A homozygous missense mutation in ERAL1, encoding a mitochondrial rRNA chaperone, causes Perrault syndrome

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Abstract

Perrault syndrome (PS) is a rare recessive disorder characterized by ovarian dysgenesis and sensorineural deafness. It is clinically and genetically heterogeneous, and previously mutations have been described in different genes, mostly related to mitochondrial proteostasis. We diagnosed three unrelated females with PS and set out to identify the underlying genetic cause using exome sequencing. We excluded mutations in the known PS genes, but identified a single homozygous mutation in the ERAL1 gene (c.707A>T; p.Asn236Ile). Since ERAL1 protein binds to the mitochondrial 12S rRNA and is involved in the assembly of the small mitochondrial ribosomal subunit, the identified variant represented a likely candidate. In silico analysis of a 3D model for ERAL1 suggested that the mutated residue hinders protein-substrate interactions, potentially affecting its function. On a molecular basis, PS skin fibroblasts had reduced ERAL1 protein levels. Complexome profiling of the cells showed an overall decrease in the levels of assembled small ribosomal subunit, indicating that the ERAL1 variant affects mitochondrial ribosome assembly. Moreover, levels of the 12S rRNA were reduced in the patients, and were rescued by lentiviral expression of wild type ERAL1. At the physiological level, mitochondrial respiration was markedly decreased in PS fibroblasts, confirming disturbed mitochondrial function. Finally, knockdown of the C. elegans ERAL1 homologue E02H1.2 almost completely blocked egg production in worms, mimicking the compromised fertility in PS-affected women. Our cross-species data in patient cells and worms support the hypothesis that mutations in ERAL1 can cause PS and are associated with changes in mitochondrial metabolism.
Introduction

Perrault syndrome (PS) (MIM 233400) is a rare disorder that is inherited in an autosomal recessive manner. It is clinically characterized by sensorineural hearing loss in both male and female patients, while females also present with ovarian dysgenesis, which results in amenorrhea and infertility (1). Some patients also present with neurological manifestations, including ataxia, mild mental retardation and peripheral neuropathy (2–4). Because of the clinical heterogeneity, the disorder has been classified into type I, which is static and without neurological manifestations, and type II that includes progressive neurological symptoms (5).

The clinical heterogeneity of PS may partly be due to its genetic heterogeneity; to date, mutations in five different genes have been identified as disease-causing in different cases of PS. The first mutations were reported in HSD17B4, a gene encoding a peroxisomal enzyme involved in fatty acid β-oxidation and steroid metabolism (5). Later on, mutations in two genes encoding the mitochondrial aminoacyl-tRNA synthetases HARS2 and LARS2 (6,7) were identified, which are components of the mitochondrial translation machinery. Finally, two more genes encoding mitochondrial proteins—the peptidase CLPP involved in mitochondrial protein homeostasis (8,9) and the helicase Twinkle that is required for mitochondrial DNA (mtDNA) maintenance (10)—were found mutated in some PS patients. Altogether, four out of five PS-causing genes are involved in mitochondrial gene expression and proteostasis, suggesting that mitochondria play a critical role in the development of the disease.

Here, we report a homozygous c.707A>T (p.Asn236Ile) missense mutation in the ERAL1 gene (NM_005702.2) identified by exome sequencing in two unrelated patients, and found a third PS patient with the same variant during the course of our study. The human ERAL1 protein (UniProtKB O75616) has been described as an rRNA chaperone indispensable for the assembly of the 28S subunit of the mitochondrial ribosome through binding to the 2542 rRNA chaperone 1.

In order to identify the underlying genetic cause for the disease, we performed whole exome sequencing (WES), which excluded the presence of mutations in the known PS genes HSD17B4 (5), HARS2 (6), LARS2 (7), CLPP (8) and C10orf2 (10). Because both patients originate from a small village, a known genetic isolate, recessive inheritance and shared genetic cause were suspected. Subsequent filtering of all variants found in WES for homozygous variants that were shared in both patients, and with a minor allele frequency of <1% in public or in house databases yielded only one variant: a homozygous nucleotide substitution at position c.707A>T (p.Asn236Ile) in ERAL1 (NM_005702.2), a gene encoding the Era-like 12S mitochondrial rRNA chaperone 1.

The substituted residue is highly conserved in vertebrates as well as in fruit flies and in C. elegans (Fig. 1A). Here, we report a homozygous missense mutation in the ERAL1 gene (NM_005702.2) identified by exome sequencing in two unrelated patients, and found a third PS patient with the same variant during the course of our study. The human ERAL1 protein (UniProtKB O75616) has been described as an rRNA chaperone indispensable for the assembly of the small 28S subunit of the mitochondrial ribosome (11,12). In line with this, patient skin fibroblasts and C. elegans with knock-down of the ERAL1 homologue display mitochondrial dysfunction, strongly suggesting that the identified mutation in ERAL1 is the cause of PS in our patients.

In order to identify the underlying genetic cause for the disease, we performed whole exome sequencing (WES), which excluded the presence of mutations in the known PS genes HSD17B4 (5), HARS2 (6), LARS2 (7), CLPP (8) and C10orf2 (10). Because both patients originate from a small village, a known genetic isolate, recessive inheritance and shared genetic cause were suspected. Subsequent filtering of all variants found in WES for homozygous variants that were shared in both patients, and with a minor allele frequency of <1% in public or in house databases yielded only one variant: a homozygous nucleotide substitution at position c.707A>T (p.Asn236Ile) in ERAL1 (NM_005702.2), a gene encoding the Era-like 12S mitochondrial rRNA chaperone 1.

Results

Identification of a homozygous missense mutation in the ERAL1 gene in three PS patients

Two women of Dutch ancestry, unrelated but both from the same village, presented at our clinic with symptoms of PS. In PS patient 1 (aged 66 years) hearing loss was diagnosed when she was 20 years old, but probably started much earlier and appeared to be progressive. She had normal menarche at 11 years of age, with irregular menses until the age of 27, when menopause occurred. One sister also had sensorineural hearing loss and premature ovarian failure, but did not participate in our study. PS patient 2 (aged 38 years) was diagnosed at 4 years of age with sensorineural hearing loss, which was more severe in the high frequencies and slowly progressive. At the age of 18 years she presented with primary amenorrhea and underdeveloped secondary sexual characteristics. Abdominal ultrasound revealed streak ovaries and a small uterus. An ovary biopsy showed fibrous tissue without primordial follicles. Her father had sensorineural hearing loss since childhood, but no fertility problems. Her mother and two sisters were healthy.

Because previously described mutations causing PS are mostly in genes related to mitochondrial homeostasis—two of which are directly involved in mitochondrial translation (6,7), one in mtDNA maintenance (10) and one in mitochondrial proteostasis (8)—we hypothesized that the identified ERAL1 variant...
is likely to cause PS in our patients. To test this hypothesis, we set out to investigate the effects of the ERAL1 variant at a cellular as well as at an organismal level.

**ERAL1 protein levels and assembly of the 28S ribosomal subunit are compromised in PS patients**

To test whether the sequence variant identified in our patients affects ERAL1 protein levels, we performed a Western blot on lysates from cultured skin fibroblasts of PS patients and control subjects. We observed that in both PS patients, ERAL1 protein levels were decreased when compared to fibroblasts from healthy controls (Fig. 1E and F).

Since ERAL1 is involved in the assembly of the small 28S mitochondrial ribosomal subunit (11,12), we next investigated whether the PS patient cells showed an impaired assembly of the 28S subunit. We assessed the abundance of assembled mitochondrial ribosomal subunits using complexome profiling (14). With this technique migration profiles and relative abundance of protein complexes are identified using a combination of blue native electrophoresis and shotgun proteomics. Although the migration profiles of the proteins composing the small (28S) and large (39S) mitochondrial ribosomal subunits were similar between PS patients and controls (Fig. 2A and B), we observed a remarkable decrease (30–40%) in the overall abundance of proteins composing the small 28S subunit in the PS patients compared to healthy controls (Fig. 2A and C). In contrast, the abundance of proteins composing the large 39S subunit was comparable between cells from PS patients and controls (Fig. 2B and D).

We also analyzed the steady state protein expression of MRPS22, a protein of the small 28S subunit, and found it to be decreased in PS patient skin fibroblasts compared to controls (Fig. 2E and F). On the contrary, levels of the large 39S subunit protein MRPL54 were similar between patients and controls (Fig. 2E and F).

These observations led us to conclude that the overall abundance of assembled small mitochondrial ribosomal subunits is lower in cells from the PS patients compared to those from healthy controls, suggesting that the identified ERAL1 variant perturbs proper assembly of the small 28S mitochondrial ribosomal subunit.

**12S rRNA levels are low in PS patients, and rescued after ERAL1 lentiviral expression**

Because ERAL1 acts as a chaperone to the mitochondrial 12S RNA of the small ribosomal subunit by protecting it from degradation (12), we hypothesized that levels of 12S rRNA are reduced in the patients, while the 16S rRNA of the large ribosomal subunit remains unaffected. We therefore employed qPCR and measured the 12S/16S rRNA ratio in patient and control fibroblasts. In support of our hypothesis, we found that this ratio was significantly decreased in the two patients compared to healthy controls (Fig. 3A).

Next, we asked whether the ectopic expression of wild type ERAL1 in the patient cells would be sufficient to reconstitute 12S rRNA levels. To address this question, we infected patient fibroblasts with lentiviral particles overexpressing either ERAL1 or GFP as a control. We found that overexpression of ERAL1 in both patients (Fig. 3B) rescued the 12S/16S rRNA ratio (Fig. 3C). These data demonstrate that the identified mutation leads to reduced 12S rRNA levels, a condition that is reversible after overexpression of wild type ERAL1.

**Mitochondrial function is impaired in PS patients**

Disturbed assembly of the mitochondrial ribosome may lead to inefficient translation of mitochondrial DNA (mtDNA)-encoded proteins, which form core subunits of the oxidative phosphorylation (OXPHOS) complexes. To test whether mitochondrial translation is disturbed, we quantified the ratio between the mtDNA-encoded cytochrome c oxidase subunit 1 (MT-CO1) and the nuclear DNA (nDNA)-encoded succinate dehydrogenase complex, subunit A (SDHA) (15) in the PS cells. Indeed, the abundance of MT-CO1 was found lower, while SDHA was unaffected (Fig. 4A and B). We next measured mitochondrial activity by means of oxygen consumption rate (OCR) in the cells using Seahorse respirometry. Basal respiration was decreased in the PS cells when compared to controls (Fig. 4C). After injection of the uncoupler FCCP, maximal respiratory capacity of the PS fibroblasts was also impaired (Fig. 4C). These findings confirm that mitochondrial function is compromised in the PS cells.

**Knockdown of the ERAL1 homologue in C. elegans compromises fertility**

In order to test the phenotypic effects of low ERAL1 levels at an organismal level, we used the nematode C. elegans as a model organism and specifically knocked down the expression of the worm ERAL1 homologue E02H1.2. We used the RNAi-sensitive nematode strain rrf-3(pk1426) (16). Worms exposed to E02H1.2 RNAi had approx. 50% knockdown (Fig. 5A), developed normally and had a normal lifespan compared to control HT115 worms (data not shown). Strikingly, we observed that the animals undergoing E02H1.2 RNAi were not carrying eggs throughout adulthood (Fig. 5B). We quantified the number of eggs laid during the first four days of adulthood, which is the fertile period of C. elegans, and confirmed that the E02H1.2 RNAi fed animals hardly laid any eggs (Fig. 5C).

Because the PS skin fibroblasts presented with decreased mitochondrial respiration, we also measured OCR in the worms with E02H1.2 knockdown. Those worms demonstrated significantly decreased OCR (Fig. 5D). Collectively, these results demonstrate that low ERAL1 expression impairs mitochondrial function and compromises fecundity in C. elegans, mimicking the defects we observe in our PS patients.

**Discussion**

Three women from three different families presented with signs of PS, including deafness and ovarian dysgenesis. We employed exome sequencing and identified a homozygous missense mutation in the ERAL1 gene, c.707A>T. The mutation leads to a damaging p.Asn236Ile substitution that is likely to interfere with the GTP binding capacity of the ERAL1 protein. Specifically, the observation that the assembly of the small mitochondrial ribosomal subunit—for which ERAL1 is known to play a pivotal role (11,12)—is compromised in the PS cells, implies that the identified mutation affects the proper function of ERAL1 protein. Importantly, the impaired 12S rRNA levels in PS fibroblasts and their rescue upon expression of wild type ERAL1 underlines the causal link between the mutation and mitochondrial function. Additionally, the decreased respiration that was noted in the PS fibroblasts demonstrates disturbed mitochondrial function.
mitochondrial function, confirming that the identified mutation affects mitochondria and is pathogenic. Finally, we performed RNAi experiments in the nematode *C. elegans* to demonstrate the role of ERAL1 in fertility. Knockdown of the worm ERAL1 homologue E02H1.2 indeed impaired egg production presenting a crucial role of this protein in the nematode’s fecundity. Our cross-species findings on both patient fibroblasts and the model organism *C. elegans* identified the ERAL1 variant as the cause for the clinical symptoms in our three PS patients.

The identification of ERAL1 as a causative gene for PS extends the mutational spectrum for this disease. So far, mutations in five different genes have been described to cause ovarian dysgenesis and sensorineural deafness,

sensorineural deafness

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**Figure 1.** Three individuals diagnosed with PS, carry the same homozygous missense mutation in the ERAL1 gene. (A) The mutated residue is highly conserved as evidenced by sequence alignment using the Alamut software. (B) Pedigrees of the two initial PS patients (indicated by P1 and P2, as annotated in the remainder of the paper) in whom WES was performed and skin fibroblasts were analyzed. Individuals that were sequenced for the mutation are indicated with asterisks, and the results are annotated (wt: wildtype ERAL1, mut: mutated ERAL1). (C) Pedigree (left panel) and sequencing results (right panel) of a third PS patient that was found homozygous for the same mutation. (D) Left panel: Structural Alignment of modelled human ERAL1 (gray) and crystallized *Aquifex aeolicus* ERA (dark blue), showing the high similarity between the two structures. Ligands of ERA are shown in yellow. Middle panel: Active center of modelled wild type ERAL1 in complex with a non-hydrolyzable GTP analog (GNP, phosphoaminophosphonic acid-guanylate ester) (yellow sticks) and magnesium (yellow sphere). Hydrogen bonds between asparagine 236 (magenta sticks), GNP and alanines 124 and 310 (cyan) are shown as red dashes. Right panel: Active center of modelled N236I mutated ERAL1 in complex with GNP (yellow sticks) and magnesium (yellow sphere), showing that no interactions can be made between the mutated amino acid, the ligand and the two flanking alanines (cyan). (E) Western blot of skin fibroblasts from PS patients and controls; both PS patients present with decreased ERAL1 protein levels. P1: PS patient 1, P2: PS patient 2. (F) Bar graph depicting the levels of ERAL1 in patient and control fibroblasts normalized to tubulin, as quantified from the blot in panel E.
Figure 2. Cells from PS patients show defected assembly of the small 28S mitochondrial ribosomal subunit. (A-B) Heat map representation of migration profiles in blue native gels of proteins of the small 28S (A) and large 39S (B) mitochondrial ribosomal subunits isolated from PS patient and control skin fibroblasts. (C-D) Graphs depicting the average normalized relative abundance of proteins of the 28S (C) and 39S (D) mitochondrial ribosomal subunits spanning the blue native gel. Protein abundance was determined by label-free quantitation using the composite iBAQ intensity values determined by MaxQuant (31) and normalized as in (30) considering multiple migration profiles of individual proteins, that is taking into account iBAQ values from all 180 gel slices (60 slices per sample). Both patients show decreased levels of assembled small mitochondrial ribosomal subunit (A, C), while the levels of assembled large subunit remain unaffected (B, D). P1: patient 1, P2: patient 2. (E) Western blot of skin fibroblasts from PS patients and controls; both PS patients present with decreased MRPS22 protein levels, while MRPL54 levels are unaffected. (F) Bar graphs depicting the levels of MRPS22 (left) and MRPL54 (right) in patient and control fibroblasts normalized to tubulin, as quantified from the blot in panel E. Data are represented as the mean ± SEM. *p < 0.05 as calculated by a 2-tailed student’s t-test.
Perrault syndrome. These include the HARS2 (6) and LARS2 (7) genes, both encoding mitochondrial tRNA synthetases, the CLPP (8) that codes for a protease of the mitochondrial matrix and the C10orf2 (10) encoding the mitochondrial helicase Twinkle. These findings imply that mitochondria are involved in the development of the disease. Interestingly, recent work on CLPP deficient mice (17) elegantly links ERAL1 mechanistically to CLPP by demonstrating that removal of ERAL1 from the assembled 28S ribosomal subunit is essential for the final maturation of the whole 55S mitochondrial ribosome, and that this removal requires CLPP. Our identification of ERAL1 as a Perrault syndrome gene further ties CLPP and ERAL1 together, as mutations in either of them lead to similar pathology in humans.

The involvement of mitochondrial dysfunction in development of deafness is already well established (18-20). On the other hand, the role of mitochondria in infertility is less known and is only recently starting to emerge. Studies in mice have shown that perturbations of mitochondrial function can affect oocyte maturation (21,22). In humans, age-related decline in oocyte quality and quantity has been associated with mitochondrial dysfunction and impaired OXPHOS, while dietary supplementation with the OXPHOS enhancer CoQ10 improved ovulation in aged women (23). In C. elegans, knockdown of the mitochondrial tRNA synthetase lars-1 led to smaller gonads and to loss of fertility, which was partly due to germ cell apoptosis (6). Similarly, the lars-2 mutant worm had underdeveloped gonads and was completely sterile (7). Also, sub-lethal disruptions in OXPHOS subunits cause sterility in worms (24-26). Our findings of compromised fertility and mitochondrial respiration after knockdown of the worm ERAL1 homologue serve as additional proof for the importance of mitochondria in fertility. The exact mechanisms through which mitochondria regulate fertility, however, remain to be resolved.

Collectively, our results indicate that mutations in another mitochondrial gene, ERAL1, can cause sensorineural deafness and ovarian dysgenesis of PS, strengthening the notion that this syndrome is caused by dysfunctional mitochondria. Detailed functional mitochondrial assays with cells from other unresolved cases of PS may shed more light on the molecular mechanisms underlying the disease and reveal other PS-causing genes.

Materials and Methods
All three PS patients visited the outpatient clinic of the AMC and signed informed written consent for this study. Information regarding the medical history was obtained by interviewing the patients and from their medical files.

Whole exome sequencing
Whole exome sequencing (WES) of PS patients was conducted using the SeqCap EZ Human Exome Library v3.0 (Roche NimbleGen) and a 5500 SOLiDTM instrument (Life Technologies). Samples were prepared using standard SOLiD 75x35 paired end
sequencing protocols. Alignment of sequence reads to the human reference genome (hg19) was done using Lifescope 2.5.1, and variants were called using the GATK2.5 software package. Mean target coverage was 86x for PS patient 1 and 66x for PS patient 2. Coverage of targeted regions at 10x read depth (after removal of duplicate reads) was 87% for patient 1 and 84% for patient 2.

Prioritization of variants identified with WES was done using the Cartagenia BENCHlab NGS software (Cartagenia NV). Public databases used for determining the frequency of the identified variants in the general population were: 1000 genomes (1000 Genomes Phase 3 release v5.20130502), dbSNP (dbSNP build 141 GRCh37.p13), the ESP6500 dataset (http://evs.gs.washington.edu/EVS/; date last accessed April 27, 2017), and the GoNL database (498 Dutch individuals, http://www.nlgenome.nl/; date last accessed April 27, 2017). Variants were further characterized using Alamut version 2.3 (Interactive Biosoftware, Rouen, France).

**Sanger sequencing of ERAL1**

Confirmation of the mutation in the PS patients and genotyping of family members of PS patient 2 was done by Sanger sequencing. Primers were designed to amplify exon 6 of ERAL1 using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/; date last accessed April 27, 2017). Amplification was performed with M13-tagged primers using HOT FIREPol™ DNA polymerase (Solys Biodyne) and a touchdown PCR program. PCR fragments were sequenced using the Bigdye kit v1.1 (Applied Biosystems). Reactions were run on an ABI3700 or ABI3730XL genetic analyzer (Applied Biosystems) and sequences were analyzed using Sequence Pilot (JSI Medical systems) or CodonCode aligner (CodonCode Corporation).

**Genotyping TaqMan**

Genotyping of 350 individuals from the same village as the PS patients was done by a TaqMan assay on a Roche LC480 lightcycler. For the SNPs, Sanger sequencing confirmed homozygous reference, heterozygous and homozygous mutant samples were used as standards for SNP calling.

**ERAL1 3D model**

The crystallized structure of Aquifex aedilis ERA (PDB code: 3IEV) was selected as a template to build a model for human ERAL1 using SWISS-MODEL server (http://swissmodel.expasy.org).
org/; date last accessed April 26, 2017) (27), as it is the most similar crystallized structure available in the database (32% identity, 0.35 similarity). UCSF Chimera package (Version 1.10.2, Computer Graphics Laboratory, University of California, San Francisco) was used to align and compare the two 3D structures in order to predict the possible implications of the mutated amino acid on the function of ERAL1 protein.

**Cell culture**

Primary skin fibroblasts from PS patients and healthy controls were cultured in DMEM or Ham’s F-10 with L-glutamine (Bio-Whittaker) growth media, supplemented with 10% fetal bovine serum (Bio-Whittaker), 25 mM HEPES buffer (BioWhittaker), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 250 ng/ml Fungizone (Life Technologies) in a humidified atmosphere with 5% CO₂ at 37 °C.

**Immunoblot analysis**

For protein extraction, cultured skin fibroblasts were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% Sodium deoxycholate, 1% Triton X-100) with the addition of Complete mini protease inhibitor cocktail (Roche). Samples were sonicated to ensure complete lysis, and briefly centrifuged to discard debris. Protein concentrations were measured using the BCA protein assay kit (Pierce). For immunoblot analysis, lysates were diluted in NuPAGE LDS Sample Buffer and Sample Reducing Agent (Life Technologies) and heated to 70 °C. Protein extracts were separated on pre-cast NuPAGE 4–12% Bis-Tris gels (Life Technologies), and transferred to a nitrocellulose membrane. Membranes were blocked with 3% (w/v) milk powder in PBS containing 0.1% (v/v) Tween-20 (PBS-T), and incubated overnight at 4 °C with the primary antibody. The following day membranes were washed with PBS-T and incubated for 1 h at room temperature with the secondary antibody. Odyssey imaging system (LI-COR) was used for imaging of membranes incubated with IRDye secondary antibodies (LI-COR). Membranes incubated with HRP-linked secondary antibodies were detected using the ECL prime western blotting detection reagent (Amersham) and imaged with the ImageQuant (Qiagen) software.

**Quantitative PCR (qPCR)**

For qPCR, RNA was isolated using the TRizol reagent (Invitrogen) and cDNA synthesis was performed with 1μg RNA using the QuantiTect Reverse Transcription Kit (Qiagen). qPCR was performed on a Roche Lightcycler 480 using Roche SYBR-green mastermix. For measurement of the mitochondrial ribosomal RNAs, cDNA samples were heated for 10 min at 95 °C, followed by 36 cycles of 15 s at 95 °C (denaturation), 10 s at 56 °C (annealing) and 15 s at 72 °C (amplification). To ensure that no genomic rRNA is amplified, samples without reverse transcriptase were also included in the qPCR run. For measurement of E02H1.2 knockdown efficiency in C. elegans, cDNA samples were heated for 6 min at 95 °C, followed by 40 cycles of 10 s at 95 °C (denaturation), 5 s at 65 °C (annealing) and 15 s at 72 °C (amplification). E02H1.2 transcript levels were normalized to the geometrical mean of three different housekeeping genes (CDC42, ACT, F35G12.2).

**List of primers**

| Gene     | Forward | Reverse |
|----------|---------|---------|
| MT-RNR1  |         |         |
| MT-RNR2  |         |         |
| E02H1.2  |         |         |
| cdc42    |         |         |
| F35G12.2 |         |         |
| Act-1    |         |         |

**Cloning and viral transfection**

For the functional complementation assay, cells were infected with viral particles for stable overexpression of either ERAL1 or GFP (pLenti/ERAL1, pLenti/GFP). The pLenti/ERAL1 vector was constructed using the Gateway technology (Invitrogen). Specifically, the ERAL1 ORF PCR product containing attB sites was cloned to the entry plasmid pDONR221 (Invitrogen) and further cloned to the destination vector pLENTI 6.3/TO/V5-DEST (Invitrogen) using the LR Clonase enzyme mix (Invitrogen) and cDNA synthesis was performed with 1 μg RNA using the QuantiTect Reverse Transcription Kit (Qiagen). For virus production, HEK293 cells at 50% confluency were transfected with viral supernatant was collected and filtered 48 and 72 h post-transfection. Patient fibroblasts at ~70% confluency were infected with the viral supernatant, grown under blasticidin selection (10 μg/ml) for 2 weeks, and further expanded in blasticidin-free medium for four more passages. Cells were regularly checked for GFP expression. Overexpression of ERAL1 was confirmed by Western blot analysis.

**Complexome profiling**

Mitochondrial pellets (200 μg protein) were solubilized with 6 mg digitonin/mg protein and separated by 4–16% gradient Blue native PAGE (BN-PAGE) (29). Complexome profiling was
done according to Heide et al. (14). Protein identification and data analysis was done essentially as previously described (30) using MaxQuant (version 1.5.0.25 (31)).

Protein abundancies were determined by label-free quantitation using the composite iBAQ values determined by MaxQuant (31) and normalized considering multiple migration profiles of individual proteins, that is taking into account iBAQ values from all 180 gel slices (60 slices per sample). The profiles were hierarchically clustered by distance measures based on Pearson correlation coefficient (uncentered) and the average linkage method and further analyzed by manual correlation profiling. The clustering and the visualization and analysis of the heat maps were done with the NOVA software v0.5 (32).

**Respiration assays**

Oxygen consumption was measured using the Seahorse XF96 analyzer (Seahorse Bioscience). Primary skin fibroblasts were plated in 96-well Seahorse plates at a density of 10,000 cells per well and incubated overnight under normal cell culture conditions. The following day, medium was replaced by DMEM (Sigma, D5030) containing 25 mM glucose (Sigma), 1 mM sodium pyruvate (Lonza), and 2 mM L-Glutamine (Life technologies). Basal respiration was measured three times, followed by measurements after addition of 1.5 μM oligomycin, 1 μM FCCP and 2.5 μM antimycin A and 1.25 μM rotenone. For respiration assays in worms, animals were transferred in 96-well Seahorse plates (20 worms per well) and basal oxygen consumption was measured six times.

**C. elegans** culturing and RNAi experiments

The C. elegans RNAi-sensitive strain nrf-3(pk1426) was obtained from The Caenorhabditis Genetics Center (CGC, University of Minnesota). Worms were cultured and maintained as described previously (33) in 20 °C incubators and fed with E. coli OP50 strain, also obtained from the CGC. RNAi experiments were carried out as previously described (34). The RNAi clone targeting the ERAL1 worm orthologue (E02H1.2) is an E. coli HT115 strain and was a kind gift of Dr. Yelena Budovskaya (SILS, Science Park, University of Amsterdam).

For the fertility assays, hermaphrodite worms were treated with E02H1.2 RNAi from L4 stage of development, and allowed to lay eggs. Their progeny (F1) was continuously exposed to the RNAi treatment, and observed throughout development. The number of F2 progeny from individual F1 worms (n = 30) was counted daily for the 4 first days of adulthood. Images of F1 worms before and after reaching adulthood were taken with a Leica DFC320 camera, using an Axio Observer.A1 (Zeiss) microscope and a 10x magnification objective. For imaging, worms were placed on top of a 2% agarose pad on slides, and anesthetized using 1 mM Levamisole in M9 buffer. For the respiration assays, F1 worms at day 1 of adulthood were used.

**Acknowledgements**

The authors thank members of the Houtkooper team as well as Prof. U. Brandt for support and helpful suggestions. I.A.C. is supported by a PhD Scholarship from the Academic Medical Center of Amsterdam. S.G.C. is a postdoctoral fellow in the laboratory of Prof. U. Brandt in Radboud University Medical Center. Some C. elegans strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

**Conflict of Interest statement.** None declared.

**Funding**

Academic Medical Center of Amsterdam, VIDI grant from the Netherlands Organisation for Health Research and Development (no. 9171305). Ecole Polytechnique Fédérale de Lausanne, and NIH Office of Research Infrastructure Programs (P40 OD010440). Funding to pay the Open Access publication charges for this article was provided by the Netherlands Organisation for Health Research and Development (VIDI grant no. 9171305; to RHH).

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