Supplemental Information

Pathogenic TFG Mutations Underlying Hereditary Spastic Paraplegia Impair Secretory Protein Trafficking and Axon Fasciculation

Erin L. Slosarek, Amber L. Schuh, Iryna Pustova, Adam Johnson, Jennifer Bird, Matthew Johnson, E.B. Frankel, Nilakshee Bhattacharya, Michael G. Hanna, Jordan E. Burke, David A. Ruhl, Kyle Quinney, Samuel Block, Jennifer L. Peotter, Edwin R. Chapman, Michael D. Sheets, Samuel E. Butcher, Scott M. Stagg, and Anjon Audhya
Figure S1. The TFG coiled coil domain plays a key role in the assembly of TFG octameric ring structures, Related to Figure 1. (A, B, E, and F) Direct estimation of the radius of gyration, Rg, and the extrapolated intensity.

(A) Guinier Fit
750 μM TFGWT
Rg = 35.1 +/- 0.1 Å
I(0) = 0.026 +/- 0.000

(B) Guinier Fit
150 μM TFGWT
Rg = 38.0 +/- 0.3 Å
I(0) = 0.028 +/- 0.000

(C) Direct estimation of the radius of gyration, Rg, and the extrapolated intensity

(D) Top View
Side View

TFQ1\textsuperscript{1-138} SAXS envelope
TFG1\textsuperscript{1-193} EM density

(E) Guinier Fit
750 μM TFG\textsuperscript{R106C}
Rg = 41.6 +/- 1.0 Å
I(0) = 0.031 +/- 0.000

(F) Guinier Fit
150 μM TFG\textsuperscript{R106C}
Rg = 41.5 +/- 0.9 Å
I(0) = 0.031 +/- 0.000

(G) UV Absorbance (AU)

(H) Light Scattering (AU)

(I) pI

(J) Ab initio Models
TFG\textsuperscript{1-96}
at zero scattering angle, I(0) for wild type TFG (amino acids 1-138) (A and B) and TFG (p.R106C) (E and F) was based on Guinier analysis conducted at two protein concentrations (n=3 each). Aggregation is not observed, and the residuals exhibit a linear behavior in all cases. (C) Pair distance distribution function plots of wild type TFG and TFG (p.R106C) at two protein concentrations. (D) An averaged ab initio model of the TFG amino-terminus (amino acid 1-138) (blue) is overlaid with the electron microscopy density of TFG (amino acids 1-193) defined previously (EMDataBank accession code EMD-6076). (G) Purified recombinant TFG (amino acids 1-96) was separated over a gel filtration column that was coupled to a multi-angle light scattering device (n=3). Both the UV absorbance (red) and the light scattering (blue) profiles are shown and a sample of the purified protein separated by SDS/PAGE and stained using Coomassie Blue is included. (H) Representative Kratky scattering profiles generated from the analysis of two concentrations of wild type TFG (amino acids 1-96) following SAXS. Experiments were conducted in 50 mM Tris-HCl, pH 7.6, 10 mM DTT, and 100 mM NaCl (n=3). (I) Pair distance distribution function plots of wild type TFG (amino acids 1-96) at two protein concentrations. (J) Ab initio models of the TFG amino-terminus (amino acid 1-96). Twenty structures were generated using the program DAMMIF, and two overlaid dummy atom models are shown (left). The twenty structures were then averaged with DAMAVER, yielding a normalized spatial discrepancy (NSD) of 0.90. The averaged SAXS envelope for wild type TFG (amino acids 1-96) is depicted (right).
Figure S2. The TFG coiled coil domain plays a key role in the assembly of TFG octameric ring structures, Related to Figure 2. (A) Recombinant TFG (amino acids 1-193) harboring 6 leucine to valine mutations within its
coiled coil domain were imaged by negative stain electron microscopy following purification in 50 mM MES, pH 5.5, and 100 mM NaCl. A higher magnification view of individual particles is shown (right). Scale bars, 100 nm (left) and 25 nm (right). (B) A purified recombinant form of TFG (p.R106K; amino acids 92-138) fused to a monomeric SUMO tag was separated over a gel filtration column coupled to a multi-angle light scattering device. Eluted fractions were separated by SDS/PAGE and stained using Coomassie Blue (n=3). Molecular mass was calculated using ASTRA software. (C-E) Recombinant TFG (amino acids 1-193) harboring various mutations (p.R106K (C), p.R22W (D), or p.K14A (E)) were imaged by negative stain electron microscopy following purification in either 50 mM MES, pH 5.5, or 50 mM HEPES, pH 7.6. Higher magnification views of individual particles are shown (right). Scale bars, 100 nm (left) and 25 nm (right). (F, I, and K) Representative RPE-1 cells treated with siRNAs targeting the 3’UTR of endogenous TFG were induced to ectopically express wild type TFG or a mutant TFG isoform, followed by fixation and staining using antibodies directed against Sec31A and TFG. Maximum intensity projections are shown. RFP expression, also induced by doxycycline, was used to identify cells with similarly low levels of TFG expression. Arrows in panel I highlight the presence of large TFG (p.R106C)-positive structures that have little associated Sec31 staining. Scale bars, 10 µm and 1 µm (inset). (G) Fluorescence intensity (I) of TFG relative to Sec31A was quantified in RPE-1 cells ectopically expressing various TFG isoforms following depletion of endogenous TFG. Error bars represent mean +/- SEM; n > 30 cells per condition; 3 biological replicates each. **p < 0.01 (compared with control), calculated using an ANOVA test. (H) Representative immunoblot analysis of control cells and TFG depleted cells ectopically expressing various TFG isoforms (n=3). (I) Quantification of the percentage of TFG-labeled structures that exhibit Sec31A staining in TFG depleted cells induced to express various forms of TFG following treatment with doxycycline (3 ng/mL). Error bars represent mean +/- SEM; n > 30 cells per condition; 3 biological replicates each. *p < 0.05, calculated using an ANOVA test.
Figure S3. Incorporation of the TFG (p.R106C) mutation into human iPSCs, Related to Figure 3. (A) Human iPSCs harboring heterozygous or homozygous TFG p.R106C mutations (Clones B, C, E, and F) were fixed and stained using antibodies directed against Oct4, Nanog, and SSEA4 together with Hoechst 33342 (n=3 each). Maximum intensity projections are shown. Scale bar, 100 µm. (B) Giemsa banding analysis was performed on iPSCs harboring a mutation in TFG (Clones B, C, E, and F), revealing no changes in karyotype that resulted from CRISPR-mediated genome editing.
Figure S4. Characterization of human iPSCs harboring the TFG (p.R106C) mutation following differentiation to fibroblast and melanocyte fates, Related to Figures 4 and 5. (A) Representative control iPSCs
and iPSC-derived cell types (fibroblasts and melanocytes) were fixed and stained using antibodies directed against Oct4, Nanog, tyrosinase, and/or vimentin (n=3 each). Maximum intensity projections are shown. Scale bars, 10 µm.

(B) Representative immunoblot analysis of control iPSC-derived fibroblasts (IMR90-4 and Clone G) and fibroblasts harboring the homozygous TFG (p.R106C) mutation (Clones A-C) using antibodies directed against TFG and β-actin. (C) Densitometry analysis was performed to quantify changes in TFG levels relative to β-actin in both control and homozygous TFG (p.R106C) expressing iPSC-derived fibroblasts and melanocytes (n=3). No statistically significant difference was found, as calculated using a paired t test. (D) Fluorescence intensity (I) of TFG relative to Sec31A in iPSC-derived fibroblasts and melanocytes from control (IMR90-4 and Clone G) and homozygous TFG (p.R106C) expressing mutants (Clones A-C). Error bars represent mean +/- SEM; n > 30 cells per condition; 3 biological replicates each. *p < 0.05 (compared with control), calculated using a paired t test. (E) Yeast co-expressing plasmids encoding Sec23A (bait fusion) and several unique prey constructs were plated (10-fold dilutions, left to right) on either selective (−Ura, −Leu, −His) or histidine supplemented medium for 48 hours (n=3). (F) Quantification of changes in luciferase-dependent luminescence relative to control (stably transduced IMR90-4 and Clone G) upon addition of tunicamycin (up to 5 µg/mL overnight) or in cells harboring the homozygous TFG (p.R106C) mutation (Clone A). Error bars represent mean +/- SEM; 3 biological replicates each. ***p < 0.005, **p < 0.01, or *p < 0.05 (compared with control), calculated using a paired t test.
Figure S5. The TFG (p.R106C) mutation does not impair neuronal differentiation, Related to Figure 6. (A-C) Flow cytometry was used to determine the percentage of cells expressing Tbr1 (A), Tau (B), and Nanog (C) in control (IMR90-4 and Clone G) or homozygous TFG (p.R106C) mutants (Clones A-C) after initiation of neuronal differentiation (C) or 12 weeks after neuronal differentiation (A and B). Error bars represent mean +/- SEM; 3 biological replicates each. No statistically significant difference was observed between conditions, based on a t test. Representative images of control (Clone G) or homozygous TFG (p.R106C) iPSC-derived neurons (Clone A) after 16 weeks of neuronal differentiation subsequent to fixing and staining with antibodies directed against Tau (B, right). Maximum intensity projections are shown. Scale bar, 50 µm. (D) Representative immunoblot analysis of control iPSC-derived cortical neurons (IMR90-4 and Clone G) and neurons harboring the homozygous TFG (p.R106C) mutation (Clones A-C) using antibodies directed against TFG and β-actin (left). Densitometry analysis was performed to quantify changes in TFG levels relative to β-actin in both control and homozygous TFG (p.R106C) expressing iPSC-derived neurons (right, n=3). Error bars represent mean +/- SEM. No statistically significant difference was found, as calculated using a paired t test. (E) Quantitative PCR was used to measure the relative expression of TFG in wild-type iPSC-derived neurons (IMR90-4) as compared to control (Clone G) and homozygous TFG (p.R106C) mutant neurons (Clones A-C) (n=3 each). Error bars represent mean +/- SEM. No statistically significant difference was found, as calculated using an ANOVA test.
Figure S6. The TFG (p.R106C) mutation does not affect the electrophysiology of iPSC-derived neurons, Related to Figure 6. (A) Example voltage clamp traces showing spontaneous synaptic activity from control (Clone

G) and clone (G). (B) Example voltage clamp traces showing spontaneous synaptic activity from control (Clone G) and clone (G). (C) Summary statistics for 

Cm, Rm, RMP, and AP amplitude. (D) GM130 (Golgi) and TFG staining in control (Clone G) and clone (G) neurons. (E) Mitochondrial staining in control (Clone G) and clone (G) neurons. (F) Mitochondrial motility in control (Clone G) and clone (G) neurons. (G) LAMP-1 volume in control (Clone G) and clone (G) neurons. (H) Western blot analysis of LC3-I, LC3-II, and β-actin in control (Clone G) and clone (G) neurons.
G; top) and homozygous TFG (p.R106C) mutant (Clone A; bottom) neurons. (B) Example membrane potential traces from control (Clone G; left) and homozygous TFG (p.R106C) mutant (Clone A; right) neurons. Smaller trace (center) shows applied current steps. Action potentials were observed in 40% of control neurons and 45% of homozygous TFG (p.R106C) mutant neurons. Occasional repetitive spiking (inset, far right) was also observed. (C) Quantification of membrane properties across cells (Cm, specific capacitance; Rm, resistance; R.M.P., resting membrane potential; A.P., action potential). No significant differences were observed between conditions, based on t tests (n = 17 cells, Clone G, and 11 cells, Clone A, from > 3 preparations). (D) Representative images of control (Clone G) and homozygous TFG (p.R106C) mutant (Clone A) neurons that were fixed and stained using antibodies directed against GM130 (red) and TFG (green). Maximum intensity projections are shown. Scale bar, 20 µm. (E) Representative images of control (Clone G) and homozygous TFG (p.R106C) mutant (Clone A) iPSC-derived neurons that were labeled with MitoTracker Red live and subsequently fixed and stained using antibodies directed against Tau (green). Maximum intensity projections are shown. Scale bar, 20 µm. The volumes of mitochondria imaged under these conditions were calculated using Imaris software. No statistically significant difference was found between control (IMR90-4 and Clone G) and homozygous TFG (p.R106C) mutant (Clones A-C) neurons, as calculated using a paired t test (n > 30 neurons per condition; 3 biological replicates each). (F) Particle tracking was used to measure the velocity of motile mitochondria in control (Clone G) and homozygous TFG (p.R106C) mutant (Clone A) iPSC-derived neurons that had been labeled with Rhodamine 123. No statistically significant difference was found, as calculated using a paired t test (n > 30 neurons per condition; 3 biological replicates each). (G) The volumes of LAMP-1 positive structures in control (Clone G) and homozygous TFG (p.R106C) mutant (Clone A) iPSC-derived neuronal cell bodies were calculated using Imaris software. A scatter plot of all data is shown. No statistically significant difference was found, as calculated using a paired t test (n > 30 neurons per condition; 3 biological replicates each). (H) Representative immunoblot analysis of control iPSC-derived cortical neurons (Clone G) and neurons harboring the homozygous TFG (p.R106C) mutation (Clone A) using antibodies directed against LC3 and β-actin. Densitometry analysis was performed to quantify changes in LC3-I vs. LC3-II levels relative to β-actin in both control and homozygous TFG (p.R106C) expressing iPSC-derived neurons (n=3). No statistically significant difference was found, as calculated using a paired t test.
Figure S7. ER stress induced by tunicamycin impairs axon outgrowth and maintenance, Related to Figures 6 and 7. Control iPSC-derived neurospheres were plated onto glass coverslips and grown in the presence or absence of tunicamycin (2.5 µg/mL). Axon outgrowth was examined using brightfield imaging at various timepoints, as tunicamycin-treated neurons could not withstand fixation and immunostaining. Scale bar, 50 µm.
Supplementary Experimental Procedures

Small angle x-ray scattering (SAXS) analysis and modeling

SAXS data were processed using Primus and Gnom (ATSAS) (Konarev et al., 2003) to determine the R_g and maximum dimension (D_max) of the protein and to obtain regularized scattering amplitudes extrapolated to q = 0 Å⁻¹ and the pair distance distribution function (PDDF) for each sample. The latter provides a profile of all the pair distances within the molecule and is therefore descriptive of the overall shape. When computing the PDDF, D_max was adjusted by increments of 1-2 Å and optimized based on the following criteria: 1) A smooth drop-off to zero probability at D_max, 2) agreement between the R_g measured using the Guinier transform and the R_g extracted from the PDDF, and 3) agreement between the regularized scattering determined from the PDDF and the experimental data. Furthermore, the regularization parameter, alpha, was kept below 5 to avoid over-smoothing. The resulting regularized scattering amplitudes were employed to calculate more than 10 replicate ab initio dummy atom models using DAMMIF (Franke and Svergun, 2009). The quality and uniqueness of the results were further assessed by agreement between replicate models as quantitated by the mean normalized spatial discrepancy (NSD) (Kozin and Svergun, 2001). Models were superimposed with one another using the Supcomb20 algorithm (Volkov and Svergun, 2003).

TALEN-mediated genome editing and cell culture

A transcription activator-like effector nuclease (TALEN)-mediated targeting system was used to generate RPE-1 cells that could inducibly express various TFG isoforms (23). Transgenes were flanked by an internal ribosome binding site and a gene encoding RFP to enable the identification of cells with similar levels of TFG expression. Each transgene was incorporated at the AAVS1 locus following electroporation and expression was induced by the addition of doxycycline (3 ng/mL) while simultaneously depleting endogenous TFG using a siRNA directed against the 3'UTR (5'-CCAAAAGACUCCAGUACUA-3') (11). RPE-1 cells were grown in
DMEM/F12 supplemented with 10% FBS, penicillin/streptomycin and L-glutamine and maintained at 37°C and 5% CO₂ (Johnson et al., 2015).

**Stem cell maintenance and differentiation**

IMR90-4 human iPSCs were grown in mTeSR-1 on Matrigel-coated plates (Yu et al., 2007). To generate fibroblasts, pluripotent iPSCs were grown on Matrigel in E6 medium supplemented with 64 mg/L ascorbic acid, 543 mg/L sodium bicarbonate, 14 mg/L sodium selenite, 19.4 mg/L insulin, and 10.7 mg/L transferrin for 10 days, and then shifted into fibroblast growth medium (High glucose DMEM, 15% FBS, 2 mM L-glutamine, 1 mM HEPES Buffer, and 0.5% Penicillin-Streptomycin) for 30 additional days (Chen et al., 2011). To generate cortical glutamatergic neurons, iPSCs were treated similarly for the first 10 days, but shifted into neural differentiation medium (E6 medium supplemented with 1 µM cyclic AMP, 10 ng/ml brain-derived neurotrophic factor (BDNF), and 10 ng/ml glial-derived neurotrophic factor (GDNF)) for 30 days. The medium was subsequently changed to cortical neural differentiation medium (E6 medium, 1 µM cyclic AMP, 10 ng/ml BDNF, 10 ng/ml GDNF, 100 ng/ml Insulin-like growth factor-I, and 2% B27 supplement) for an additional 30 days (Brennand et al., 2011). Cortical glutamatergic neurons were differentiated for 16 weeks in total, prior to analyses. To generate melanocytes, iPSCs were grown on laminin in E6 medium for 10 days and then shifted into melanocyte growth medium (Medium 254 plus HMGS-2 Supplement) for 30 days (Fang et al., 2006). Transfections of ManII-GFP into iPSC-derived fibroblasts were carried out using GeneJuice Transfection Reagent (EMD Millipore), while iPSC-derived melanocytes were transfected using TransIT-2020 Transfection Reagent (Mirus Bio). MitoTracker Red (Thermo Fisher) and Rhodamine 123 (Sigma-Aldrich) staining were conducted on live iPSC-derived cortical neurons, as per the recommendations of the manufacturers, and followed by fixation and immunostaining using antibodies directed against Tau where indicated.
**Electrophysiology**

Human iPSC-derived neurons were maintained in culture for 16 weeks prior to being transferred to a recording chamber that was continually perfused with ACSF (128 mM NaCl, 5 mM KCl, 25 mM HEPES, 30 mM D-glucose, 1 mM MgCl$_2$ and 2 mM CaCl$_2$, pH 7.4, adjusted to 300-310 mOsm with D-glucose). Borosilicate glass pipettes, pulled to a resistance of 3-7 MΩ, were filled with an intracellular solution containing 130 mM K-gluconate, 10 mM HEPES, 5 mM Na-phosphocreatine, 2 mM Mg-ATP, 1 mM EGTA, and 0.3 mM Na-GTP (pH 7.4). Whole-cell patch-clamp recordings were made with an Axon MultiClamp 700b amplifier (Molecular Devices). Isolated target cells were identified visually by their neuron-like morphology. Typical seal resistances after achieving the patch were 15 MΩ, neurons showing significant (+/-10%) fluctuations in seal quality during recording were excluded from the analysis. Resting membrane potentials were made immediately after achieving whole-cell mode. The calculated liquid junction potential of 17 mV was corrected offline. Membrane voltage was monitored in response to 500 ms current injections, beginning at -100 pA, and increasing to 500 pA in 10 pA increments. Action potentials were identified as rapidly depolarizing spikes crossing 0 mV. Cells were voltage clamped at -60 mV for measurement of spontaneous synaptic activity.

**Flow cytometry and luminescence measurements**

Cells were harvested with Accutase and fixed in paraformaldehyde (1.5%) for 10 minutes, prior to blocking (PBS, 2% fetal bovine serum, and 0.1% Triton X-100) and incubation with directly-labeled antibodies (1 μg/mL) for 1 hour at room temperature. Samples were examined using a BD LSRFortessa cell analyzer, and data analysis was carried out using FlowJo Single Cell Data Analysis software. For luciferase activity measurements, extracts were prepared from cells using Cell Culture Lysis Reagent (Promega) and stored at -80ºC. After thawing and equilibrating to room temperature, 10 μl of each cell extract was mixed with 100 μl of Firefly Luciferase
Reagent (Promega). Samples were analyzed for luciferase activity using 10-second measurements and a Berthold Lumat 9501 luminometer.

**Immunoblotting and immunostaining**

Extracts were prepared by lysing cells directly in 3X SDS sample buffer (0.25 mM Tris-HCl, pH 6.8, 30% glycerol, 6% SDS, 0.02% bromophenol blue, and 5% β-mercaptoethanol) after harvesting them using Accutase or Trypsin-EDTA. Following separation by SDS-PAGE and transfer to nitrocellulose, immunoblotting was carried out using antibodies directed against TFG (Lifespan Biosciences, LS-B11074), L1CAM (Sigma-Aldrich, L4543), phospho-IRE1 (Novus, NB100-2323), LC3 (GeneTex, GTX127375), and β-actin (Sigma, A1978). Densitometry measurements were carried out using ImageJ software. Commercially available antibodies used for immunofluorescence studies include Sec31A (BD Biosciences, 612351), GM130 (BD Biosciences, 610822), TFG (Lifespan Biosciences, LS-B11074), Tau (Sigma-Aldrich, SAB5500182), Oct4 (EMD-Millipore, AB3209), Nanog (Abcam, ab21624), SSEA4 (Abcam, ab16287), vimentin (R&D System, MAB2105), tyrosinase (Santa Cruz, sc-7833), and LAMP-1 (Developmental Studies Hybridoma Bank, H4A3). For confocal microscopy studies, images were acquired at 0.2 µm intervals in Z, and datasets shown are maximum intensity projections of full cell volumes unless otherwise noted. Co-localization studies were carried out based on individual confocal sections, and intensity measurements were conducted on maximum projections of exactly 1.4 µm in Z, as described previously (Johnson et al., 2015). Staining with antibodies directed against L1CAM (Sigma-Aldrich, L4543) was conducted prior to fixation, so that only the surface population of the protein was labeled.

**Yeast Two-Hybrid Studies**
Yeast transformed with a bait plasmid encoding human Sec23A and the indicated prey plasmids were grown in the absence of uracil and leucine overnight at 30°C with shaking, diluted to an OD$_{600}$ of 0.25 in synthetic medium lacking amino acids, and spot-plated onto selective medium. Plates were incubated at 30°C for 48 hours and then imaged.
Supplementary References

Brennand, K.J., Simone, A., Jou, J., Gelboin-Burkhart, C., Tran, N., Sangar, S., Li, Y., Mu, Y., Chen, G., Yu, D., McCarthy, S., Sebat, J., and Gage, F.H. (2011). Modelling schizophrenia using human induced pluripotent stem cells. Nature 473, 221-225.

Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Dietl, N.R., Propson, N.E., Wagner, R., Lee, G.O., Antosiewicz-Bourget, J., Teng, J.M., and Thomson, J.A. (2011). Chemically defined conditions for human iPSC derivation and culture. Nat. Methods 8, 424-429.

Fang, D., Leishear, K., Nguyen, T.K., Finko, R., Cai, K., Fukunaga, M., Li, L., Bravour, P.A., Kulp, A.N., Xu, X., Smalley, K.S., and Herlyn, M. (2006). Defining the conditions for the generation of melanocytes from human embryonic stem cells. Stem Cells 24, 1668-1677.

Franke, D., and Svergun, D.I. (2009). DAMMIF, a program for rapid ab-initio shape determination in small-angle scattering. J.Appl. Crystallogr. 42, 342-346.

Konarev, P.V., Volkov, V.V., Sokolova, A.V., Koch, M.H.J., and Svergun, D.I. (2003). PRIMUS: a Windows PC-based system for small-angle scattering data analysis. J. Appl. Crystallogr. 36, 1277–1282

Kozin, M., and Svergun, D. (2001). Automated matching of high- and low-resolution structural models. J. Appl. Crystallogr. 34, 33-41.
Volkov, V.V., and Svergun, D.I. (2003). Uniqueness of ab initio shape determination in small-angle scattering. J. Appl. Crystallogr. 36, 860–864.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I., and Thomson, J.A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917-1920.