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Supplementary Materials for

ER-mitochondria cross-talk is regulated by the Ca^{2+} sensor NCS1 and is impaired in Wolfram syndrome

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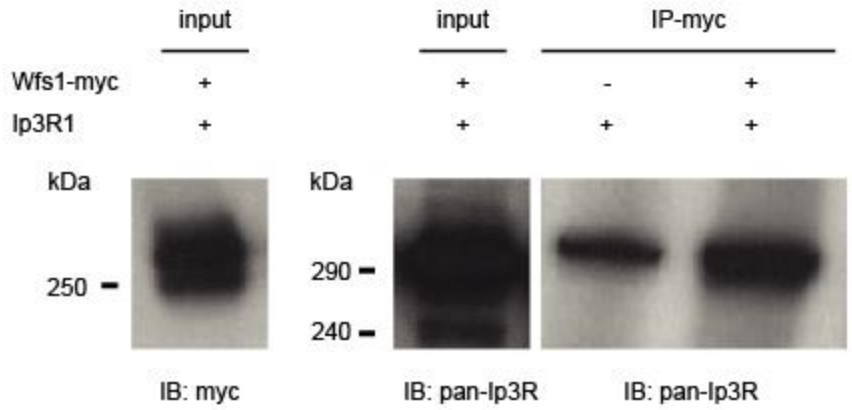

Fig. S1. WFS1 interacts with IP_3R. Co-immunoprecipitation examining binding of Wfs1 with Ip3R1. HEK293T were transfected with Wfs1-myc. By co-immunoprecipitation with myc antibody, Ip3R1 can be mostly detected in the presence of Wfs1. Immunoprecipitates were analysed with antibody against pan-Ip3R. Input represents cell lysate. n=3 independent experiments.

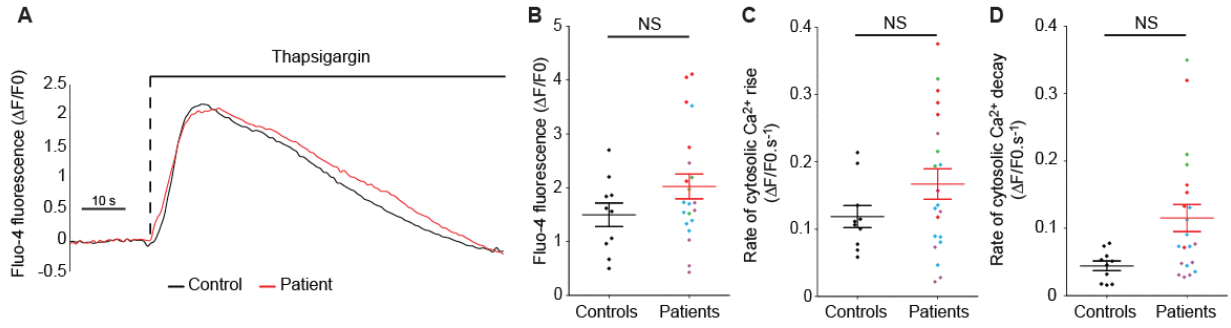

Fig. S2. Ca^{2+} imaging in thapsigargin-treated cells. (**A**) Cytosolic Ca^{2+} measured by Fluo-4 fluorescence in control (black line, n=10) and in patient (red line, n=21) cells under thapsigargin treatment. (**B**) Maximum amplitude of Fluo-4 fluorescence in control (n=10) and patient (P1 red dots, n=5; P2 green dots, n=3; P3 blue dots, n=7; P4 purple dots, n=6) cells. (**C**) Rate of cytosolic Ca^{2+} rise in control (n=10) and patient (P1 red dots, n=5; P2 green dots, n=3; P3 blue dots, n=7; P4 purple dots, n=6) cells. (**D**) Rate of cytosolic Ca^{2+} decay in control (n=10) and patient (P1 red dots, n=5; P2 green dots, n=3; P3 blue dots, n=7; P4 purple dots, n=6) cells. Data are presented as mean ± SEM. NS: not significant.

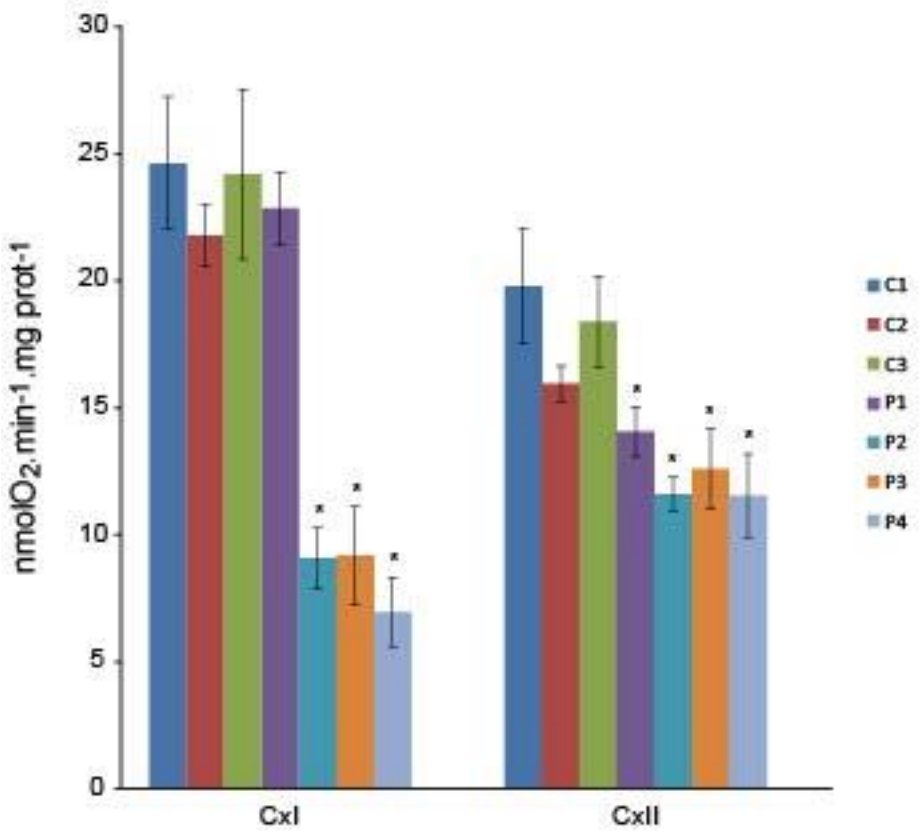

Fig. S3. Analysis of mitochondrial respiratory rate.
Mitochondrial complex I and complex II-dependent respiratory rate in control (C1, C2, C3) and patient (P1, P2, P3, P4) fibroblasts. Respiratory rate: nmol oxygen consumed/min/mg protein. (mean ± SEM, n=5 experiments).

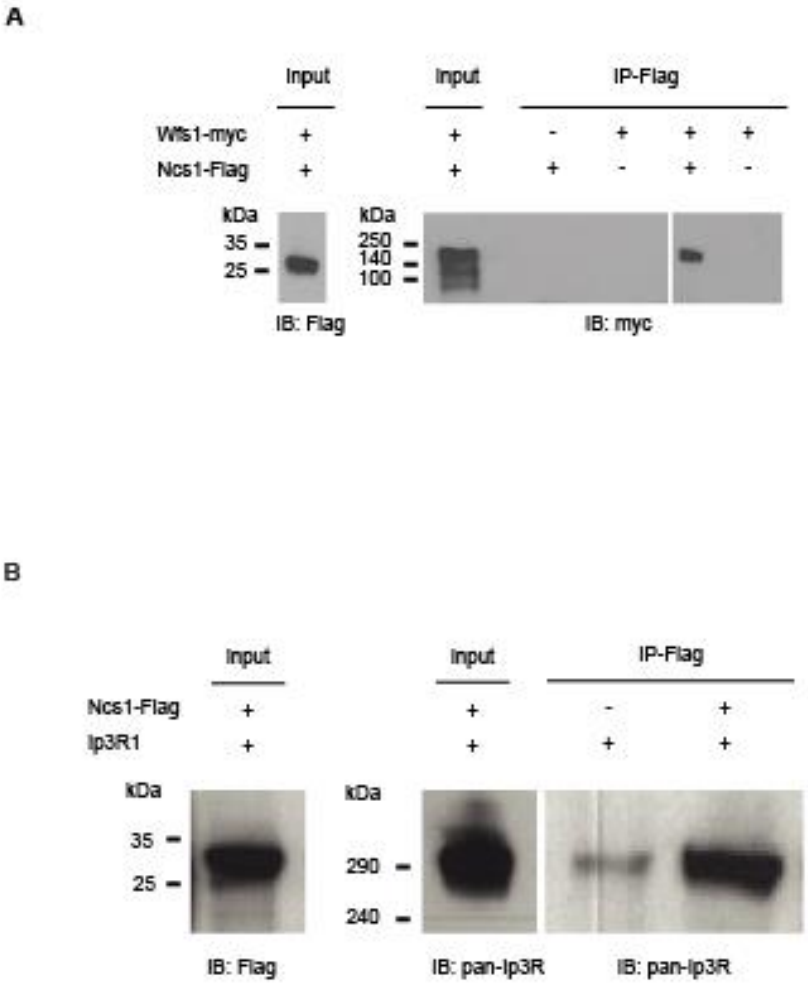

Fig. S4. NCS1 interacts with WFS1 and IP_3R. (**A**) Co-immunoprecipitation of Wfs1-myc with Ncs1-Flag with antibody against Flag from lysates of transfected HEK293T. Immunoprecipitates were analysed by western blotting with antibodies against Flag and myc. Input represents cell lysate. n= 3 independent experiments. (**B**) Co-immunoprecipitation examining the interaction of Ncs1 with Ip3R1. HEK293T were transfected with Ncs1-Flag. By co-immunoprecipitation with Flag antibody, Ip3R1 can be mostly detected in the presence of Ncs1. Immunoprecipitates were analysed with antibody against pan-Ip3R. Input represents cell lysate. n=3 independent experiments.

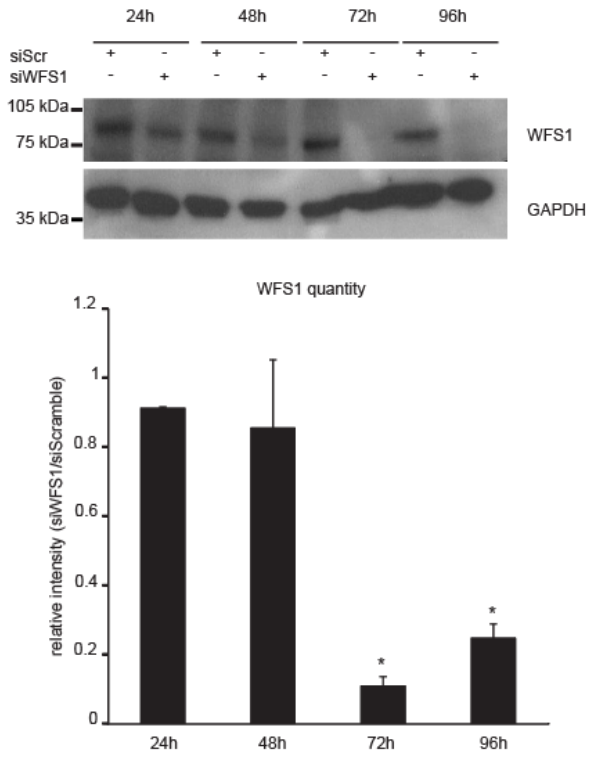

Fig. S5. Time course of siRNA-mediated knockdown of WFS1. Control fibroblasts were transfected with WFS1 siRNA (siWFS1) or a scrambled siRNA (siScr). Protein were isolated at the indicated times and the expression intensity was normalized against GAPDH. Data represent mean ± SEM. (n=4 experiments, *p<0.05).

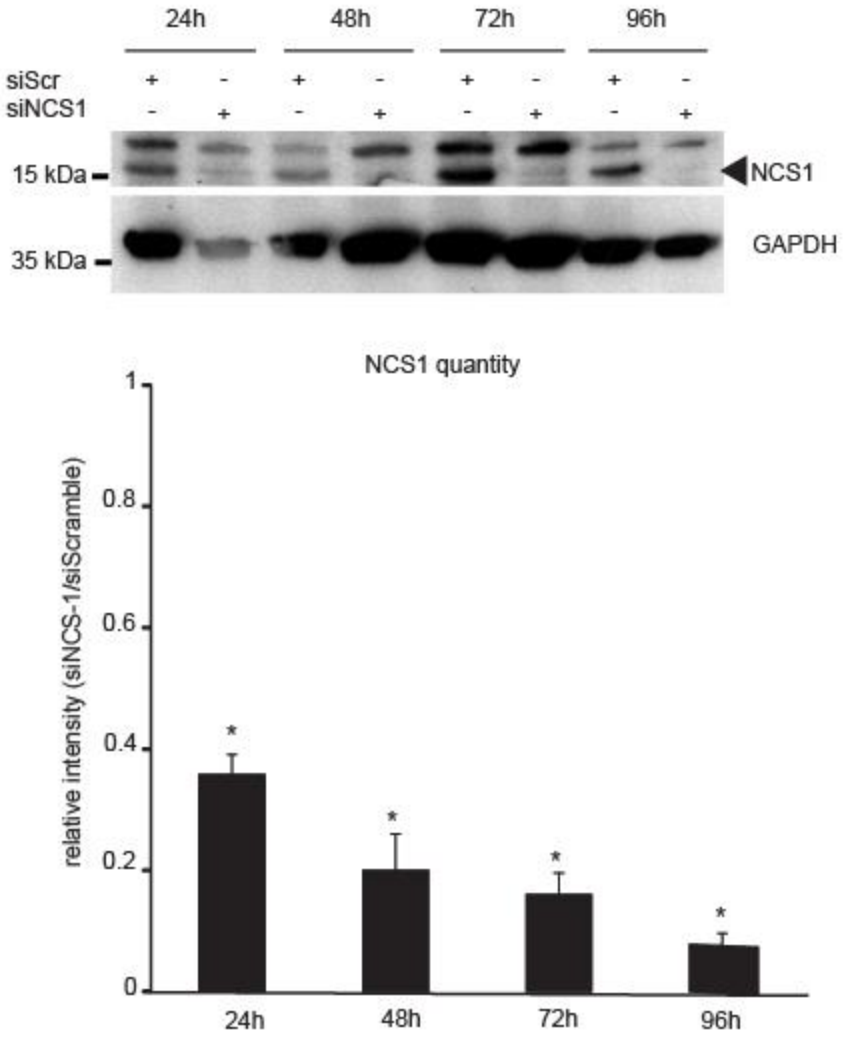

Fig. S6. Time course of siRNA-mediated knockdown of NCS1. Control fibroblasts were transfected with NCS1 siRNA (siNCS1) or a scrambled siRNA (siScr). Proteins were isolated at the indicated times and the expression intensity was normalized against GAPDH. Data represent mean ± SEM. (n=4 experiments, *$p<0.05$).

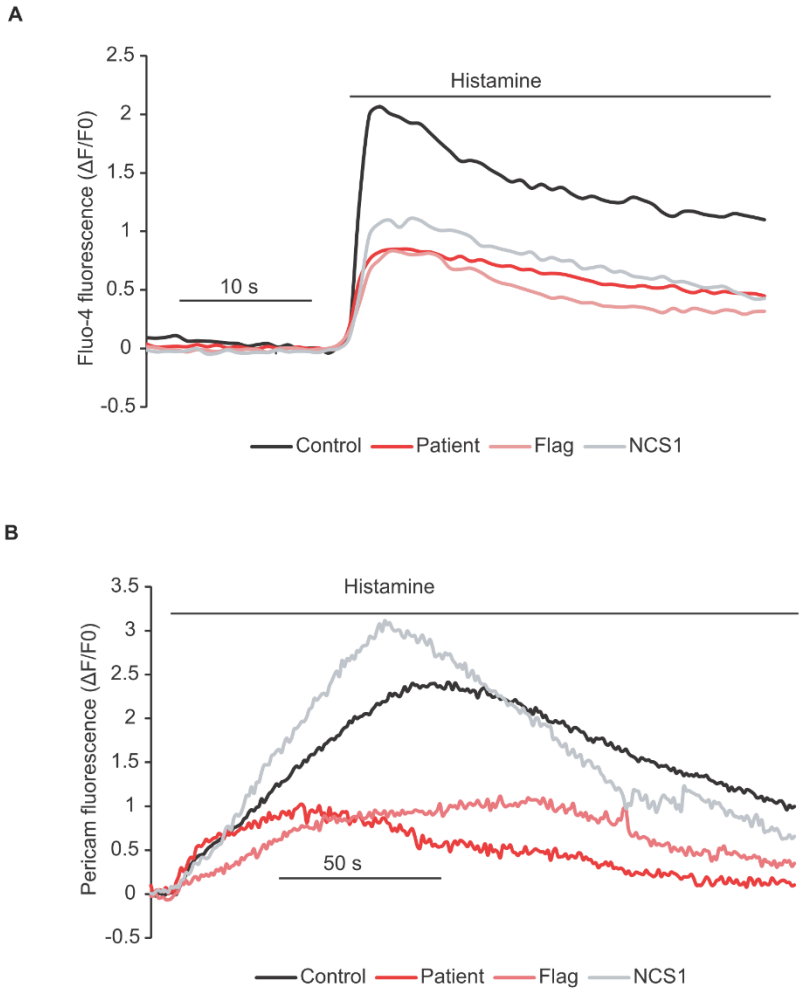

Fig. S7. Representative cytosolic and $[Ca^{2+}]_m$ in control and patient fibroblasts expressing Flag or NCS1-Flag. (**A**) Representative cytosolic Ca^{2+} measured by Fluo-4 fluorescence in control fibroblasts, P3 patient fibroblasts, P3 patient fibroblasts expressing Flag alone and P3 patient fibroblasts expressing NCS1-Flag under histamine stimulation. (**B**) Representative mitochondrial Ca^{2+} measured by pericam fluorescence in control fibroblasts, P3 patient fibroblasts, P3 patient fibroblasts expressing Flag alone and P3 patient fibroblasts expressing NCS1-Flag under histamine stimulation.

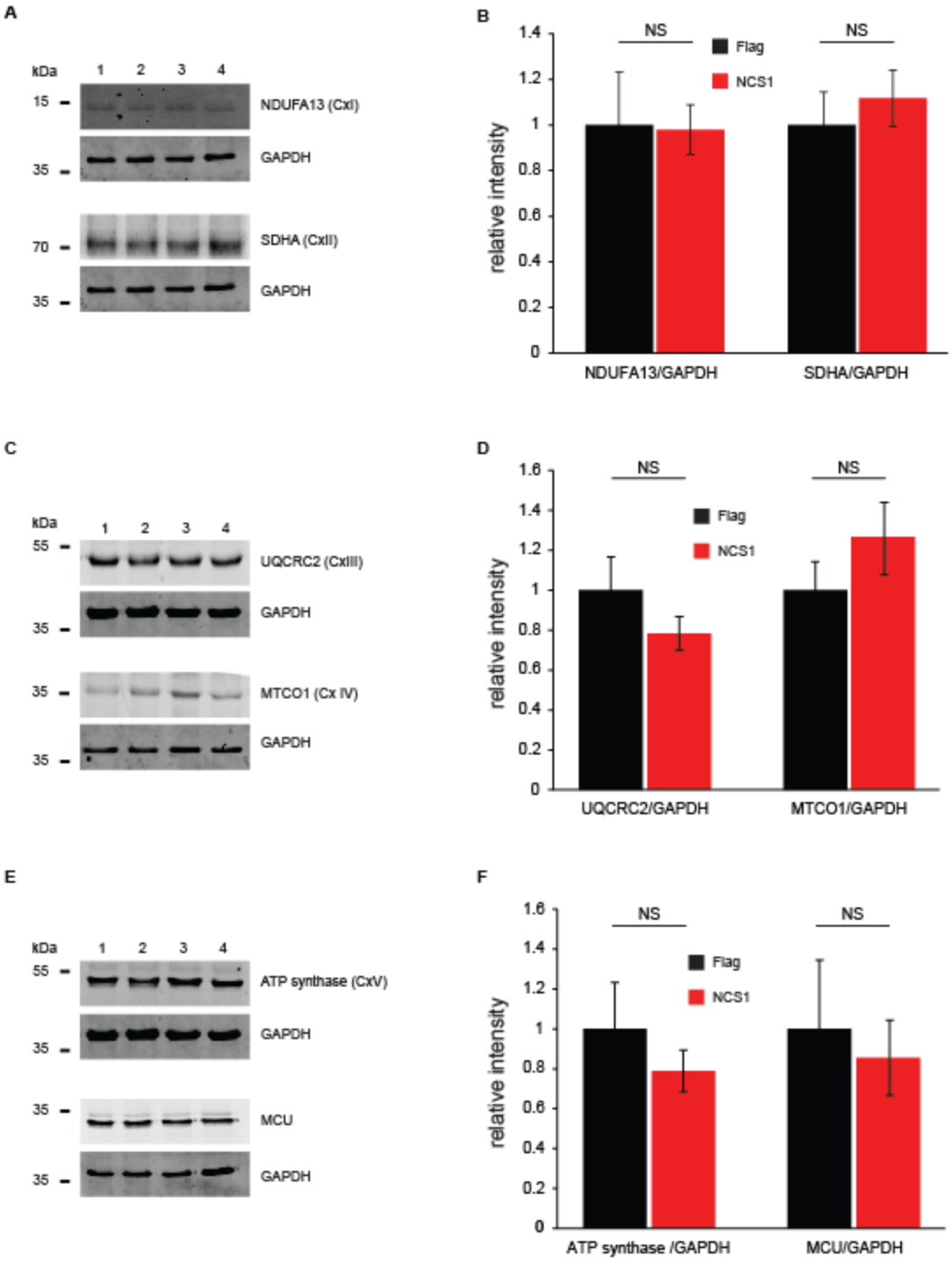

Fig. S8. Mitochondrial protein abundance in patient fibroblasts overexpressing NCS1. (**A**) Western blot of NDUFA13 (complex I) and SDHA (complex II) in patient fibroblasts transfected with Flag alone (lanes 1 and 3) or NCS1-Flag (lanes 2 and 4). GAPDH was used as a loading control. (**B**) Densitometric analysis of NDUFA13 and SDHA expression in patient cells transfected with Flag alone (Flag) or NCS1-Flag (NCS1) (mean ± SEM, n=4 independent experiments). (**C**) Western blot of UQCRC2 (complex III) and MTCO1 (complex IV) in patient

fibroblasts transfected with Flag alone (1, 3) or NCS1-Flag (2, 4). GAPDH was used as a control of specificity and loading. (**D**) Densitometric analysis of UQCRC2 and MTCO1 expression in patient cells transfected with Flag alone (Flag) or NCS1-Flag (NCS1) (mean ± SEM, n=4 independent experiments). (**E**) Western blot of ATP synthase (complex V) and MCU in patient fibroblasts transfected with Flag alone (1, 3) or NCS1-Flag (2, 4). GAPDH was used as a loading control. (**F**) Densitometric analysis of ATP synthase and MCU expression in patient cells transfected with Flag alone (Flag) or NCS1-Flag (NCS1) (mean ± SEM, n=4 independent experiments). NS: not significant. The same GAPDH loading control was used for NDUFA13 and SDHA, and for UQCRC2 and ATP synthase.

Table S1. Clinical features of control subjects and patients with Wolfram syndrome. NA: Not Available.

Clinical features	Patient 1 (P1)	Patient 2 (P2)	Patient 3 (P3)	Patient 4 (P4)	Control 1 (C1)	Control 2 (C2)	Control 3 (C3)
Age	22	18	21	19	22	22	22
Sex	Male	Male	Male	Female	Male	Male	Female
Mutations	V509-Y513del F882fsX950	V509-Y513del F882fsX950	L432V L432V	c.1-43G>T W867X	No	No	No
Optic neuropathy	Yes	Yes	Yes	Yes	No	No	No
Diabetes mellitus	Yes	Yes	Yes	Yes	No	No	No
Diabetes insipidus	*Yes*	Yes	NR	NA	No	No	No
Deafness	Yes	Yes	Yes	NA	No	No	No
Neurologic signs	Epilepsy Myoclonic seizures Tonic-clonic seizures	Epilepsy Myoclonic seizures Tonic-clonic seizures	No	Dysphagia	No	No	No
Psychiatric signs	No	Yes	Nevrosis	No	No	No	No
Urological signs	Yes	Yes	Yes	No	No	No	No
Other signs	Hypertrophic cardiomyopathy Renal tubulopathy	No	No	No	No	No	No

Table S2. TEM image analysis results. Top: Parameters of mitochondria of control (C1, C2, C3) and patient (P1, P2, P3, P4) fibroblasts. Bottom: Mitochondrial perimeter in contact with ER in control and patient fibroblasts. Nb, Number. Pic, Picture. Mito, Mitochondria. ER, Endoplasmic Reticulum.

	Nb Pic	Nb Mito	Nb Mito /pic	Total Mito Perimeter (nm)	Mean Mito Perimeter (µm)	Mean Cyto Area / pic (μm^2)	Mean Mito Area / pic (μm^2)	% mean Cyto Area occupied / Mito / pic
C1	19	140	7.37	118299.86	0.845	5.16	0.281	5.80
C2	20	121	6.05	121214.08	1.002	4.97	0.326	6.93
C3	20	142	7.10	130219.33	0.917	5.84	0.318	6.34
Total	59	403						
Mean			**6.84**	**123244.42**	**0.921**	**5.32**	**0.308**	**6.36**
P1	19	129	6.79	145350.33	1.126	4.73	0.393	8.36
P2	20	138	6.90	134922.34	0.977	4.48	0.309	7.42
P3	18	172	9.56	222304.03	1.29	8.24	0.764	9.18
P4	20	220	11	267892.21	1.22	7.64	0.781	10.49
Total	77	649						
Mean			**8.56**	**192617.23**	**1.15**	**6.27**	**0.561**	**8.86**

	Nb Mito analysed (only in contact with ER)	% Mito perimeter in contact with ER
C1	76	17.90
C2	48	16.61
C3	48	17.54
Mean		**17.35**
P1	20	13.68
P2	30	12.40
P3	30	16.58
P4	116	15.61
Mean		**14.57**

Table S3. Two-hybrid screening results.

HYBRIGENICS SERVICES

Results Summary

ULTImate Y2H SCREEN Mus musculus - Wfs1 vs Mouse Inner Ear_RP1

Screen Parameters

Nature	cDNA
Reference Bait Fragment	Mus musculus - Wfs1 (aa 1-311) ; hgx3311v1
Prey Library	Mouse Inner Ear_RP1
Vector(s)	pB29 (N-bait-LexA-C fusion)
Processed Clones	29 (pB29_B)
Analyzed Interactions	54.3 millions (pB29_B)
3AT Concentration	0.0 mM (pB29_B)

Global PBS®

Global PBS (for Interactions represented in the Screen)		Nb	%
A	Very high confidence in the interaction	1	16.7%
B	High confidence in the interaction	1	16.7%
C	Good confidence in the interaction	1	16.7%
D	Moderate confidence in the interaction This category is the most difficult to interpret because it mixes two classes of interactions : - False-positive interactions - Interactions hardly detectable by the Y2H technique (low representation of the mRNAin the library, prey folding, prey toxicity in yeast)	1	16.7%
E	Interactions involving highly connected (or relatively highly connected) prey domains, warning of non-specific interaction. The total number of screens performed on each organism is taken into account to set this connectivity threshold: 20 interactions to different bait proteins in our entire database for Human, 10 for Mouse, Drosophila and Arabidopsis and 6 for all other organisms. They can be classified in different categories: - Prey proteins that are known to be highly connected due to their biological function - Proteins with a prey interacting domain that contains a known protein interaction motif or a biochemically promiscuous motif	1	16.7%
F	Experimentally proven technical artifacts	1	16.7%
Non Appliable			
N/A	The PBS is a score that is automatically computed through algorithms and cannot be attributed for the following reasons : - All the fragments of the same reference CDS are antisense - The 5p sequence is missing - All the fragments of the same reference CDS are either all OOF1 or all OOF2 - All the fragments of the same reference CDS lie in the 5' or 3' UTR		

Prey Fragment Analysis

Symbols	Means
*	The fragment contains the full length CDS
	Fragment is fully in 5' UTR
	Fragment is fully in 3' UTR
×	Fragment contains at least one In Frame STOP codon
[NR]	Fragment was found to be non relevant (poor quality, high N density)
IF OOF1 OOF2	With regard to the theoretical frame of each corresponding CDS (GeneBank),fragments are cloned in frame (IF) if they are in the same frame as Gal4AD.In general, polypeptides synthesized from OOF fragments are not considered of biological interest, unless found together with another frame. However, some of the proteins expressed from an OOF fragment can be translated in the correct frame,due to the existence of natural frameshift events during translation in yeast
??	Unidentified frame when : - The clone sequence is antisense - The 5p sequence is missing
N	Antisense
Start...Stop	Position of the 5p and 3p prey fragment ends, relative to the position of the ATG start codon (A=0)

Clone Name	Type Seq	Gene Name (Best Match)	Start..Stop (nt)	Frame	Sense	%Id 5p	%Id 3p	PBS
pB29_B-28	5p	Mus musculus - A	-25	IF		93.4		B
pB29_B-19	3p	Mus musculus - A	..536	??			86.6	B
pB29_B-12	3p	Mus musculus - A	..594	??			85.7	B
pB29_B-20	5p/3p	Mus musculus - A	3..594 ×	IF		99.3	96.9	B
pB29_B-21	5p/3p	Mus musculus - A	3..594 ×	IF		96.8	88.7	B
pB29_B-5	3p	Mus musculus - B	..1076	??			94.8	A
pB29_B-33	3p	Mus musculus - B	..1282	??			82.3	A
pB29_B-8	3p	Mus musculus - B	..1240	??			81.4	A
pB29_B-16	3p	Mus musculus - B	..1276	??			73.8	A
pB29_B-1	5p/3p	Mus musculus - B	234..1075	IF		93.5	84.9	A
pB29_B-2	5p/3p	Mus musculus - B	501..1076	IF		93.4	87.1	A

pB29_B-27	5p	Mus musculus - B	516	IF		91.4		A
pB29_B-6	5p/3p	Mus musculus - B	528..1125	IF		95.8	87.1	A
pB29_B-24	5p	Mus musculus - B	528	IF		88.8		A
pB29_B-31	5p/3p	Mus musculus - B	528..1125	IF		88.5	91.2	A
pB29_B-15	5p	Mus musculus - B	534	IF		87.4		A
pB29_B-18	5p	Mus musculus - B	534	IF		90.7		A
pB29_B-26	5p	Mus musculus - B	534	IF		96.3		A
pB29_B-32	5p/3p	Mus musculus - B	534..1107	IF		89.8	97.2	A
pB29_B-29	5p/3p	Mus musculus - B	534..1107	IF		82.3	82.4	A
pB29_B-23	5p/3p	Mus musculus - C	-13..687	IF		97.1	83.6	F
pB29_B-30	3p	Mus musculus - C	..746	??			88.5	F
pB29_B-4	5p	Mus musculus - C	108	IF		95.3		F
pB29_B-10	5p	Mus musculus - Ncs1	21	IF		96.2		D
pB29_B-7	3p	Mus musculus - D	..3190	??			84.7	E
pB29_B-22	5p	Mus musculus - D	1530	IF		91.1		E
pB29_B-14	5p	Mus musculus - D	1578	IF		97.2		E
pB29_B-9	5p	Mus musculus - E	438	IF		90.1		C
pB29_B-17	5p/3p	Mus musculus - E	438..1209	IF		92.1	95.7	C