Supplementary methods

Molecular genetic analysis

STAT1 and its flanking introns were amplified by PCR and sequenced. Total RNA was extracted from peripheral blood mononuclear cells (PBMCs) with ISOGEN (NIPPON GENE CO, Japan), and complementary DNA (cDNA) was synthesized by reverse transcription. The wild-type (WT) and Y701C mutant alleles were amplified by PCR, spanning the entire coding region of *STAT1*. The PCR products were inserted into the pGEM-T Easy vector (Promega, USA) and their sequences were confirmed. We generated constructs encoding the L706S, Q463H, and K673R mutant forms of STAT1, by performing PCR-based mutagenesis of the WT construct with mismatched PCR primers. The resulting fragments were inserted, in frame, between the *Bam*HI and *Eco*RV sites of the pcDNA-C-Flag mammalian expression vector. Primer sequences and PCR conditions are available on request.

Analysis of gene expression

Epstein-Barr virus-transformed B (EBV-B) cells from control individuals, the patient from Kindred A and patients carrying L706S *STAT1* mutations were maintained in RPMI1640 supplemented with 20% fetal bovine serum (FBS) (Thermo Scientific, USA).

U3C STAT1-null fibrosarcoma cells were maintained in DMEM supplemented with 10% FBS. The plasmids carrying the WT and each of the mutant STAT1 genes were transferred, individually, into U3C cells by lipofection (Invitrogen, USA). EBV-B cells and transfected U3C cells were left unstimulated or were stimulated with 10^5 IU/ml of IFNs for 15 minutes and subjected to immunoblot analysis. Pervanadate was prepared by mixing orthovanadate with H_2O_2 and incubating for 15 minutes at 22°C. U3C transfectants were treated with pervanadate (0.8 mM orthovanadate mixed with 0.2 mM H_2O_2) for five minutes before stimulation with IFN-γ. We investigated the nuclear and cytoplasmic fractions, by stimulating U3C transfectants with IFN-γ and then isolating these fractions with NE-PER Nuclear and Cytoplasmic Extraction Regents (Thermo Scientific), according to the manufacturer's protocol. Information about the antibodies used is provided in Supplementary Table S2.

Immunostaining

U2OS human bone osteosarcoma epithelial cells stably expressing a Flag-tagged WT or mutant STAT1 construct were generated by lipofection and selected on 1 mg/ml G418 (Calbiochem, Germany). Immunostaining was carried out as previously described (16). Images were acquired with a BZ-8000 fluorescence microscope (KEYENCE, Japan).

All immunostaining images were obtained with identical exposure times, to optimize comparison.

Luciferase reporter assay

Reporter plasmids (CignalTM GAS or ISRE Reporter Assay Kit; SABiosciences, Germany) and WT and/or mutant STAT1-encoding plasmids were transferred into U3C cells by lipofection. Six hours after transfection, the cells were transferred to a medium containing 1% FBS, in which they were cultured for 18 hours. Transfectants were treated with 10^3 IU/ml IFN-γ for 16 h or IFN-α for 12 h. Luciferase assays were then carried out with the Dual-Glo luciferase assay system (Promega). Experiments were performed in triplicate and the data are expressed in relative luciferase units (RLU).

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as previously described (18). WT and/or mutant STAT1 plasmids were introduced into U3C cells by lipofection, with analysis 36 h after transfection. U3C transfectants or EBV-B cells were stimulated with 10^5 IU/ml IFNs for 20 minutes and a nuclear extract was then prepared as previously described.(18) We incubated 20 μg (10 μg for analyses of EBV-B cells) of nuclear extract with ^{32}P-labeled (α-dATP)

GAS (generated under control of the *FCGR1* promoter) or ISRE (*ISG15* promoter) probe for 30 minutes.

Quantitative reverse transcription-PCR

EBV-B cells were stimulated with 10^3 IU/ml of IFNs for 2 or 6 hours. Total RNA was then extracted with an RNeasy Mini Kit (QIAGEN, Germany). The cDNA was synthesized directly with random primers, by reverse transcription. *IRF1*, *CXCL9* and *ISG15* mRNA levels were determined by quantitative PCR (qPCR) on the cDNA, with the CFX96 Touch Real-Time PCR Detection System (Bio Rad, USA) and Taqman probes (Applied Biosystems). The results were normalized with respect to the values obtained for the endogenous *GAPDH* cDNA.

CD14-positive monocytes were purified from PBMCs by magnetic sorting (BD Biosciences, USA) and stimulated with 10^3 IU/ml IFN-γ for 2 or 6 hours. The stimulated cells were then subjected to qPCR analysis, for the detection of *IRF1* and *CXCL9* mRNA.

Cytokine determinations

CD14-positive monocytes were used to seed 96-well plates (1.0×10^5/well). They were

added to RPMI 1640 containing 10% FBS and cultured for 48 hours in the presence of 100 ng/ml LPS and various concentrations of IFN-γ (10, 100 and 1000 IU/ml). TNF-α concentration in the supernatant was determined in duplicate, with a human TNF-α antibody bead kit (Invitrogen) and the Luminex 100 system (Luminex, USA).

Flow cytometry

PBMCs were stimulated with 10^4 IU/ml IFN-γ for 15 minutes in the presence of FITC-conjugated anti-CD14 antibody (BD Biosciences). The cells were fixed by incubation with 200 µl BD Cytofix buffer (BD Biosciences) for 10 min at 37°C, permeabilized by incubation with 400 µl BD Phosflow Perm Buffer III (BD Biosciences) on ice for at least 30 min, and then stained with PE-conjugated anti-Stat1 antibody (pY701) (BD Biosciences). The cells were analyzed on a FACS Calibur apparatus (Becton Dickinson, USA).

Statistical analysis

Data are expressed as mean ± Standard Deviation (SD). Statistical significance was calculated using analysis of variance followed by Tukey's post hoc analysis and SPSS software. $P<0.05$ was considered statistically significant.

Supplementary Table S1 Laboratory data (Specific antibody) of the patients

Specific antibody	P1	P2	Negative range
Mumps IgG (EIA)	25.1	7.5	<2.0
Measles IgG (EIA)	<2.0	17.9	<2.0
Rubella IgG (EIA)	<2.0	34.3	<2.0
VZV IgG (EIA)	63.2	18.8	<2.0
Adeno (CF)	<4	<4	<4
Influenza (CF)	128	<4	<4
RSV (CF)	<4	<4	<4
EB VCA IgG (FA)	<10	80	<10
EBNA (FA)	<10	40	<10

Supplementary Table S2

Antibody	Species	Vendor
Phosphorylated STAT1 (pY701)	rabbit	BD Bioscience
	rabbit	Cell Signaling
STAT1	rabbit	Cell Signaling
FLAG	mouse	Sigma-Aldrich
β-actin	mouse	Sigma-Aldrich
Lamin A/C	mouse	Cell Signaling
GAPDH	mouse	Sigma-Aldrich

Figure S1A

Reverse

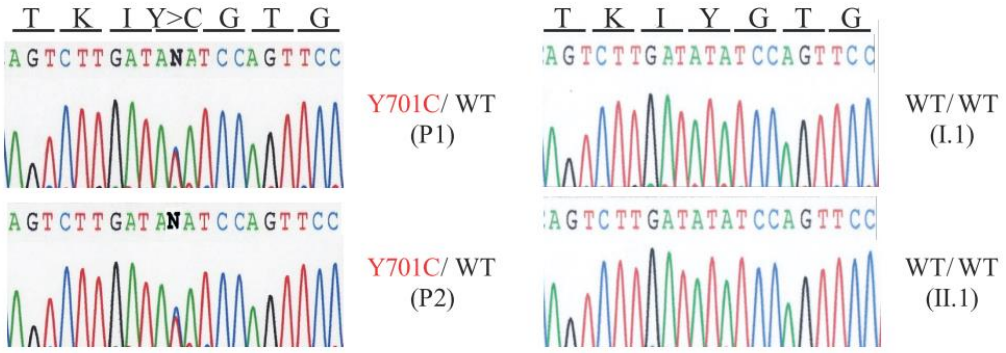


Figure S1B

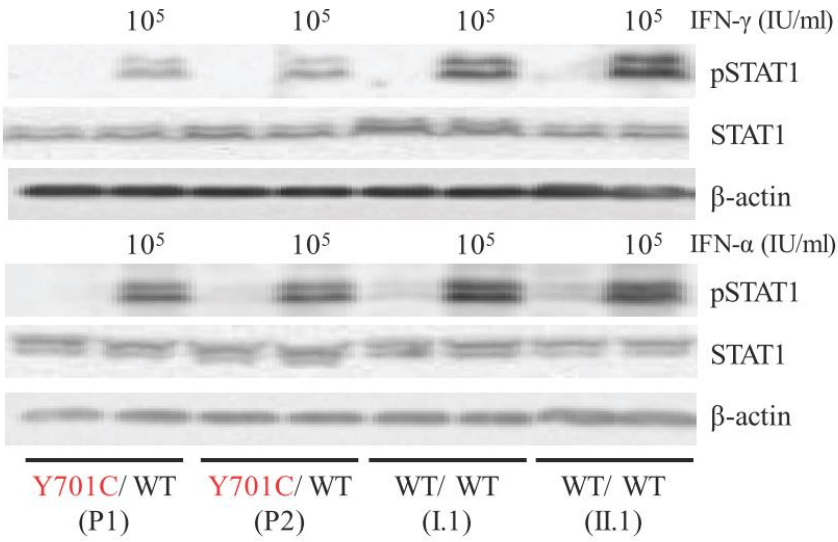
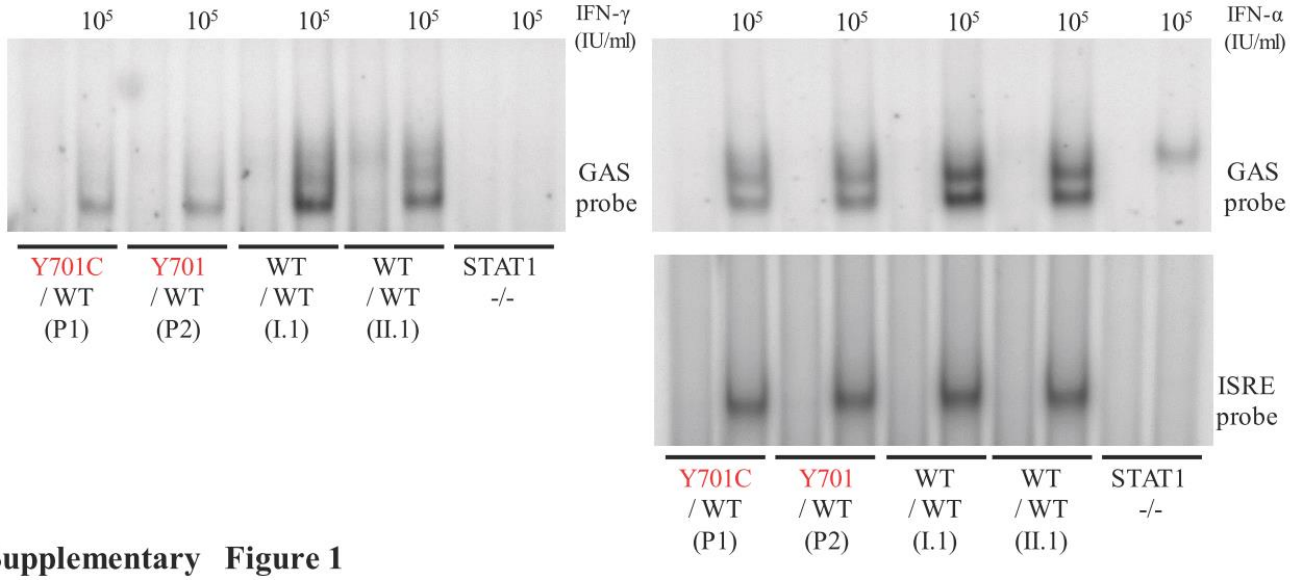


Figure S1C



Supplementary Figure 1

Figure S2A

Before IFN-γ stimulation

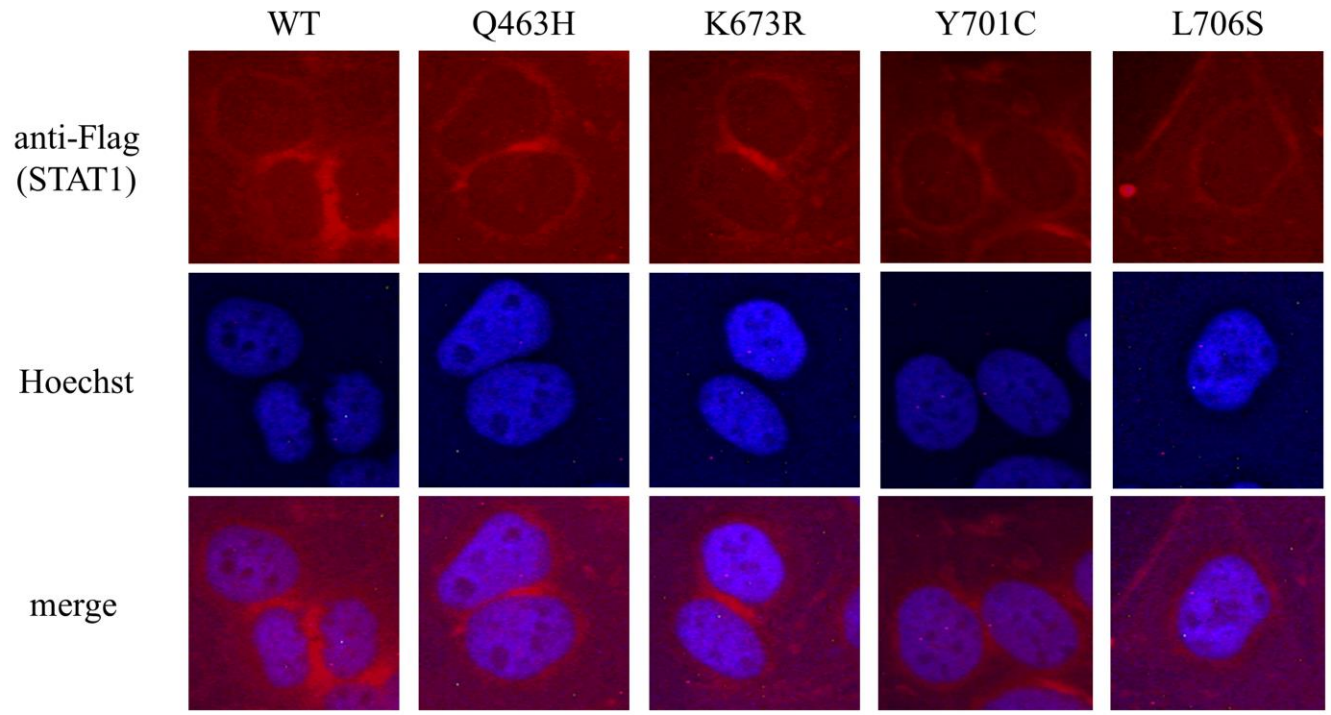
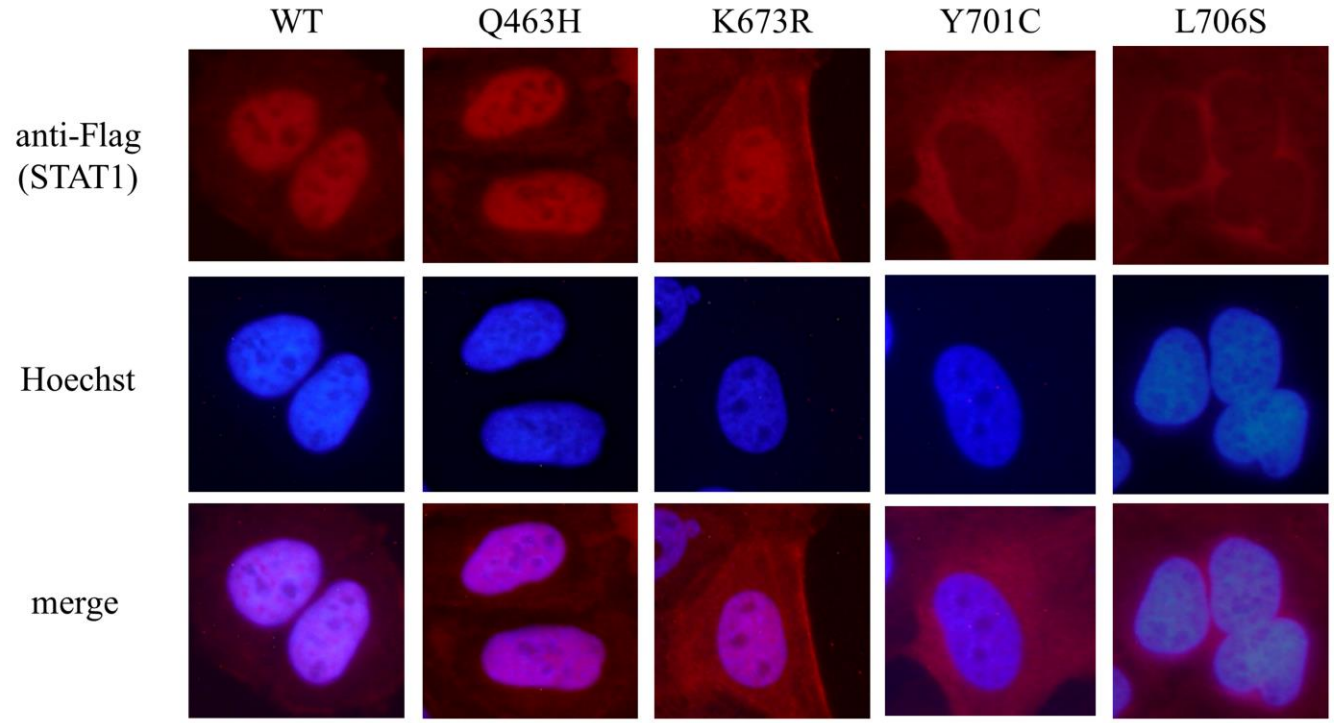


Figure S2B

After IFN-γ stimulation



Supplementary Figure 2

Figure S3A

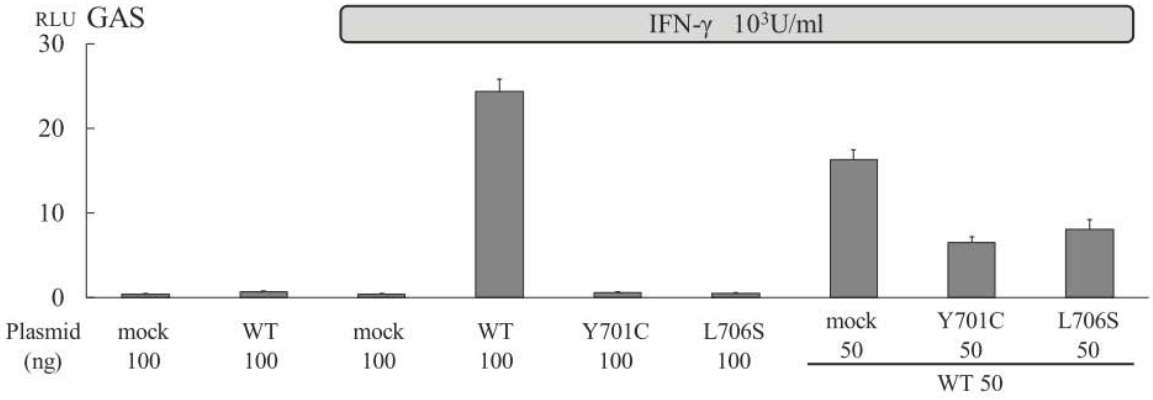


Figure S3B

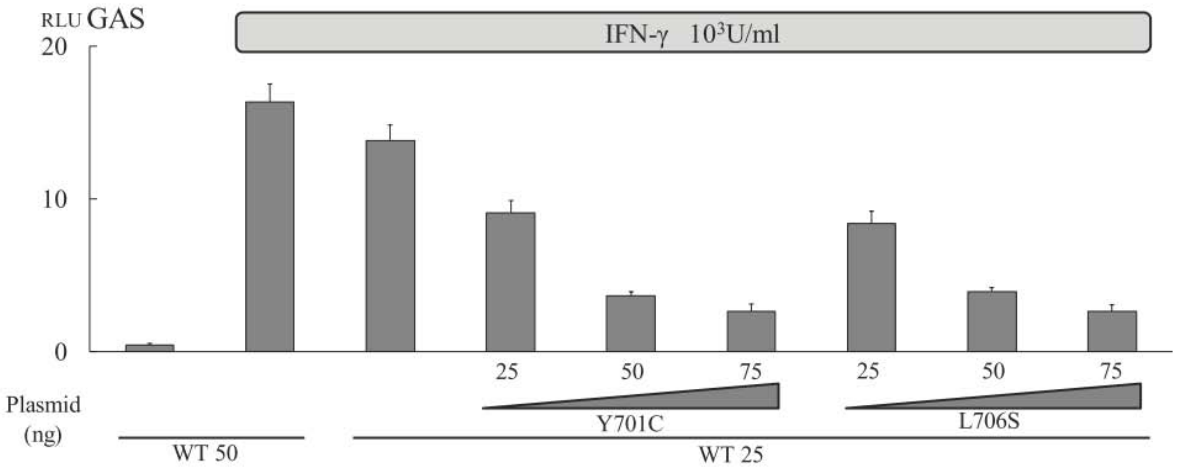


Figure S3C

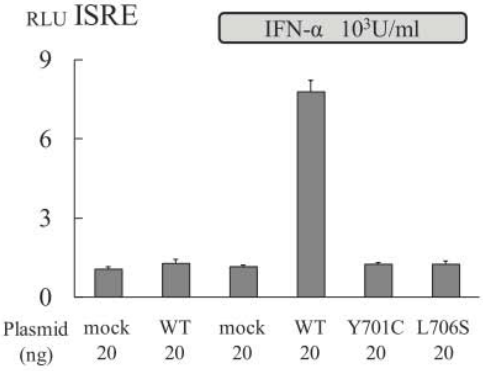
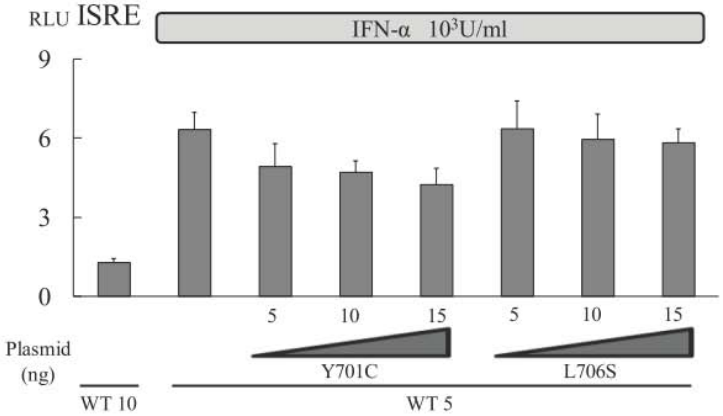


Figure S3D



Supplementary Figure 3

Supplementary Figure Legends

Figure S1.

(A) A heterozygous 2102 T>C (Y701C) mutation was detected in the patient (II.2) and his mother (I.2). (B) EBV-B cells from the patient (P1), his mother (P2) carrying Y701C mutation and other family members (I.1, II.1) were stimulated with 10^5 IU/ml IFNs for 15 min and subjected to immunoblotting. Cells from the patients (P1, P2) displayed an impairment of STAT-1 phosphorylation that was particularly marked following stimulation with IFN-γ. (C) EBV-B cells from the patient (P1), his mother (P2) carrying the Y701C mutation and other family members (I.1, II.1) were subjected to EMSA with GAS and ISRE probes. Cells from the patients (P1, P2) displayed an impairment of GAF binding to DNA in response not only to IFN-γ, but also in response to IFN-α. ISGF3-DNA binding levels upon IFN-α stimulation were almost normal in the patients' cells. At least two independent experiments were performed.

Figure S2. Immunostaining of WT and mutant STAT1-producing cells

U2OS cells stably expressing Flag-tagged WT, Q463H, K673R Y701C, or L706S STAT1 were incubated without (A) or with (B) IFN-γ for 30 min and stained with FITC-tagged anti-Flag antibody for STAT1 protein and Hoechst 33342 (DAPI) for

nuclei. (A) WT and mutant STAT1 proteins were present mostly in the cytoplasm before IFN-γ stimulation. (B) After IFN-γ stimulation, the WT and Q463H STAT1 proteins were found mostly in the nucleus. Nuclear translocation was severely impaired in cells producing the Y701C and L706S STAT1 proteins. The K673R STAT1 mutant was observed in both the nucleus and cytoplasm, suggesting incomplete nuclear translocation. Two independent experiments were carried out.

Figure S3. Effect of *STAT1* mutation on transcriptional activity

U3C cells were transiently cotransfected with plasmids carrying the WT and/or mutant *STAT1* and with GAS (A, B) or ISRE (C, D) reporter plasmids. The quantities of the plasmids used are indicated under the bar. The cells were stimulated with IFN-γ (for 16 hours) or IFN-α (for 12 hours), 24 hours after transfection. (A) The Y701C and L706S *STAT1* mutations abolished GAS transcriptional activity. Cotransfection experiments revealed that cells producing mutant STAT1 proteins had just under a quarter the GAS transcriptional activity of cells producing the WT protein. (B) A dose-dependent negative effect on WT protein activity was observed in cells cotransfected with Y701C and L706S constructs. (C, D) The Y701C and L706S *STAT1* mutations abolished ISRE transcriptional activity. A negative effect was suspected in cells cotransfected with the

Y701C construct, but no clear dominant-negative effects on ISRE transcriptional activity were demonstrated. The error bars indicate the SD of one experiment carried out in triplicate. At least two independent experiments were performed.

Figure S1A

Reverse

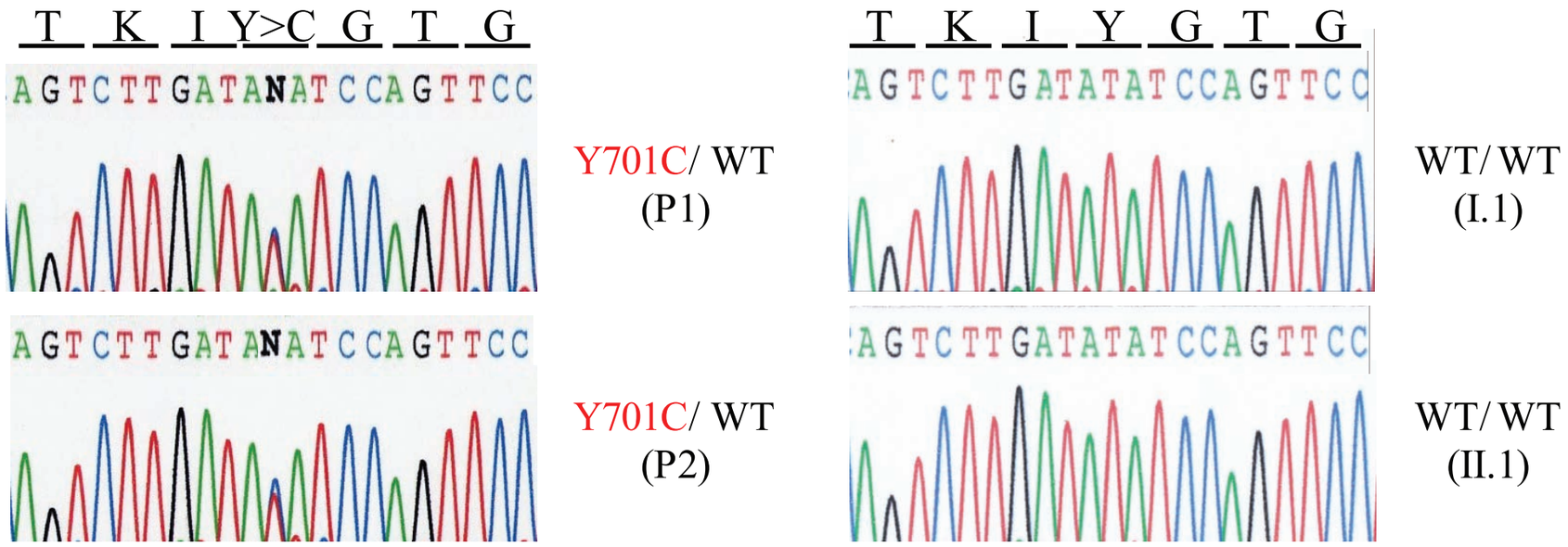


Figure S1B

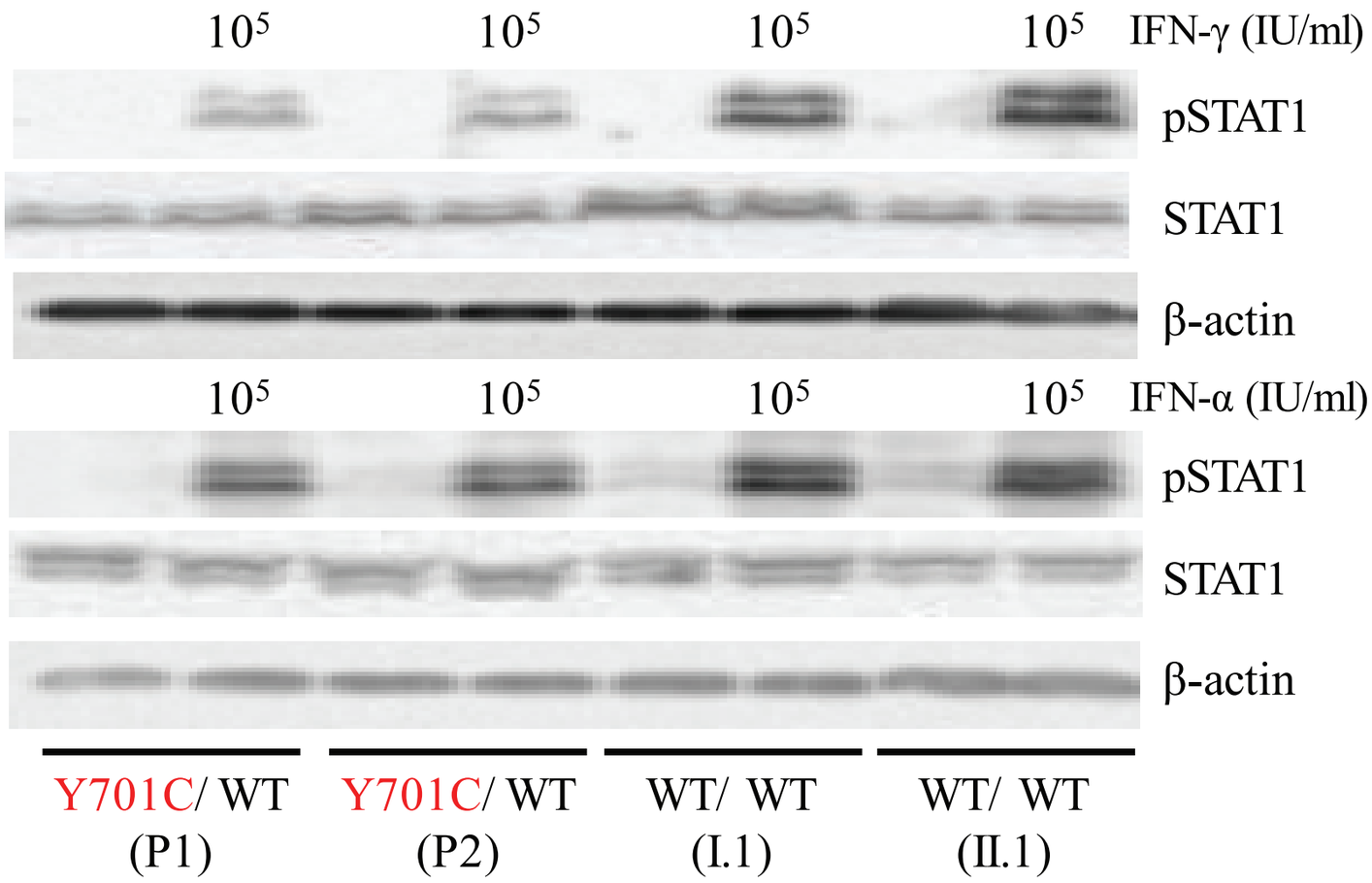
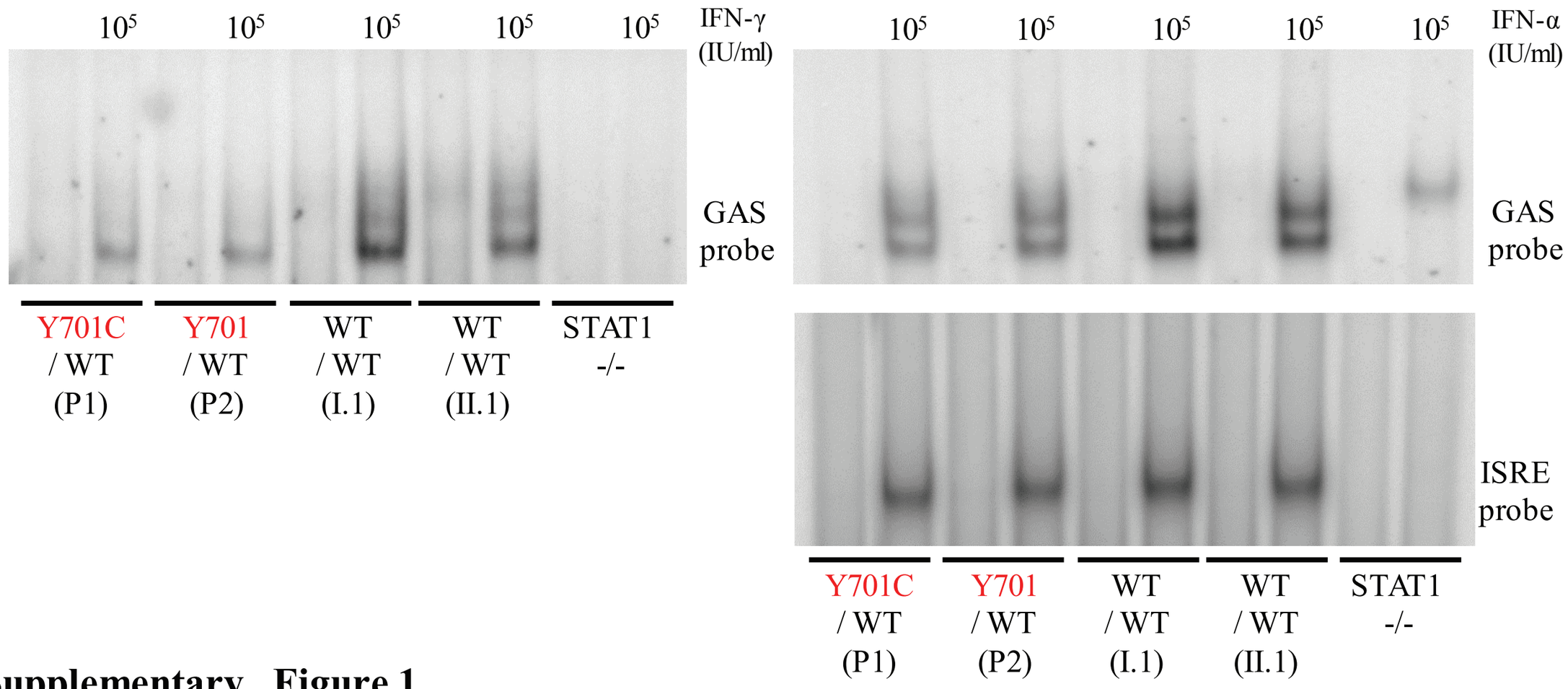


Figure S1C



Supplementary Figure 1

Figure S2A

Before IFN-γ stimulation

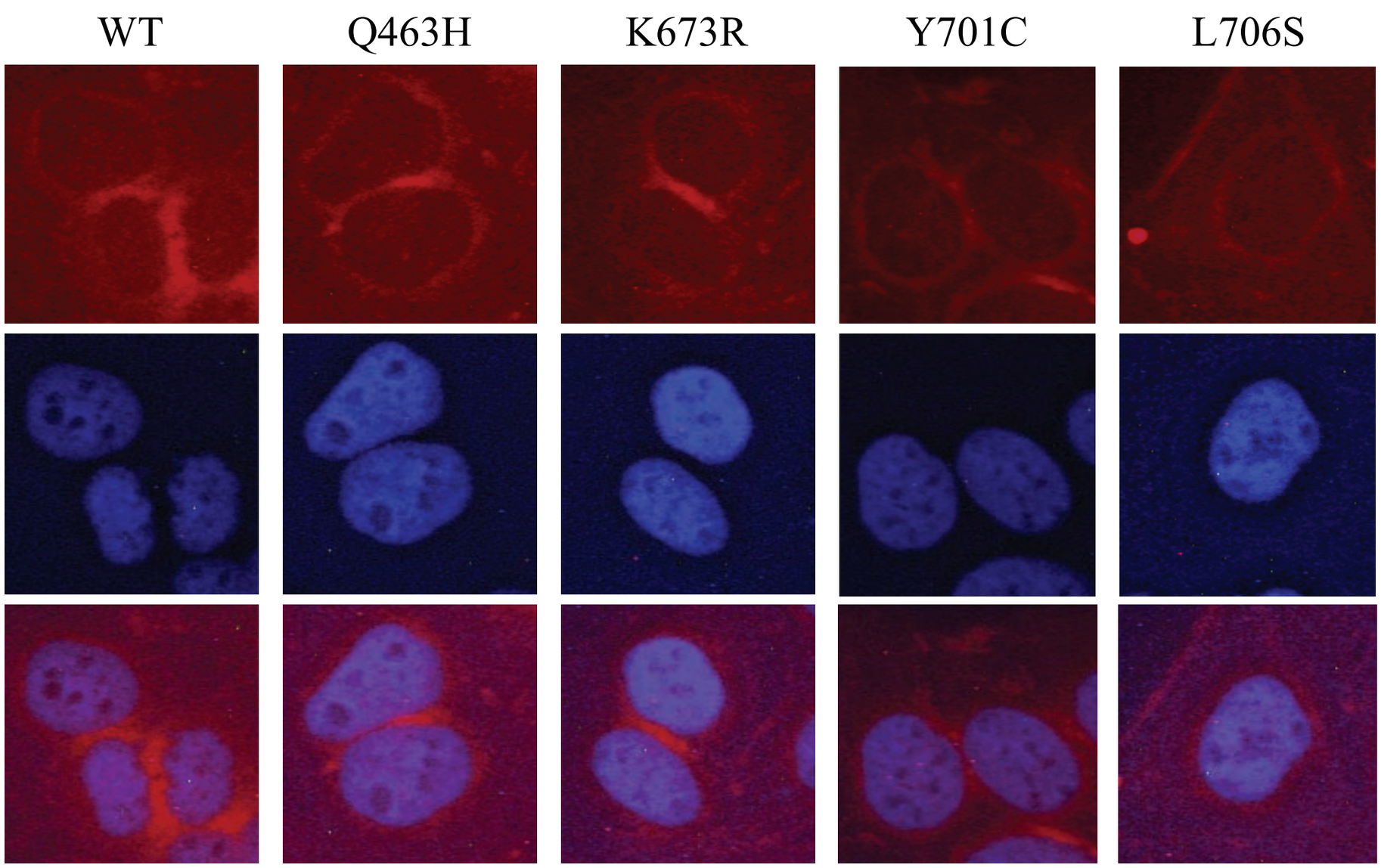
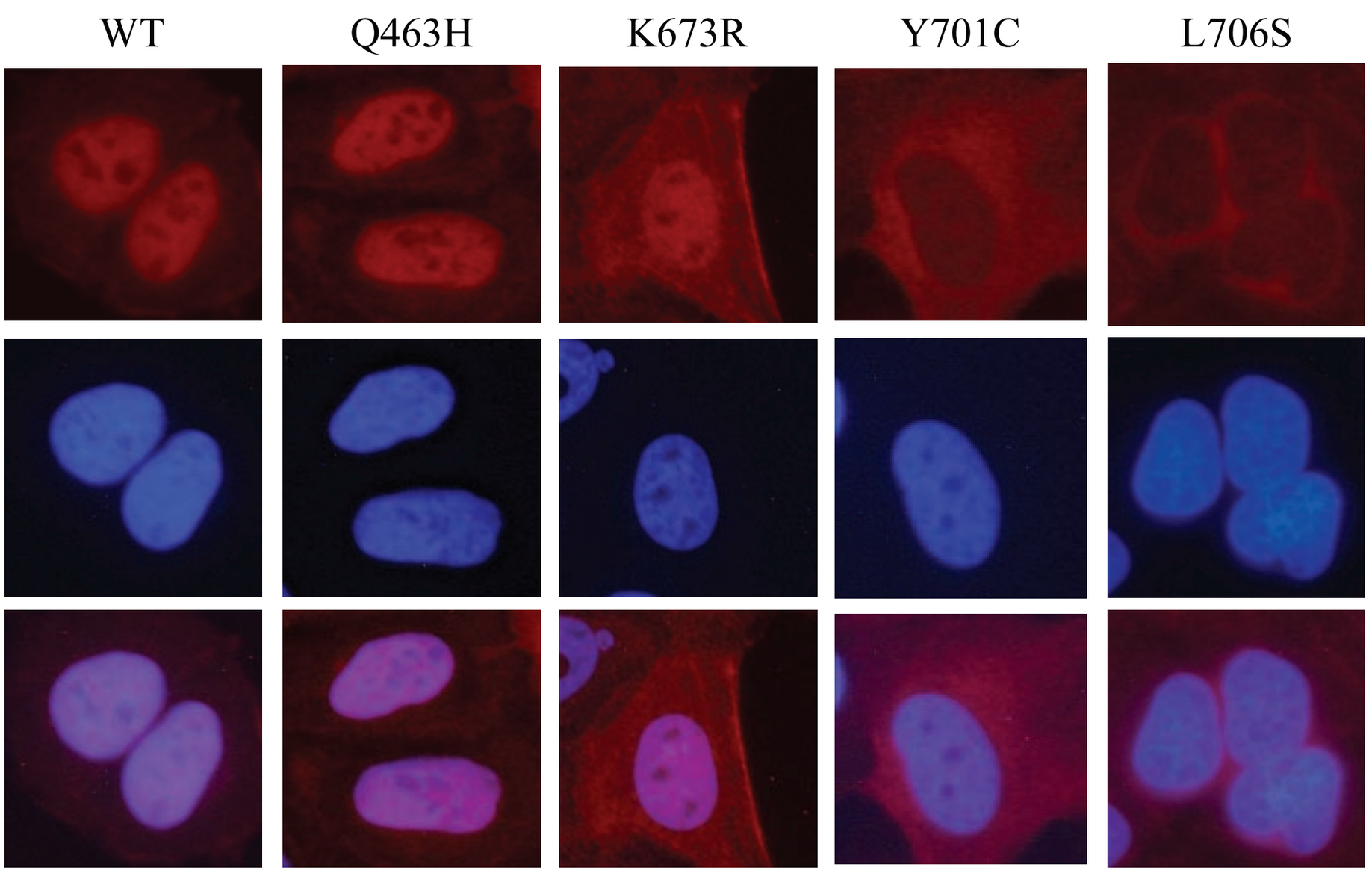


Figure S2B

After IFN-γ stimulation



Supplementary Figure 2

Figure S3A

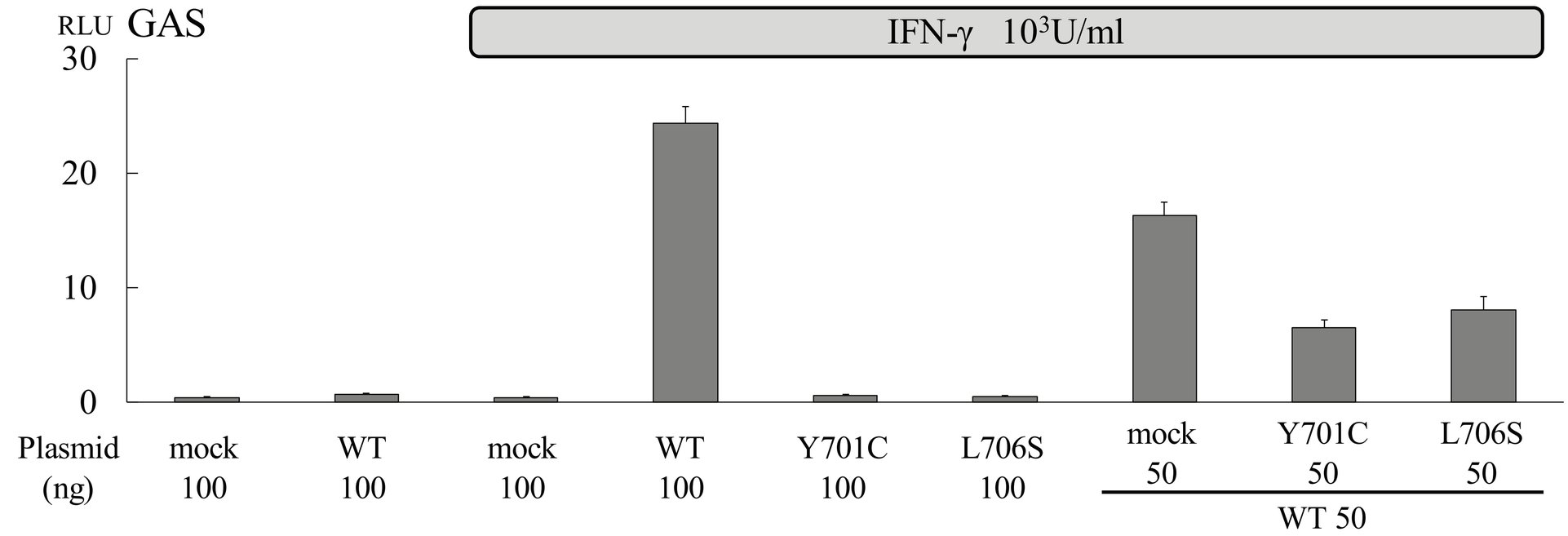


Figure S3B

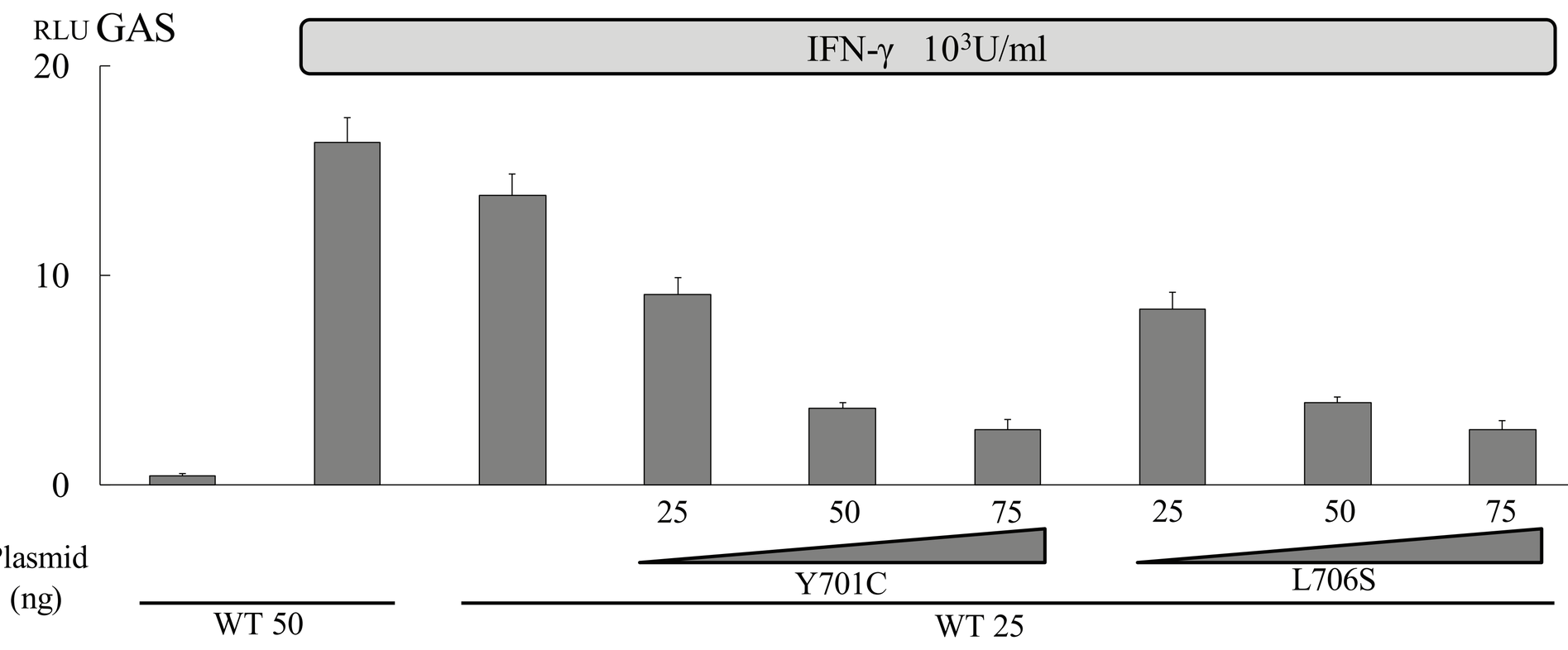


Figure S3C

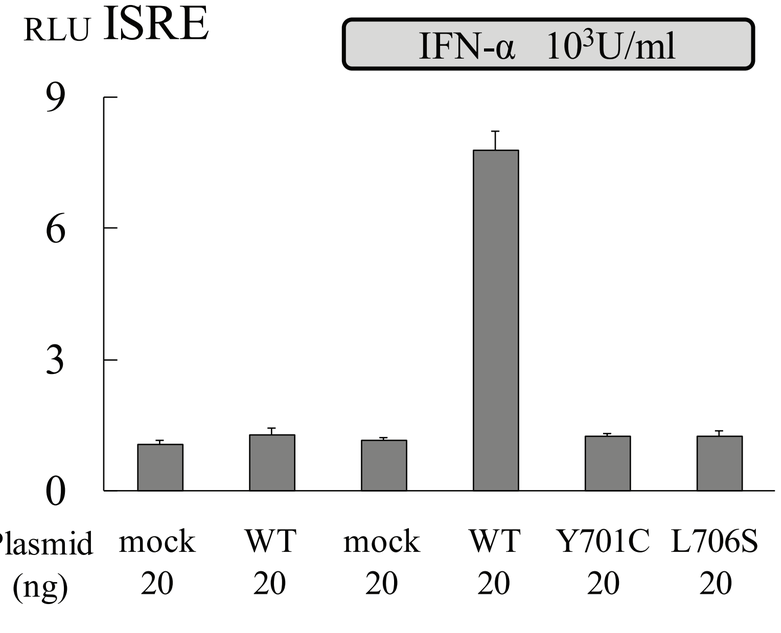
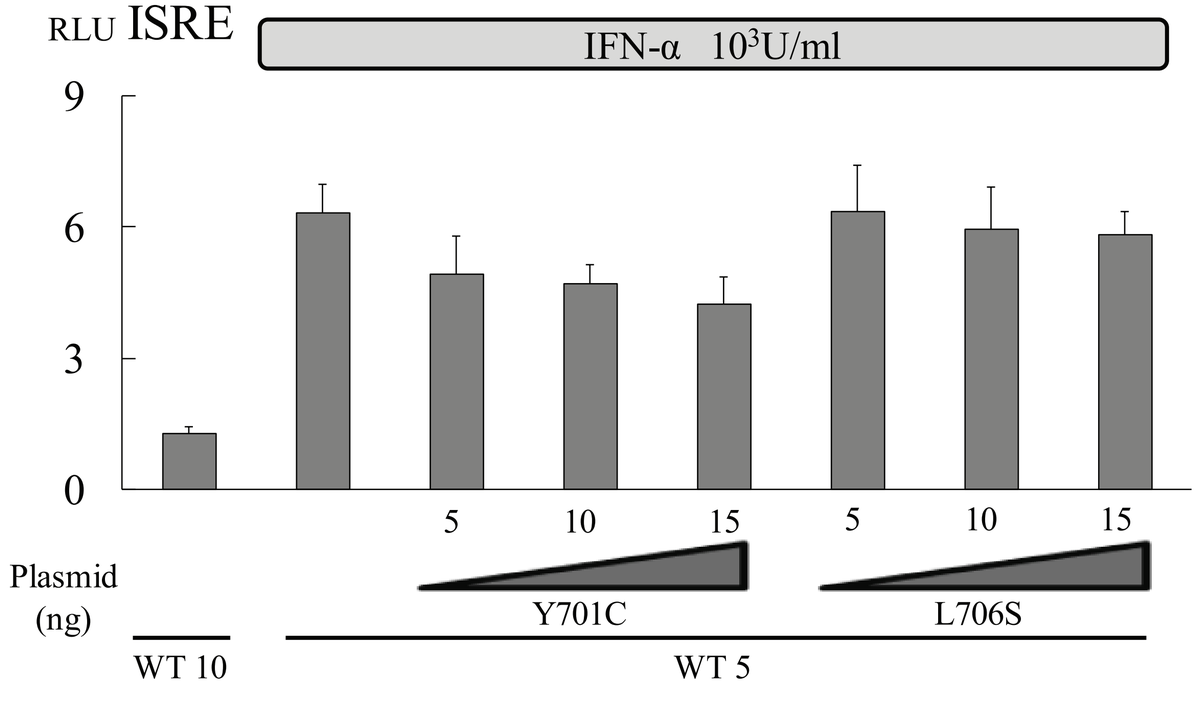


Figure S3D



Supplementary Figure 3