Supplementary Information

Partial IFN-γR2 deficiency is due to protein misfolding and can be rescued by inhibitors of glycosylation

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Supplemental Table S1. Results of whole exome sequencing of P2 and P3.

		P2	P3
Nonsense (Stop-gained)	**Total**	72	84
	Novel homozygous	0	0
	Novel heterozygous	10	16
Readthrough (Stop-lost)	**Total**	11	10
	Novel homozygous	0	0
	Novel heterozygous	0	0
Missense	**Total**	9203	7386
	Novel homozygous	15	16
	Novel heterozygous	308	237
Silent	**Total**	10432	8449
	Novel homozygous	8	134
	Novel heterozygous	179	15
Frameshift	**Total**	89	69
	Novel homozygous	15	4
	Novel heterozygous	6	20
Inframe (Del and Ins)	**Total**	206	92
	Novel homozygous	36	9
	Novel heterozygous	41	12
Splice	**Total**	111	65
	Novel homozygous	3	1
	Novel heterozygous	9	8

Only variations in coding regions are displayed.

Supplemental Material and Methods

Case reports

Kindred A. The patient P1 (A.III.1) is a Mexican boy born to parents not known to be consanguineous. He was vaccinated with BCG at the age of four months and presented axillary adenopathies and a purulent cutaneous lesion at the vaccination site at the age of six months. His plasma IFN-γ concentration was found to be high (44 pg/ml). A tuberculin skin test (TST) with the purified protein derivative was positive, producing indurations of 20 mm x 20 mm; chest X ray was highly suggestive of miliary tuberculosis. No acid-fast bacilli were detected and cultures for bacteria, mycobacteria, viruses and fungi were negative. The infection was resolved by six months of treatment with isoniazid, rifampicin and pyrazinamide. At 30 months of age, the patient presented an enlarged supraclavicular lymph node. Biopsy was performed without positive bacterial identification, but histological examination revealed granuloma formation. The patient recovered following treatment with isoniazid, rifampicin and pyrazinamide. At the age of nine years, he presented symptoms consistent with peritoneal tuberculosis. The microbe involved was not identified, but the patient was nonetheless given anti-tuberculosis treatment. At 10 years of age, the patient was admitted to hospital with seizures. A few months before admission, he presented cutaneous lesions associated with fever. Serological tests for cytomegalovirus, Epstein-Barr virus, parvovirus B19, HIV, brucellosis and leptospirosis were negative. Magnetic resonance imaging (MRI) revealed frontotemporal arachnoiditis and a left frontal mass suggestive of tuberculoma. Brain biopsy revealed the presence of a granuloma, for which no microbial identification (fungi, mycobacteria, bacteria, viruses) was possible by culture. However, a polymerase chain reaction (PCR) on cerebrospinal fluid was positive for

Mycobacterium tuberculosis complex (which includes BCG, an attenuated *M. bovis* strain). A daily regimen of isoniazid, rifampicin, ethambutol and pyrazinamide was initiated and has been maintained ever since. The patient also received subcutaneous recombinant interferon gamma. Corticosteroids were administered over a period of 12 months, for arachnoiditis. The patient is now 12 years old and is responding well to antibiotic treatment.

Kindred B. The patient P2 (B. III.2) is a Turkish boy, fourth child of second degree consanguinous healthy parents. He was vaccinated with BCG at the age of two months old and developed left preaxillary enduration one month later. Biopsy was performed and *Mycobacterium elephantis* was isolated. The patient was treated with isoniazid, rifampicin, clarithromycine and ciprofloxacin for 9 months. He presented high plasma IFN-γ concentration (111 pg/ml). At 6 years old, he developed hepatosplenomegaly. The patient has three healthy siblings. Two brothers and one sister died from an unknown etiology. The patient is now 12 years old and doing well with continuous therapy (Rifabutin, ciprofloxacine, claritromycine and salazoprine) without any infections, diarrhea or autoimmune findings.

Kindred C. The patient P3 (C.IV.3) is a Turkish boy, born to second degree consanguinous healthy parents. He has two healthy siblings. He was vaccinated with BCG at the age of two months and presented left preaxillary enduration at the age of eight months. Ultrasonography revealed 38*30 cm in diameter semisolid mass and chest computed tomography (CT) revealed a soft tissue mass, not causing any destructive lesion on neighbouring ribs, located on left upper hemithorax leading to preaxillary region. The patient underwent an excisional biopsy. Histopathology of biopsy specimen showed noncaseating granuloma containing epiteloid

histiocytes and fibroblasts, a few langhans giant cells and profound neutrophilic inflammation. The diagnosis was tuberculosis lymphadenitis (without isolation of the mycobacteria) and anti-mycobacterial treatment (isoniazid, rifampicin, and pyrazinamide) was started. After 3 months, the treatment was continued with 2 anti-mycobacterial medications. There was no endobronchial pathology. Chest and abdominal CT revealed hilar and mediastinal lympadenopathy, bronchiolitis obliterans, and right iliac wing and fifth rib osteomyelitis. He also presented detected level of IFN-γ (20 pg/ml) on plasma. The patient is now 8 years old and doing well with continuous therapy (isoniazid, rifampicin, and pyrazinamide).

Cell culture, stimulation and the extraction of DNA and RNA

Lines of EBV-transformed B cells (EBV-B cells) and SV40-transformed fibroblasts (SV40-fibroblasts) were established as previously described[1,2]. Briefly, cells were stimulated with IFN-γ (Imukin; Boehringer Ingelheim) and IFN-α2B (IntronA, Schering Plough), at the indicated doses, for 20 minutes. Genomic DNA was isolated from unstimulated EBV-B cells and leucocytes from a healthy control, the patients, parents and some relatives, by phenol/chloroform extraction. RNA was isolated, with Trizol (Invitrogen), from unstimulated and stimulated EBV-B cells from the patients, three healthy controls, a patient with complete IFN-γR2 deficiency (218delAA/218delAA)[3], a patient with partial recessive IFN-γR2 deficiency (R114C/R114C)[4] and a patient with dominant partial IFN-γR1 deficiency (818del4/WT)[5].

Electrophoretic mobility shift assay (EMSA)

EMSA was carried out as previously described[6]. Briefly, EBV-B cells were either left unstimulated or were stimulated with various doses of IFN-γ or IFN-α for 20 min. Nuclear

proteins were extracted and quantified by the Bradford method. We then incubated 10 µg (stimulated with IFN-γ) or 5 µg (stimulated with IFN-α) of nuclear protein with a radiolabeled GAS probe corresponding to the Fc-γR1 promoter and subjected the mixture to electrophoresis in a polyacrylamide gel. For supershift assays, 10 µg of nuclear protein from WT/WT and P1 cells were first incubated with excess of unlabeled probe or 2 µg of antibodies against STAT1 (sc345), STAT2 (sc476), STAT3 (sc7179) or p48/IRF-9 (sc496) (Santa Cruz) for 30 minutes before the addition of the radiolabeled GAS probe, to determine the composition of the GAS-binding protein.

Whole-exome sequencing, PCR and sequencing

The method used to sequence the whole exome has been described elsewhere[7-9]. For Sanger sequencing, we amplified the seven coding and flanking intron regions of *IFNGR1* and *IFNGR2* with *Taq* polymerase (Applied Biosystems). The conditions used and the sequences of the primers are available on request. PCR products were analyzed by electrophoresis in agarose gels, purified on Sephadex, sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems) and analyzed on an ABI Prism 3730 (Applied Biosystems). The S124 and G141 residues of IFN-γR2 have been conserved through evolution and both Polyphen II[10] and SIFT[11,12] predicted both mutations to be "probably damaging".

Estimation of the age of the most recent common ancestor to G141R carriers

Founder-effect analysis was carried out on two apparently unrelated patients (P2 and P3) bearing the same homozygous G141R *IFNGR2* mutation and living in the same city of Turkey. Genotypes were derived from the patient's exomes. Known dbSNP variants that were called for

both individuals with a phred-scaled score of more than 30 were scanned for continuous stretches of homozygosity upstream and downstream from the *IFNGR2* locus on chromosome 21q22.11. Comparison of the two patients' genotypes revealed the limits of their shared haplotype and thus the positions of recombination break points. The likelihood-based ESTIAGE method was used to estimate the age of the most recent common ancestor (MRCA) for this mutation from the observed shared haplotypes; recombination rates were obtained from the HapMap Project and haplotype frequencies were derived from the 1,000 genome project's phase 1 integrated call set version 3[13-15].

Flow cytometry

We evaluated the expression of IFN-γR1 and IFN-γR2 on the cell surface by carrying out flow cytometry on EBV-B cells and SV40-fibroblasts, as previously described[4,16,17]. Briefly, we treated 5 x 10^5 EBV-B cells with FcR blocking reagent for 10 min (Miltenyi Biotec), washed them twice and incubated them for 30 min in the dark at 4°C with monoclonal antibodies against IFN-γR1-PE (GIR-94, BD Pharmingen), IFN-γR2-APC (FAB773A, R&D Systems) or with the corresponding isotypic antibodies. For SV40-fibroblasts, the same protocol was used except that the cells were not treated with FcR blocking reagent. The cells were then washed three times, and the signals were read with a BD LSRII flow cytometer and analyzed with Cellquest software (Becton Dickinson Immunocytometry Systems), gating on live cells. Files were analyzed with FlowJo v8.2 software (TreeStarInc) and the results were compared with those for the controls, as indicated.

Relative real-time PCR

We evaluated the induction of *CXCL9, CXCL10* and *IRF8* by either leaving EBV-B cells unstimulated or stimulating them for two or eight hours with IFN-γ (10^3 IU/ ml, Imukin; Boehringer Ingelheim). The induction of *BiP* was evaluated by either leaving SV40-fibroblasts untreated or treated them for six hours with 1μg/ml of brefeldin A. Total RNA was extracted from the cells with Trizol. The RNA was treated with DNase and reverse-transcribed with random hexamer primers and the SuperScript III First-Strand Synthesis System (Invitrogen). We used the *CXCL9* (Hs00970538_m1), *CXCL10* (Hs99999049_m1), *IRF8* (Hs01128710_m1) and *BiP* (Hs00607129_gH) *Taq*man probes to quantify specific mRNA levels and the results were normalized against values for endogenous *GUS* gene expression (β-glucuronidase, a housekeeping gene) (*Taq*man Gene expression assays, Applied Biosystems).

RT-PCR of XBP1 splicing

HEK293 cells were transiently transfected with of mock vector, WT, G185R [18]and C194X-*ELA2*[19], WT *IFNGR2*, R114C, S124F, G141R, G227R, 382-387dup, T168N and 278delAG mutant constructs in the presence of Lipofectamine™ Reagent (Invitrogen), as described by the manufacturer. The cells were analyzed 48 h after transfection. SV40-fibroblasts were left untreated or treated them for six hours with 1μg/ml of brefeldin A (BD GolgiPlug). cDNA was prepared and PCR for *XBP1* splicing was evaluated using the following primers 5'-TTACGAGAGAAAACTCATGGCC-3' and 5'- GGGTCCAAGTTGTCCAGAATGC-3'. *GAPDH* served as internal control and was amplified using the following primers 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'. The PCR products were resolved on 3% agarose gel.

Induction of HLA-DR in response to IFN-γ in SV40-fibroblasts

The method used to detect the expression of HLA-DR on the cell surface has been described elsewhere[4,17]. Briefly, 5 x 10^5 SV40-fibroblasts were incubated with 10 and 10^3 IU/ml of IFN-γ for 48 h. HLA-DR levels on the cell surface were then evaluated by flow cytometry with an anti-human HLA-DR FITC antibody (G46-6, BD Pharmingen). For chemical complementation assays, 3 x 10^4 SV40-fibroblasts were incubated in complete culture medium with or without 1.5 mM NB-DNJ (Toronto Research Chemicals), 2 mM castanospermine (Toronto Research Chemicals) or 160μM kifunensine (Toronto Research Chemicals) for 24 h. They were then left untreated or were treated with 2.4 × 10^4 IU/ml IFN-γ or 10^2 IU/ml IFN-γ (WT and P3) and. HLA-DR levels were analyzed 48 h later, by flow cytometry.

Whole-blood assay of the IL-12/IFN-γ circuit

Whole-blood assays were performed as previously described[20]. Briefly, whole blood diluted 1:2 in RPMI was either not stimulated or was stimulated with BCG+IFN-γ. Supernatants were recovered 48 h later and levels of IL-12p40 and IL-12p70 production were assessed by ELISA.

Biotinylation of cell surface proteins

Two days after transfection, SV40-fibroblasts from an IFN-γR2-deficient patient (278delAG/278delAG)[21], either untreated or treated with 166μM kifunensine (Toronto Research Chemicals), were washed twice with cold PBS. Cell surface biotinylation was then performed with the Pierce® Cell Surface Protein Isolation kit (Thermo Scientific), according to a slightly modified version of the manufacturer's protocol. Briefly, the cell surface proteins were labeled

by incubation of the cells, with gentle shaking, for 30 min at 4°C in phosphate-buffered saline (PBS; pH 7.2) with 0.25 mg/ml EZ-Link Sulpho-NHS-SS-Biotin. The cells were then washed twice with cold PBS. The cross-linking reaction was stopped by adding 50 mM NH_4Cl in PBS and incubating for 10 min at room temperature, with gentle shaking. The cells were then washed twice with cold PBS and lysed in 1%-NP40 lysis buffer. An aliquot of 20 µg of total lysate was retained for western blotting (total fraction) and the rest of the lysate was incubated overnight at 4°C with NeutrAvidin agarose under rotation. The supernatant (flow-through/cytoplasmic fraction) was collected by centrifugation, the beads were washed thoroughly with 1%-NP40 lysis buffer and the precipitated proteins were eluted in SDS-PAGE sample buffer and analyzed by western blotting with HRP-conjugated anti-V5 (Invitrogen) and integrin β1 (Cell Signaling) antibodies, used as cell-surface receptor control.

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Supplemental Figure titles and legends

Supplemental Figure 1

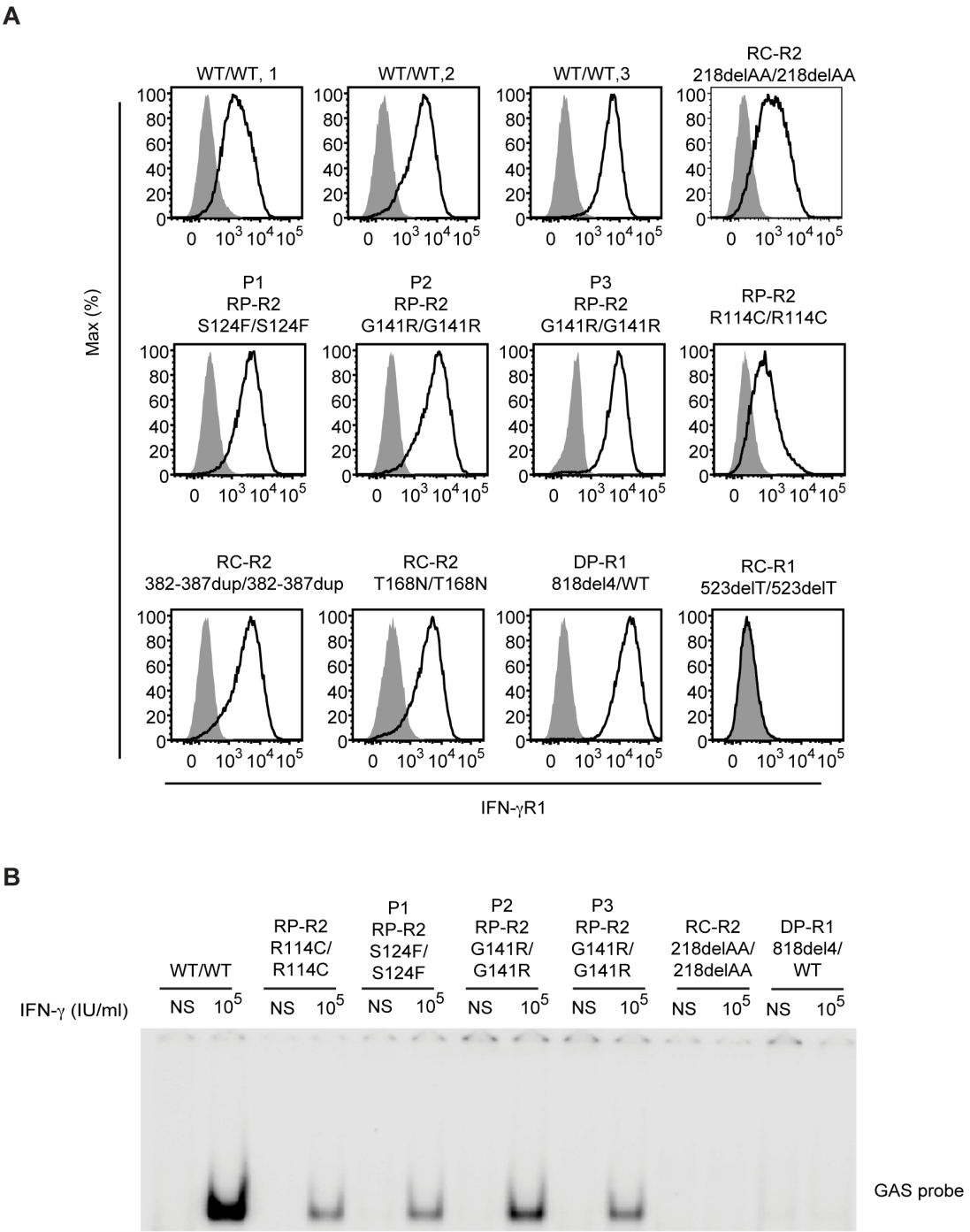

Figure S1. IFN-γR1 expression and impaired STAT1-DNA-binding activity in response to IFN-γ stimulation of the patients' cells. (A) Flow cytometry analysis of IFN-γR1 was compared in EBV-B cells from three healthy controls, a patient with recessive complete IFN-γR2 deficiency (RC-R2, 218delAA/218delAA), the patient P1 with the recessive S124F mutation conferring partial deficiency (RP-R2, S124F/S124F), the patients P2 and P3 with the recessive G141R mutation (RP-R2, G141R/G141R), a patient with recessive partial IFN-γR2 deficiency (RP-R2, R114C/R114C), two patients with complete recessive IFN-γR2 deficiency and surface expression (RC-R2, 382-387dup/382-387dup and RC-R2, T168N/T168N), a patient with dominant partial IFN-γR1 deficiency (DP-R1, 818del4/WT), and a patient with complete recessive IFN-γR1 deficiency (RC-R1, 523delT/523delT). These results are reproducible and representative of at least three independent experiments. The histograms represent the expression of IFN-γR1 (bold- line) and the isotype control (grey filled). **(B)** Response of EBV-B cells to IFN-γ (10^5 IU ml for 20 min), as determined by EMSA of GAS probe-binding nuclear proteins from a healthy control (WT/WT), the patients P1 with the S124F mutation (RP-R2, S124F/S124F), P2 and P3 with the G141R mutation (RP-R2, G141R/G141R) studied here, a patient with recessive partial IFN-γR2 deficiency (RP-R2, R114C/R114C), a patient with recessive complete IFN-γR2 deficiency (RC-R2, 218delAA/218delAA), and a patient with dominant partial IFN-γR1 deficiency (DP-R1, 818del4/ WT).

Supplemental Figure 2

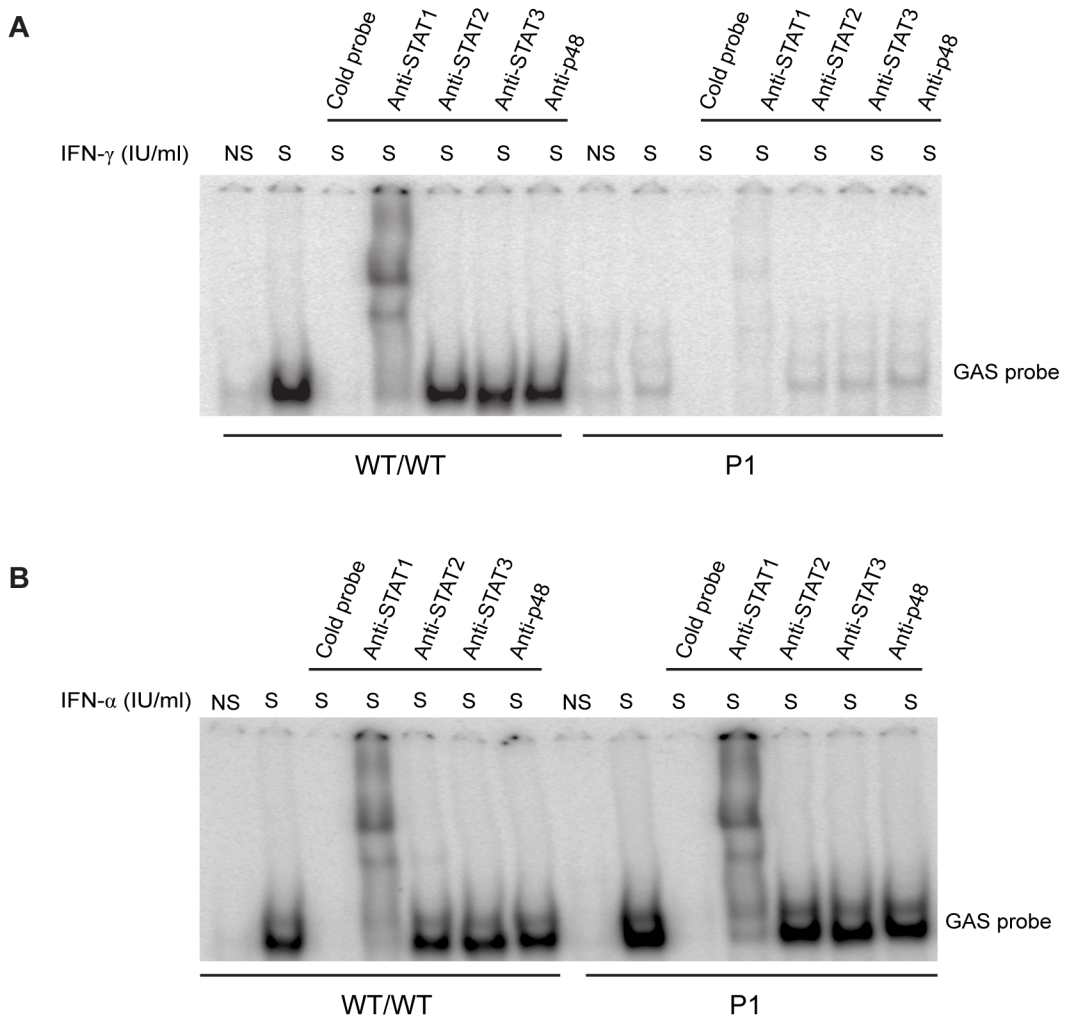

Figure S2. Supershift assay of the GAS-binding proteins. EBV-B cells from a healthy control (WT/WT) and P1 were or were not stimulated with **(A)** 10^5 IU/ml IFN-γ or **(B)** 10^5 IU/ml IFN-α. Antibodies against STAT1, STAT2, STAT3 and IRF9/p48 were added to the nuclear extract to determine the composition of the GAS-binding protein. Only the STAT1 antibody induced a supershift of the GAS binding protein in both WT/WT and P cells. NS: No stimulation, S: Stimulation.

Supplemental Figure 3

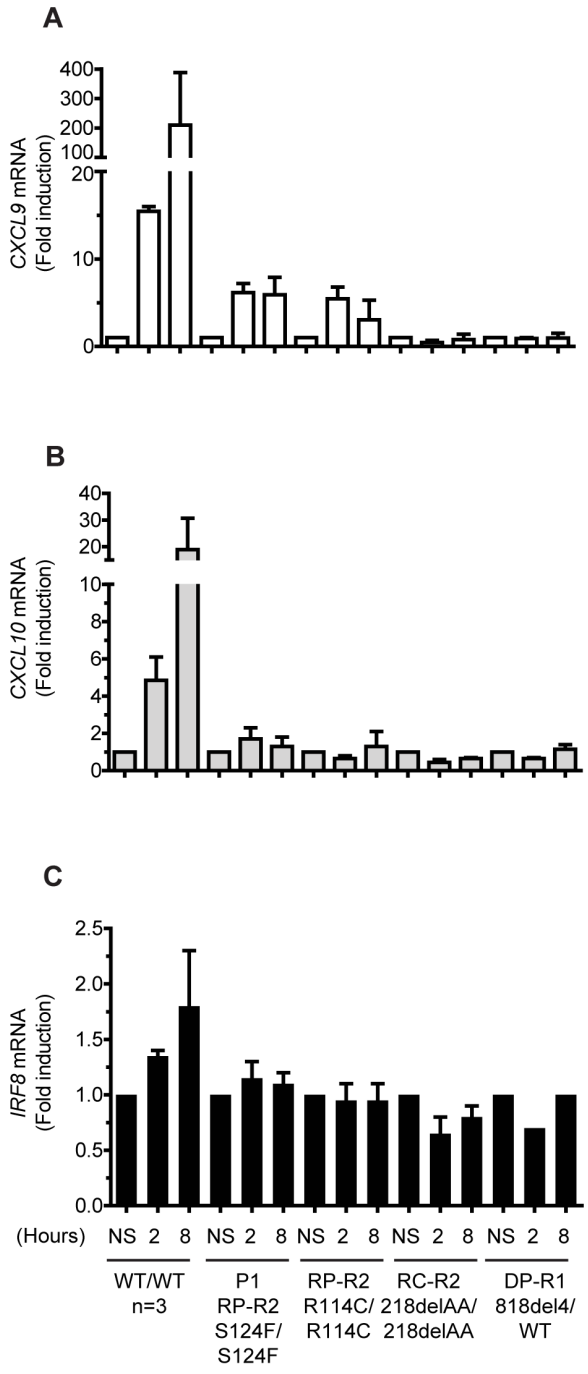

Figure S3. Impaired induction of *CXCL9, CXCL10* and *IRF8* in P1 and R114C cells in response to IFN-γ. RT-qPCR was used to assess the induction of the **(A)** *CXCL9*, **(B)** *CXCL10*

and **(C)** *IRF8* mRNA in EBV-B cells after stimulation with IFN-γ (10^3 IU/ ml for 2 and 8 h) in healthy controls (*n*=3), a patient with recessive partial IFN-γR2 deficiency (RP-R2, R114C/R114C), the patient P1 with the S124F mutation (RP-R2, S124F/S124F), a patient with recessive complete IFN-γR2 deficiency (RC-R2, 218delAA/218delAA) and a patient with dominant partial IFN-γR1 deficiency (DP-R1, 818del4/WT). The values shown are mean values ± SD, calculated from two independent experiments.

Supplemental Figure 4

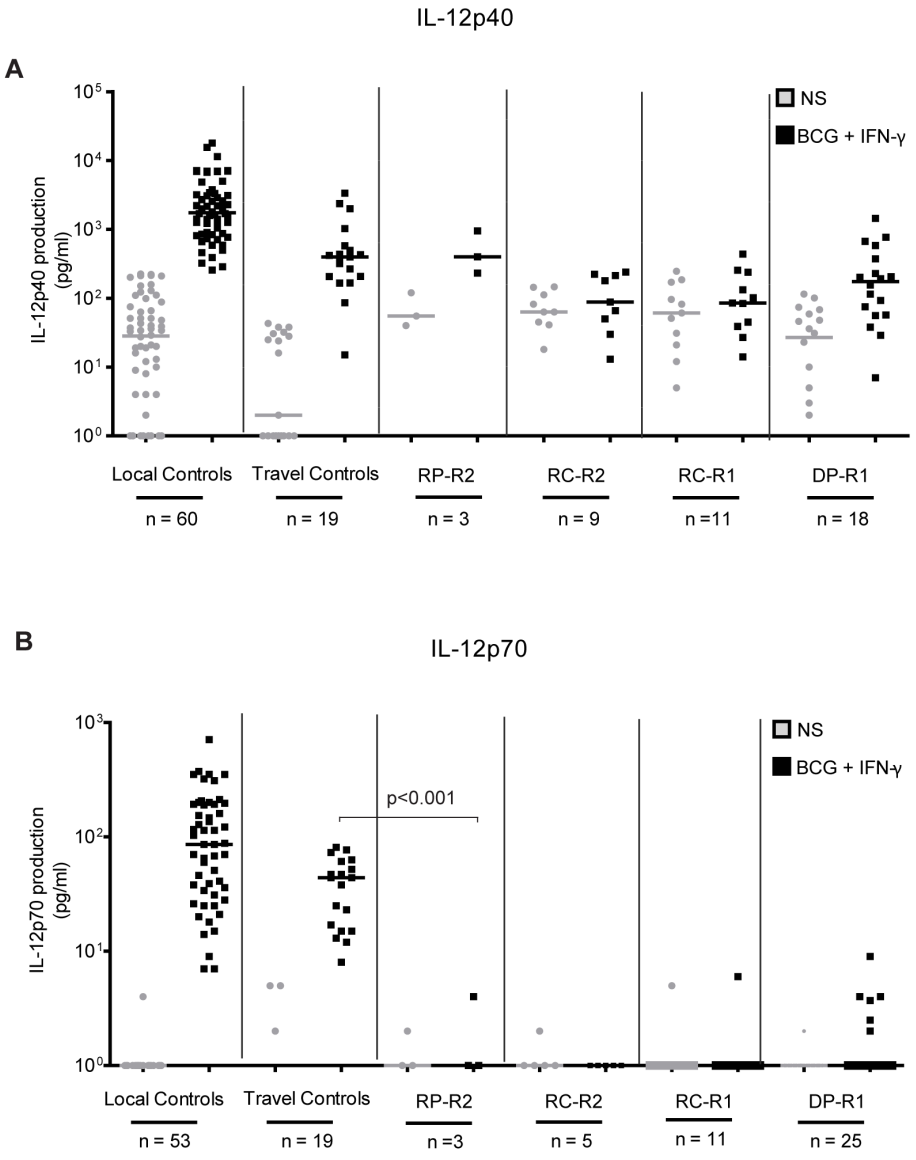

Figure S4. Impaired response to IFN-γ in terms of the production of IL-12p70 in the whole-blood supernatant from the patients. Production of **(A)** IL-12p40 and **(B)** IL-12p70 in whole blood cells from local controls, travel controls, the patients P1, P2 and P3 (RP-R2), patients with complete IFN-γR2 deficiency (RC-R2), patients with complete IFN-γR1 deficiency (RC-R1), and patients with dominant partial IFN-γR1deficiency (DP-R1) either not stimulated (NS) or

stimulated for 48 hours with live BCG alone or live BCG plus IFN-γ, as assessed by ELISA. Each symbol represents a value from an independent test and horizontal bars represent medians.

Supplemental Figure 5

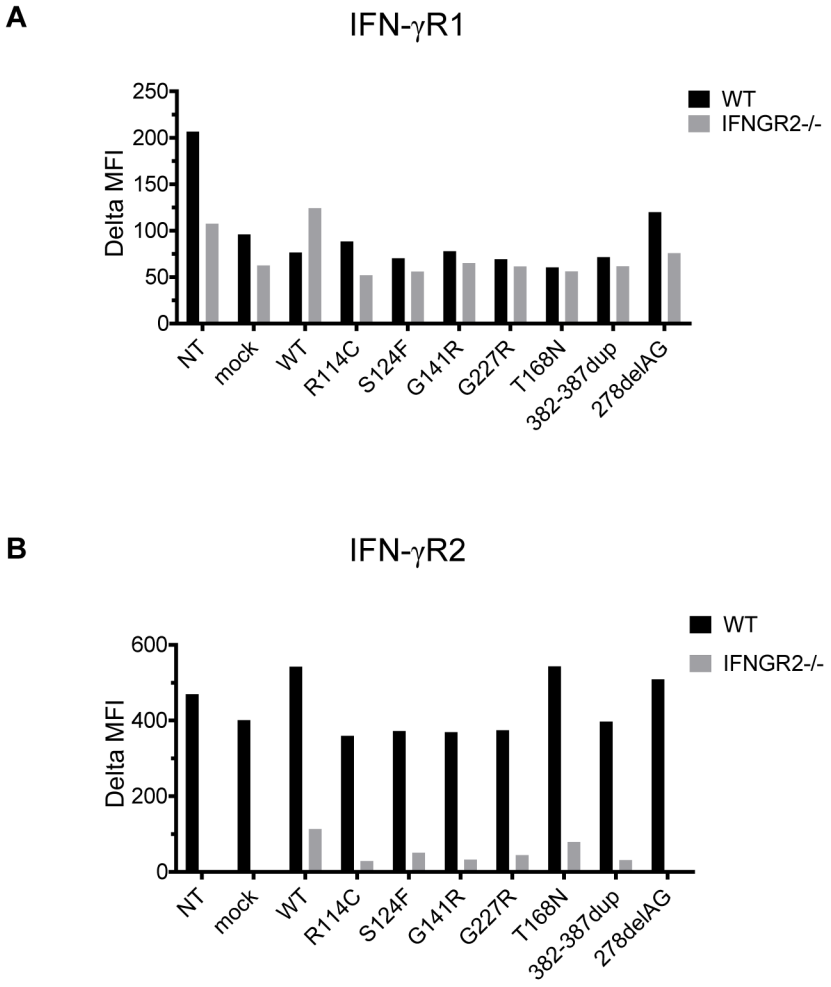

Figure S5. Surface expression of IFN-γR1 and IFN-γR2 on SV40- fibroblasts from WT cells and cells with complete IFN-γR2 deficiency, after transfection. SV40-transformed fibroblasts from a healthy control (WT, black bar) and an IFN-γR2-deficient patient (278delAG/278delAG, gray bar) were left non transfected (NT), or were transiently transfected with mock vector, WT,

R114C, S124F, G141R, G227R, T168N, 382-387dup and 278delAG *IFNGR2*-tagged V5 constructs. Transfected cells were left unstimulated and the surface expression of **(A)** IFN-γR1 and **(B)** IFN-γR2 was evaluated by flow cytometry. Results are presented as the delta mean fluorescence intensity (ΔMFI) of IFN-γR1 and IFN-γR2.

Supplemental Figure 6

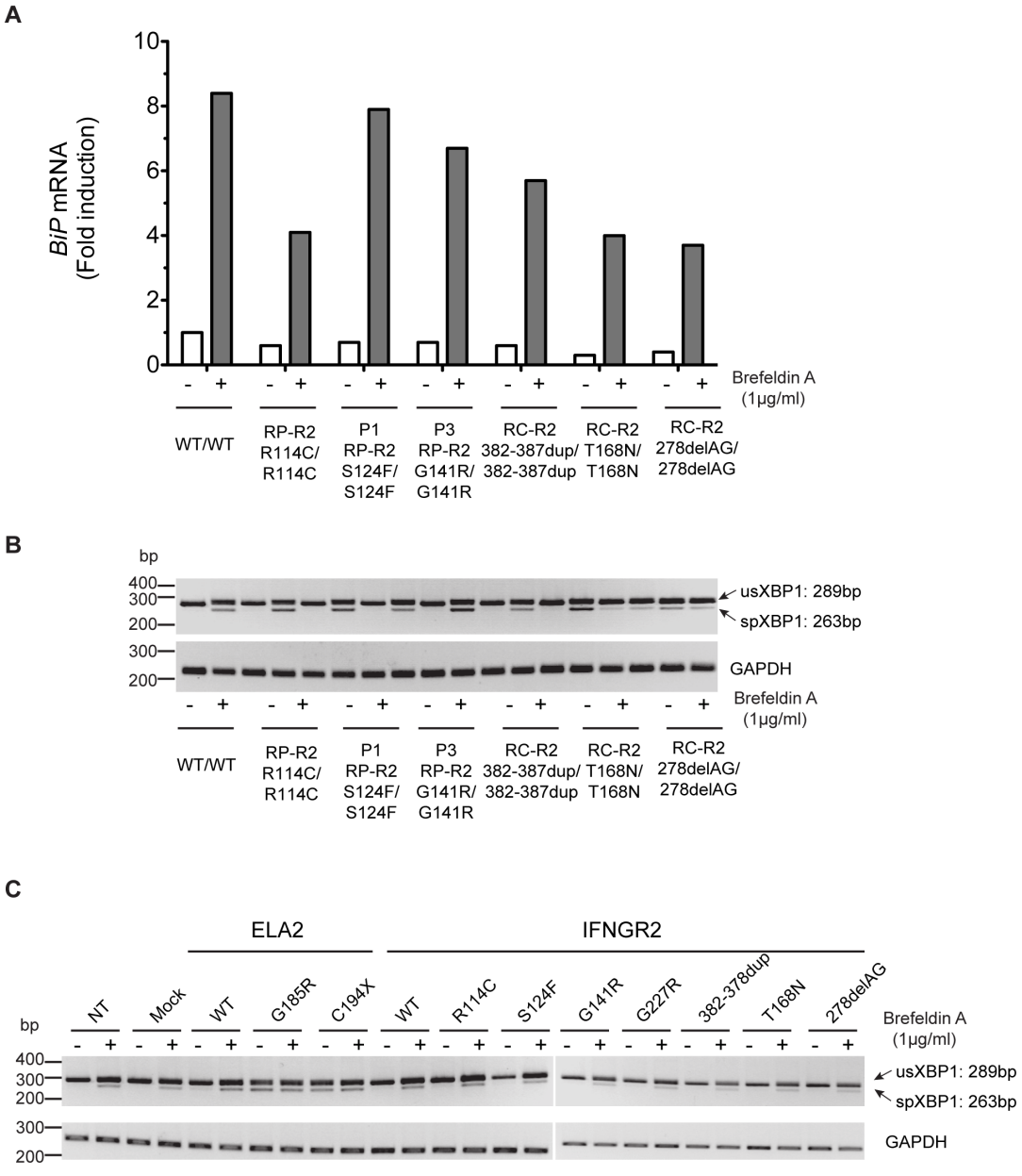

Figure S6. No activation of the UPR response in the patient's SV40- fibroblasts or transfected HEK293. (A and B) SV40-fibroblasts from a healthy control (WT/WT), a patient with recessive partial IFN-γR2 deficiency (RP-R2, R114C/R114C), the patients P1 (RP-R2,

S124F/S124F) and P3 (RP-R2, G141R/G141R), and three patients with recessive complete IFN-γR2 deficiency (RC-R2, 278delAG/278delAG), (RC-R2, 382-387dup/382-387dup), (RC-R2-T168N/T168N were left untreated (-) or treated (+) for 6 h with 1μg/ml Brefeldin A as ER stress inductor. Activation of UPR was evaluated by measuring **(A)** mRNA levels of *BiP* relative to *GUS* by qRT-PCR and **(B-C)** detection of spliced *XBP-1* mRNA by RT-PCR. Unspliced (us*XBP-1*) and spliced *XBP-1* (sp*XBP-1*) products are shown. *GAPDH* was used as a housekeeping control gene. PCR products were run on 3% agarose gels. **(C)** HEK293 cells were transiently left untransfected (NT) or transiently transfected with WT, R114C, S124F, G141R, G227R, 382-387dup, T168N and 278delAG *IFNGR2*-tagged V5 constructs in addition to WT and G185R and C194X-*ELA2* alleles, as a positive control of a mutant protein that activate UPR and detection of spliced *XBP-1* mRNA by RT-PCR was evaluated. Vertical lines have been inserted to indicate a repositioned gel lane.