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Supplemental information

cGAS and DDX41-STING mediated intrinsic immunity spreads intercellularly to promote neuroinflammation in SOD1 ALS model

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FIGURE S1

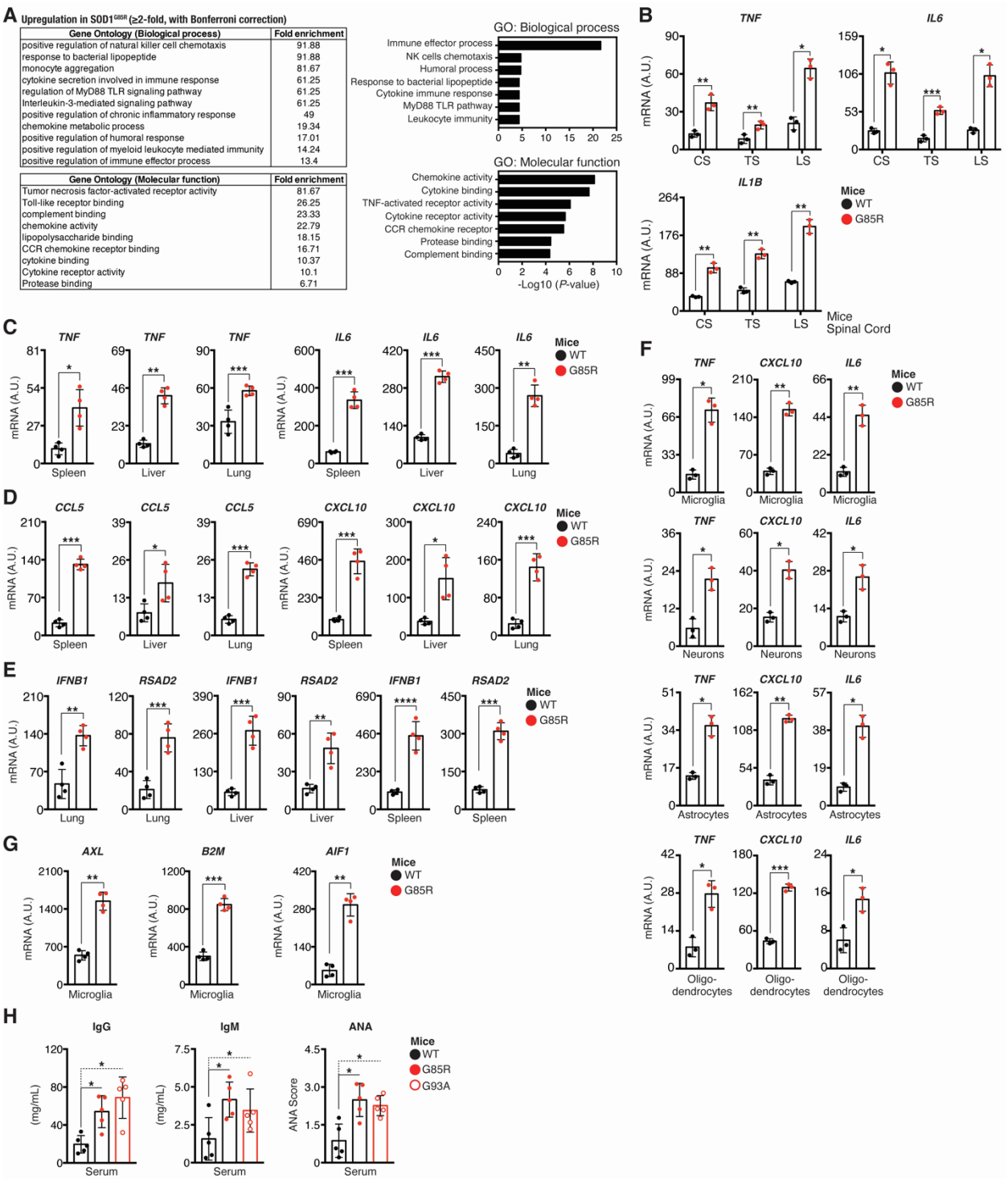
A
Upregulation in SOD1G85R (≥2-fold, with Bonferroni correction)
Gene Ontology (Biological process) | Fold enrichment
positive regulation of natural killer cell chemotaxis | 91.88
response to bacterial lipopeptide | 91.88
monocyte aggregation | 81.67
cytokine secretion involved in immune response | 61.25
regulation of MyD88 TLR signaling pathway | 61.25
Interleukin-3-mediated signaling pathway | 61.25
positive regulation of chronic inflammatory response | 49
chemokine metabolic process | 19.34
positive regulation of humoral response | 17.01
positive regulation of myeloid leukocyte mediated immunity | 14.24
positive regulation of immune effector process | 13.4
Gene Ontology (Molecular function) | Fold enrichment
Tumor necrosis factor-activated receptor activity | 81.67
Toll-like receptor binding | 26.25
complement binding | 23.33
chemokine activity | 22.79
lipopolysaccharide binding | 18.15
CCR chemokine receptor binding | 16.71
cytokine binding | 10.37
Cytokine receptor activity | 10.1
Protease binding | 6.71
GO: Biological process
Immune effector process
NK cells chemotaxis
Humoral process
Response to bacterial lipopeptide
Cytokine immune response
MyD88 TLR pathway
Leukocyte immunity
GO: Molecular function
Chemokine activity
Cytokine binding
TNF-activated receptor activity
Cytokine receptor activity
CCR chemokine receptor
Protease binding
Complement binding
-Log10 (P-value)
B
TNF
IL6
IL1B
mRNA (A.U.)
CS
TS
LS
Mice
WT
G85R
Spinal Cord
C
TNF
IL6
mRNA (A.U.)
Spleen
Liver
Lung
Mice
WT
G85R
D
CCL5
CXCL10
mRNA (A.U.)
Spleen
Liver
Lung
Mice
WT
G85R
E
IFNB1
RSAD2
mRNA (A.U.)
Lung
Liver
Spleen
Mice
WT
G85R
F
TNF
CXCL10
IL6
mRNA (A.U.)
Microglia
Neurons
Astrocytes
Oligo-dendrocytes
Mice
WT
G85R
G
AXL
B2M
AIF1
mRNA (A.U.)
Microglia
Mice
WT
G85R
H
IgG
IgM
ANA
(mg/mL)
ANA Score
Serum
Mice
WT
G85R
G93A

Figure S1. Immune-profiling of ALS mutants SOD1-G85R mice. (Related to Figure 1)
(**A**) Differentially expressed genes with at least two-fold upregulation were subjected to unbiased gene ontology analysis using PANTHER. Categories of molecular function and biological process are shown. (**B**) Spinal cord from age-matched SOD1 WT or G85R mice were dissected into three sections: cervical (CS), thoracic (TS), and lumbar (LS) sections. The indicated mRNA was analyzed by RT-qPCR. (**C**-**E**) The RT-qPCR analysis of the indicated mRNAs in lung, spleen and liver organ lysates obtained from age-matched WT or G85R mice. (**F**-**G**) RT-qPCR analysis of the indicated mRNA in primary cells extracted from either WT or G85R mice on day 10 using an immunopanning approach. (**H**) Total IgG and IgM concentrations and antinuclear antibodies in the serum of mice of the indicated genotypes were measured using ELISA or indirect fluorescence antibody techniques, respectively.
All RT-qPCR was performed using β-actin as the internal control. Each dot represents a mouse of similar age. Data represent the mean of at least three independent experiments (mean±SEM). **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001; (**B**-**H**: two-tailed, unpaired Student's *t* test). A.U.=arbitrary units.

FIGURE S2

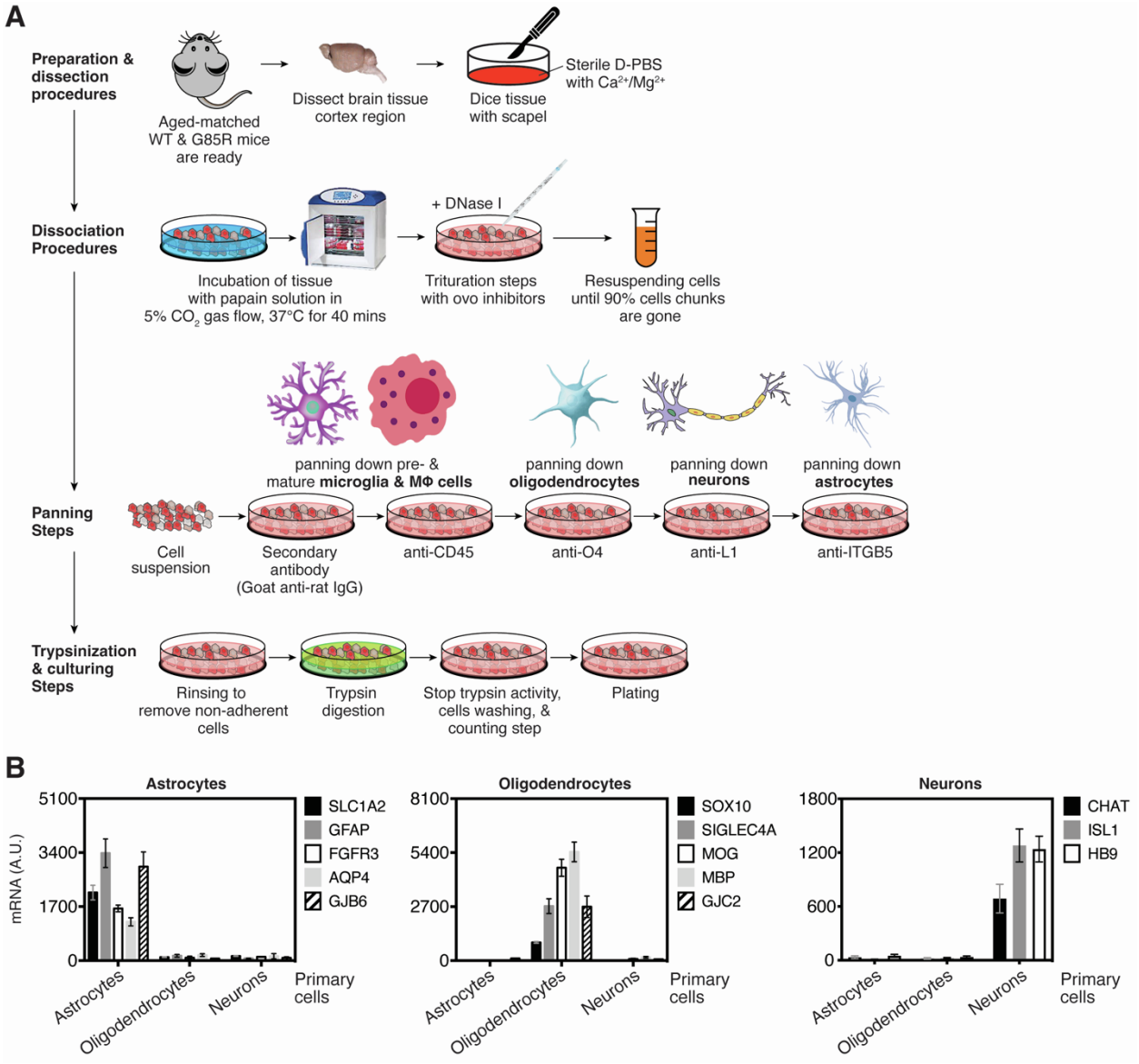

Figure S2. Immunopanning of primary cells from SOD1-G85R mice. (Related to Figure 1) (**A**) Schematic diagram for the purification of primary cells from 10-day-old SOD1 WT and G85R mice using a modified immunopanning protocol involving lineage-specific antibodies. (**B**) The expression of well-described markers for astrocytes, oligodendrocytes, and neurons measured using RT-qPCR with β-actin as an internal control. Data represent the mean of three independent experiments (mean±SEM).

FIGURE S3

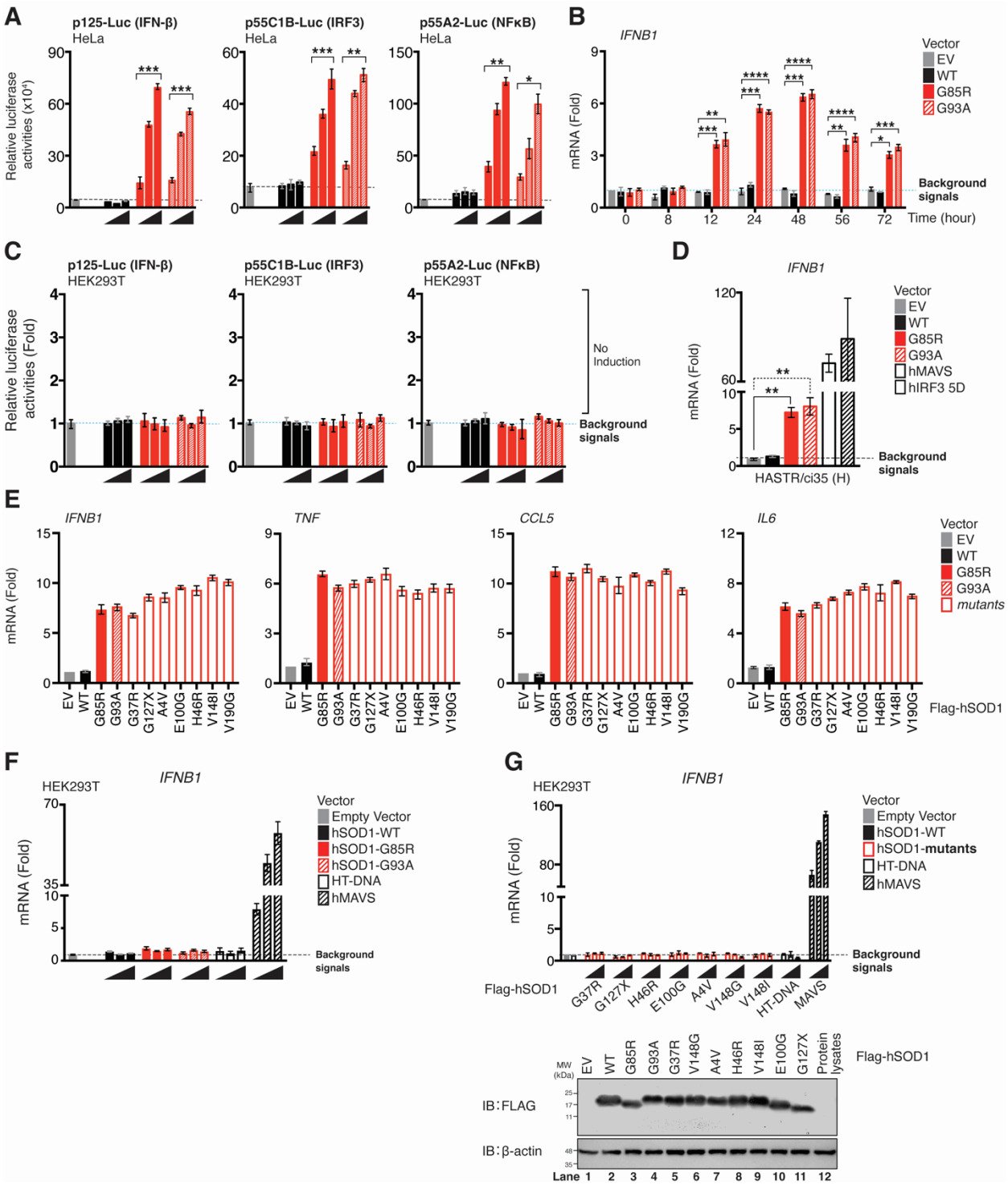
A
p125-Luc (IFN-β)
HeLa
p55C1B-Luc (IRF3)
HeLa
p55A2-Luc (NFκB)
HeLa
Relative luciferase activities (x10⁴)
B
IFNB1
mRNA (Fold)
Vector
EV
WT
G85R
G93A
Background signals
Time (hour)
C
p125-Luc (IFN-β)
HEK293T
p55C1B-Luc (IRF3)
HEK293T
p55A2-Luc (NFκB)
HEK293T
Relative luciferase activities (Fold)
No Induction
Background signals
D
IFNB1
mRNA (Fold)
Vector
EV
WT
G85R
G93A
hMAVS
hIRF3 5D
Background signals
HASTR/ci35 (H)
E
IFNB1
TNF
CCL5
IL6
mRNA (Fold)
Vector
EV
WT
G85R
G93A
mutants
EV WT G85R G93A G37R G127X A4V E100G H46R V148I V190G
Flag-hSOD1
F
HEK293T
IFNB1
mRNA (Fold)
Vector
Empty Vector
hSOD1-WT
hSOD1-G85R
hSOD1-G93A
HT-DNA
hMAVS
Background signals
G
HEK293T
IFNB1
mRNA (Fold)
Vector
Empty Vector
hSOD1-WT
hSOD1-mutants
HT-DNA
hMAVS
Background signals
Flag-hSOD1
G37R G127X H46R E100G A4V V148G V148I HT-DNA MAVS
MW (kDa)
EV WT G85R G93A G37R V148G A4V H46R V148I E100G G127X Protein lysates
Flag-hSOD1
IB:FLAG
IB:β-actin
Lane 1 2 3 4 5 6 7 8 9 10 11 12

Figure S3. Overexpression of ALS SOD1-mutants does not activate the IFN-I response in HEK293T cells. (Related to Figure 2)

(**A**) HeLa WT cells co-transfected with plasmids encoding EGFP-, human SOD1-WT, -G85R, or -G93A mutant, together with the indicated reporter plasmids (0.01, 0.025 or 0.05 μg). 36 h after transfection, cell lysates were collected and analyzed for luciferase activity. (**B**) The RT-qPCR analysis of *Ifnb1* mRNA in HeLa cells after transfection with 0.05 μg of the indicated plasmids for the indicated time-points. (**C**) Similar conditions in panel **A** but repeated using HEK293T cells. (**D**) Similar conditions in **Figure 2D** but performed using HASTR/ci35 cells. (**E**) RT-qPCR analysis of the indicated mRNA in C20 cells after transfection with 0.02 μg of the indicated SOD1-ALS mutant plasmids for 36 h. (**F**,**G**) HEK293T cells transfected with the indicated plasmids (0.01, 0.025 or 0.05 μg) for 36 h, or HT-DNA (0.1, 0.25, 0.5 μg/mL) for 8 h. *Ifnb1* mRNA assessed via RT-qPCR with β-actin as the internal control. Protein expression for 0.05 μg transfected samples of each plasmid blotted with the indicated antibodies.

Data represent the mean of three independent experiments (mean±SEM). **p*<0.05, ***p*<0.01, ****p*<0.001; (**A**: one-way ANOVA test; **B, D**: two-tailed, unpaired Student's *t* test).

FIGURE S4

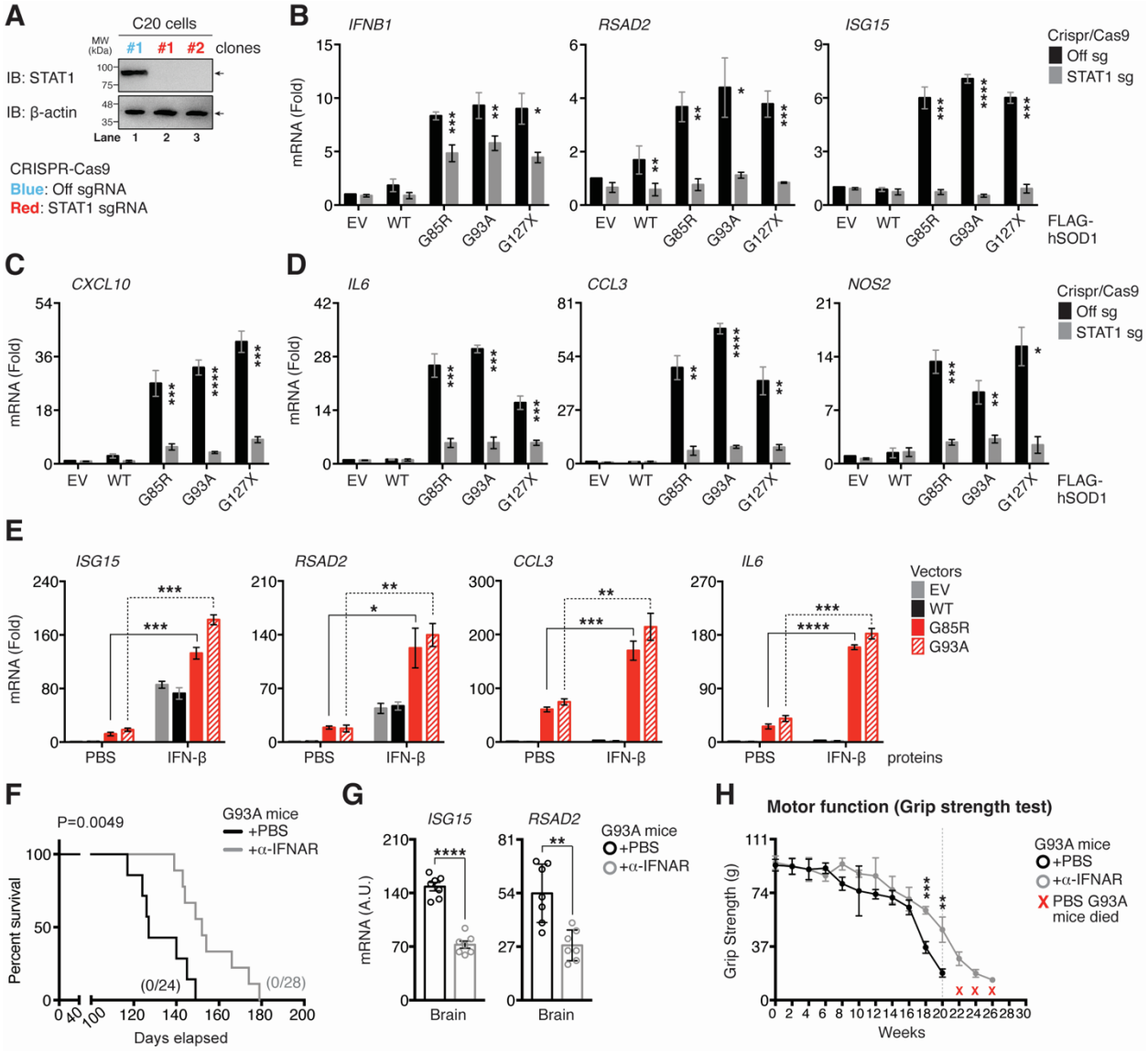
A
C20 cells
MW (kDa)
#1 #1 #2 clones
IB: STAT1
IB: β-actin
Lane 1 2 3
CRISPR-Cas9
Blue: Off sgRNA
Red: STAT1 sgRNA
B
IFNB1
RSAD2
ISG15
mRNA (Fold)
Crispr/Cas9
Off sg
STAT1 sg
EV WT G85R G93A G127X
FLAG-hSOD1
C
CXCL10
D
IL6
CCL3
NOS2
E
ISG15
RSAD2
CCL3
IL6
Vectors
EV
WT
G85R
G93A
PBS IFN-β
proteins
F
P=0.0049
G93A mice
+PBS
+α-IFNAR
Percent survival
(0/24)
(0/28)
Days elapsed
G
ISG15
RSAD2
mRNA (A.U.)
Brain
H
Motor function (Grip strength test)
Grip Strength (g)
Weeks
PBS G93A mice died

Figure S4. The ALS SOD1 mutants-dependent pro-inflammatory response requires signaling via the IFNAR receptor. (Related to Figure 3)

(**A**) Immunoblotting analysis of WT or STAT1-CRISPR/Cas9-deficient C20 cells. (**B**-**D**) WT or STAT1-CRISPR/Cas9-deficient C20 cells transfected with 0.01 μg of Flag-SOD1 WT, G85R, G93A, or G127X plasmids for 36 h, and the indicated mRNA was assessed via RT-qPCR. (**E**) WT C20 cells treated with PBS, or IFN-β recombinant protein (10 ng/mL) for 24 h, followed by transfection with 0.01 μg of Flag-SOD1 WT, G85R, or G93A plasmids. RNA was purified and the indicated mRNA was measured via RT-qPCR. (**F**) Survival data of G93A mice in response to challenge with PBS or IFNAR-neutralizing antibody (i.p., 500 μg, twice per week). The number of mice in each condition is indicated. (**G**) Basal gene expression of *Isg15* or *Rsad2* mRNA *in vivo* in brain homogenates isolated from G93A mice challenged with vehicle (PBS) or IFNAR-neutralizing antibody at Week 21 were analyzed by RT-qPCR. (**H**) Grip strength test of G93A mice after challenge with either PBS or IFNAR-neutralizing antibody.

All RT-qPCR of RNA samples was normalized to β-actin. Data represent the mean of three independent experiments (mean±SEM). **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001; (**B**-**E**, **G**-**H**: unpaired Student's *t* test; **F**: Log-rank Mantel-Cox test).

FIGURE S5

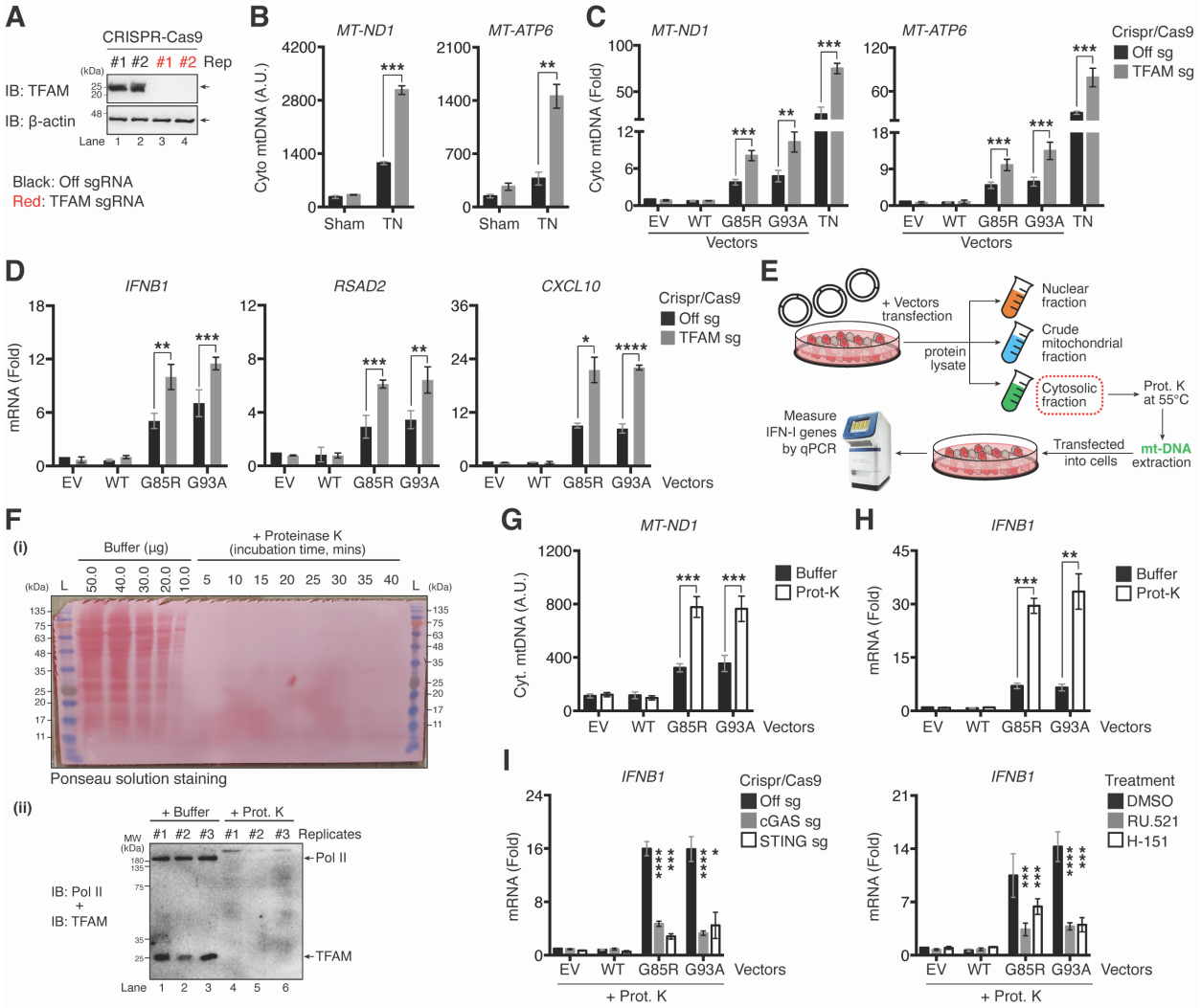
A
CRISPR-Cas9
#1 #2 #1 #2 Rep
IB: TFAM
IB: β-actin
Lane 1 2 3 4
Black: Off sgRNA
Red: TFAM sgRNA
B
MT-ND1
MT-ATP6
Cyto mtDNA (A.U.)
Sham TN
C
MT-ND1
MT-ATP6
Cyto mtDNA (Fold)
EV WT G85R G93A TN
Vectors
Crispr/Cas9
Off sg
TFAM sg
D
IFNB1
RSAD2
CXCL10
mRNA (Fold)
EV WT G85R G93A Vectors
E
+ Vectors transfection
protein lysate
Nuclear fraction
Crude mitochondrial fraction
Cytosolic fraction
Prot. K at 55°C
mt-DNA extraction
Transfected into cells
Measure IFN-I genes by qPCR
F
(i)
Buffer (μg)
+ Proteinase K (incubation time, mins)
Ponseau solution staining
(ii)
+ Buffer
+ Prot. K
Replicates
IB: Pol II + IB: TFAM
Pol II
TFAM
G
MT-ND1
Cyt. mtDNA (A.U.)
Buffer
Prot-K
H
IFNB1
mRNA (Fold)
I
IFNB1
Crispr/Cas9
Off sg
cGAS sg
STING sg
Treatment
DMSO
RU.521
H-151
+ Prot. K

Figure S5. TFAM-deficiency promotes ALS SOD1-mutants induced IFN-I responses in a cGAS-STING-dependent fashion. (Related to Figure 5)

(**A**) TFAM-deficient C20 cells generated through the CRISPR-Cas9 system. Knockout efficiencies were examined via immunoblotting. (**B**-**C**) WT or TFAM-deficient C20 cells treated with 1.0 μg/mL of tunicamycin for 8 h, or transfected with plasmids encoding EGFP-, SOD1-WT, SOD1-G85R, or SOD1-G93A mutant. After 36 h of transfection, cells were subjected to subcellular fractionation as described in **Figure 5A**. Total DNA was harvested from cytosolic and nuclear fractions. Cytosolic mtDNA genes expression as assessed via RT-qPCR. (**D**) WT or TFAM-deficient C20 cells either transfected with plasmids encoding EGFP-, SOD1-WT, SOD1-G85R, or SOD1-G93A mutants. After 36 h of transfection, RNA was purified, and the indicated mRNAs were measured. (**E**) Schematic diagram for proteinase K treatment experiments. (**F**) C20 cells subjected to the experimental conditions described in **E** were (i) stained with Ponceau solution for total protein profiling, or (ii) immunoblotted with indicated antibodies. (**G**) Cytosolic mtDNA genes was assessed via RT-qPCR in C20 cells subjected to the experimental design in **E**. (**H**) Samples from **G** were re-transfected into WT C20 cells for 8 h. *Ifnb1* mRNA was measured. (**I**) Proteinase K-treated extracts from **E** were re-transfected into either WT, cGAS or STING-deficient C20 cells for 8 h (*left*), WT C20 cells treated either with vehicle (DMSO), RU.521 or H-151 (*right*). RNA was purified and *Ifnb1* mRNA was measured via RT-qPCR.

FIGURE S6

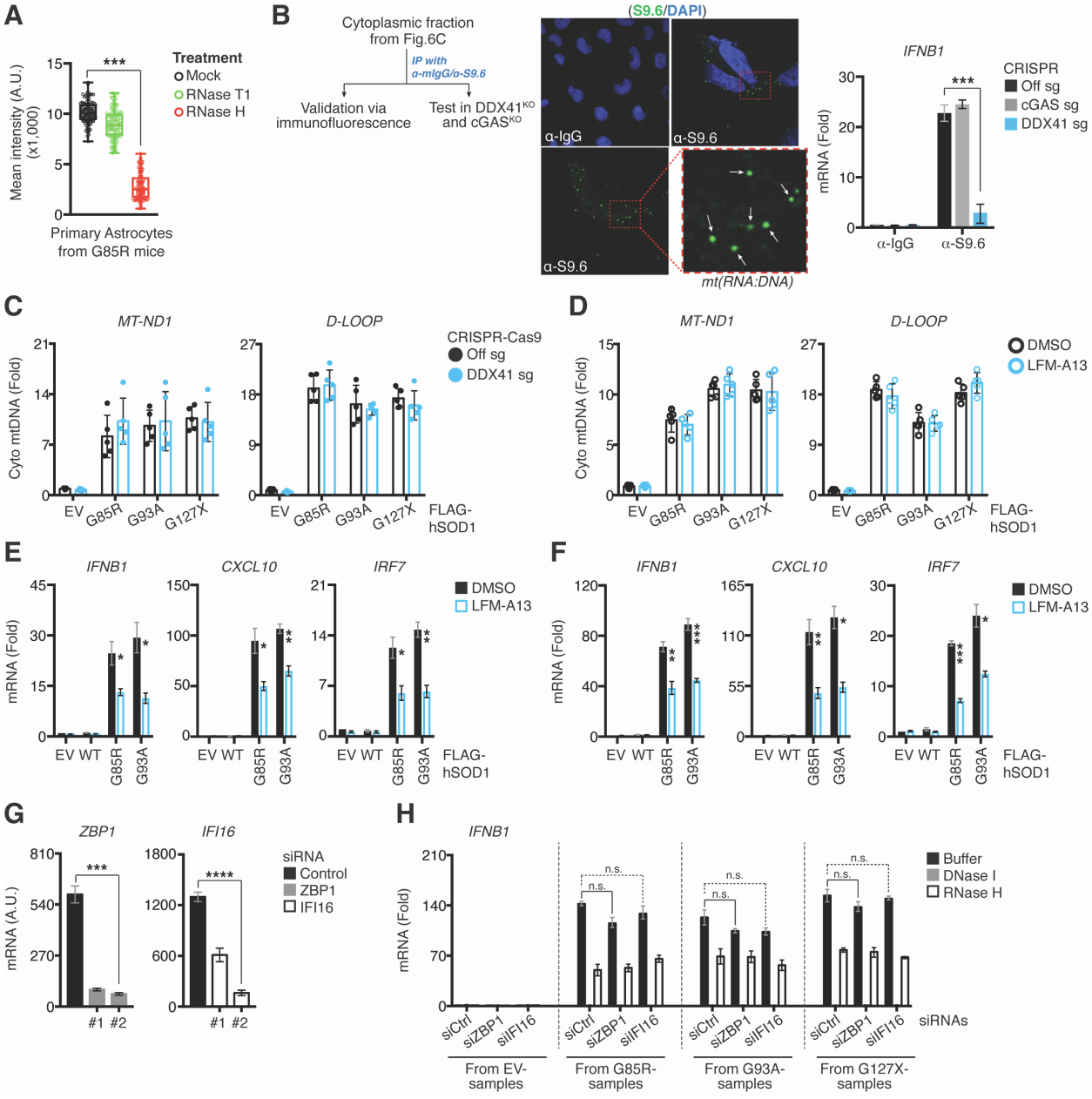
A
Mean intensity (A.U.) (x1,000)
***
Treatment
Mock
RNase T1
RNase H
Primary Astrocytes from G85R mice
B
Cytoplasmic fraction from Fig.6C
IP with α-mIgG/α-S9.6
Validation via immunofluorescence
Test in DDX41KO and cGASKO
(S9.6/DAPI)
α-IgG
α-S9.6
α-S9.6
mt(RNA:DNA)
IFNB1
mRNA (Fold)
***
CRISPR
Off sg
cGAS sg
DDX41 sg
α-IgG
α-S9.6
C
MT-ND1
D-LOOP
Cyto mtDNA (Fold)
CRISPR-Cas9
Off sg
DDX41 sg
EV
G85R
G93A
G127X
FLAG-hSOD1
D
MT-ND1
D-LOOP
Cyto mtDNA (Fold)
DMSO
LFM-A13
EV
G85R
G93A
G127X
FLAG-hSOD1
E
IFNB1
CXCL10
IRF7
mRNA (Fold)
DMSO
LFM-A13
EV WT
G85R
G93A
FLAG-hSOD1
F
IFNB1
CXCL10
IRF7
mRNA (Fold)
DMSO
LFM-A13
EV WT
G85R
G93A
FLAG-hSOD1
G
ZBP1
IFI16
mRNA (A.U.)
***
****
siRNA
Control
ZBP1
IFI16
#1 #2
H
IFNB1
mRNA (Fold)
n.s.
Buffer
DNase I
RNase H
siCtrl
siZBP1
siIFI16
siRNAs
From EV-samples
From G85R-samples
From G93A-samples
From G127X-samples

Figure S6. DAI/ZBP1 and IFI16 is not essential for mtDNA and mt(RNA:DNA) hybrid-dependent IFN-I responses in human microglia cells. (Related to Figure 6)

(**A**) PFA-fixed primary astrocytes obtained from G85R mice (**Figure 6A**) were pre-treated either with mock, RNase T1, or RNase H for 1 h at room temperature. Cells (*n*=100) were subjected to single-cell FACS-sorting to quantify the mean S9.6 intensities for individual cells. (**B**) Pure cytosolic fraction prepared from the experimental design in **Figure 6C** were subjected to immunoprecipitation with IgG antibody or S9.6. Small aliquots of pull-down lysates re-transfected into HeLa cells and immunoassayed with S9.6 and validation of pull-down efficiency and specificity is shown (*left*). mt(RNA:DNA) hybrid indicated by white arrows in box indicated by the enlarged, red-dashed line (*middle*). The remaining pull-down lysates were re-introduced into WT, cGAS-, or DDX41-deficient human microglia for *Ifnb1* mRNA quantification (*right*). (**C**) WT or DDX41-deficient human microglia transfected with the indicated plasmids were subjected to subcellular fractionation to determine the amount of cytoplasmic mtDNA. (**D**) Human microglia transfected with the indicated plasmids in the presence or absence of LFM-A13 (100 μM) were subjected to subcellular fractionation to determine the amount of cytoplasmic mtDNA. (**E**,**F**) Human HASTR/ci35 and murine NSC-34 cells were either transfected with 0.01 μg of the indicated plasmids for 36 h in the presence or absence of LFM-A13 (100 μM). RNA was purified and the indicated mRNA was measured using RT-qPCR. (**G**) WT human microglia were transfected with the indicated siRNAs (10 nM) for 48 h. Knockdown efficiency of the indicated genes was examined using RT-qPCR. (**H**) Pure cytosolic fractions deriving from digitonin extracts of WT C20 cells transfected with 0.01 μg of Flag-tagged-empty vector (EV), -G85R, -G93A, or -G127X plasmids were treated with DNase I or RNase H. Samples were then re-transfected into WT C20 cells deficient in genes using the indicated siRNAs. *Ifnb1* mRNA was measured using RT-qPCR.

Quantification of mtDNA genes was normalized to genomic DNA, *TERT*. All RT-qPCR of RNA samples were normalized to β-actin. Data represent the mean of three independent experiments (mean±SEM). **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001; (**A**-**B**,**E-H**: unpaired Student's *t* test). A.U.=arbitrary unit; n.s.=non-significant.

FIGURE S7

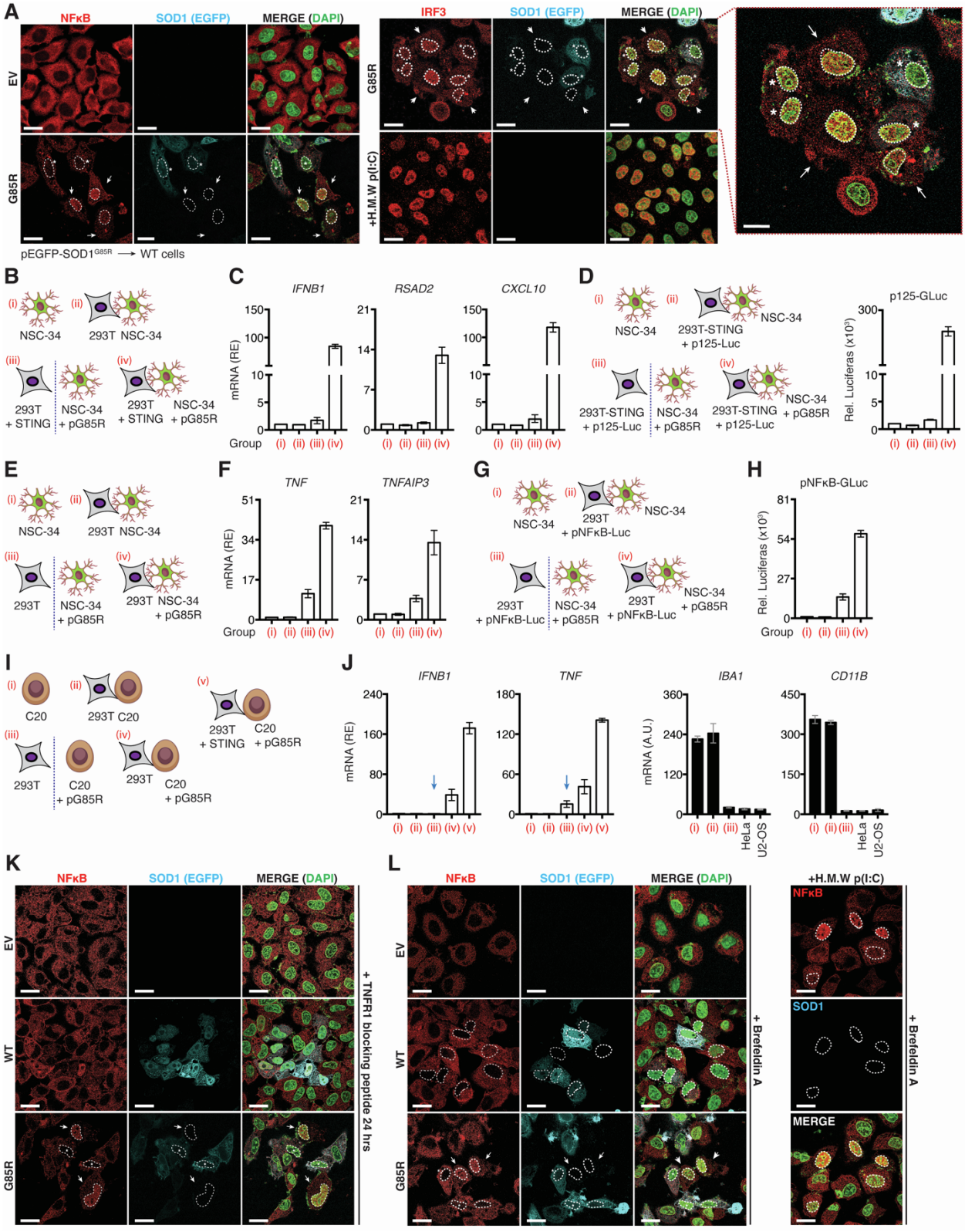
A
NFκB
SOD1 (EGFP)
MERGE (DAPI)
EV
G85R
IRF3
+H.M.W p(I:C)
pEGFP-SOD1G85R ⟶ WT cells
B
(i) NSC-34
(ii) 293T NSC-34
(iii) 293T + STING
NSC-34 + pG85R
(iv) 293T + STING
NSC-34 + pG85R
C
IFNB1
RSAD2
CXCL10
mRNA (RE)
Group (i) (ii) (iii) (iv)
D
(i) NSC-34
(ii) 293T-STING + p125-Luc
NSC-34
(iii) 293T-STING + p125-Luc
NSC-34 + pG85R
(iv) 293T-STING + p125-Luc
NSC-34 + pG85R
p125-GLuc
Rel. Luciferas (x10³)
E
(i) NSC-34
(ii) 293T NSC-34
(iii) 293T
NSC-34 + pG85R
(iv) 293T NSC-34 + pG85R
F
TNF
TNFAIP3
mRNA (RE)
Group (i) (ii) (iii) (iv)
G
(i) NSC-34
(ii) 293T + pNFκB-Luc
NSC-34
(iii) 293T + pNFκB-Luc
NSC-34 + pG85R
(iv) 293T + pNFκB-Luc
NSC-34 + pG85R
H
pNFκB-GLuc
Rel. Luciferas (x10³)
Group (i) (ii) (iii) (iv)
I
(i) C20
(ii) 293T C20
(iii) 293T
C20 + pG85R
(iv) 293T
C20 + pG85R
(v) 293T + STING
C20 + pG85R
J
IFNB1
TNF
IBA1
CD11B
mRNA (RE)
mRNA (A.U.)
(i) (ii) (iii) (iv) (v)
HeLa
U2-OS
K
NFκB
SOD1 (EGFP)
MERGE (DAPI)
EV
WT
G85R
+ TNFR1 blocking peptide 24 hrs
L
NFκB
SOD1 (EGFP)
MERGE (DAPI)
EV
WT
G85R
+ Brefeldin A
+H.M.W p(I:C)
NFκB
SOD1
MERGE
+ Brefeldin A

Figure S7. Cell-cell contact amplifies the propagation of ALS SOD1-mutants induction of IFN-I and pro-inflammatory responses to bystander cells. (Related to Figure 7)

(**A**) C20 cells expressing empty vector (EV) or pEGFP-SOD1 G85R were co-cultured with WT cells and immunoassayed with the indicated antibodies. Cells transfected with 0.1 μg of high molecular weight poly(I:C) [H.M.W p(I:C)] served as the negative control. Cells expressing EGFP-SOD1 mutants are marked by as asterisk (*). Bystander cells with nuclear translocation of NF-κB or IRF3 are indicated by with white arrows. The enlarged box (*right*) shows the merge WT co-cultured cells with microglia expressing SOD1-G85R. (**B**,**C**) Schematic view of the experimental design is shown. HEK293T cells or NSC-34 cells in the indicated conditions were co-cultured in the presence or absence of the trans-well system, and the indicated mRNA were measured via RT-qPCR. (**D**) Schematic view of the experimental design is shown. HEK293T cells expressing STING were co-transfected with p125-luciferase reporter 24 h before NSC-34 donor cells were added in the presence or absence of trans-well system. After 18 h, the luciferase activity was assessed. (**E**-**H**) WT HEK293T cells co-cultured with NSC-34 cells in various conditions are shown. The NF-κB luciferase activity and the relative induction of *Ifnb1*, or *Tnf* mRNA was measured. (**I-J**) WT HEK293T cells co-cultured with C20 cells in various conditions is shown. The relative induction of *Tnf,* and *Tnfaip3* mRNA was measured via RT-qPCR. (**K,L**) EV, WT, or EGFP-SOD1-G85R-expressing microglia were co-cultured with WT cells in the presence of TNFR1 blocking peptide (2.0 μg/mL, 24 h) or Brefeldin A (BFA, 0.1 μM, 2 h). Cells were fixed with 4% PFA and immuno-stained with antibodies targeting NF-κB. Cells expressing EGFP-SOD1 WT or G85R are marked by an asterisk (*). Bystander cells with nuclear translocation of NF-κB are indicated with white arrows. WT cells transfected with H.M.W. p(I:C) served as the negative control.

All RT-qPCR of RNA samples was normalized to β-actin. Data represent the mean of at least three independent experiments (mean±SEM). White scale bars correspond to 10 μm. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

FIGURE S8

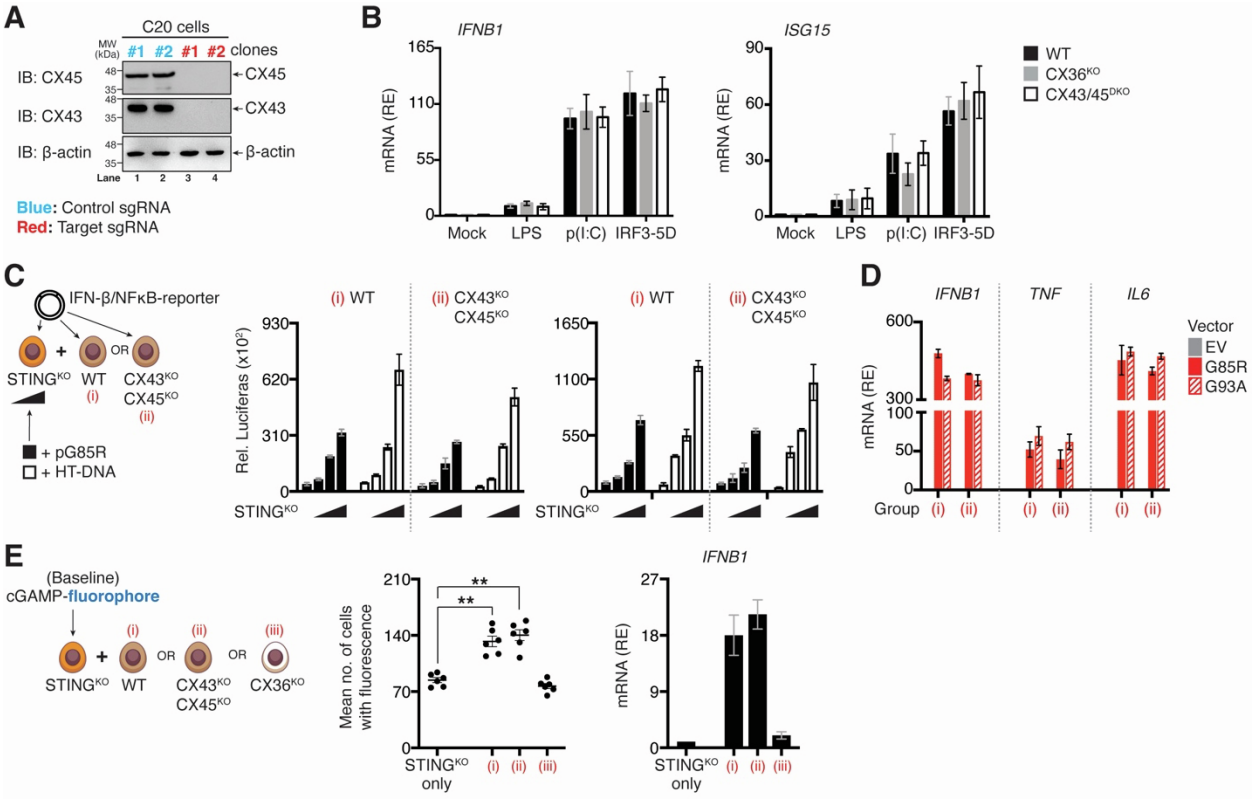

Figure S8. Connexin-43 and -45 are dispensable for cGAMPs transfer in human microglia cells. (Related to Figure 7)

(**A**) Knockout efficiency of CX43, CX45 through CRISPR-Cas9 system was verified via immunoblotting. (**B**) WT, CX36-, or CX43/45-double deficient microglia cells were either stimulated with LPS (0.1 μg/mL), transfected with poly(I:C) [p(I:C), 0.1 μg] or Flag-tagged-IRF3-5D plasmid (1.0 μg). Relative expression of *Ifnb1* or *Isg15* mRNA were measured via RT-qPCR. (**C**,**D**) WT or CX43/45-double deficient microglia cells co-cultured with STING-deficient microglia cells (ratios ranging from 1:0.5 to 1:0.125) containing G85R or HT-DNA (0.1 μg/mL), followed by transfection with p125-Gluc (IFN-β) or NF-κB-GLuc, luciferase reporter activity (**C**) Expression of indicated mRNA was assessed via RT-qPCR after 24 h (**D**). (**E**) STING-deficient human microglia cells pre-loaded with cGAMP-labeled fluorophore co-cultured either with WT, CX36, or CX43/45-double deficient cells in equal ratios (*n*=100 cells for each genotype). 24 h later, cells were trypsinized and subjected to FACS-sorting analysis of single cells positive for fluorescence. Cells from each group were collected from sorter and subjected to RT-qPCR analysis of *Ifnb1* mRNA. All RT-qPCR of RNA samples normalized to β-actin. Data represent the mean of at least three independent experiments (mean±SEM). ***p*<0.01. (**E**: unpaired Student's *t* test).