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Supplemental information

STAT6 in mitochondrial outer membrane impairs mitochondrial fusion by inhibiting MFN2 dimerization

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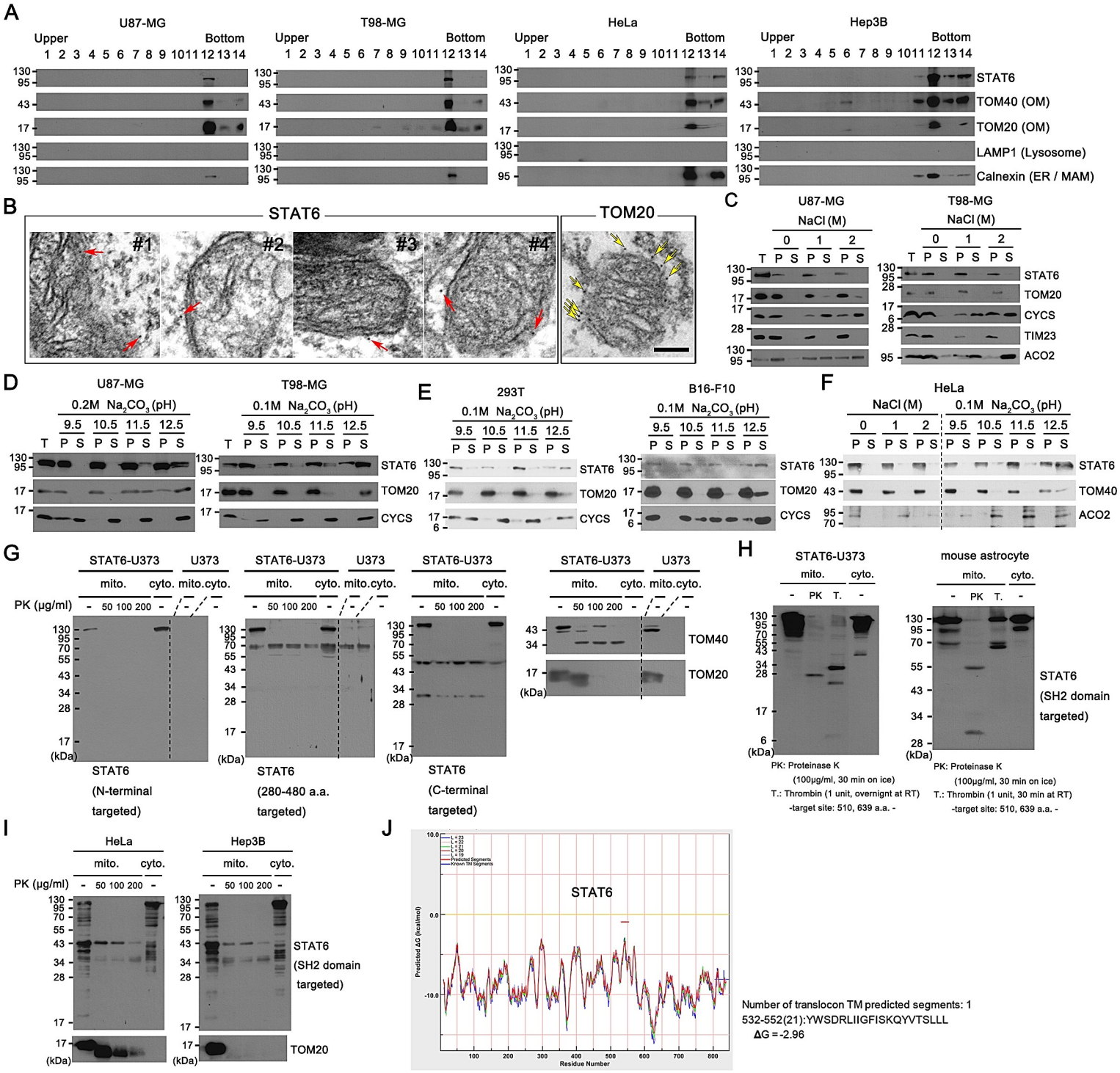

Figure S1. OMM localization of STAT6 in malignant glioma cell lines and other cell lines, related to Figure 1

(A) Sucrose-density gradient centrifugation analysis of mitochondria isolated from U87-MG, T98-MG, HeLa, and Hep3B. TOM20 and TOM40: mitochondrial marker. LAMP1: lysosomal marker. Calnexin: ER and MAM marker. (B) Immunogold electron microscopy analysis with antibodies against anti-STAT6 (#1-#4) or anti-TOM20 antibody. Red arrowhead: STAT6. Yellow arrowhead: TOM20. Scale bar: 100 nm

(C-F) High salt (C and F) and Sodium carbonate extraction at different pH (D, E, and F) of mitochondria isolated from malignant glioma and other cell lines. T: total mitochondrial fraction. P: pellet. S: supernatant. TOM20: signal-anchored outer membrane protein. CYCS: soluble protein in intermembrane space or peripherally associated membrane protein in inner membrane. TIM23: integral inner membrane protein. ACO2: soluble matrix protein.

(G-J) Approximate identification of cytosol-facing STAT6 residues in OMM by protease digestion. (G) Proteinase K protection assay of mitochondria isolated from STAT6-expressing U373-MG (STAT6-U373) using STAT6 antibodies targeted to N-terminal, 280-480 residues, and C-terminal. PK: proteinase K. Mito.: mitochondrial fraction. Cyto.: cytoplasmic fraction. (H) Proteinase K and Thrombin (T.) treatment on mitochondria isolated from STAT6-U373 and astrocytes using STAT6 antibody targeted to SH2 domain. (I) Proteinase K protection assay of mitochondria isolated from HeLa and Hep3B using STAT6 antibody targeted to SH2 domain. (J) Predicted TM (transmembrane) segments analyses of STAT6, based on translocon-mediated TM helix assembly. The predicted segments were analyzed using the membrane protein explorer (MPEx) programme (ver. 3.3).

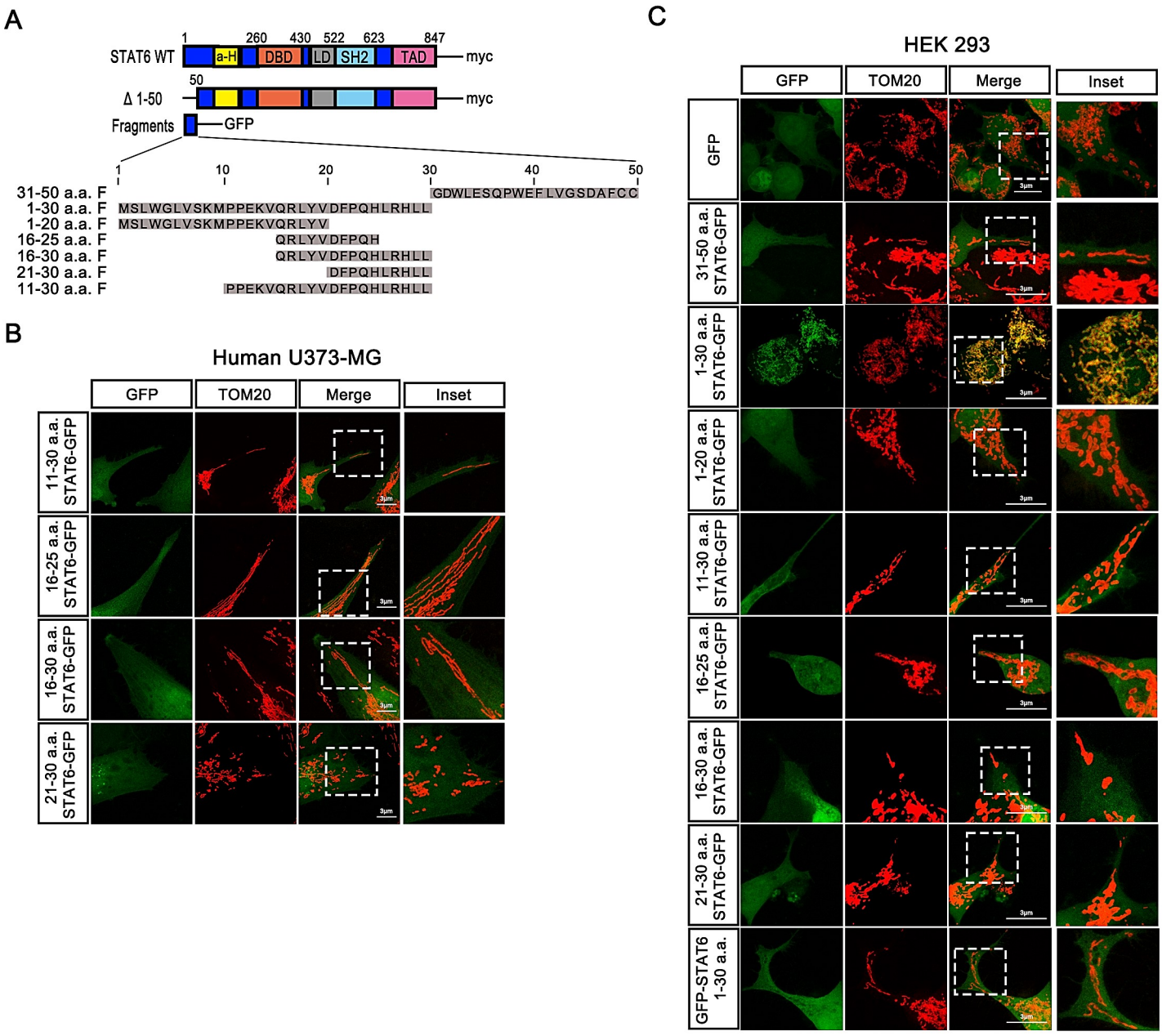

Figure S2. Identification of mitochondrial targeting sequences in STAT6, related to Figure 2

(A) Schematic of human STAT6-WT, Δ1-50 with myc-tag, and n-terminal fragments with GFP-tag. (B and C) Confocal images of mitochondria in human U373-MG (B) and HEK 293 (C) transfected with GFP or human STAT6 n-terminal fragments with GFP were stained with TOM20 (mitochondrial marker, red). The inset shows a magnification of the selected mitochondrial region (white square). Scale bar, 3μm.

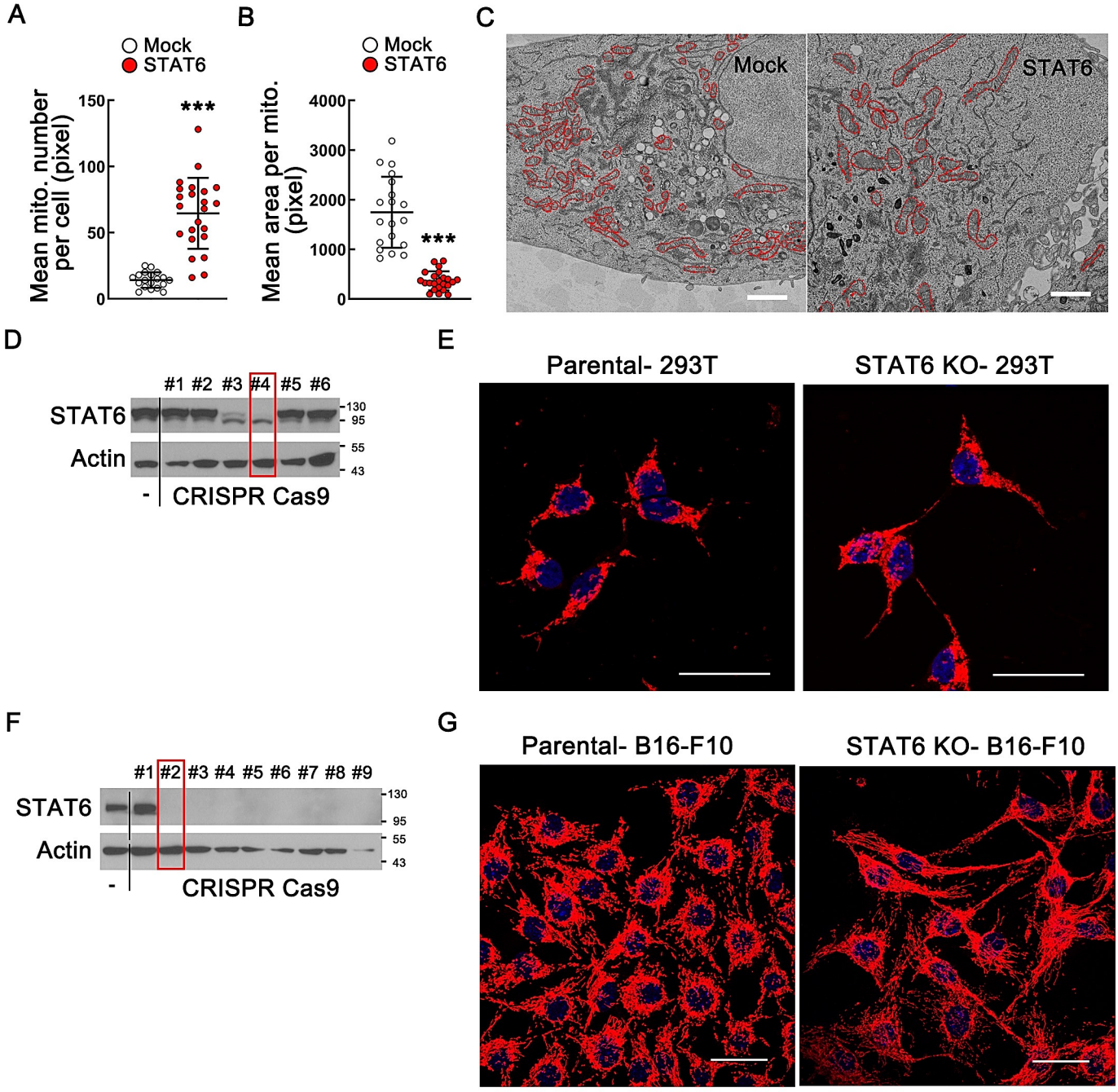

Figure S3. Mitochondrial morphology regulated by STAT6, related to Figure 5

(A and B) The mitochondrial number per cell (A) and mean area per mitochondrion (B) in U373-MG transfected with GFP or STAT6-GFP were quantified using Image J. (GFP, n= 19 and STAT6-GFP, n= 23) Data are presented as means ±SD of three independent experiments (***p<0.001), unpaired Student t-test. (C) EM images of mitochondrial morphology in Empty vector- or STAT6-expressing U373-MG stable cell lines. Scale bar, 2μm. (D and E) We attempted to engineer STAT6 KO in 293T using CRISPR Cas9 system, obtained several clones, and screened by immuno blot. Although we did not identify completely knocked out-STAT6 clone, among the clones obtained, one clone (#4) that expressed truncated STAT6 with low residual level (D) was used to the experiments. (E) Confocal images of mitochondrial morphology in parental-293T or #4 clone (STAT6 KO-293T), stained with TOM20 (red, mitochondrial marker). Scale bar: 10 μm. (F and G) STAT6 KO stable cell lines were generated from parental B16-F10 and screened by immune blot (F). Among the clones obtained from B16-F10, #2 clone was used to the experiments. (G) Confocal images of mitochondrial morphology in parental- B16-F10 or #2 clone (STAT6 KO- B16-F10), stained with TOM20 (red, mitochondrial marker). Scale bar: 10 μm.

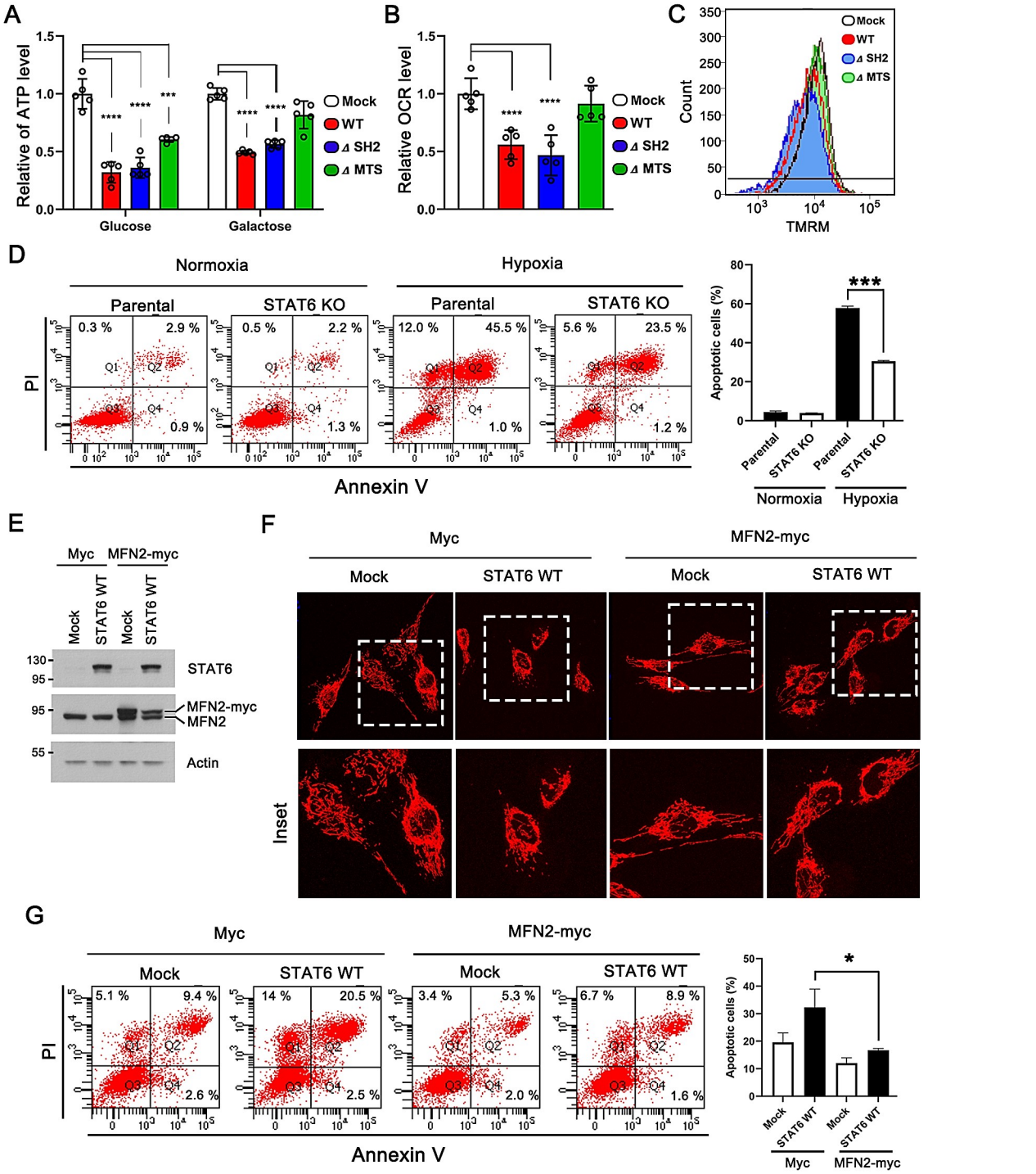

Figure S4. Mitochondrial STAT6 regulates ATP production, mitochondrial membrane potential, and cell death, related to Figure 6 (A) ATP assay in Empty vector-, STAT6 WT, ΔSH2, and ΔMTS-expressing 293T stable cell lines. 5 independent experiments (***p<0.001 and ****p<0.0001). One-way ANOVA with Scheffe's post-hoc test. (B) OCR assay in Empty vector-, STAT6 WT, ΔSH2, and ΔMTS-expressing 293T stable cell lines. 5 independent experiments (****p<0.0001). One-way ANOVA with Scheffe's post-hoc test. (C) Flow cytometry analysis of mitochondrial membrane potential in Empty vector-, STAT6 WT, ΔSH2, and ΔMTS-expressing 293T stable cell lines exposed to hypoxic condition (0.1% O2, 48hr) and stained with TMRM. (D) Flow cytometry dot plots of annexin V and propidium iodide (PI) staining in parental-293T and STAT6 KO-293T exposed to normoxic or hypoxic condition. Data are presented as means ±SD of 3 independent experiments. (*p<0.05). (E-G) MFN2 inhibits the apotic cell death induced by STAT6. To determine whether STAT6-induced mitochondrial fragmentation was reversed by MFN2 overexpression, Myc- or MFN2-myc was transfected to mock- or STAT6 WT-expressing stable cell line. (E) Western blot analysis using mock- or STAT6 WT-expressing stable cell line transfected with Myc- or MFN2-myc. (F) Confocal images of mitochondrial morphology in mock- or STAT6 WT-expressing stable cell line transfected with Myc- or MFN2-myc, stained with TOM20 (red, mitochondrial marker). (G) Flow cytometry dot plots of annexin V and propidium iodide (PI) staining in mock- or STAT6 WT-expressing stable cell line transfected with Myc- or MFN2-myc in hypoxic condition. Data are presented as means ±SD of 3 independent experiments. (*p<0.05).

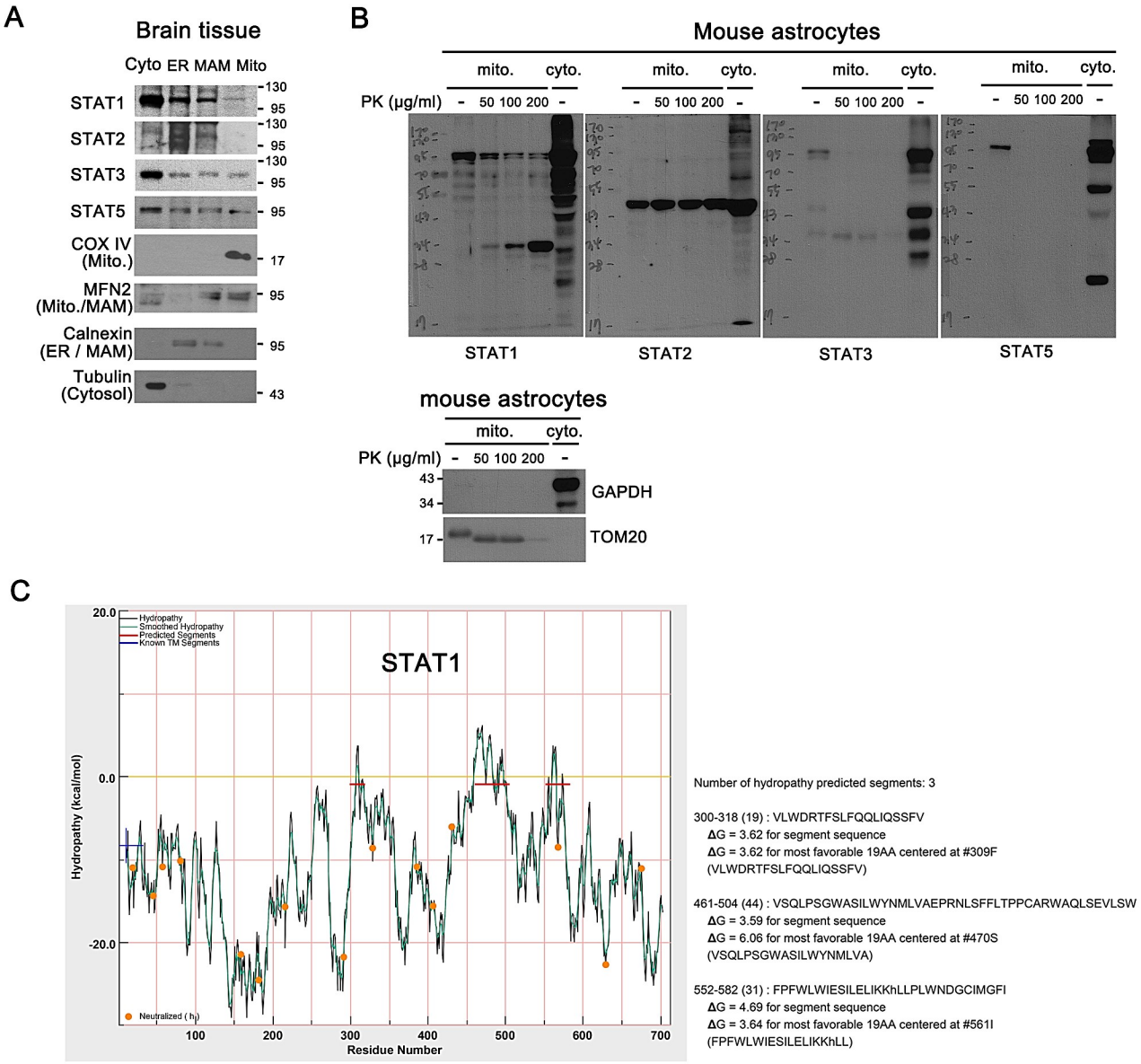

Figure S5. Mitochondrial localization of other STAT family members in brain, related to Figure 1

(A) Western blot analysis of subcellular fractionations from mouse brain tissue with the indicated antibodies. Cyto: cytosol. MAM: mitochondria-associated membrane. Mito: mitochondria. COX IV: Cytochrome C Oxidase IV. (B) Proteinase K protection assay of mitochondria isolated from mouse astrocytes. (C) Hydropathy plots of STAT1. The hydrophobicity of STAT1 was analyzed using the MPEx programme.

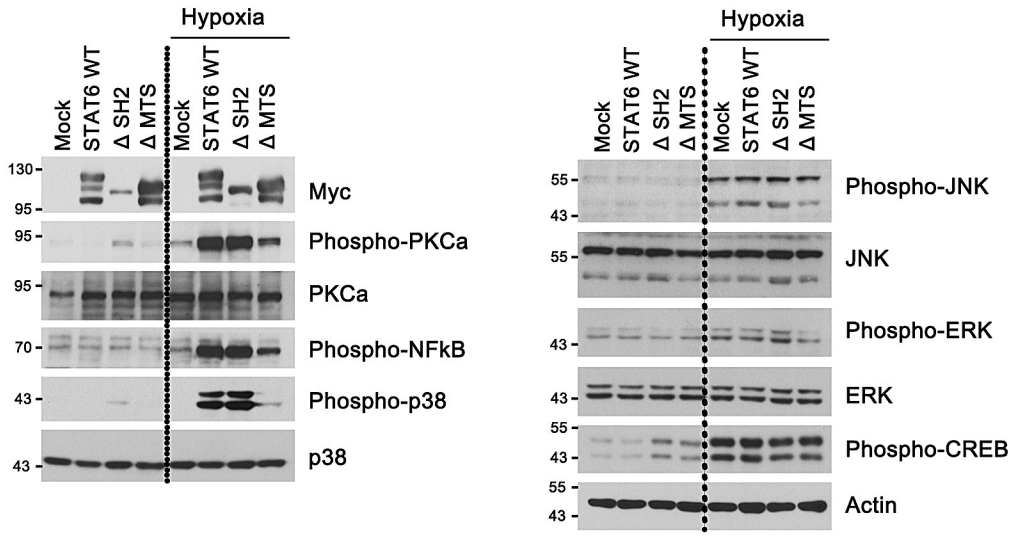

Figure S6. Mitochondrial retrograde signaling triggered from STAT6 in OMM with hypoxia, related to Figure 6

Western blot in Empty vector-, STAT6 WT, ΔSH2, and ΔMTS-expressing 293T exposed to hypoxia with the indicated antibodies. ΔMTS decreased phosphorylation of PKCa, NF-kB, and p38 that induced by STAT6 WT and ΔSH2, suggesting that mitochondrial retrograde signaling was driven through STAT6 in OMM.

Table S1. A list of mitochondrial candidates that bind to STAT6 in mouse brain, Related to Figure 3

Uniprot ID	Protein name
Q3UHD9	ArfGAP with GTPase domain, ankyrin repeat and PH domain 2(Agap2)
Q9CPP6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 5(Ndufa5)
Q8R0Y6	aldehyde dehydrogenase 1 family, member L1(Aldh1l1)
Q3UL36	arginine and glutamate rich 1(Arglu1)
P31786	diazepam binding inhibitor(Dbi)
P19096	fatty acid synthase(Fasn)
O88741	ganglioside-induced differentiation-associated-protein 1(Gdap1)
P48318	glutamate decarboxylase 1(Gad1)
P28798	granulin(Grn)
O08756	hydroxysteroid (17-beta) dehydrogenase 10(Hsd17b10)
P51660	hydroxysteroid (17-beta) dehydrogenase 4(Hsd17b4)
Q922D8	methylenetetrahydrofolate dehydrogenase (NADP+ dependent), methenyltetrahydrofolate cyclohydrolase, formyltetrahydrofolate synthase(Mthfd1)
Q6PCP5	mitochondrial fission factor(Mff)
Q80U63	mitofusin 2(Mfn2)
O55125	nipsnap homolog 1 (C. elegans)(Nipsnap1)
Q8BGT8	phytanoyl-CoA hydroxylase interacting protein-like(Phyhipl)
Q8BKZ9	pyruvate dehydrogenase complex, component X(Pdhx)
Q62465	vesicle amine transport 1(Vat1)