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Supplemental Information

The tyrosine kinase c-Abl potentiates interferon-mediated antiviral immunity by STAT1 phosphorylation

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Supplemental Information

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Figure S1

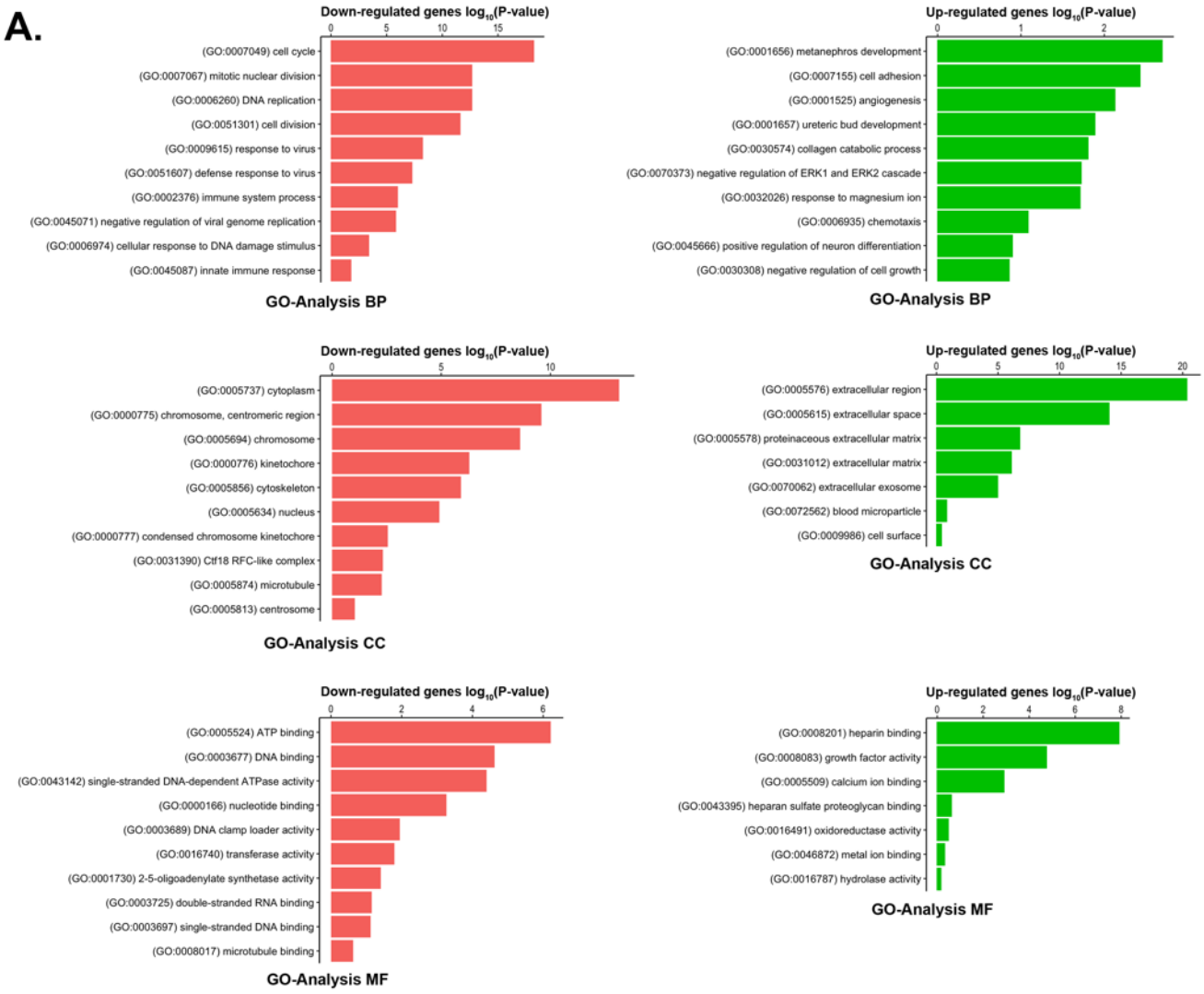

A.
Down-regulated genes log10(P-value)
(GO:0007049) cell cycle
(GO:0007067) mitotic nuclear division
(GO:0006260) DNA replication
(GO:0051301) cell division
(GO:0009615) response to virus
(GO:0051607) defense response to virus
(GO:0002376) immune system process
(GO:0045071) negative regulation of viral genome replication
(GO:0006974) cellular response to DNA damage stimulus
(GO:0045087) innate immune response
GO-Analysis BP
Up-regulated genes log10(P-value)
(GO:0001656) metanephros development
(GO:0007155) cell adhesion
(GO:0001525) angiogenesis
(GO:0001657) ureteric bud development
(GO:0030574) collagen catabolic process
(GO:0070373) negative regulation of ERK1 and ERK2 cascade
(GO:0032026) response to magnesium ion
(GO:0006935) chemotaxis
(GO:0045666) positive regulation of neuron differentiation
(GO:0030308) negative regulation of cell growth
GO-Analysis BP
Down-regulated genes log10(P-value)
(GO:0005737) cytoplasm
(GO:0000775) chromosome, centromeric region
(GO:0005694) chromosome
(GO:0000776) kinetochore
(GO:0005856) cytoskeleton
(GO:0005634) nucleus
(GO:0000777) condensed chromosome kinetochore
(GO:0031390) Ctf18 RFC-like complex
(GO:0005874) microtubule
(GO:0005813) centrosome
GO-Analysis CC
Up-regulated genes log10(P-value)
(GO:0005576) extracellular region
(GO:0005615) extracellular space
(GO:0005578) proteinaceous extracellular matrix
(GO:0031012) extracellular matrix
(GO:0070062) extracellular exosome
(GO:0072562) blood microparticle
(GO:0009986) cell surface
GO-Analysis CC
Down-regulated genes log10(P-value)
(GO:0005524) ATP binding
(GO:0003677) DNA binding
(GO:0043142) single-stranded DNA-dependent ATPase activity
(GO:0000166) nucleotide binding
(GO:0003689) DNA clamp loader activity
(GO:0016740) transferase activity
(GO:0001730) 2-5-oligoadenylate synthetase activity
(GO:0003725) double-stranded RNA binding
(GO:0003697) single-stranded DNA binding
(GO:0008017) microtubule binding
GO-Analysis MF
Up-regulated genes log10(P-value)
(GO:0008201) heparin binding
(GO:0008083) growth factor activity
(GO:0005509) calcium ion binding
(GO:0043395) heparan sulfate proteoglycan binding
(GO:0016491) oxidoreductase activity
(GO:0046872) metal ion binding
(GO:0016787) hydrolase activity
GO-Analysis MF
B.
Log2FC
C.
Lysates
MEF
DKO
0 0 0.8 3.2 12.5 50 Ad-Abl (MOI)
kDa 50 50 0 0 0 0 Ad-Vector (MOI)
130-
←c-Abl
IB: anti-c-Abl
45-
←β-actin
IB: anti-β-actin

Figure S1. DEGs Analysis of the gene expression profiles, related to Figure 1. (A)-(B) GO analysis of genes regulated ≥4-fold (A) and the changes in ISG expression (B) in *c-abl/arg* double-knockout MEFs compared to wild-type MEFs determined by RNA-seq. (C) Lysates of wild-type and *c-abl/arg* double-knockout MEFs infected with adenovirus at the indicated MOI were analyzed by immunoblotting.

Figure S2

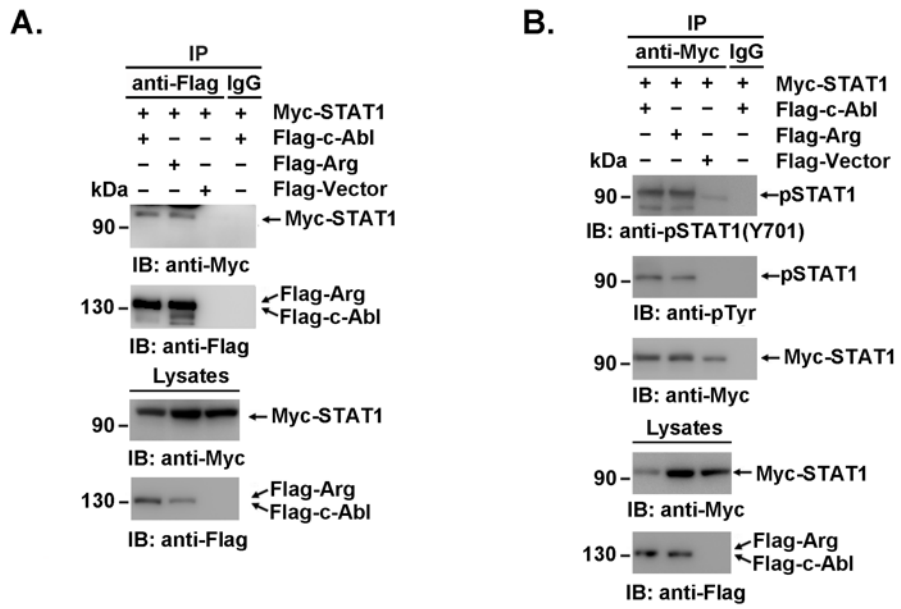


Figure S2. Arg phosphorylates STAT1, related to Figure 2 and Figure 3. (A)-(B) Lysates of 293T cells transfected with the indicated plasmids were subjected to anti-Flag or IgG immunoprecipitation. The immunoprecipitates were analyzed by immunoblotting.

Figure S3

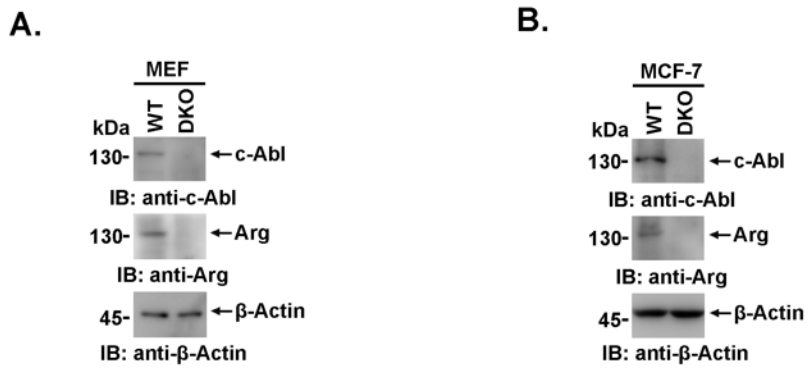
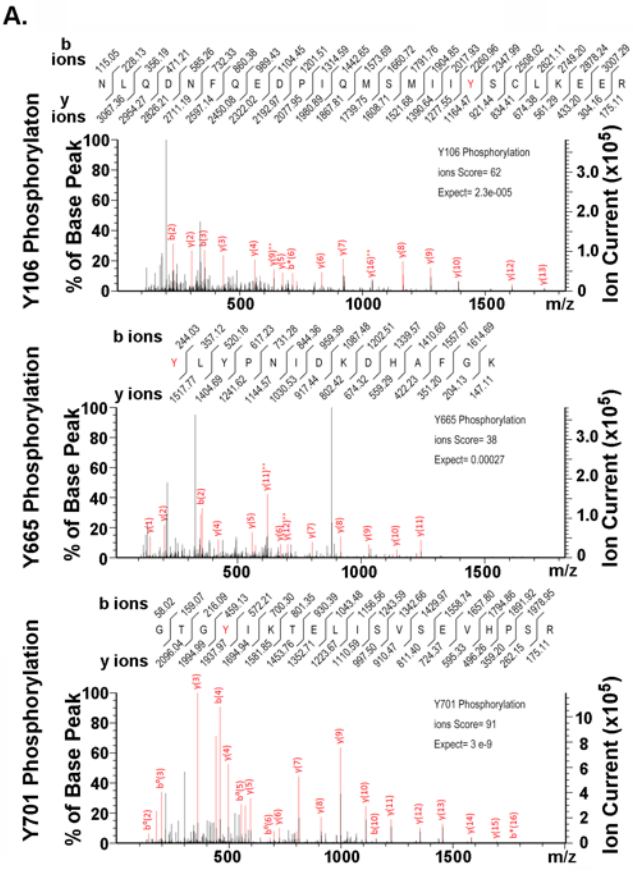
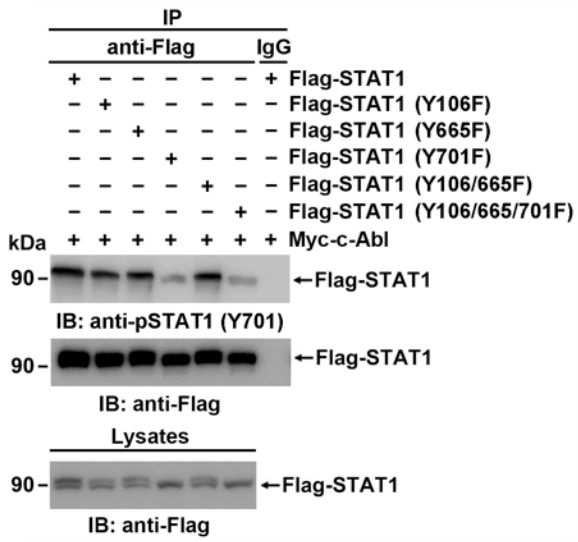


Figure S3. The validation of *c-abl/arg* double-knockout in MEFs or MCF-7 cells, related to Figure 1 and Figure 4. (A)-(B) Lysates of wild-type and *c-abl/arg* double-knockout MEFs (A) or MCF-7 cells (B) were analyzed by immunoblotting.

Figure S4


A.
Y106 Phosphorylaton
Y665 Phosphorylation
Y701 Phosphorylation
% of Base Peak
Ion Current (x10^5)
m/z
Y106 Phosphorylation
ions Score= 62
Expect= 2.3e-005
Y665 Phosphorylation
ions Score= 38
Expect= 0.00027
Y701 Phosphorylation
ions Score= 91
Expect= 3 e-9


B.
IP
anti-Flag
IgG
Flag-STAT1
Flag-STAT1 (Y106F)
Flag-STAT1 (Y665F)
Flag-STAT1 (Y701F)
Flag-STAT1 (Y106/665F)
Flag-STAT1 (Y106/665/701F)
kDa
Myc-c-Abl
90
←Flag-STAT1
IB: anti-pSTAT1 (Y701)
IB: anti-Flag
Lysates

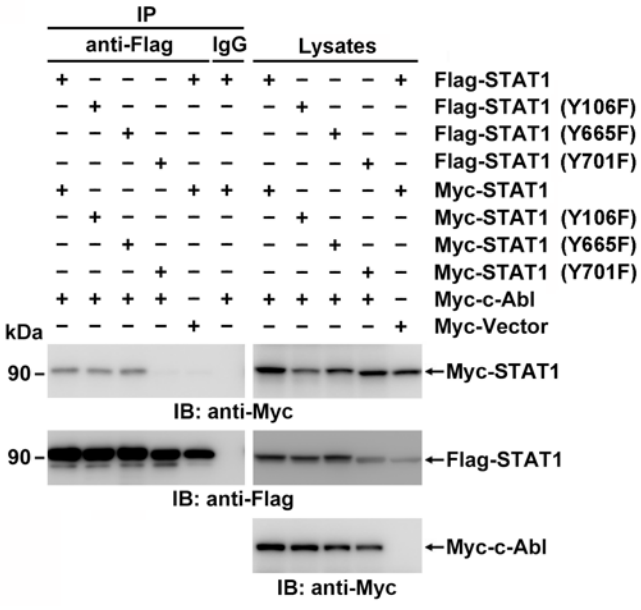

C.
IP
anti-Flag
IgG
Lysates
Flag-STAT1
Flag-STAT1 (Y106F)
Flag-STAT1 (Y665F)
Flag-STAT1 (Y701F)
Myc-STAT1
Myc-STAT1 (Y106F)
Myc-STAT1 (Y665F)
Myc-STAT1 (Y701F)
Myc-c-Abl
Myc-Vector
kDa
90
←Myc-STAT1
IB: anti-Myc
←Flag-STAT1
IB: anti-Flag
←Myc-c-Abl
IB: anti-Myc

Figure S4. Identification of STAT1 tyrosine phosphosites, related to Figure 3. (A) Myc-STAT1 coexpressed with Flag-c-Abl was purified via anti-Flag immunoprecipitation and SDS-PAGE and then subjected to chymotrypsin digestion and LC-MS/MS analysis. Monophosphorylated peptides containing PO_3^-modified Y106, Y665 and Y701 were identified. (B)-(C) Lysates of 293T cells transfected with the indicated plasmids were subjected to anti-Flag or IgG immunoprecipitation. The immunoprecipitates were analyzed by immunoblotting.

Figure S5

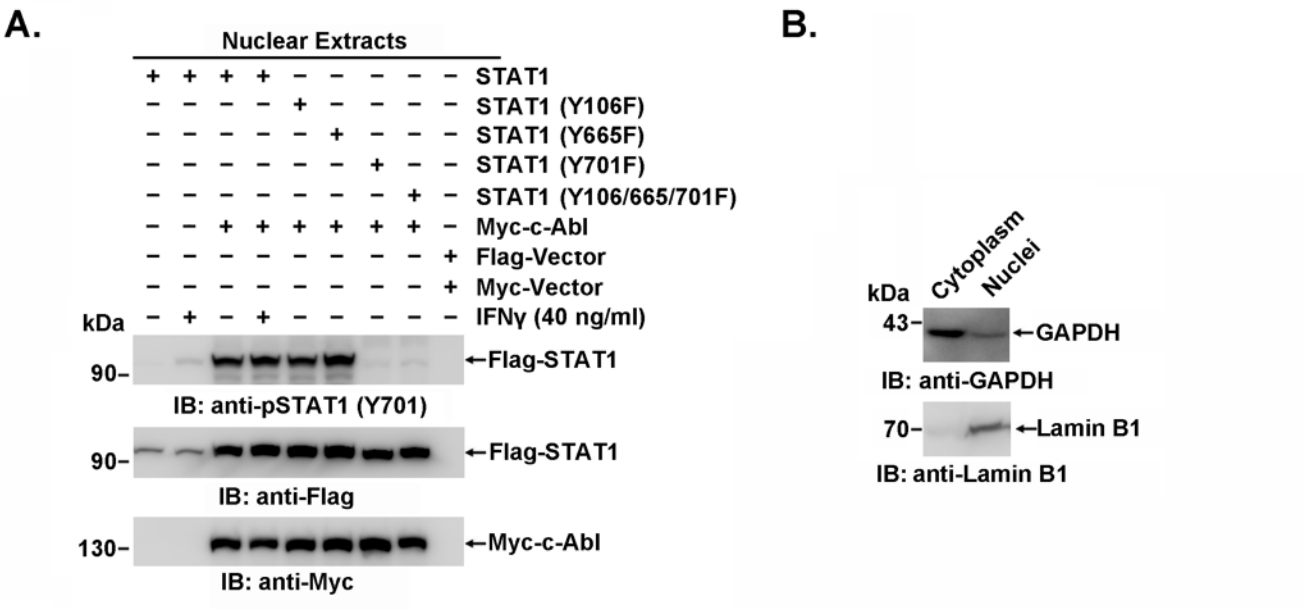


Figure S5. The indicated gene expression and the validation of nuclear extracts isolation, related to Figure 4. (A) Lysates of 293T cells transfected with the indicated plasmid were analyzed by immunoblotting. (B) Nuclear and cytoplasmic extracts were analyzed by immunoblotting.

Figure S6

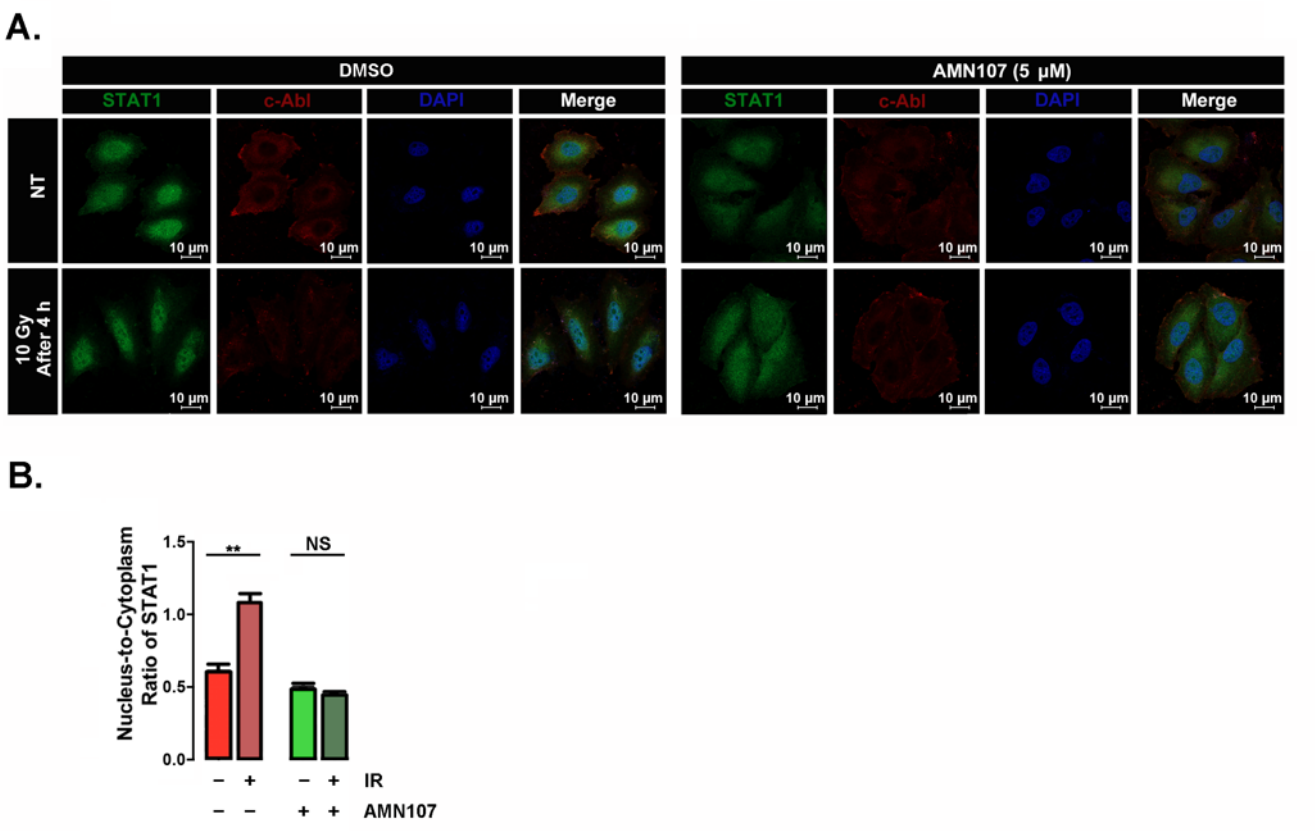


Figure S6. The STAT1 nuclear translocation induced by ionizing radiation, related to Figure 4. (A) MCF-7 cells treated with or without AMN107 were subjected to 10 Gy γ-irradiation. Then, the localization of endogenous STAT2 (green) and c-Abl (red) was detected via immunofluorescence microscopy. Nuclei were stained with DAPI (blue). (B) The nuclear localization of STAT1 in C was measured by the ratio of fluorescence intensity in the nucleus to that in the cytoplasm in the same cell quantified by ImageJ software. At least 15 cells were calculated, and the results are expressed as the mean ± SD (unpaired Student's t-test). **P<0.01.

Figure S7

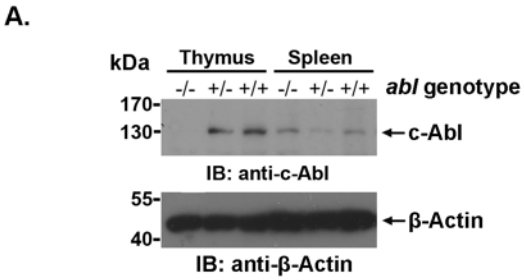


Figure S7. Identification of *abl* genotype in mice, related to Figure 6. (A) c-Abl expression in T cells separated from the thymuses and spleens of mice with the *c-abl*$^{fl/fl}$*; Lck-Cre*, *c-abl*$^{wt/fl}$*; Lck-Cre* and *c-abl*$^{wt/wt}$*; Lck-Cre* genotypes was analyzed by immunoblotting. β-actin was used as an equal loading control.

Figure S8

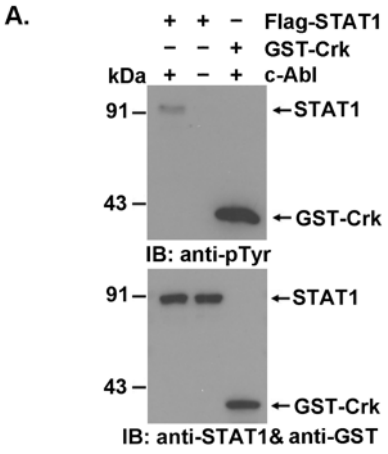


Figure S8. c-Abl phosphorylates STAT1 *in vitro*, related to Figure 3. (A) Purified Flag-STAT1 or GST-Crk (as a positive control) was incubated with or without 10 ng of human Abl protein (Upstate Biotechnology) in the presence of 0.2 mM ATP. The reaction products were analyzed by immunoblotting.

Figure S9

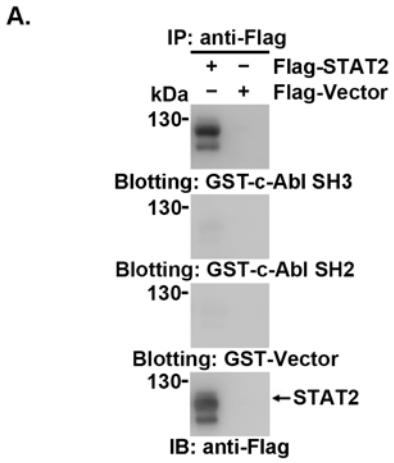


Figure S9. The Abl kinase domain responsible for STAT2 association, related to Figure 2. (A) Anti-Flag or IgG immunoprecipitates prepared from cells transfected with the indicated plasmids were subjected to SDS-PAGE, and the proteins were blotted onto a PVDF membrane. The PVDF membrane was incubated with soluble GST-c-Abl SH2, GST-c-Abl SH3 or GST only for 2 h and then analyzed with an anti-GST antibody.

Figure S10

A.

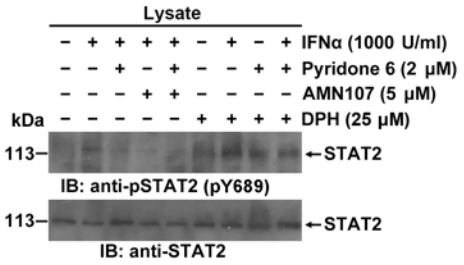


Figure S10. JAK and Abl kinase-mediated STAT2 activation, related to Figure 3. (A) Lysates of MCF-7 cells subjected to the indicated treatments were analyzed by immunoblotting.

Figure S11

A.

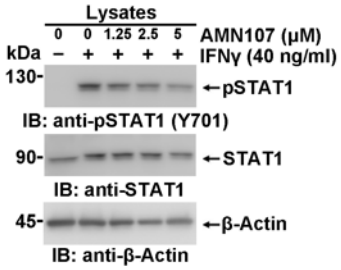
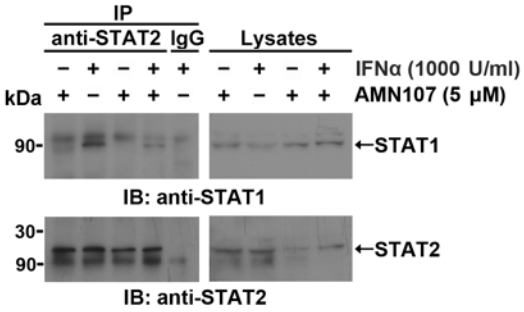


Figure S11. IFNγ-induced STAT1 phosphorylation is dependent on Abl kinase activity, related to Figure 3. (A) Lysates of MCF-7 cells treated with AMN107 at different concentrations were analyzed by immunoblotting.

Figure S12

A.



B.

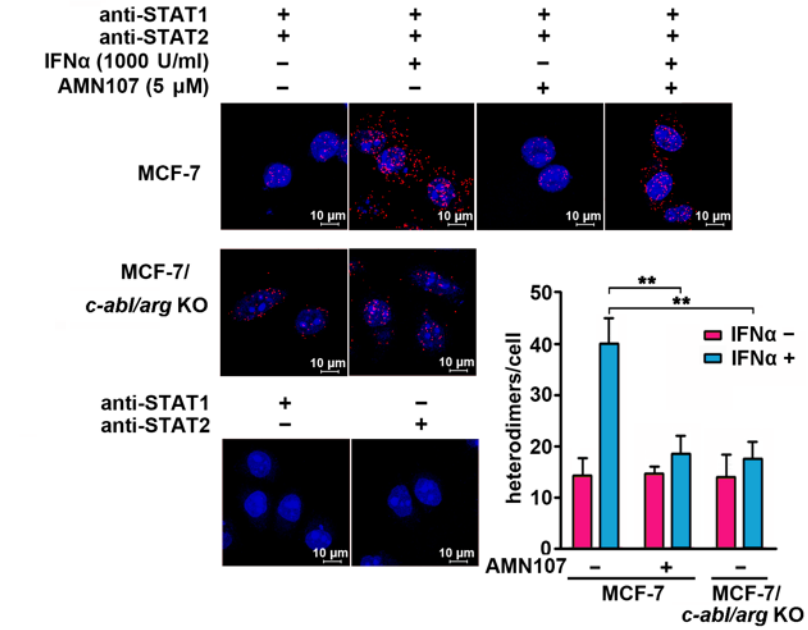


Figure S12. c-Abl potentiates IFNα-induced STAT1/STAT2 heterodimerization, related to Figure 4. (A) Anti-STAT2 or normal rabbit IgG immunoprecipitates prepared from lysates of MCF-7 cells subjected to the indicated treatments were analyzed by immunoblotting. (B) *In situ* PLAs were performed on wild-type and *c-abl/arg* double-knockout MCF-7 cells with the indicated treatments using anti-STAT1 and anti-STAT2 antibodies. PLA signals were shown as red spots, and nuclei were stained with DAPI (blue). Cells stained with anti-STAT1 or anti-STAT2 antibody alone were used as negative controls. The red spots of each cell were counted, and at least 15 cells were analyzed. All quantitative data are shown as the mean ± SD (unpaired Student's t-test). **P<0.01.

Table S1. Primer sequences for quantitative PCR, related to Figure 1 and Figure 5.

TARGETS	QPCR PRIMERS
HOMO-CCL5	5'- CCTGCTGCTTTGCCTACATTGC-3'
	5'- CCTGCTGCTTTGCCTACATTGC-3'
HOMO-CXCL10	5'- GGTGAGAAGAGATGTCTGAATCC-3'
	5'- GTCCATCCTTGGAAGCACTGCA-3'
HOMO-CXCL11	5'- AAGGACAACGATGCCTAAATCCC-3'
	5'- CAGATGCCCTTTTCCAGGACTTC-3'
HOMO-GBP2	5'- GTTCCTACATCCTCAGCCATTCC -3'
	5'- CCACTGCTGATGGCATTGACGT -3'
HOMO-IDO1	5'- GCCTGATCTCATAGAGTCTGGC -3'
	TGCATCCCAGAACTAGACGTGC -3'
HOMO-IFI35	5'- CACGATCAACATGGAGGAGTGC -3'
	5'- GGCAGGAAATCCAGTGACCAAC -3'
HOMO-IRF1	5'- GAGGAGGTGAAAGACCAGAGCA -3'
	5'- TAGCATCTCGGCTGGACTTCGA -3'
HOMO-ISG15	5'- CTCTGAGCATCCTGGTGAGGAA -3'
	5'- AAGGTCAGCCAGAACAGGTCGT -3'
HOMO-OASL	5'- GTGCCTGAAACAGGACTGTTGC -3'
	5'- CCTCTGCTCCACTGTCAAGTGG -3'
HOMO-PSMB9	5'- CGAGAGGACTTGTCTGCACATC -3'
	5'- CACCAATGGCAAAAGGCTGTCG -3'
HOMO-TAP1	5'- GCAGTCAACTCCTGGACCACTA -3'
	5'- CAAGGTTCCCACTGCTTACAGC -3'
MUS-CXCL10	5'- ATCATCCCTGCGAGCCTATCCT -3'
	5'- GACCTTTTTTGGCTAAACGCTTTC -3'
MUS-DDX58	5'- AGCCAAGGATGTCTCCGAGGAA -3'
	5'- ACACTGAGCACGCTTTGTGGAC -3'
MUS-DDX60	5'- CGCAAGCCAGACAGTCCTACAA -3'
	5'- AAACATCGCCCTGTCTCACGGA -3'
MUS-GBP1	5'-ACATGCCCACAGAAACCCTCCA-3'
	5'-AGGCATCTCGTTTGGCTTCCAG-3'
MUS-GBP2	5'- AGATGCCCACAGAAACCCTCCA -3'
	5'- AAGGCATCTCGCTTGGCTACCA -3'
MUS-GBP7	5'- CGTGTCATCACAGCAGACGAGT -3'
	5'- CCGTCTTGGAAAGAAGTGCCTG -3'
MUS-IFI44	5'- ATGCACTCTTCTGAGCTGGTGG -3'
	5'- TCAGATCCAGGCTATCCACGTG -3
MUS-IRF9	5'- CAACATAGGCGGTGGTGGCAAT -3'
	5'- GTTGATGCTCCAGGAACACTGG -3'
MUS-ISG15	5'- CATCCTGGTGAGGAACGAAAGG -3'
	5'- CTCAGCCAGAACTGGTCTTCGT -3'
MUS-OASL2	5'- CCAAAACGAGGTCGTCAGGAAC -3'

	5’- AGCCACCTGTTCCCATCCCTTT -3’
MUS-PSMA5	5’- ATTGGCTCTGCTTCTGAGGGTG -3’
	5’- TGATGAGCGAGGACTTGATGGC -3’
MUS-PSMB9	5’- TACCGTGAGGACTTGTTAGCGC -3’
	5’- GGCTGTCGAATTAGCATCCCTC -3’
MUS-TAP1	5’- GACTCCTTGCTCTCCACTCAGT -3’
	5’- AACGCTGTCACCGTTCCAGGAT -3’

Transparent Methods

Ethics statement

This study followed the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All mice protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Laboratory Animal Center, Academy of Military Medical Sciences, China.

Mice

c-abl$^{fl/fl}$ mutant mice in which loxP sites flanked exons 5-6 of *abl* oncogene 1 were purchased from the Jackson Laboratory, and Lck-Cre mice on a C57BL/6J background were kindly donated by Prof. Xiao Yang (Beijing Proteome Research Center). These mice were generated by crossing mice bearing loxP-flanked *c-abl* alleles (*c-abl*$^{fl/fl}$) with a transgenic mouse line expressing Cre recombinase under the control of a modified distal promoter of the gene encoding *Lck* kinase (*Lck*-Cre), which was specifically expressed in thymocytes (Fig. S5B).

Female mice (18-22 g, 6-8-wk-of-age) were used in the experiments.

Cell culture and transfection

293T cells, MCF-7 cells and MEFs derived from WT and *c-abl*$^{-/-}$*arg*$^{-/-}$ littermates (Koleske et al., 1998) were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries). 2C4 (parental) and γ2A (JAK2-deficient) cell lines were grown in the presence of 400 μg/ml G418 (Roche). RAW 264.7 and B3Z hybridoma cell lines specific for SIINFEKL were cultivated in RPMI-1640

medium (Gibco). C57BL/6-derived JAWS II dendritic cells were obtained from the American Type Culture Collection and grown as described in the manual. Cells were treated with AMN107 (Selleck), ruxolitinib (Selleck) or IFNα/γ (PeproTech) as noted in the text. Transient transfection was performed with Lipofectamine 3000 (Thermo Fisher) and the TransIT-X2™ Dynamic Delivery System (Mirus).

Generation of gene knockout cell lines via CRISPR/Cas9.

We used the pSpCas9(BB)-2A-Puro plasmid (Addgene plasmid ID: 48139) for Abl/Arg and JAK2 gene targeting. The sgRNAs (*abl*: 5'-TGTGATTATAGCCTAAGACC-3'; *arg*: 5'-AGTTCGCTCTAAGAATGGGC-3'; jak2-1: 5'-AATGAAGAGTACAACCTCAG-3'; jak2-2: 5'-CTGAGCGAACAGTTTCCATC-3') were designed using the online tool developed by F. Zhang's lab (http://crispr.mit.edu/). The plasmid carrying a specific sgRNA sequence was transfected into parental cells. After 24 h, the cells were treated with 0.25 μg/ml puromycin (Life Technologies) for at least 7 days. Cell clones were selected to expanded culture followed with genomic sequencing identification and immunoblot analysis.

Vectors and epitope tagging of proteins

Flag-tagged STAT1, c-Abl, and Arg and their mutants were expressed by cloning the genes into the pcDNA3-based Flag vector (Invitrogen). Myc-tagged STAT1, c-Abl, and mutants were prepared by cloning the genes into pCMV-Myc (Clontech). Plasmids

encoding EGFP-tagged STAT1 were constructed by cloning STAT1 into pEGFP-C1 (Clontech). GST- and His-tagged STAT1 fusion proteins in pGEX4T-1 (Amersham Biosciences) and pET-22b (+) (Novagen) were generated by expression in *Escherichia coli* BL21(DE3).

Reporter assay

MEFs were transfected with a luciferase plasmid that contained the Psmb9 and Tap1 bidirectional promoters. 24 h after transfection, the cells were treated with AMN107 (5 mM) for 18 h and lysed with passive lysis buffer (Promega, E1910). Reporter activity was analyzed with a Dual-Luciferase® Reporter Assay System kit (Promega, E1910) and a TD-20/20 luminometer (Turner Designs).

Immunoprecipitation analysis and immunoblotting

Cell lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 1 mM DTT, 10 mM NaF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 10 mg/ml pepstatin A) containing 1% Nonidet P-40. Soluble proteins were immunoprecipitated using anti-Flag (M2, Sigma-Aldrich), anti-Myc (E6654, Sigma-Aldrich), anti-STAT1 (06-501, Millipore), and anti-mouse IgG (Sigma-Aldrich) antibodies. An aliquot of the total lysate (5%, v/v) was included as a control. Immunoblotting was performed with horseradish peroxidase (HRP)-conjugated anti-Myc (Sigma-Aldrich), HRP-conjugated anti-Flag (Sigma-Aldrich), anti-c-Abl (K-12, Santa Cruz), anti-c-Abl pY412 (07-788, Millipore), anti-STAT2 (06-502, Millipore), anti-STAT1 pY701 (07-307, Millipore), anti-STAT2 pY689 (07-224), anti-β-actin (Sigma-Aldrich), HRP-conjugated anti-pTyr (4G10, Millipore), anti-JAK2 (3230, CST), anti-His (Proteintech), anti-GST (B-14, Santa Cruz), and anti-BCR (SC-104, Santa Cruz)

antibodies. The resultant antigen-antibody complexes were visualized via an enhanced chemiluminescence (ECL) system (GE Healthcare). A PageRuler Western marker (Thermo Fisher) was used as a molecular weight standard.

Protein binding assays

In GST pulldown experiments, the lysates of cells subjected to the indicated transfection were incubated with 5 mg of GST or GST fusion proteins conjugated with glutathione beads for 2 h at 4 °C. After lysis buffer washing, the adsorbates were subjected to SDS-PAGE and immunoblot analysis. An aliquot of the total lysate (5%, v/v) was included in SDS-PAGE as a loading control.

In direct binding assays (Far-Western blot assays), anti-Flag immunoprecipitates were separated via SDS-PAGE, and proteins were then transferred onto PVDF membranes. The membranes were subsequently incubated with purified GST fusion proteins in TBST for 2 h at room temperature. The binding of GST fusion proteins on PVDF membranes was detected by immunoblotting with an anti-GST antibody.

Kinase assays

Purified His-STAT1 or GST-STAT1 was incubated with active Abl protein expressed in *E. coli* (Merck, 14-529) in protein kinase buffer for 30 min at 30 °C. The reaction products were analyzed via SDS-PAGE and immunoblotting.

LC-MS/MS analysis

Anti-Flag immunoprecipitates prepared from Myc-c-Abl and Flag-STAT1 cotransfected 293T cell lysates were separated via SDS-PAGE, and the protein bands were subsequently excised for chymotrypsin digestion, LC-ESI-MS/MS-resolved peptides were analyzed using a Q-TOF 2 system (Micromass), and the data were compared against SWISS-PROT using the Mascot search engine (http://www.matrixscience.com) to detect phosphorylation.

***In situ* PLA**

Duolink *in situ* PLA (Proximity Ligation Assay, Sigma-Aldrich) was applied to detect the interactions between STAT1 and c-Abl in MEFs. In brief, cells on glass coverslips were permeabilized with 0.2% Triton X-100 in PBS for 15 min after blocking with 4% PFA. Antibodies against STAT1 (06-501, Millipore) and Abl (K-12, Santa Cruz) were used according to the manufacturer's instructions for the PLA. The red fluorescent spots generated from the DNA amplification-based reporter system were detected by the Zeiss LSM 800 confocal microscope.

Semiquantitative RT-PCR analyses

Total cellular RNA was extracted from cells (10^5 cells) using an RNeasy Mini Kit (Qiagen, 74126) and cDNA was subsequently synthesized using a GoScript™ Reverse Transcription System (Promega, A5001). RT-PCR was performed using GoTaq® qPCR Master Mix (Promega, A6001). Primers used for genes detection were listed in Table S1, of which β-actin was used as a control to normalize mRNA levels.

EMSAs

EMSAs were performed using a LightShift Chemiluminescent EMSA Kit (Thermo). The IRF1 probe employed in this assay contained a previously reported STAT1-binding sequence (Aaronson and Horvath, 2002). Unlabeled oligonucleotides were added to the reaction mixtures for DNA binding competition.

The sequences of the oligonucleotides were as follows:

5'-TAGCTCTACAACAGCCTGATTTCCCCGAAATGACGGCACGCAGCCGGC-3'

5'-GCCGGCTGCGTGCCGTCATTTCGGGGAAATCAGGCTGTTGTAGAGCTA-3'

Microarray analysis

RNA was extracted with an RNeasy Mini Kit (Qiagen, 74126) and verified according to the RNA integrity number (RIN). After first-strand cDNA synthesis and second-strand cDNA synthesis, the cDNA was labeled with biotin and fragmented. Then, the fragmented RNA was hybridized to a Mouse Genome 430 2.0 GeneChip (Affymetrix), scanned with a GeneChip Scanner 3000 (Affymetrix) and analyzed with Affymetrix GeneChip Command Console (AGCC) software.

Antiviral assay

A total of 5×10^3 MEFs pretreated with or without AMN107 (5 μM) and *c-Abl/Arg*-knockdown MEFs pre-infected with or without an adenovirus expressing Abl were seeded into 96-well plates containing four-fold serial dilutions of IFNγ. After 20-24 h of incubation

with the cytokine, the cells were infected with the Indiana strain of VSV at a multiplicity of infection (MOI) of 10. When a significant cytopathic effect (CPE) was observed in the VSV-infected positive control after infection, cell viability was measured using a CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS) (Promega), and the absorbance at 492 nm was measured using a multilabel reader (PerkinElmer EnVision).

For the NDV-GFP complementation assay, A549 cells transfected with 2 μg of plasmid DNA or treated with AMN107 were seeded into a 24-well microplate. After 24 h of incubation, the cells were washed twice with phosphate-buffered saline and infected with NDV bearing the GFP gene at an MOI of 10 at 37 °C for 2 h. The viral inoculum was removed from the cells, and 0.5 ml of DMEM containing 10% FBS was added to the cells. The cells were incubated for 24 h at 37 °C, and the green infected cells (GFP-expressing cells) were counted by fluorescence-activated cell sorting (FACS) analysis (BD FACSCalibur).

Analysis of nuclear-cytoplasm distribution

Cells on glass coverslips were fixed and permeabilized as described previously. Anti-STAT1 and anti-c-Abl antibodies were applied to detect endogenous STAT1 (green) and c-Abl (red) in MCF-7 cells via immunofluorescence microscopy (Zeiss LSM 800). The nuclear level of STAT1 was calculated by the ratio of fluorescence intensity in the nucleus to that in the cytoplasm in the same cell using ImageJ software. At least 15 cells were examined, and the results are expressed as the mean±SD.

FACS analysis

For GFP fluorescence analysis, A549 cells were harvested and subsequently washed with PBS containing 2% FBS. After centrifugation, the cell pellets were resuspended and analyzed using an ImageStreamX Mark II flow cytometer (Millipore).

Antigen presentation assays

Antigen presentation by JAWS II dendritic cells was analyzed as described by Mangala Rao et al. (Steers et al., 2009). Briefly, JAWS II cells were pretreated with or without AMN107 (5 μM), washed, diluted to 2×10^6 cells/ml in serum-free medium in the presence of 0.1 mg/ml OVA in a total volume of 0.2 ml and incubated for 90 min at 37 °C. The cells were then washed three times in serum-free medium and cocultured for 18 h with 5×10^3 B3Z cells/well (B3Z is a murine $CD8^+$ T cell hybridoma line specific for SIINFEKL). At the end of the coculture period, the supernatants were collected, and the levels of IL-2 in the supernatants were measured in triplicate using a Mouse IL-2 ELISA kit (eBioscience) according to the manufacturer's instructions.

Viral infection

Infection of 8- to 10-week-old mice was carried out via intranasal administration of 0.1 LD_{50} mouse-adapted UI182 IAV derived from A/Changchun/01/2009. WT mice were treated with AMN107 (200 mg/kg/d) or vehicle for 1 week. Groups of mice were monitored for survival for 12 days after infection.

Statistical analysis

Mean values quantified from at least three independent experiments were analyzed by Student's t-test or log-rank test (survival curves) using GraphPad Prism software. Differences with p-values<0.05 were considered significant.

Supplemental References

Aaronson, D.S., and Horvath, C.M. (2002). A road map for those who don't know JAK-STAT. Science *296*, 1653-1655.

Koleske, A.J., Gifford, A.M., Scott, M.L., Nee, M., Bronson, R.T., Miczek, K.A., and Baltimore, D. (1998). Essential roles for the Abl and Arg tyrosine kinases in neurulation. Neuron *21*, 1259-1272.

Steers, N.J., Peachman, K.K., McClain, S.R., Alving, C.R., and Rao, M. (2009). Human immunodeficiency virus type 1 Gag p24 alters the composition of immunoproteasomes and affects antigen presentation. Journal of virology *83*, 7049-7061.