Supporting Information

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SI Materials and Methods

Reagents. Thapsigargin, tunicamycin, calpeptin dantrolene, ryanodine, and cycloheximide were obtained from SIGMA. RPMI-1640 and DMEM were from Invitrogen. Neural induction media, neural proliferation media were from Stemcell Technologies. MitoProbe DilC1(5) mitochondrial membrane potential assay kit, Annexin V Alexa Flour488 conjugate, Fluo-4, and Fura-2 calcium indicators were obtained from Invitrogen. Caspase-glo 3/7 protease assay kit and calpain-glo protease assay kit was purchased from Promega. Mito Stress test kit was from Seahorse Bioscience. Anti-WFS2 antibody, and anti-WFS1 antibody were purchased from Proteintech, anti-Caspase 3, anti-CAPN2 antibodies were obtained from Cell Signaling Technology, anti-CAPNS1 and anti-alpha II spectrin antibody were obtained from Millipore. Anti-actin antibody was purchased from SIGMA. Anti-Myelin basic protein antibody was from Santa Cruz Biotechnology. Anti-Calpain 2 antibody, which detects both CAPN2 and CAPNS1, was raised in rabbits against bacterially expressed rat calpain 2.

Plasmids and siRNA. pCMV-SPORT6-WFS2 expression plasmid was purchased from Open Biosystems. pDsRed2-ER vector was purchased from Clontech. FLAG-tagged WFS2 plasmids were constructed by inserting FLAG sequences into the N and C termini of the expression plasmid. GST-WFS2 plasmid was generated by inserting WFS2 sequence into pEBG mammalian expression plasmid. A CAPN2 expression plasmid was generated in pLenti-CMV-puro plasmid provided by E. Campeau (1). Lipofectamine2000 (Invitrogen) was used to transfect small interfering RNA (siRNA) directed against WFS2 and CAPN2 into cells. siRNAs were designed and synthesized at QIAGEN as follows: mouse WFS2 CAACAGAAGGAUAGCUUG, human WFS2 CGAAAGUAGUGAAUGAAA, human CAPN2 CCGAGGAGGUUGAAAGUA, rat WFS1 GUUUGACCGCUACAAGUUU. Cells were incubated in media overnight after siRNA transfection, and then additional treatments were performed, including ER stress induction.

Cell Culture. Neuro-2a, NSC34, HEK293, MEFs, and COS7 cells were cultured in DMEM containing 10% (vol/vol) FBS and penicillin and streptomycin. MIN6 cells were grown in DMEM containing 15% FBS and penicillin and streptomycin. INS-1 832/13 cells were cultured in RPMI containing 10% FBS before measurement. Neural progenitor cells were maintained in STEMdiff Neural Progenitor Medium from Stemcell Technologies.

iPS Cell and Neural Progenitor Cell Generation. To generate iPS cells, we obtained fibroblasts from nonaffected controls and patients with Wolfram syndrome. Integration-free iPS cells were generated via Sendai viral delivery of the four reprogramming factors, Oct4, Sox2, Klf4, and c-Myc using Life Technologies' Cytotune reagents and protocol. All WFS- and control-iPSCs showed silencing of the four transgenes, exhibited characteristic human embryonic stem cell morphology, expressed pluripotency markers including ALP, NANOG, SOX2, SSEA4, and TRA-1-81, and had a normal karyotype. To generate neural progenitor cells, iPSCs were counted and plated ~50,000 per well in a 96-well plate to form uniform embryoid bodies. After 5 d, embryoid bodies were suspended in neural induction media and replated as adherent cultures. Fresh media were applied every day for 7 d. Neural rosettes formed in these cultures were selected and plated. Plated rosettes were fed with neural induction media every day for 4–7 d to obtain neural progenitor cells.

MALDI-TOF Mass Spectrometry. HEK293 cells were transfected with GST-WFS2 plasmid and empty GST plasmid. Cell lysates were collected and immunoprecipitated with glutathione beads in lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Hepes, 1 mM EDTA, 1 mM DTT, pH 7.5). The precipitated proteins from both samples were resolved by SDS/PAGE and stained with Coomassie blue staining. The distinct bands that only appear in GST-WFS2 lane but not GST lane were analyzed by MALDI-TOF tandem mass spectrometry on a Shimadzu Axima TOF2 mass spectrometry at University of Massachusetts Medical School Proteomics and mass spectrometry facility.

Immunostaining. Cells were fixed in 4% paraformaldehyde for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 for 2 min. The fixed cells were washed with PBS/Tween 0.1%, blocked with Image-It FX signal enhancer (Invitrogen) for 1 h, and incubated in primary antibody overnight at 4 °C. The cells were washed three times in PBS/Tween 0.1% and incubated with secondary antibody for 1 h at room temperature. Images were obtained with a Zeiss LSM 5 PASCAL confocal microscope with LSM Image software.

FACS Analyses. For flow cytometry analyses, neural progenitor cells or NSC34 cells were plated in 24-well plates. After staining, cells were washed and resuspended in PBS. Flow cytometry analyses were performed with LSRII (BD) at the FACS core facility of Washington University School of Medicine. The results were analyzed by FlowJo ver.7.6.3.

Quantitative Real-Time PCR. Total RNA was extracted by RNeasy kits (Qiagen). Reverse transcriptase PCR was performed using High capacity reverse transcription kit (Applied Biosystems) and quantitative PCR was demonstrated with Applied Biosystems ViiA7 using SYBR green dye.

2D Fluorescence Difference Gel Electrophoresis. Proteins were extracted from cerebellums from WFS1 knockout mice and control mice. Equal amount of protein extract from paired samples were labeled by CyDye DIGE fluors. The spectrally resolvable dyes enabled simultaneous coseparation and analysis of samples on a single multiplexed gel. These paired samples were simultaneously separated on a single 2D gel, using isoelectric focusing (IEF) in the first dimension and SDS polyacrylamide gel electrophoresis (SDS/PAGE) in the second dimension. After electrophoresis, the gel was scanned using a Typhoon image scanner. Each scan revealed one of the CyDye signals (Cy3 and Cy5). ImageQuant software was used to generate the image presentation data including the single and overlay images. The comparative analysis of all spots was done by the DeCyder analysis software. The protein expression ratios between WFS1 knockout and control mice were generated, and differentially expressed spots were analyzed by MALDI-TOF mass spectrometry.

SERCA Activity Assay. The SERCA activity assay was performed as described in ref. 2. HEK293 cells were homogenized in hypotonic buffer, consisting of 10 mM $NaHCO_3$, 250 mM sucrose, 5 mM NaN_3, and 0.1 mM PMSF. ER fractions were isolated using differential centrifugation. 125–300 μg of ER protein fractions were added to the assay mixture [100 mM KCL, 30 mM imidazole-histidine (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 5 mM $(COOK)_2$, 5 mM NaN_3, and 50 μM $CaCl_2$ (10 μCi/μmol[^{45}Ca]; $CaCl_2$ American Radiolabeled Chemicals)] heated to 37 °C for 15 min. (The amount of protein we used in this study was 125 μg per sample). The reaction was stopped by the addition of 250 mM KCl and 1 mM

$LaCl_3$. The mix was then vacuum filtered through a 0.2-μm HT Tuffryn membrane (Sigma). SERCA-dependent calcium transport was measured by comparing calcium transport in the presence or absence of 10 μM thapsigargin, a SERCA inhibitor.

1. Campeau E, et al. (2009) A versatile viral system for expression and depletion of proteins in mammalian cells. *PLoS ONE* 4(8):e6529.

2. Funai K, et al. (2013) Muscle lipogenesis balances insulin sensitivity and strength through calcium signaling. *J Clin Invest* 123(3):1229–1240.

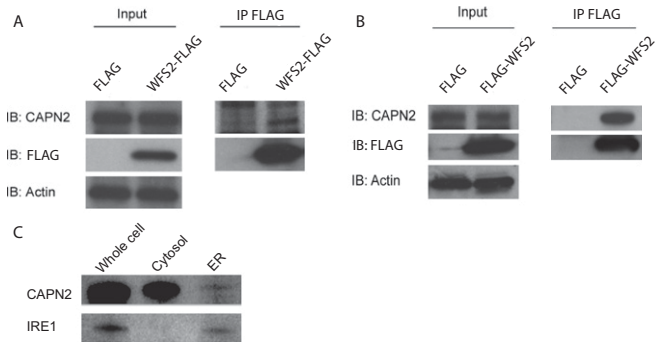

Fig. S1. FLAG-tagged WFS2 was immunoprecipitated from HEK293 cells transfected with WFS2 expression plasmids with either an N-terminal (*A*) or a C-terminal FLAG-tag (*B*), and the immunoprecipitates were analyzed for CAPN2 by immunoblotting with anti-CAPN2 antibody. (*C*) Cells were fractionated into cytosolic and ER fractions. Localization of CAPN2 and IRE1 were determined by immunoblotting.

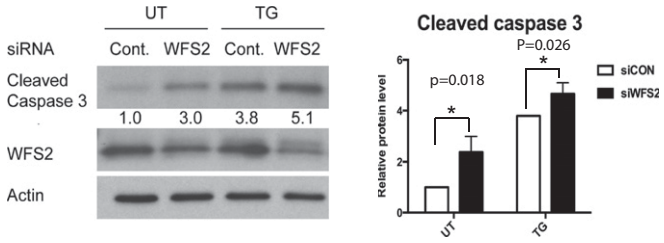

Fig. S2. MIN6 cells were transfected with control scrambled siRNA (Cont) or siRNA directed against WFS2, and then treated with 0.5 μM thapsigargin (TG) for 6 h or untreated (UT). Expression levels of cleaved caspase 3, WFS2, and actin were measured by immunoblotting (*Left*). (*Right*) Quantification of cleaved caspase 3 is indicated ($n = 3$, $^*P < 0.05$).

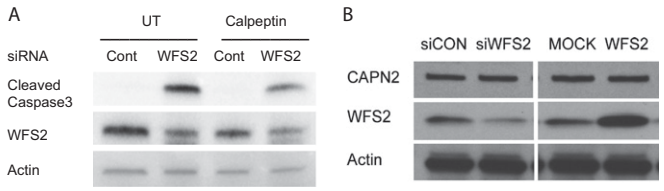

Fig. S3. (*A*) MIN6 cells were transfected with scrambled siRNA (Cont) or WFS2 siRNA. Thirty-six h after transfection, cells were treated with or without 100 μM calpeptin for 12 h. Cleaved caspase 3, WFS2, and actin levels were monitored by immunoblotting. (*B*) CAPN2, WFS2 and actin levels were assessed by immunoblotting in HEK293 cells transfected with scrambled siRNA (siCON), WFS2 siRNA (siWFS2), empty expression plasmid (Mock), or WFS2 expression plasmid (WFS2).

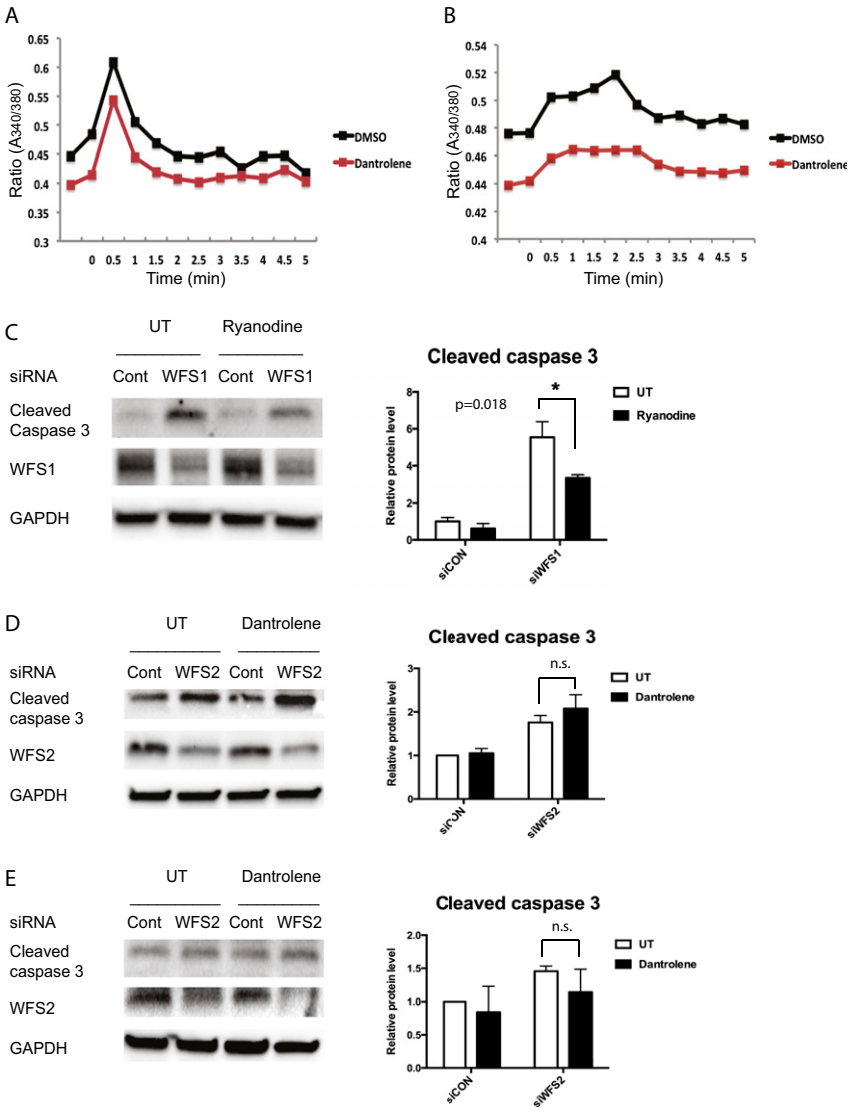

Fig. S4. INS-1 832/13 (*A*) and NSC34 (*B*) cells were pretreated with or without 10 μM dantrolene for 24 h. Cytoplasmic calcium levels were measured by Fura-2 calcium indicator over a period. Thapsigargin was added at 0 min time point ($n = 6$, the experiment was repeated six independent times). (*C*) INS-1 832/13 cells were transfected with scrambled siRNA (Cont) or WFS1 siRNA. Twenty-four h after transfection, cells were treated with or without 2 μM ryanodine for 24 h. Cleaved caspase 3, WFS1, and GAPDH levels were monitored by immunoblotting (*Left*) and quantified (*Right*) ($n = 3$, $^*P < 0.05$). (*D* and *E*) INS-1 832/13 cells (*D*) and NSC34 cells (*E*) were transfected with scrambled siRNA (Cont) and WFS2 siRNA. Twenty-four h after transfection, cells were treated with or without 10 μM dantrolene for 24 h. Cleaved caspase 3, WFS2 and GAPDH levels were monitored by immunoblotting. Quantification of cleaved caspase 3 protein levels are shown to the right ($n = 3$, $^*P < 0.05$).

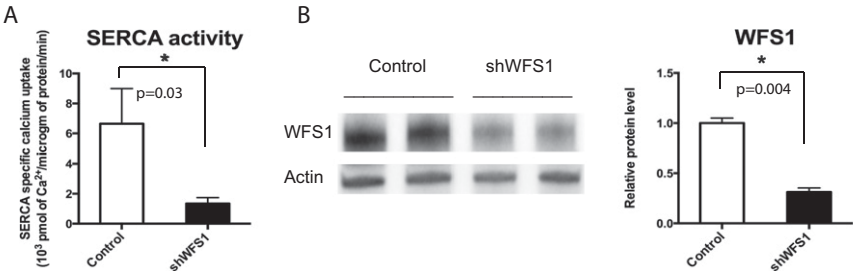

Fig. S5. (*A*) SERCA activity was measured in HEK293 cells stably expressing shRNA directed WFS1 or a scrambled sequence (Control). All values are means ± SEM. For the wild-type condition, $n = 6$; the experiment was repeated six independent times. For the WFS1 knockdown condition, $n = 7$; the experiment was repeated seven independent times. $^*P < 0.05$. (*B*) Protein levels of WFS1 and actin were monitored in HEK293 cells stably expressing shRNA directed against WFS1 or a scrambled shRNA (Control). Quantification is shown on the right ($n = 3$, $^*P < 0.05$).

Table S1. GST-WFS2 interacting proteins

Order on gel	Gene symbol	Full name	Molecular weight, KDa
1	PRKDC	DNA dependent protein kinase catalytic subunit	450
2	COPA	Coatomer Subunit alpha	140
3	IPO7, 4, 9	Importin 7, 4, and 9	120
4	XPO1, 2	Exportin 1, 2	110
5	MMS19	MMS19 nucleotide excision repair	110
6	CNX	Calnexinp	67
7	CAPN2	calpain-2	80
8	GRP78	GRP78	78
9	TUBA TUBB	Alpha and Beta Tubulin	50

Table S2. Information on genotypes and phenotypes of Wolfram syndrome and control subjects

iPSC line	Source	Clinical diagnosis	WFS1 mutation	Sex	Age at biopsy	Age at onset of DM	Age at onset of OA	Deafness	DI
Wolf-2010–5	Washington University Wolfram Clinic	WFS	H313Y	F	15	3.8	12	1.7	NA
Wolf-2010–9	Washington University Wolfram Clinic	WFS	A126T; W613X	M	16	10.8	11	NA	14
Wolf-2010–11	Washington University Wolfram Clinic	WFS	A126T; W613X	M	10	7.5	6	8	10
Wolf-2010–13	Washington University Wolfram Clinic	WFS	L200fs286Stop; E752Stop	F	7	4.8	5.2	6	7.5
GM01610	Corriell Research Institute	WFS	W648X; G695V	F	11	NA	NA	NA	NA
BJ CRL-2522	ATCC	Control	NA	M	Newborn	NA	NA	NA	NA
Wolf-2010–5-MO	Washington University Wolfram Clinic	Control	None identified	F	41	NA	NA	No	No
Wolf-2010–9 MO	Washington University Wolfram Clinic	Control	NA	F	33	NA	NA	No	No
Wolf-2012–13-FA	Washington University Wolfram Clinic	Control	NA	M	42	NA	NA	No	No

WFS, Wolfram syndrome; DM, diabetes mellitus; OA, optic atrophy; DI, diabetes insipidus.

Table S3. Chemical compounds used for a screen targeting the ER calcium homeostasis

	Drugs	Treatment conc.
1	Nicotinamide(Vitamin B3)	10 μM
2	Valproic acid	10 μM
3	Sodium tauroursodeoxycholate(TUDCA)	10 μM
4	(-)-Riboflavin(Vitamin B2,Vitamin G)	10 μM
5	Thiamine hydrochloride(Vitamin B1 hydrochloride)	10 μM
6	Memantine hydrochloride	10 μM
7	(+-)-a-Lipoic acid	10 μM
8	Kynurenic acid	10 μM
9	Folic acid	10 μM
10	Idebenone	10 μM
11	Acetovanillone(Apocynin)	10 μM
12	Aspirin	10 μM
13	Pyridoxine hydrochloride	10 μM
14	Dextromethorphan hydrobromide	10 μM
15	2.3-Pyridinedicarboxylic acid(DPA)	10 μM
16	R-(-)-Deprenyl hydrochloride(Selegiline hydrochloride)	10 μM
17	NS-398	10 μM
18	4-Aminobenzoic acid(PABA,Vitamin Bx, Vitamin H1)	10 μM
19	Biotin	10 μM
20	D-Pantothenic acid hemicalcium salt(Vitamin B5)	10 μM
21	Chondroitin sulfate A sodium salt from bovine tracha(Glycosaminoglycans)	10 μg/mL
22	Ebselen	10 μM
23	PPBP maleate(4-PPBP maleate)	10 μM
24	Minocycline hydrochloride	10 μM
25	Pravastatin sodium salt hydrate	10 μM
26	*N*-tert-Butyl-alpha-phenylnitrine(PBN)	10 μM
27	Curcumin	10 μM
28	TRO19622(Olesoxime)	10 μM
29	Pyridoxamine dihydrochloride	10 μM
30	Pyridoxal hydrochloride	10 μM
31	Fibroblast Growth factor-Basic human	100 ng/mL
32	Bryostatin1	100 nM
33	Brain derived neurotrophic factor human	100 ng/mL
34	SRP4988(PEDF)	100 ng/mL
35	Erythropoietin	0.1 UN/mL
36	Clioquinol	10 μM
37	Kenpaullone	10 μM
38	PARP inhibitor iii,DPQ	10 μM
39	Glial Cell Line-derived Neurotrophic Factor human	100 ng/mL
40	Ciliary Neurotrophic Factor, human	100 ng/mL
41	Nitric Oxide Synthase, Neuronal Inhibitor 1(nNOS inhibitor)	10 μM
42	Riluzole	10 μM
43	Creatine	10 μM
44	Anisomycin from streptomyces griseolus	10 μM
45	NE 100 hydrochloride	10 μM
46	Phenytoin	10 μM
47	CsA	300 nM
48	FK506	300 nM
49	Rapamycin	10 μM
50	Docosahexaenoic acid	10 μM
51	GLP-1	50 nM
52	Diazoxide	300 μM
53	Glibenclamide	100 μM
54	2-APB (2-Aminoethoxydiphenyl borate)	200 nM
55	IL1RA	100 ng/mL
56	Retinol	10 μM
57	GW5015-16	10 μM
58	GW9508	10 μM
59	Etomoxir	20 μM
60	Verapamil	20 μM
61	Metformin	44 μM
62	AICAR	10 μM
63	pioglitazone	10 μM

Table S3. Cont.

	Drugs	Treatment conc.
64	Troglitazone	10 μM
65	*N*-Acetyl D-Shingosine	10 μM
66	Dihydroceramide C2	10 μM
67	Fumonisin B1	10 μM
68	Ros inhibitor	100 μM
69	SNAP	1 mM
70	Dantrolene	10 μM
71	Bcl XLBH4 human	1 μM
72	Calp. Inhibitor iii	1 μM
73	salburinal	25 μM