Supporting Information (SI Appendix):

Calpain Inhibitor and Ibudilast Rescue β-Cell Function in a Cellular Model of Wolfram Syndrome

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Supplementary methods:

Calcium imaging with Fluo-4-AM

Buffers were prepared following the same protocol as described for calcium imaging with Fura-2-AM (see method section of the manuscript). Fura-4-AM dye powder (Thermo Fisher) was dissolved to 4µM in calcium-containing HEPES buffer supplemented with 0.03% Pluronic acid (Thermo Fisher).

For measurements of ER-calcium release, cells were plated at a density of $2x10_5$ cells per coverslip. After 48 hours, calcium imaging was carried out as described for Fura-2-AM. Following stimulation with 50nM ATP, cells were imaged using sequential excitation at 488 nm, and images were acquired with emission bandwidth of 501 to 555 nm. After background subtraction, data were normalized to the first 10 sec of baseline recording. Subsequent data quantification was performed as described for Fura-2-AM. All figures depicting calcium imaging traces show the average of 5-12 coverslips, each with 40-70 cells, from at least 3 independent recordings.

For measurements of cytosolic calcium, $2.5x10_4$ cells were plated per well on a black, clear-bottom 96-well plate. After 48 hours, wells were carefully washed two times with calcium-containing HEPES-buffered saline solution. Then, cells were incubated in Fluo-4-AM dye solution for 45 min in the dark at room temperature. After washing off the dye, cells were kept in calcium-containing HEPES-buffered saline solution and imaged with a Tecan Infinite M1000 Pro microplate reader using the following setting: 2 sec linear shaking (2mm, 654 rpm), followed by imaging in fluorescence mode with 40µsec integration time.

mRNA analysis

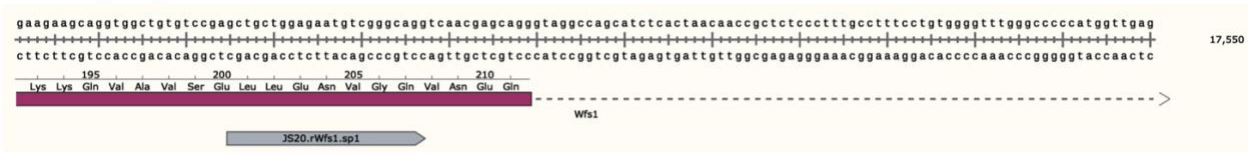
mRNA was isolated from INS1 cells grown to confluency using the RNeasy Mini kit (Qiagen) and reverse-transcribed to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time reverse transcription PCR, 40 ng of cDNA was used as template in a reaction with POWER SYBR Green MasterMix (Life Technologies) in a 7500 Fast machine (Applied Biosystems). Each sample was run as three technical replicates on a 96-well plate. Fold change in mRNA transcript levels was determined by using the ΔCt method [1]. 18S was used as a control. The following primers were used: rat *18S (fwd, 5' CATTCGAACGTCTGCCCTAT 3'; rev, 5' GTTTCTCAGGCTCCCTCTCC 3'), rat NCS1 (fwd, 5' GGAGACCCCACCAAGTTCG 3'; rev, 5' AACTCGATCCTGCCATCCTTG 3').*

Subcellular fractionation

Subcellular fractionation to obtain homogenate, membrane, cytosolic, and mitochondrial fractions was carried out following the protocol provided by Abcam (R. Patten). Lysis buffer contained 250mM Sucrose, 20mM HEPES (pH 7.4), 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, and 1mM EGTA. Cell lysate obtained from a 15cm dish of confluent INS1 cells was passed through a 25G needle and centrifuged repeatedly resulting in the different fractions step by step. Additionally, we prepared crude mitochondrial extracts from HEK293 lysate using a mitochondrial extraction kit (Thermo Fisher, #89874). A cell suspension in HEK cell media (DMEM high glucose, 5% FBS, 1% PenStrep) was collected using TrypLE and pelleted at 850xg for 2 min. Between steps of centrifuging and vortexing, the reagents provided in the kit were added to the pellet step by step. Finally, mitochondria were lysed in 2% CHAPS with tris buffer. For further analysis, fractionation samples were prepared for Western Blot (see method section of the manuscript).

Supplementary figures:

A Design of gRNA



B Details of gRNA (CRISPOR tool)

Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes + Variants (Only G- / Only GG- / Only A-)	Specificity Score	Predicted Efficiency (Show all scores) Doench '16	Mor.-Mateos	Out-of-Frame score (Click on score to show micro-homology)	Off-targets for 0-1-2-3-4 mismatches + next to PAM
157 / fw	GCTGCTGGAGAATGTCGGGC *AGG* Enzymes: *Hpy166II, HincII* Cloning / PCR primers	82	56	42	48	0 - 0 - 0 - 6 - 158 (0 - 0 - 0 - 0 - 0) 164 off-targets

C Deep sequencing results: *left*: genotyping of clones on 96-well plates, *right*: confirmation of genotype after expansion

Sample	Total	WT_g1	#1-Indel	#1-Reads(%)	#2-Indel	#2-Reads(%)
1E11	1525	0 (0.0%)	1	889 (58.3%)	32	625 (41.0%)
4F1	2022	0 (0.0%)	-4	1012 (50.0%)	1	1004 (49.7%)
7B7	1605	1513 (94.3%)	0	1578 (98.3%)		

Sample	Total	WT_g1	#1-Indel	#1-Reads(%)	#2-Indel	#2-Reads(%)
Clone 1E1	1488	0 (0.0%)	1	838 (56.3%)	32	639 (42.9%)
Clone 7B7	1229	1129 (91.9%)	0	1216 (98.9%)	-1	11 (0.9%)
Clone 4F1	1459	0 (0.0%)	1	735 (50.4%)	-4	710 (48.7%)

D Sequencing Alignments

E Verification of WFS1 knockout (KO) in INS1 cells via Western blot

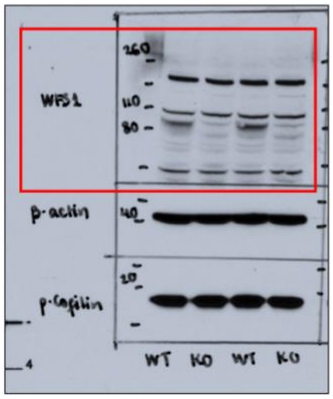


Figure S1. WFS1-WT control and WFS1-KO cell lines were generated using CRISPR-Cas by the Genome Engineering and iPSC Center at Washington University in St. Louis. (A) gRNA was designed to target an early, conserved exon of WFS1. (B) Details of gRNA design using CRISPOR. (C) Sequencing

results for the cell clones obtained. KO cell lines show out-of-frame indels (insertion or deletion mutations) in all alleles that result in immature stop codons before a.a. 230 (of 890) and lead to non-sense mediated decay of mRNA. (D) Indels aligned with WFS1-sequence. (E) Loss of WFS1 protein expression was verified using Western blot. Shown is uncropped blot of two independent preparations. In addition to the WFS1 band slightly above the 80kDa marker, the antibody used also resulted in several unspecific bands.

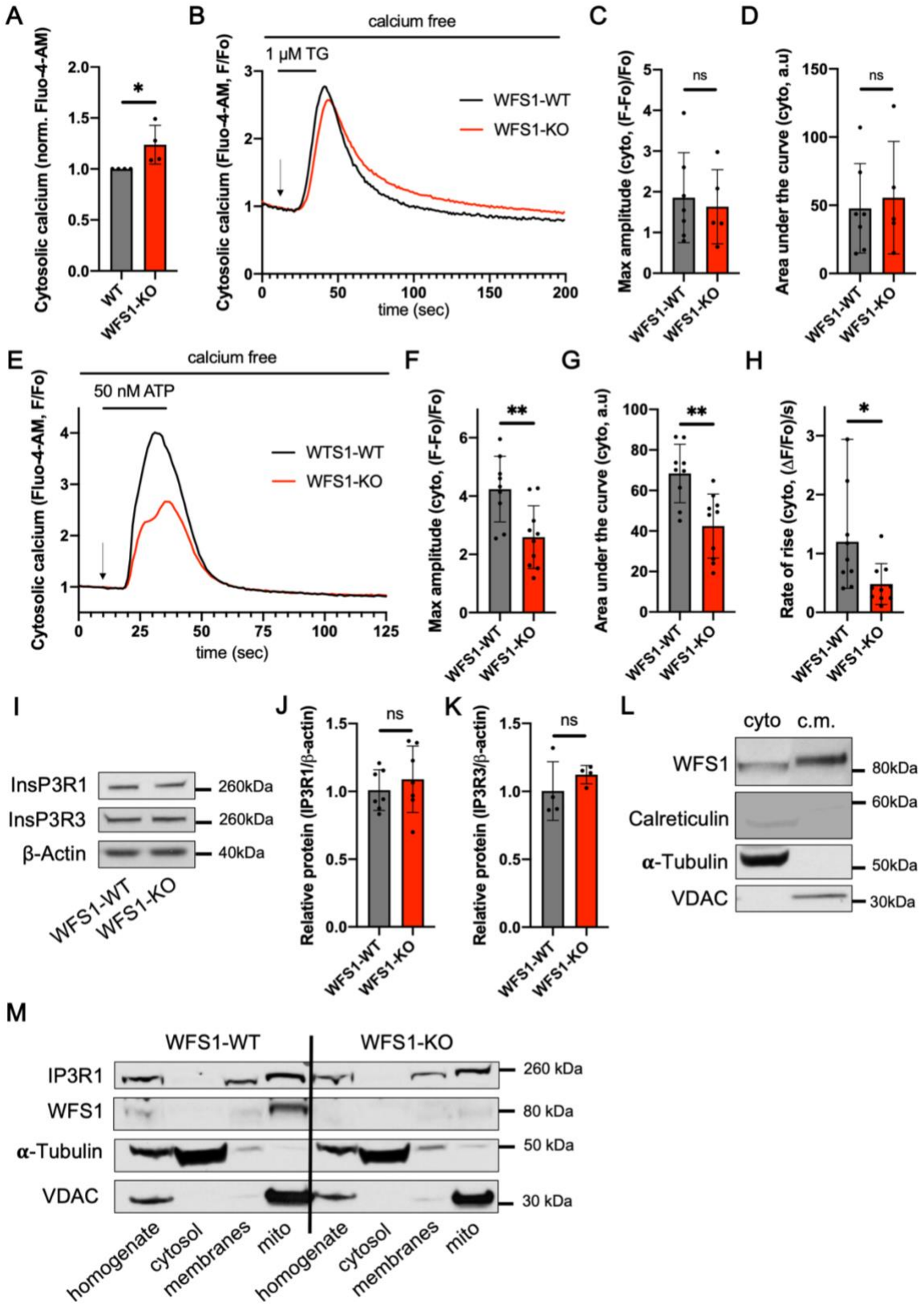

A
Cytosolic calcium (norm. Fluo-4-AM)
2.0
1.5
1.0
0.5
0.0
*
WT
WFS1-KO
B
calcium free
1 µM TG
Cytosolic calcium (Fluo-4-AM, F/Fo)
3
2
1
0
0
50
100
150
200
time (sec)
WFS1-WT
WFS1-KO
C
Max amplitude (cyto, (F-Fo)/Fo)
5
4
3
2
1
0
ns
WFS1-WT
WFS1-KO
D
Area under the curve (cyto, a.u)
150
100
50
0
ns
WFS1-WT
WFS1-KO
E
calcium free
50 nM ATP
Cytosolic calcium (Fluo-4-AM, F/Fo)
4
3
2
1
0
25
50
75
100
125
time (sec)
WTS1-WT
WFS1-KO
F
Max amplitude (cyto, (F-Fo)/Fo)
6
4
2
0
**
WFS1-WT
WFS1-KO
G
Area under the curve (cyto, a.u)
100
80
60
40
20
0
**
WFS1-WT
WFS1-KO
H
Rate of rise (cyto, (ΔF/Fo)/s)
3
2
1
0
*
WFS1-WT
WFS1-KO
I
InsP3R1
InsP3R3
β-Actin
260kDa
260kDa
40kDa
WFS1-WT
WFS1-KO
J
Relative protein (IP3R1/β-actin)
1.5
1.0
0.5
0.0
ns
WFS1-WT
WFS1-KO
K
Relative protein (IP3R3/β-actin)
1.5
1.0
0.5
0.0
ns
WFS1-WT
WFS1-KO
L
cyto
c.m.
WFS1
Calreticulin
α-Tubulin
VDAC
80kDa
60kDa
50kDa
30kDa
M
WFS1-WT
WFS1-KO
IP3R1
WFS1
α-Tubulin
VDAC
260 kDa
80 kDa
50 kDa
30 kDa
homogenate
cytosol
membranes
mito
homogenate
cytosol
membranes
mito

Figure S2. WFS1 regulates intracellular calcium homeostasis in INS1 cells. (A) WFS1-KO cells showed a significant elevation of cytosolic calcium compared to WFS1-WT cells. (B) Panel shows averaged traces of 5-6 coverslips for each cell line in response to 1µM thapsigargin. The Fluo-4-AM signal was normalized to the intensity at 10 sec. (C, D) Quantification of area under the curve and max amplitude for cytosolic calcium traces shown in B, no difference was observed. (E) Panel shows averaged traces of 9-10 coverslips for each cell type in response to 50nM ATP. The fluo-4-AM signal was normalized to the intensity at 10 sec. (F-H) Compared to WFS1-WT cells, WFS1-KO cells showed decreased area under the curve, max amplitude, and rate of rise for the cytosolic calcium traces shown in E. (I) Representative blot of InsP3R1 and InsP3R3 protein abundance in WFS1-WT and WFS1-KO cells. (J-K) Quantification of I (from 4-7 independent preparations) showed no difference in InsP3R1 and InsP3R3 expression in both cell lines. (L) Representative blot of subcellular fractionations isolated from HEK293 cells; cyto = cytosol; c.m. = crude mitochondria, containing mitochondria and MAMs. Tubulin was used as a marker for cytosolic proteins, VDAC for mitochondrial proteins, and calreticulin for non-MAM ER-proteins. (M) Representative blot of subcellular fractionations obtained from INS1 WFS1-WT and WFS1-KO cells. Same markers used as in L.

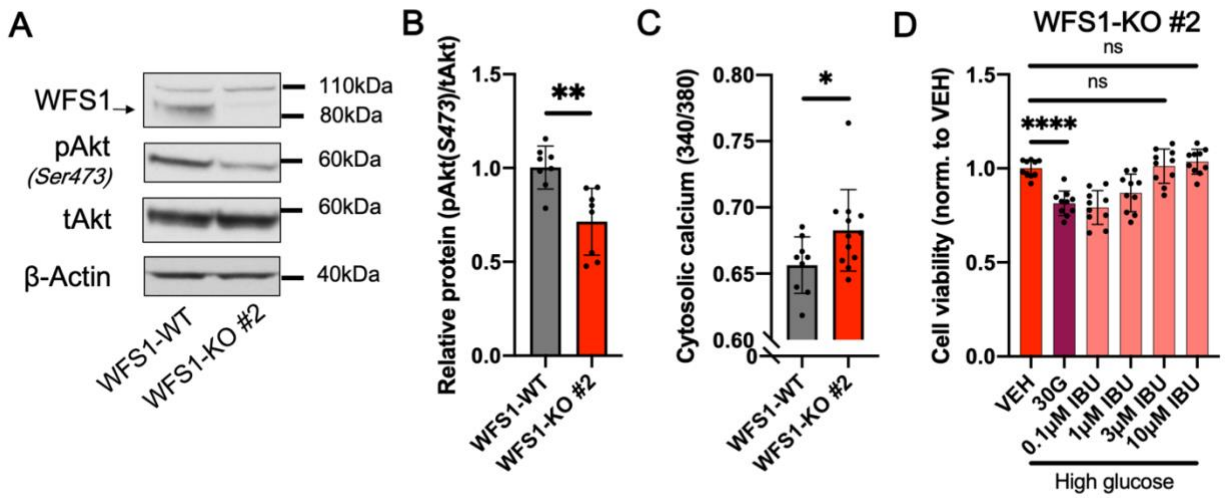


Figure S3. Validation of key findings in a second CRISPR-WFS1-KO INS1 clone. (A) Representative blot confirming the loss of WFS1 in WFS1-KO clone 2, and showing protein abundance of pAkt (S473) and tAkt. (B) Quantification of B (from 7 independent preparations). (C) WFS1-KO clone #2 cells exhibited significantly elevated cytosolic calcium compared to WFS1-WT cells. (D) High glucose-induced loss of cell viability in WFS1-KO clone #2 cells was dose-dependently reversed by ibudilast, 30G = 30 mM additional glucose, IBU = ibudilast.

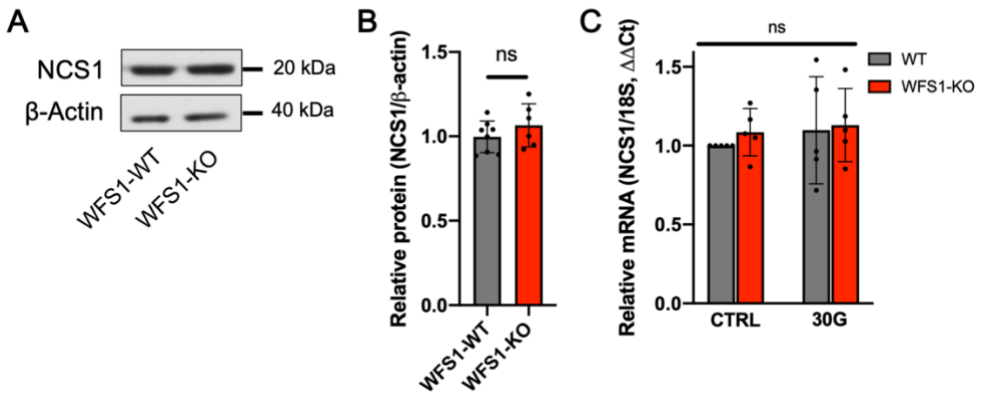


Figure S4. NCS1 protein and mRNA levels in WFS1-WT and WFS1-KO cells. (A) Representative blot showing NCS1 protein abundance. (B) Quantification of A (from 6-8 independent preparations), normalized to WT, no difference was observed between cell lines. (C) Quantification of 5 independent qPCR experiments, no difference was observed between the different conditions, 30G = 30 mM additional glucose.

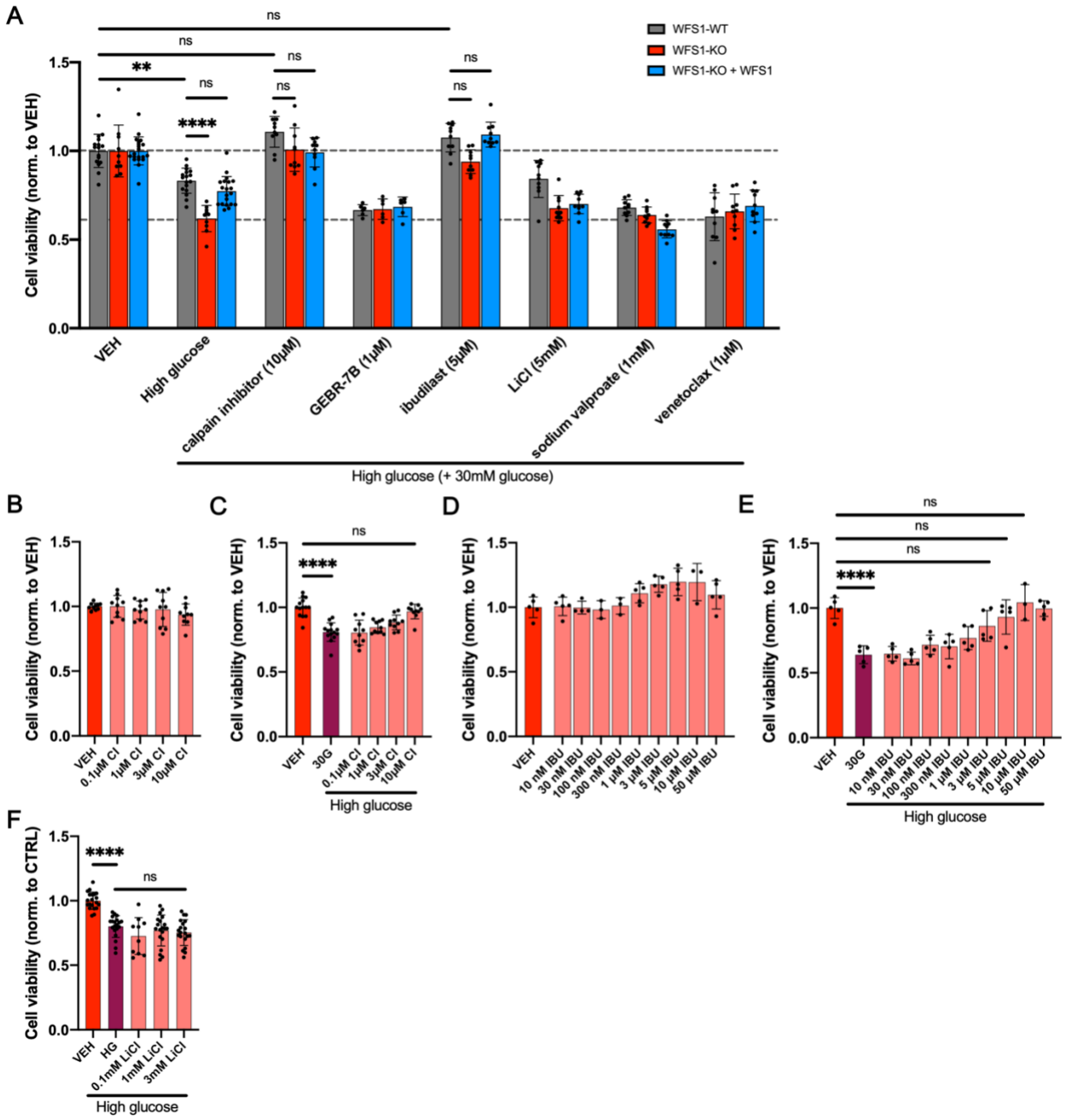


Figure S5. Drug screen for compounds that rescue cell viability in WFS1-KO cells. Compounds were selected to target WFS1, NCS1, and/or calcium signaling. (A) Preliminary screening of various drugs to prevent glucose toxicity. Following treatment with 30 mM additional glucose (30G, 48h), WFS1-WT, WFS1-KO, and WFS1-OE cells showed significant cell death as compared to cells cultured in normal medium. However, WFS1-KO cells showed more severe cell death compared to WFS1-WT and WFS1-OE cells following high glucose. Of the 7 compounds tested, calpain inhibitor XI and ibudilast rescued cell viability back to a normal level. (B) Calpain inhibitor XI did not affect cell viability in WFS1-KO cells at baseline. (C) Calpain inhibitor XI dose-dependently reversed high glucose-induced loss of cell viability in WFS1-KO cells.

(D) Ibudilast slightly raised cell viability in WFS1-KO cells at baseline. (E) Ibudilast dose-dependently reversed high glucose-induced loss of cell viability in WFS1-KO cells. (F) Lithium did not reverse hyperglycemia-induced loss of cell viability in WFS1-KO cells.

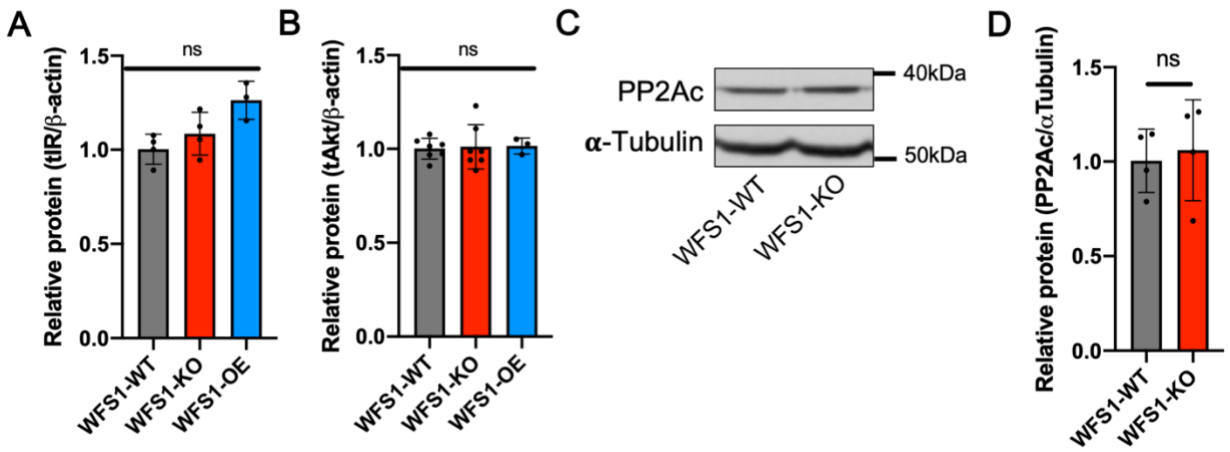


Figure S6. Total IR, total Akt, and PP2Ac protein levels in WFS1-WT and WFS1-KO cells. (A) Total IR and (B) total Akt protein levels were not changed, representative blot shown in Fig. 5 A. (C) Representative blot showing protein abundance of the catalytic subunit of protein phosphatase 2A (PP2Ac) in WFS1-WT and WFS1-KO cells. (D) Quantification of C (from 4 independent preparations), no significant difference was observed between cell lines.

Table S1: List of primary antibodies used.

Antibody	**Source**	**Identifier**
WFS1	Proteintech	11558-1-AP
β-Actin	Cell Signaling	8H10D10
NCS1	Santa Cruz	FL-190
phospho-Akt (pAkt) (S473)	Cell Signaling	#4060
phospho-Akt (pAkt) (T308)	Cell Signaling	#9275
total-Akt (tAkt)	Cell Signaling	#2920
Phospho-IGF-I Receptor β(Tyr1135/1136)/Insulin Receptor β (Tyr1150/1151) (pIRβ)	Cell Signaling	#3024
total-Insulin-Receptor (tIR)	Gift from Dr. G. Shulman	
Calreticulin	Cell Signaling	#12238
Inositol trisphosphate receptor isoform 1 (InsP3R1)	Homemade production	
Inositol trisphosphate receptor isoform 3 (InsP3R3)	BD biosciences	610312
α-Tubulin	Abcam	ab7291
VDAC	Abcam	ab34726
PP2A c subunit	Cell signaling	#2259

Table S2: Detailed statistical analysis for Figures 1-5. All data were included in statistical testing, relevant p-values are shown below.

Fig.	Statistical test	P-value	Post-hoc test pairwise comparison
1B	One-way ANOVA	p = 0.0006	WFS1-WT vs. WFS1-KO: p = 0.0004 WFS1-WT vs. WFS1-OE: p = 0.1463 WFS1-KO vs. WFS1-OE: p = 0.009
1C	Two-tailed student t-test	p = 0.0002	
1E	One-way ANOVA	p = 0.0005	WFS1-WT vs. WFS1-KO: p = 0.0004 WFS1-WT vs. WFS1-OE: p = 0.1568 WFS1-KO vs. WFS1-OE: p = 0.0096
1F	One-way ANOVA	p < 0.0001	WFS1-WT vs. WFS1-KO: p < 0.0001 WFS1-WT vs. WFS1-OE: p < 0.0001 WFS1-KO vs. WFS1-OE: p = 0.0456
1G	One-way ANOVA	p = 0.0215	WFS1-WT vs. WFS1-KO: p = 0.0345 WFS1-WT vs. WFS1-OE: p = 0.8356 WFS1-KO vs. WFS1-OE: p = 0.0420
1H	Two-tailed student t-test	p = 0.0021	
1I	Two-tailed student t-test	p = 0.0037	
1J	Two-tailed student t-test	p = 0.0001	
2A	One-way ANOVA	p = 0.0002	WFS1-WT CTRL vs. WFS1-KO CTRL: p = 0.0005 WFS1-WT CTRL vs. WFS1-WT 30G: p = 0.0319 WFS1-KO CTRL vs. WFS1-KO 30G: p = 0.9214 WFS1-WT 30G vs. WFS1-KO 30G: p = 0.1954
2C	One-way ANOVA	p < 0.0001	WFS1-WT CTRL vs. WFS1-WT 15G: p < 0.0001 WFS1-WT CTRL vs. WFS1-WT 30G: p < 0.0001 WFS1-WT 15G vs. WFS1-WT 30G: p = 0.0040 WFS1-KO CTRL vs. WFS1-KO 15G: p = 0.0219 WFS1-KO CTRL vs. WFS1-KO 30G: p < 0.0001 WFS1-KO 15G vs. WFS1-KO 30G: p = 0.6517 WFS1-WT CTRL vs. WFS1-KO CTRL: p < 0.0001 WFS1-WT 15G vs. WFS1-KO 15G: p = 0.0466 WFS1-WT 30G vs. WFS1-KO 30G: p = 0.9469

			WFS1-WT 15G vs. WFS1-KO CTRL: p = 0.9996 WFS1-WT 30G vs. WFS1-KO 15G: p = 0.9841
2D	One-way ANOVA	p < 0.0001	WFS1-WT CTRL vs. WFS1-WT 15G: p < 0.0001 WFS1-WT CTRL vs. WFS1-WT 30G: p < 0.0001 WFS1-WT 15G vs. WFS1-WT 30G: p = 0.0072 WFS1-KO CTRL vs. WFS1-KO 15G: p = 0.0062 WFS1-KO CTRL vs. WFS1-KO 30G: p < 0.0001 WFS1-KO 15G vs. WFS1-KO 30G: p = 0.5243 WFS1-WT CTRL vs. WFS1-KO CTRL: p < 0.0001 WFS1-WT 15G vs. WFS1-KO 15G: p = 0.0618 WFS1-WT 30G vs. WFS1-KO 30G: p = 0.7928 WFS1-WT 15G vs. WFS1-KO CTRL: p = 0.9998 WFS1-WT 30G vs. WFS1-KO 15G: p = 0.9946
2E	One-way ANOVA	p < 0.0001	WFS1-WT CTRL vs. WFS1-WT 15G: p = 0.1663 WFS1-WT CTRL vs. WFS1-WT 30G: p < 0.0001 WFS1-WT 15G vs. WFS1-WT 30G: p = 0.0005 WFS1-KO CTRL vs. WFS1-KO 15G: p = 0.1327 WFS1-KO CTRL vs. WFS1-KO 30G: p = 0.0006 WFS1-KO 15G vs. WFS1-KO 30G: p = 0.7635 WFS1-WT CTRL vs. WFS1-KO CTRL: p < 0.0001 WFS1-WT 15G vs. WFS1-KO 15G: p = 0.0060 WFS1-WT 30G vs. WFS1-KO 30G: p = 0.9514 WFS1-WT 15G vs. WFS1-KO CTRL: p = 0.4714 WFS1-WT 30G vs. WFS1-KO 15G: p = 0.9956

3C	One-way ANOVA	p < 0.0001	WFS1-WT CTRL vs. WFS1-WT 30G: p = 0.0003 WFS1-KO CTRL vs. WFS1-KO 30G: p = 0.0366
3F	One-way ANOVA	p < 0.0001	WFS1-WT vs. WFS1-KO: p = 0.0062 WFS1-WT vs. e.v.: p = 0.0029 WFS1-KO vs. e.v.: p = 0.8839 WFS1-WT vs. NCS1-OE: p = 0.9820 WFS1-KO vs. NCS1-OE: p = 0.0046 e.v. vs. NCS1-OE: p = 0.0006
3G	One-way ANOVA	p < 0.0001	WFS1-WT vs. WFS1-KO: p = 0.0019 WFS1-WT vs. e.v.: p = 0.0007 WFS1-KO vs. e.v.: p = 0.8635 WFS1-WT vs. NCS1-OE: p = 0.2083 WFS1-KO vs. NCS1-OE: p < 0.0001 e.v. vs. NCS1-OE: p < 0.0001
3H	One-way ANOVA	p = 0.0003	WFS1-WT vs. WFS1-KO: p = 0.0934 WFS1-WT vs. e.v.: p = 0.0354 WFS1-KO vs. e.v.: p = 0.9928 WFS1-WT vs. NCS1-OE: p = 0.9789 WFS1-KO vs. NCS1-OE: p = 0.0186 e.v. vs. NCS1-OE: p = 0.0009
3I	One-way ANOVA	p = 0.0298	WFS1-WT vs. WFS1-KO: p = 0.0449 WFS1-WT vs. NCS1-OE: p = 0.9111 WFS1-KO vs. NCS1-OE: p = 0.0462
4A	Two-tailed student t-test	p = 0.6171	
4C	One-way ANOVA	p < 0.0001	WFS1-WT CTRL vs. WFS1-WT 30G: p = 0.1259 WFS1-WT CTRL vs. WFS1-WT 30G + CI: p = 0.4930 WFS1-WT CTRL vs. WFS1-WT 30G + IBU: p > 0.9999 WFS1-KO CTRL vs. WFS1-KO 30G: p < 0.0001 WFS1-KO CTRL vs. WFS1-KO 30G + CI: p > 0.9999 WFS1-KO CTRL vs. WFS1-KO 30G + IBU: p = 0.8563 WFS1-WT 30G vs. WFS1-KO 30G: p < 0.0001 WFS1-WT CI vs. WFS1-KO CI: p > 0.9999 WFS1-WT IBU vs. WFS1-KO IBU: p = 0.9980 WFS1-WT 30G + CI vs. WFS1-KO 30G + CI: p = 0.5953

			WFS1-WT 30G + IBU vs. WFS1-KO 30G + IBU: p = 0.9861
4D	One-way ANOVA	p < 0.0001	WFS1-WT CTRL vs. WFS1-KO CTRL: p < 0.0001 WFS1-WT CTRL vs. WFS1-WT C.I.: p = 0.9940 WFS1-WT CTRL vs. WFS1-KO C.I.: p = 0.6244 WFS1-KO CTRL vs. WFS1-KO C.I.: p = 0.0007
4E	One-way ANOVA	p = 0.0029	WFS1-WT CTRL vs. WFS1-KO CTRL: p = 0.0097 WFS1-WT CTRL vs. WFS1-WT IBU: p = 0.9942 WFS1-WT CTRL vs. WFS1-KO IBU.: p = 0.9002 WFS1-KO CTRL vs. WFS1-KO IBU: p = 0.0484
5A	One-way ANOVA	p < 0.0001	WFS1-WT CTRL 2.5G vs. WFS1-KO CTRL 2.5G: p = 0.6053 WFS1-WT 9G CTRL vs. WFS1-KO 9G CTRL: p = 0.0328 WFS1-WT CTRL 2.5G vs. WFS1-WT CTRL 9G: p = 0.0008 WFS1-KO CTRL 2.5G vs. WFS1-KO CTRL 9G: p = 0.0664 WFS1-WT CTRL 2.5G vs. WFS1-WT C.I. 2.5G: p > 0.9999 WFS1-WT CTRL 9G vs. WFS1-WT C.I. 9G: p > 0.9999 WFS1-WT C.I. 2.5G vs. WFS1-KO C.I. 2.5G: p > 0.9999 WFS1-WT C.I. 9G C.I. vs. WFS1-KO C.I. 9G: p = 0.8072 WFS1-WT C.I. 2.5G vs. WFS1-WT C.I. 9G: p = 0.0006 WFS1-KO C.I. 2.5G vs. WFS1-KO C.I. 9G: p = 0.0236 WFS1-WT CTRL 2.5G vs. WFS1-WT IBU 2.5G: p = 0.0005 WFS1-WT CTRL 9G vs. WFS1-WT IBU 9G: p = 0.0244 WFS1-WT IBU 2.5G vs. WFS1-KO IBU 2.5G: p > 0.9999 WFS1-WT IBU 9G C.I. vs. WFS1-KO IBU 9G: p = 0.9998 WFS1-WT IBU 2.5G vs. WFS1-WT IBU 9G: p = 0.0355 WFS1-KO IBU 2.5G vs. WFS1-KO IBU 9G: p = 0.0010
5C	One-way ANOVA	p < 0.0001	WFS1-WT vs. WFS1-KO: p < 0.0001 WFS1-WT vs. WFS1-OE: p = 0.0003 WFS1-KO vs. WFS1-OE: p < 0.0001
5D	One-way ANOVA	p < 0.0001	WFS1-WT vs. WFS1-KO: p < 0.0001 WFS1-WT vs. WFS1-OE: p = 0.4867 WFS1-KO vs. WFS1-OE: p = 0.0005
5F	Two-tailed student t-test	p = 0.0026	

References

1. Schmittgen, T.D. and K.J. Livak, *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc, 2008. **3**(6): p. 1101-8.