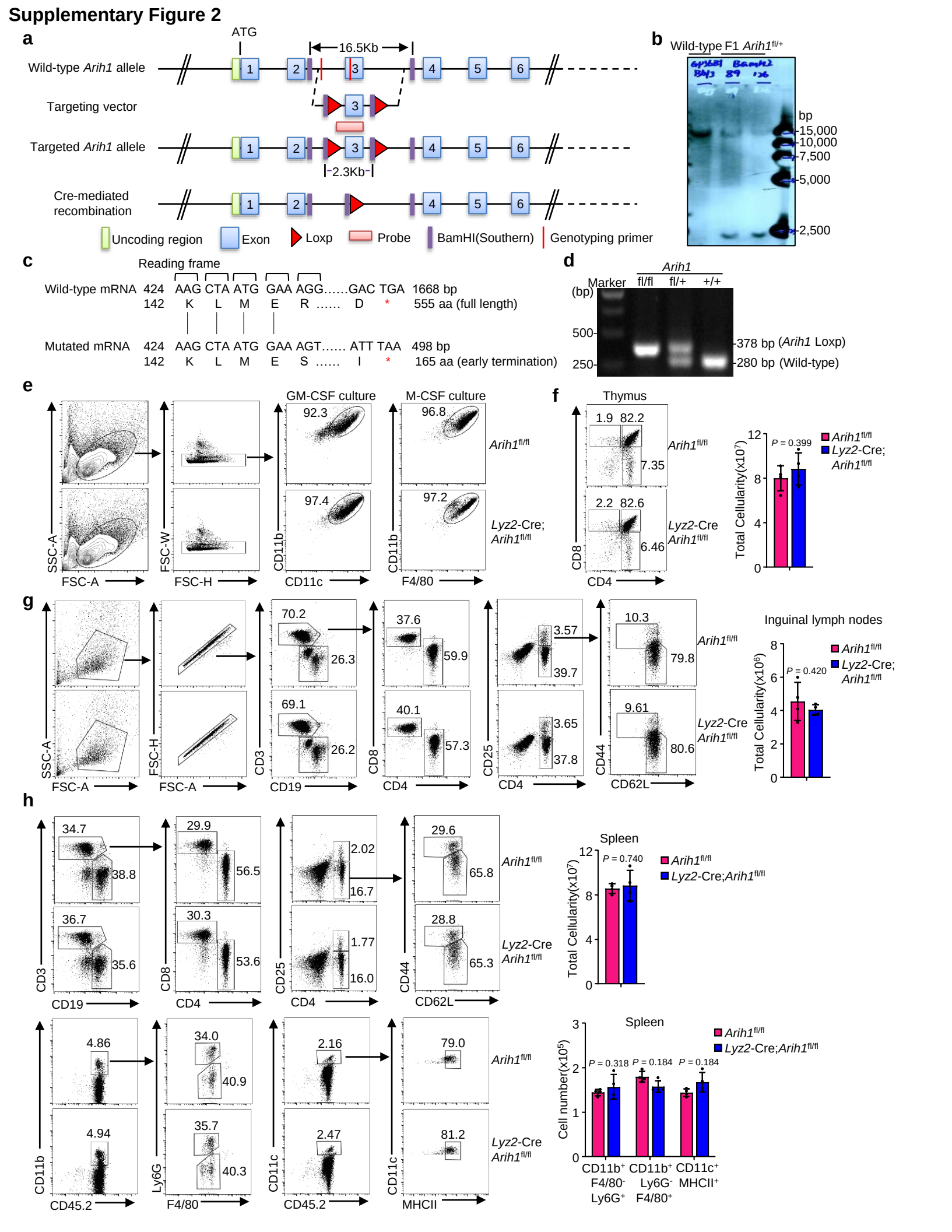
(**b**) Luciferase reporter assays analyzing ISRE promoter activity in HEK293 cells transfected with shCon, shARIH1#1 or shARIH1#2 together with the indicated plasmids for 36 h.

(**c-e**) qRT-PCR analysis of *IFNB*, *ISG56*, *CCL5*, *IP10* and *ARIH1* mRNA in THP-1 cells stably transfected with shCon or shARIH1#1 followed by infection with HSV-1 for 0-8 h **(c),** transfection with HSV60, DNA90 and HSV120 for 4 h (**d**) or transfection with cGAMP for 4 h (**e**).

(**f**) Immunoblot analysis of total and phosphorylated (p-) IRF3,TBK1 and P65, ARIH1 and GAPDH in THP-1 cells stably transfected with shCon or shARIH1#1 followed by infection with HSV-1 for 0-8 h or transfection with cGAMP for 0-4 h .

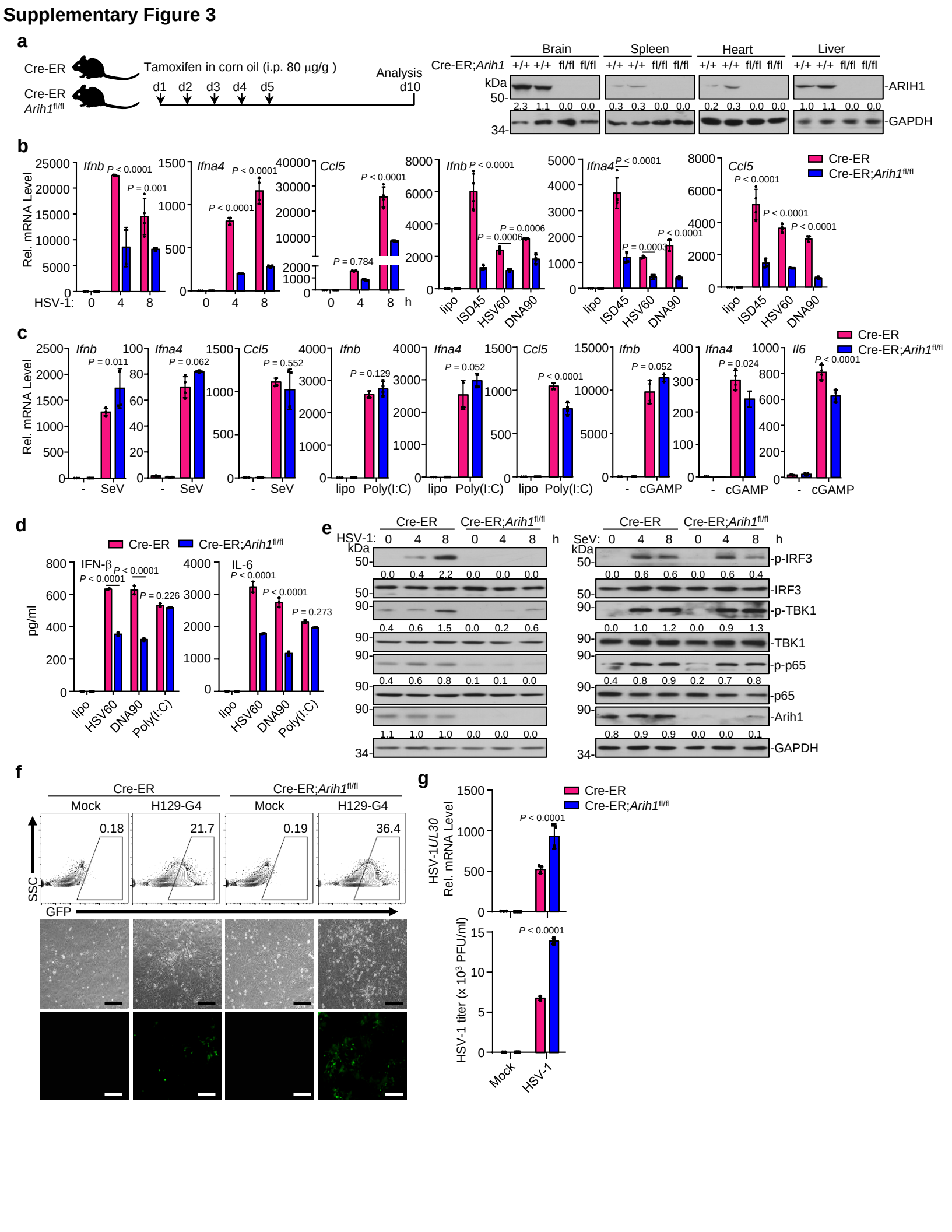
Data are representative of three (**a**, **c-e**) or two (**b**, **f**) independent experiments. Graphs show mean ± S.D. (n = 3 for **b**, or n = 4 for **c-e**, biologically independent experiments). Statistical significance was determined using two-tailed Student's *t*-test in **c-e**, or one-way ANOVA in **b**. The quantitative anaylsis is derive from the same experiment and that blots were processed in parallel in **f**. Source data are provided as a Source Data file.

Supplementary Figure 2



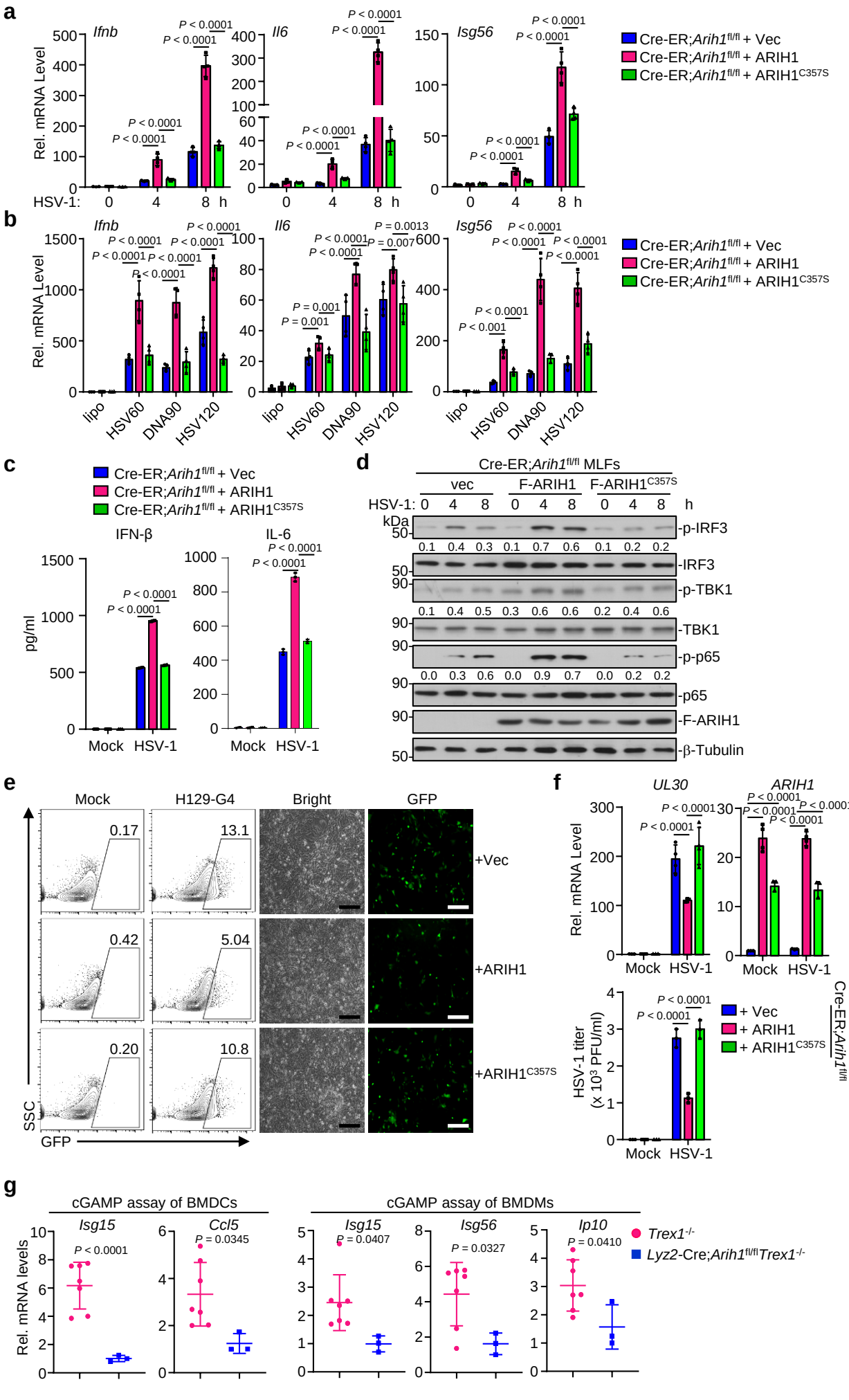
Supplementary Figure 2 Generation of *Arih1*$^{fl/+}$ mice.
(**a**) A scheme of CRIPSR/Cas9-mediated genome editing of the *Arih1* gene locus.
(**b**) Southern blot analysis of the generated F1 *Arih*$^{fl/+}$ mice.
(**c**) mRNA sequence and reading frame of wild-type and edited *Arih1* alleles.
(**d**) Genotyping analysis of the tail DNAs from *Arih*$^{fl/fl}$, *Arih*$^{fl/+}$ and unedited mice.
(**e**) Flow cytometry analysis of in vitro generated *Arih*$^{fl/fl}$ and *Lyz2*-Cre;*Arih*$^{fl/fl}$ BMDCs or BMDMs. The same Gating strategy was used in Fig. 2e and Supplementary Fig. 3f, 4e.
(**f-h**) Flow cytometry analysis of myeloid cells or lymphocytes and quantitative data in thymus (**f**), inguinal lymph nodes (**g**), and spleen (**h**) from *Arih*$^{fl/fl}$ and *Lyz2*-Cre;*Arih*$^{fl/fl}$ mice (n=4). Data are representative of two independent experiments (**e-h**). The same Gating strategy was used in Fig. 5e and Supplementary Fig. 2f, h.
Graphs show mean ± S.D. (n = 4 for **f-h**, biologically independent experiments). Statistical significance was determined using two-tailed Student's *t*-test in **f-h**. Source data are provided as a Source Data file.

Supplementary Figure 3



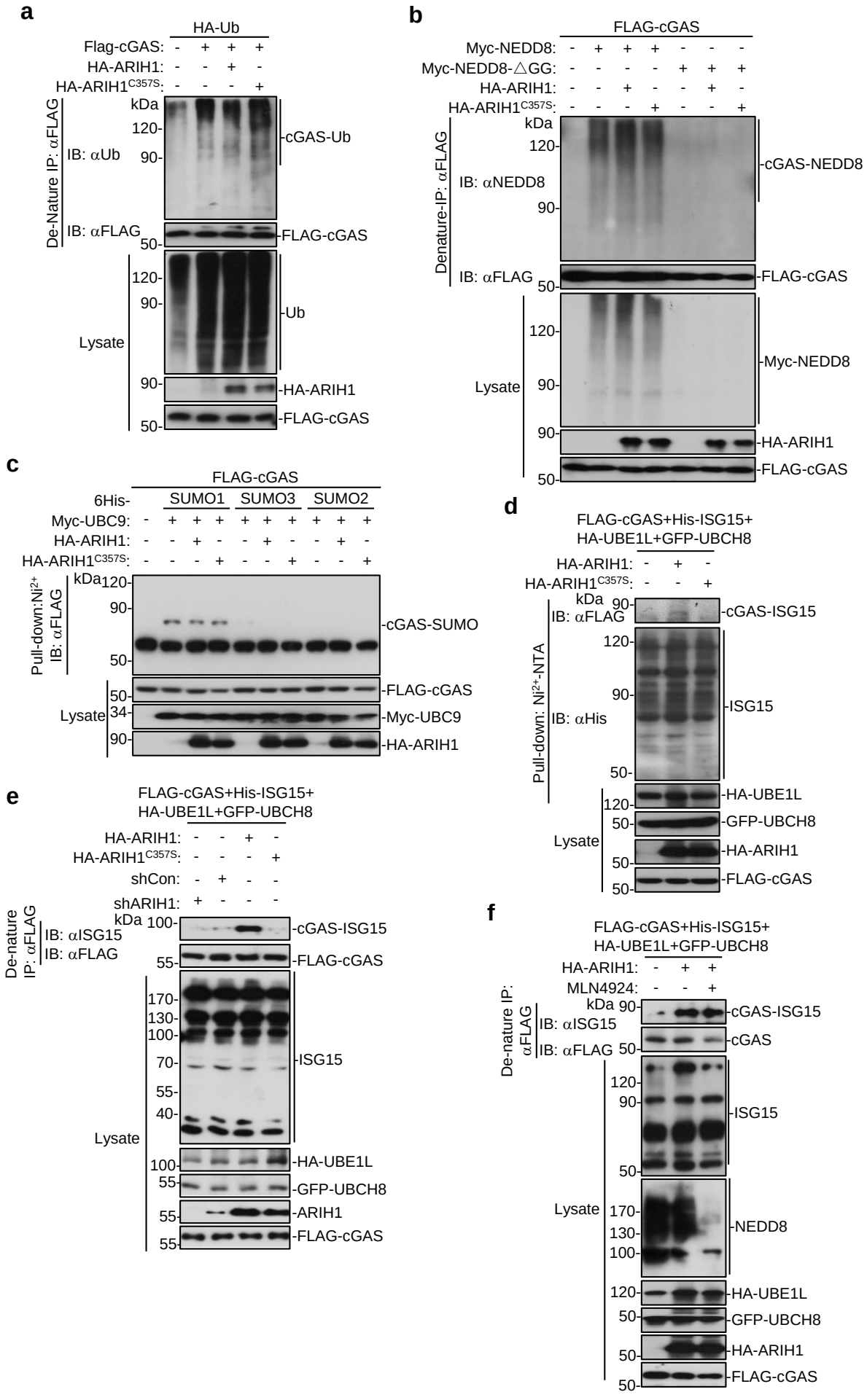
Supplementary Figure 3 Knockout of ARIH1 inhibits cellular antiviral responses against HSV-1.
(**a**) Immunblot analysis of ARIH1 and GAPDH in brains, spleens, hearts, and livers from Cre-ER and Cre-ER;*Arih*$^{fl/fl}$ mice that were intraperitoneally injected with tamoxifen (80 mg/kg in corn oil) for 5 successive days and rested for another 5 days.
(**b**) qRT-PCR analysis of *Ifnb*, *Ifna4* and *Ccl5* mRNA in Cre-ER and Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated with 4-hydroxytamoxifen (4OHT, 1 μM) for 3 days followed by infection with HSV-1 for 0-8 h.
(**c**) qRT-PCR analysis of *Ifnb*, *Ifna4* and *Ccl5* mRNA in Cre-ER and Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated as in (**a**) followed by infection with SeV for 0-8 h or transfection with poly(I:C) (1 μg) or cGAMP (1 μg) for 0-4 h.
(**d**) ELISA analysis of IFN-β and IL-6 in the supernatants of Cre-ER and Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated as in (**a**) followed by transfection with HSV60, DNA90 and poly(I:C) (1 μg) for 12 h.
(**e**) Immunoblot analysis of total and phosphorylated (p-) IRF3,TBK1 and p65, Arih1 and GAPDH in Cre-ER and Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated as in (**b**) followed by infection with HSV-1 (left panel) or SeV (right panel) for 0-8 h.
(**f**) Flow cytometric analysis (upper flow charts) and fluorescent microscopy imaging (lower images) of the replication of H129-G4 (MOI = 0.5) in Cre-ER and Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated as in (**b**) that were left uninfected or infected with H129-G4 for 1 h followed by twice PBS wash and culture in full medium for 12 h. Scale bars represent 200 μm.
(**g**) qRT-PCR analysis of HSV-1 *UL30* mRNA (upper graph) and plaque assays (lower graph) analyzing HSV-1 titers in Cre-ER and Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated as in (**b**)) that were infected with HSV-1 (MOI = 0.5) for 1 h followed by twice PBS wash and culture in full medium for 12 h.
Data are representative of three (**b**, **c**) or two (**d-g**) independent experiments. Graphs show mean ± S.D. (n = 4 for **b-c**, **g**, or n = 3 for **d**, biologically independent experiments). Statistical significance was determined using two-tailed Student's *t*-test in **b-d**, **g**. The quantitative anaylsis is derive from the same experiment and that blots were processed in parallel in **e**. Source data are provided as a Source Data file.

Supplementary Figure 4



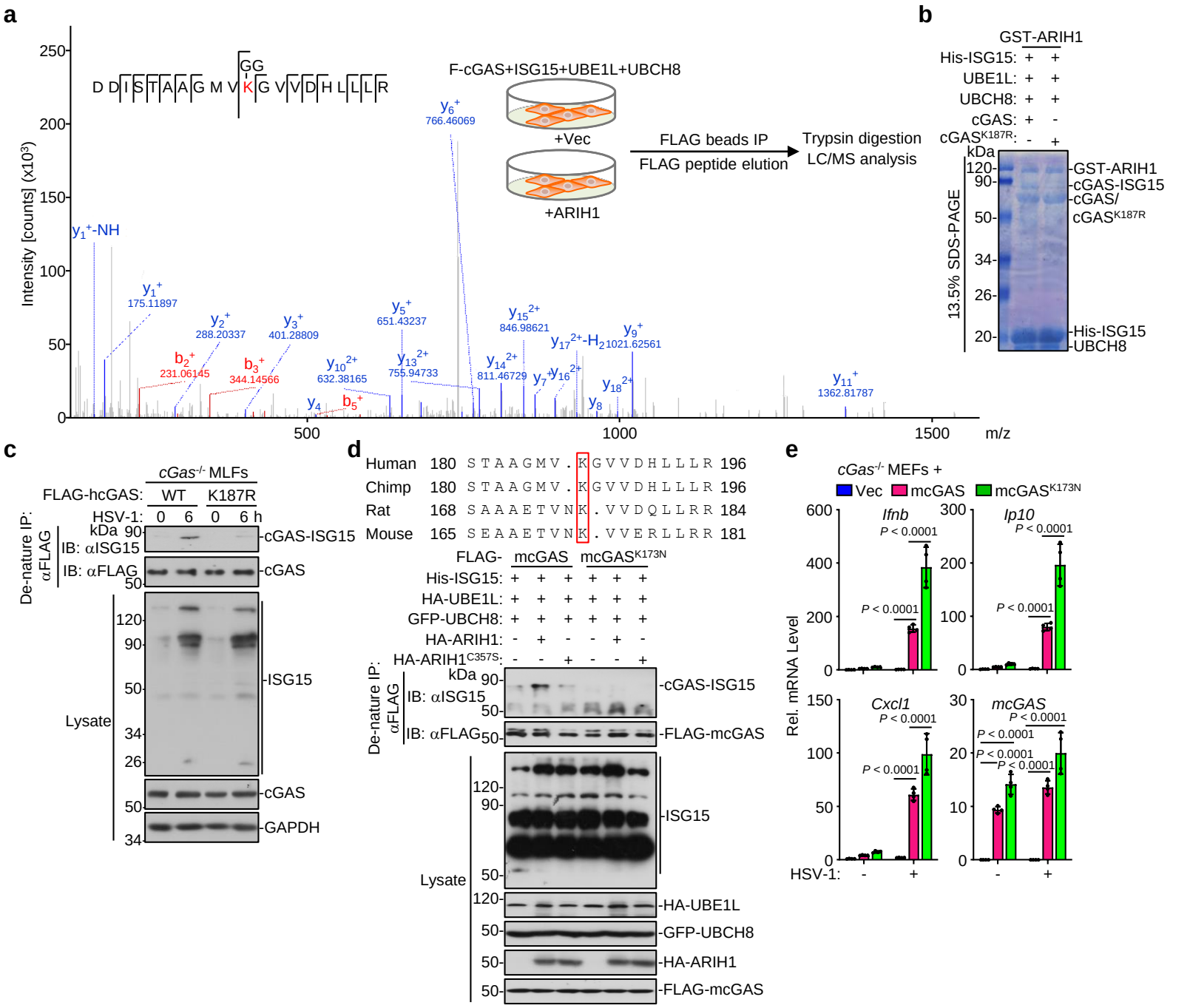
Supplementary Figure 4 The enzyme activity of ARIH1 is required for antiviral signaling.
(**a-c**) qRT-PCR analysis of *Ifnb*, *Il6* and *Isg56* mRNA (**a**, **b**) or ELISA analysis of IFN-β and IL-6 (**c**) in Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated with 4-hydroxytamoxifen (4OH Tam, 1 μM) and reconstituted with empty vector (Vec), FLAG-ARIH1 or ARIH1^{C357S} followed by infection with HSV-1 for 0-8 h (**a**) or 12 h (**c**) or transfection with HSV60, DNA90 or HSV120 (1 μg) for 8 h (**b**).
(**d**) Immunoblot analysis of total and phosphorylated (p-) IRF3,TBK1 and p65, FLAG-ARIH1 and β-tubulin in Cre-ER and Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated as in (**a**) followed by infection with HSV-1 for 0-8 h.
(**e**) Flow cytometric analysis (left flow charts) and fluorescent microscopy imaging (right images) of the replication of H129-G4 (MOI = 0.5) in Cre-ER and Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated as in (**a**) that were infected with H129-G4 for 1 h followed by twice PBS wash and culture in full medium for 12 h. Scale bars represent 200 μm.
(**f**) qRT-PCR analysis of HSV-1 *UL30* and *ARIH1* mRNA (upper graphs) and plaque assays (bottom graph) analyzing HSV-1 titers in Cre-ER and Cre-ER;*Arih1*$^{fl/fl}$ MLFs treated as in (**a**) that were infected with HSV-1 (MOI = 0.5) for 1 h followed by twice PBS wash and culture in full medium for 12 h.
(**g**) qRT-PCR analysis of *Isg15*, *Isg56* and *Ip10* in L929 cells to analyze the cGAMP activity in the homogenates of *Trex1*$^{-/-}$ and *Lyz2*-Cre;*Arih1*$^{fl/fl}$*Trex1*$^{-/-}$ BMDCs (left graphs) and BMDMs (right graphs) that were transfected with DNA120 (1 μg) for 6 h.
Data are representative of two independent experiments (**a**-**g**). Graphs show mean ± S.D. (n = 4 for **a**-**b**, **f** or n = 3 for **c**, or n = 7 and 3 for **g**, biologically independent experiments). Statistical significance was determined using two-tailed Student's *t*-test in **g**, or one-way ANOVA in **a**-**c**, **f**. The quantitative anaylsis is derive from the same experiment and that blots were processed in parallel in **d**. Source data are provided as a Source Data file.

Supplementary Figure 5



Supplementary Figure 5 ARIH1 catalyzes mono-ISGylation but not other ubiquitin-like modifications of cGAS.
(**a**) Denature-IP (with anti-FLAG) and immunoblot analysis (with anti-FLAG, anti-HA or anti-Ub) of HEK293 cells transfected with plasmids encoding an empty vector, HA-ARIH1 or ARIH1^{C357S} together with FLAG-cGAS and HA-Ub for 24 h.
(**b**) Denature-IP (with anti-FLAG) and immunoblot analysis (with anti-FLAG, anti-HA, or anti-NEDD8) of HEK293 cells transfected with plasmids encoding an empty vector, HA-ARIH1 or ARIH1^{C357S} together with FLAG-cGAS and Myc-NEDD8 or Myc-NEDD8△GG for 24 h.
(**c**) Ni^{2+}-NTA pull-down and immunoblot analysis (with anti-FLAG, anti-HA, or anti-Myc) of HEK293 cells transfected with plasmids encoding an empty vector, HA-ARIH1 or ARIH1^{C357S} together with FLAG-cGAS, Myc-UBC9 and His-SUMO1, His-SUMO2 or His-SUMO3 for 24 h.
(**d**) Ni^{2+}-NTA pull-down and immunoblot analysis (with anti-FLAG, anti-HA, anti-GFP, or anti-His) of HEK293 cells transfected with plasmids encoding an empty vector, HA-ARIH1 or ARIH1^{C357S} together with FLAG-cGAS, His-ISG15, GFP-UBCH8 and HA-UBE1L for 24 h.
(**e**) Denature-IP (with anti-FLAG) and immunoblot analysis (with anti-FLAG, anti-HA, anti-GFP or anti-ISG15) of HEK293 cells transfected with plasmids encoding an empty vector, HA-ARIH1, ARIH1^{C357S}, shCon, or shARIH1#2 together with FLAG-cGAS, His-ISG15, GFP-UBCH8 and HA-UBE1L for 24 h.
(**f**) Denature-IP (with anti-FLAG) and immunoblot analysis (with anti-FLAG, anti-HA, anti-GFP or anti-ISG15) of HEK293 cells transfected with plasmids encoding an empty vector, HA-ARIH1 or ARIH1^{C357S} together with FLAG-cGAS, His-ISG15, GFP-UBCH8 and HA-UBE1L for 8 h followed by treatment with MLN4924 for 24 h.
Data are representative of three independent experiments (**a**-**f**). Source data are provided as a Source Data file.

Supplementary Figure 6



Supplementary Figure 6 ARIH1 catalyzes mono-ISGylation of cGAS.

(**a**) Strategy of identifying the ISGylated sites of cGAS. FLAG-cGAS was purified from HEK293 cells transfected with FLAG-cGAS, His-ISG15, HA-UBE1L and GFP-UBCH8 together with a empty control vector or HA-ARIH1 and followed by immunoprecipitation (with anti-FLAG agarose) and elution (with 3 × FLAG peptide). The eluted proteins was digested by trypsin and subsequently analyzed by LC/MS.

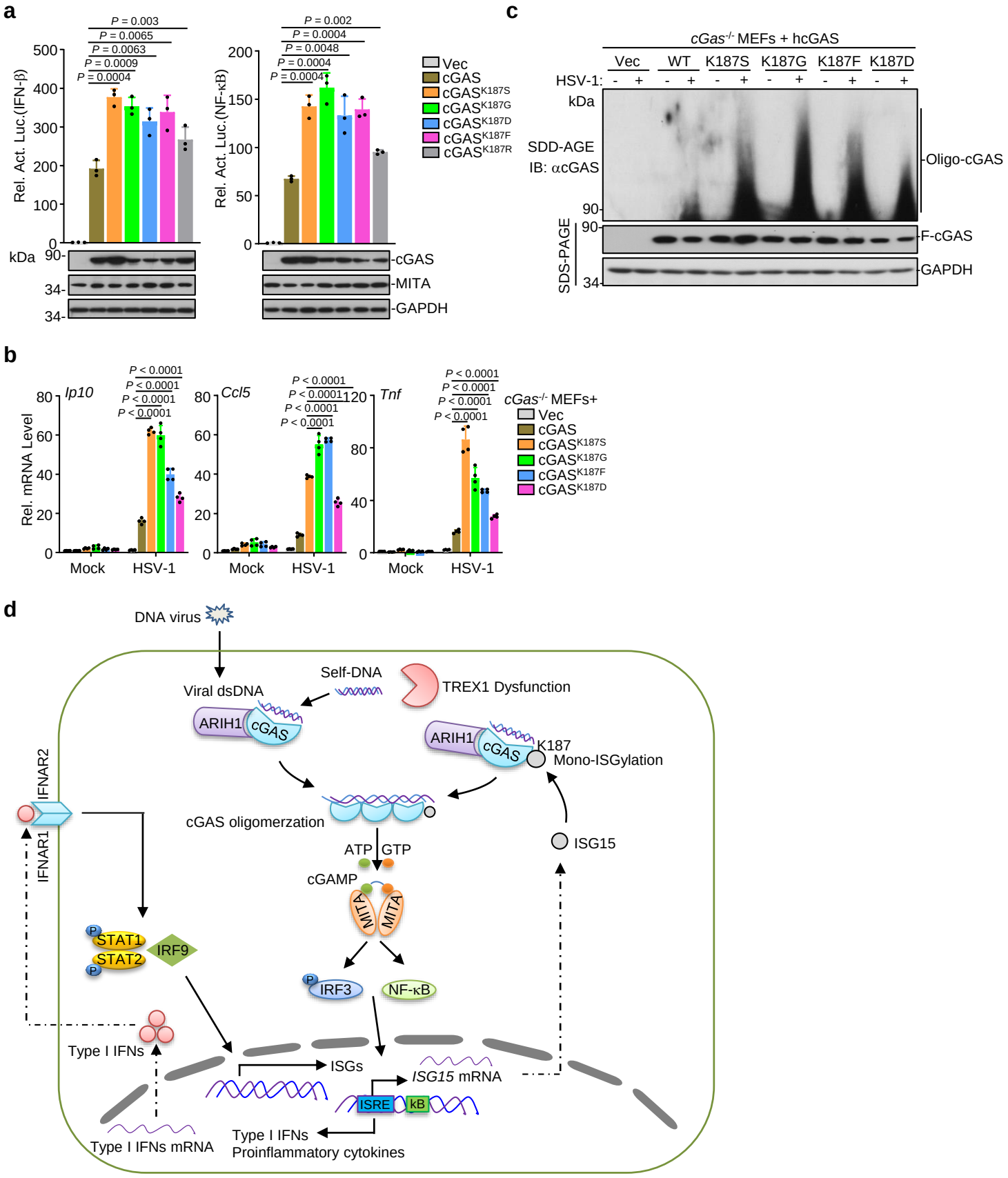
(**b**) In vitro ISGylation assay of cGAS or cGAS^{K187R}. Purified cGAS or cGAS^{K187R} (5 μg) were incubated with GST-ARIH1 (5 μg), UBE1L (0.2 μg), UBCH8 (0.5 μg) and His-ISG15 (5 μg) in the presence of ATP (5 mM) at 37°C for 2 h and subject to 13.5% SDS-PAGE followed by Coomassie brilliant blue staining.

(**c**) Denature-IP (with anti-FLAG) and immunoblot analysis (with anti-FLAG, anti-GAPDH or anti-ISG15) of *cGas*$^{-/-}$ MLFs reconstituted with FLAG-cGAS or cGAS^{K187R} followed by HSV-1 infection for 0-6 h.

(**d**) An alignment of cGAS of different species (upper image). Denature-IP (with anti-FLAG) and immunoblot analysis (with anti-FLAG, anti-HA, anti-GFP or anti-ISG15) of HEK293 cells transfected with plasmids encoding an empty vector, HA-ARIH1 or ARIH1^{C357S} together with His-ISG15, GFP-UBCH8 HA-UBE1L and FLAG-mcGAS or FLAG-mcGAS^{K173N} for 24 h (lower blots).

(**e**) qPCR analysis of *Ifnb*, *Ip10*, *Cxcl1*, and *mcGAS* mRNA in *cGas*$^{-/-}$ MEFs reconstituted with an empty vector (Vec), mcGAS or mcGAS^{K173N} followed by HSV-1 infection for 0-8 h. Data are representative of three independent experiments (**b**-**e**). Graphs show mean ± S.D. (n = 4 for **e**, biologically independent experiments). Statistical significance was determined using one-way ANOVA in **e**. Source data are provided as a Source Data file.

Supplementary Figure 7



Supplementary Figure 7 Lys 187 of cGAS inhibits its activation and oligomerization.
(**a**) Luciferase reporter assays analyzing IFN-β and NF-κB promoter activity in HEK293 cells transfected with an empty vector (Vec), cGAS, cGAS^{K187S}, cGAS^{K187G}, cGAS^{K187D}, cGAS^{K187F}, or cGAS^{K187R} together with FLAG-MITA for 24 hours.
(**b**) qRT-PCR analysis of *Ip10*, *Ccl5*, and *Tnf* in *cGas*$^{-/-}$ MEFs reconstituted with vec, cGAS, cGAS^{K187S}, cGAS^{K187G}, cGAS^{K187D}, or cGAS^{K187F} followed by treatment with HSV-1 for 8 h.
(**c**) Immunoblot assays with SDD-AGE to analyze the aggregation of cGAS and with SDS-PAGE to analyze the expression levels of the indicated proteins in cells treated as in (**b**).
(**d**) A model on ARIH1-mediated mono-ISGylation of cGAS and regulation of cGAS-dependent antiviral immunity and autoimmunity.
Data are representative of three independent experiments (**a**-**c**). Graphs show mean ± S.D. (n = 3 for **a**, or n = 4 for **b**, biologically independent experiments). Statistical significance was determined using one-way ANOVA in **a**,**e**. Source data are provided as a Source Data file.

Supplementary Table 1. antibody information

Antibody	Source & Identifier	Dilution
mouse control IgG	Santa Cruz Biotechnology, sc-2025	1/1000
rabbit control IgG	Millipore, 12-370	1/1000
HRP-conjugated goat-anti mouse or rabbit IgG	Thermo Scientific, PA1-86717 and SA1-9510	1/1000
mouse anti-FLAG	Sungene, KM8002	1/5000
anti-GFP	Sungene, KM8009	1/5000
anti-β-Actin	Sungene, KM9001	1/10000
anti-GAPDH	Sungene, KM9002	1/10000
anti-Tubulin	Sungene, KM9003	1/10000
anti-Myc	Sungene, KM8003	1/10000
anti-HA	Covance, MMS-101R	1/10000
anti-Ubiquitin	Santa Cruz Biotechnology, sc-8017	1/1000
anti-TBK1	Abcam, 96328-11	1/5000
anti-p-TBK1	Abcam, 109272	1/1000
anti-IRF3	Santa Cruz Biotechnology, sc-33641	1/5000
anti-p-IRF3	Cell Singling Technologies, 4947S	1/1000
anti-p65	Santa Cruz Biotechnology, sc-8008	1/2000
anti-p-p65	Cell Singling Technologies, 3033S	1/1000
anti-hcGAS	Cell Singling Technologies, 83623	1/1000
anti-mcGAS	Cell Singling Technologies, 31659	1/1000
anti-ARIH1	Abcam, ab3891	1/1000
anti-ISG15	Abclonal, A2416	1/1000
anti-NEDD8	Abclonal, A1163	1/1000
anti-CD3	Biolegend, 100312	1/300
anti-CD19	Biolegend, 152404	1/300
anti-GL7	Biolegend, 144610	1/300
anti-FAS	Biolegend, 152608	1/300
anti-CD4	Biolegend, 558107	1/300
anti-CD8	Biolegend, 100734	1/300
anti-CD44	Biolegend, 103012	1/300
anti-CD62L	Sungene, M10621-02E	1/300
anti-CD25	Sungene, M10251-09B	1/300
anti-CD11b	Sungene, M10117-02E	1/300
anti-CD45.2	Biolegend, 109806	1/300
anti-F4/80	Biolegend, 123110	1/300
anti-Ly6G	biolegend, 127624	1/300
anti-CD11c	Biolegend, 117328	1/300
anti-MHCII	Sungene, M100M2-11C	1/300
anti-CD16/32	Sungene, M10161-02B	1/300

Supplementary Table 2. sequences of DNA ligands

DNA ligands	Forward	Reverse
ISD45	TACAGATCTACTAGTGATCTATGACTGAT	TGTAGATCATGTACAGATCAGTCATAGAT
	CTGTACATGATCTACA	CACTAGTAGATCTGTA
HSV60	TAAGACACGATGCGATAAAATCTGTTTGT	GCTAGGGCAATTTGTACCCTTAATAAATT
	AAAATTTATTAAGGGTACAAATTGCCCTAGC	TTACAAACAGATTTTATCGCATCGTGTCTTA
DNA90	TACAGATCTACTAGTGATCTATGACTGATC	TGTAGATCATGTACAGATCAGTCATAGATC
	TGTACATGATCTACATACAGATCTACTAGT	ACTAGTAGATCTGTATGTAGATCATGTACA
	GATCTATGACTGATCTGTACATGATCTACA	GATCAGTCATAGATCACTAGTAGATCTGTA
HSV120	AGACGGTATATTTTTGCGTTATCACTGTCCC	AAGTCCTCCAAAAAACCCGCCACAAATAAA
	GGATTGGACACGGTCTTGTGGGATAGGCAT	AAGGGGTTAACCCAATATGCCTTCTGGGCAT
	GCCCAGAAGGCATATTGGGTTAACCCCTTT	GCCTATCCCACAAGACCGTGTCCAATCCGGG
	TTATTTGTGGCGGGTTTTTTGGAGGACTT	ACAGTGATAACGCAAAAATATACCGTCT

Supplementary Table 3. sequences of qPCR primers

Gene	Forward	Reverse
β-Actin	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT
cGAS	AGAAGGCCTGCGCATTCAAA	GCCGCCATGTTTCTTCTTGGA
CXCL1	AGCTTGCCTCAATCCTGCATCC	TCCTTCAGGAACAGCCACCAGT
IFN-β	TTGTTGAGAACCTCCTGGCT	TGACTATGGTCCAGGCACAG
IL6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
IP10	GGTGAGAAGAGATGTCTGAATCC	GTCCATCCTTGGAAGCACTGCA
ISG15	AGGACAGGGTCCCCCTTGCC	CCTCCAGCCCGCTCACTTGC
ISG56	TCATCAGGTCAAGGATAGTC	CCACACTGTATTTGGTGTCTAGG
ARIH1	ACTTTGATGGAAACCTGGAGAA	GCCAGTGAAATACGAGTTAGGG
CCL5	GGCAGCCCTCGCTGTCATCC	GCAGCAGGGTGTGGTGTCCG
TNF	GCCGCATCGCCGTCTCCTAC	CCTCAGCCCCCTCTGGGGTC
UL30	CATCACCGACCCGGAGAGGGAC	GGGCCAGGCGCTTGTTGGTGTA
m*Isg56*	GAAGTCCTTTGCTTGGAGGA	AGCCCAAGAAGGCTGGTACT
m*Ifnb*	TACAACAGATACGCCTGGAT	AGTCCGCCTCTGATGCTTAA
m*Il6*	ACAAAGCCAGAGTCCTTCAGA	TCCTTAGCCACTCCTTCTGT
m*Ccl5*	ATATGGCTCGGACACCACTC	CCACTTCTTCTCTGGGTTGG
m*cGAS*	TGTCAGTGTGGAGAAGGAAAAA	CAGCCAGCCTTGAATAGGTAG
m*Arih1*	GGTGAAACAGAAGATGGAGGAG	TTTGAGGTAGAAAGCGAAGACA
m*Ifna4*	AGGATCACTGTGTACCTGAGA	TCTCCACACTTTGTCTCAGGA
m*Ip10*	GTGAGAATGAGGGCCATAGG	TTTTTGGCTAAACGCTTTCAT
m*Isg15*	GGCCACAGCAACATCTATGA	ACTGGGGCTTTAGGCCATAC
m*Cxcl1*	CACTCAAGAATGGTCGCGAG	GTTGTCAGAAGCCAGCGTTC
m*β-actin*	ACGGCCAGGTCATCACTATT	TGGCATAGAGGTCTTTACGGA