

## Online supplemental materials

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## Supplemental methods

### *Yeast model*

The yeast strains used in this work were the haploid strain BY4741 *etr1Δ* (*Mata*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, *etr1::KanMX4*) from Euroscarf collection (<http://www.euroscarf.de/index.php?name=Description>) and W303-1B (*Mata*, *ade2-1*, *leu2-3*, *ura3-1*, *trp1-1*, *his3-11*, *can100*) (Thomas & Rothstein, 1989). All experiments were performed in YP medium (0.5% yeast extract, ForMedium, UK, and 1% peptone, ForMedium). YP medium was supplemented with different carbon sources (Carlo Erba Reagents) as indicated in the text. The transformation was performed in SD medium (0.69 % yeast nitrogen base without amino acids, ForMedium) supplemented with amino acids necessary to complement the auxotrophies and with 2% glucose. Solidification of the medium was obtained by adding 20 g/L agar (ForMedium). Oligos used in this study are reported in online supplemental table 1. (±)-α-Lipoic acid (Merck), hereafter lipoic acid or LA, was dissolved in 96% ethanol at a concentration of 20 mg/ml. Stabilized 30% (w/w) H<sub>2</sub>O<sub>2</sub> (Merck) was added to the medium when necessary.

*ETR1* deletion in strain W303-1B was performed using the one-step gene disruption through amplification of the *etr1::KanMX4* cassette from the genomic DNA extracted from the strain BY4741 *etr1Δ* (Wach et al., 1994). Transformation and selection on YP medium supplemented with 2% glucose and 200mg /l G-418 sulfate (ForMedium, UK) were performed to obtain W303-1B *etr1Δ*, according to the “LiAc/SS carrier DNA/PEG high-efficiency method” (Gietz, 2014).

*ETR1* was PCR-amplified and cloned under its endogenous promoter into the *Bam*HI-*Pst*I-digested centromeric vector pFL38 (Bonneaud et al., 1991). Human *MECR* (*MECR*<sup>wt</sup>) cDNA (GenBank ID: BC001419.2), purchased from Horizon Discovery was PCR-amplified and cloned in the *Not*I-digested episomal vector YepLac112TEToff (Cappuccio et al., 2021), under the strong and doxycycline-repressible TEToff promoter. Since complementation occurred and no toxicity was observed even without adding doxycycline, antibiotics which repressed partially the expression from TEToff promoter were not added to the medium for the following assay. The mutant *MECR* R258W mutant allele (*MECR*<sup>R258W</sup>) was constructed using the QuikChange® PCR (Agilent) with KOD Hot Start DNA Polymerase (Merck). All plasmids were introduced through transformation in the W303-1B *etr1Δ* strain using the “LiAc/SS carrier DNA/PEG quick method” (Gietz, 2014).

Growth spot assays were performed on YP medium, supplemented with different carbon sources, by plating 5 μL of yeast cells suspension diluted at 1, 10<sup>-1</sup> and 10<sup>-2</sup> OD<sub>600</sub>/mL. Cells were grown at 28°C for 2 to 4 days. Oxidative growth was measured by pre-growing the cell in liquid YP medium supplemented with 2% glucose (YPD) until the early stationary phase and inoculating the cells in liquid YP medium supplemented with 2% ethanol, with or without LA. Cells were grown under shaking and the cell concentration was spectrophotometrically measured after 48, 72 and 96 hours

at 600 nm. Oxygen consumption rate (OCR) was measured at 30°C using a Clark-type oxygen electrode (Oxygraph System Hansatech Instruments) in a stirred chamber, containing 1 ml of air-saturated respiration buffer (0.1 M phthalate–KOH, pH 5.0) and 0.5% glucose. The reaction started by adding 50 µL of concentrated yeast cell suspensions, cultured under shaking in YP medium supplemented with 0.5% glucose for 18 hours at 28°C until the glucose was exhausted. OCR values were normalized to the dry weight of the cells. Proteins were extracted after growth in the same conditions used for the measurement of OCR with the trichloroacetic acid (TCA) method. (Del Dotto et al., 2018). Protein corresponding to 1.5 OD<sub>600</sub> of the original cells was loaded on 12% SDS-PAGE and electroblotted into nitrocellulose filters which were then incubated with rabbit anti-MECR (CusaBio, 1:2000), rabbit anti-Lipoic acid antibody (Abcam, 1:2000) and mouse anti-yeast Por1 (1:10000) antibodies. Blots were subsequently incubated with the specific secondary antibody, anti-Rabbit StarBright Blue 700 (Bio-Rad, 1:7500) and anti-Mouse StarBright Blue 520 (Bio-Rad, 1:10000). After this incubation, the fluorescent signals were detected with a Chemidoc MP imager (Bio-Rad). Signals were quantified with the Image Lab software and, for each sample, the ratio between the proteins of interest and Por1 was calculated.

To highlight the deficit associated with oxidative stress, a spot assay was set up, in which serial dilutions of the wild type, mutant, and deleted strains were plated on YPD medium with or without 2 mM H<sub>2</sub>O<sub>2</sub>. The plates were incubated at 28 °C. To quantify the oxidative stress induced by the exposure to H<sub>2</sub>O<sub>2</sub>, a viability assay was performed. The cells were pre-inoculated in YPD medium for 24h, then exposed to different concentrations of H<sub>2</sub>O<sub>2</sub> (0.5, 1 or 2 mM) for different times (2 and 3 hours). At the end of the treatment, the cells were washed to avoid the residual effects of H<sub>2</sub>O<sub>2</sub>. Different amounts of cells were plated on YPD medium (2000, 1000, 500 cells per Petri dish, at least 5000 cells for each strain/condition) and after incubation at 28°C for three days, the colony-forming units (CFU) were counted to calculate the survival rate. The same protocol was slightly modified to evaluate the protective effect of LA against oxidative stress. In this case, the cells were pre-cultured for 24h in YPD medium and subsequently inoculated in the same medium supplemented with LA at different concentrations (0.4, 4 or 40 µg/ml) and incubated at 28°C for 18h under shaking. Cells were washed twice with sterile water and, once collected, 10<sup>7</sup> cells/ml were inoculated in YPD medium supplemented with 1 mM H<sub>2</sub>O<sub>2</sub> for 2 hours. The viability was assessed as previously described.

#### *Fibroblast cell lines and culture conditions*

Fibroblasts were established from skin biopsies, after having obtained informed and written consent from patients and controls for the study (Ethical Committee code #211/2018/SPER/AUSLBO). Fibroblasts were generated from two patients (P1 and P2) carrying c.772C>T variant in the *MECR* gene, leading to missense change p.Arg258Trp in the protein (F-MECR<sup>R258W</sup>), and compared with age and sex-matched fibroblasts derived from two healthy donors (F-MECR<sup>WT</sup>). Control and mutant cell lines are cultured in Dulbecco Modified Eagle Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO), 2 mmol/l L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, at 37 °C in a 5% CO<sub>2</sub> humidified incubator.

### *Mitochondrial respiration evaluation*

Oxygen consumption rate (OCR) was measured using the XFe24 Extracellular Flux Analyzer (Seahorse, Agilent Technologies).  $2.5 \times 10^4$  cells/well were seeded in 5 wells for each line of the XFe24 plate with 250  $\mu$ L/well of glucose-free DMEM supplemented with 5 mM galactose, 5 mM Na-Pyruvate, 2 mM L-Glutamine and 5% FBS (galactose-containing medium) and incubated at 37 °C and 5% CO<sub>2</sub>. After 48 hours, galactose-containing medium was replaced with XFe medium supplemented with 5 mM galactose, 5 mM sodium pyruvate and 1 mM L-glutamine at pH 7.4. For temperature and pH equilibration, cells were incubated at 37 °C without CO<sub>2</sub> for 1 hour. After three OCR baseline measurements, 1  $\mu$ M oligomycin, 1  $\mu$ M carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 1  $\mu$ M rotenone, and 1  $\mu$ M antimycin A were sequentially added to each well. At the end of the assay, the medium was removed, and a sulforhodamine B (SRB) assay was performed to determine the protein content for normalization of OCR. Briefly, plates were incubated with 10% trichloroacetic acid (TCA) for 1 h at 4 °C to fix the cells. Five washes in water were carried out. Once the plates were dried, proteins were stained by incubation with 0.4% SRB for 30 min at RT. Then, SRB was solubilized with 10 mM Tris, and the absorbance at 560 nm was determined using an EnSpire® Multimode Plate Reader (Perkin-Elmer). Basal respiration, ATP-linked respiration, maximal respiration and CI-linked respiration parameters were evaluated as described in (Divakaruni et al., 2014).

### *Western blot and antibodies*

Total lysates were prepared using RIPA lysis buffer following a standard protocol. Proteins were separated on pre-cast NuPAGE 4–12% Bis–Tris Glycine gels (Life Technologies) and then transferred on nitrocellulose membranes, using the XcellSure Lock (Life Technologies) apparatus. After blocking with 5% milk, membranes were blotted with primary antibodies specific for MECR (Proteintech 51027-AP, 1:250), OXPHOS cocktail (Abcam AB110411, 1:500), GAPDH (Sigma-Aldrich G8795, 1:1000). Fluorescent secondary antibodies anti-rabbit or anti-mouse (Licor, catalog 926–32,210 and 926–68,071, 1:5000) were used for immunodetection using the Odyssey Fc instrument (Licor).

### *Respiratory supercomplexes assembly by BN-Page and CI-IGA*

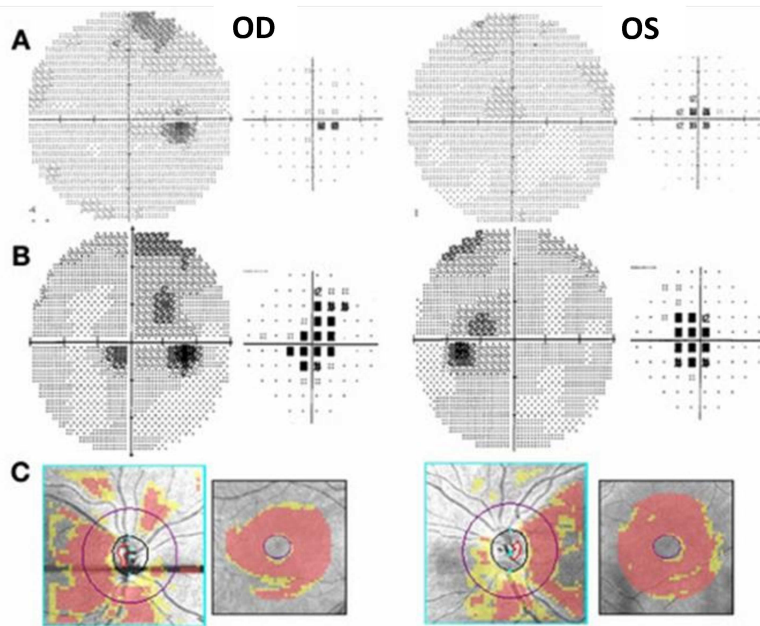
Supercomplexes assembly was assessed in mitochondria-enriched fractions (mitoplasts) obtained from cell pellets (approximately  $10^6$  cells) using digitonin (final concentration 50  $\mu$ g/mL). After centrifugation, the pellet was suspended (5 mg protein/mL) in 340 mM K-Acetate, 70 mM HEPES (pH 7.4), 25% glycerol, 2.3 mM PMSF, and 5% (w/v) digitonin and incubated on ice for 30 min. Samples were centrifuged for 5 min at 600g and the supernatant, with supercomplexes sample buffer (5% Serva G Blue in 750 mM aminocaproic acid), was loaded on pre-cast NativePAGE™ 3-12 % Bis-Tris Protein Gels (Life Technologies). Gels were analyzed for CI-In Gel Activity (CI-IGA) by the NADH/MTT reductase activity, incubating the gels with 5 mM Tris-HCl (pH 7.4), 0.15 mM NADH, and 2.5 mg/mL MTT at room temperature.

**Online supplemental table 1. List of oligonucleotides used in this work.**

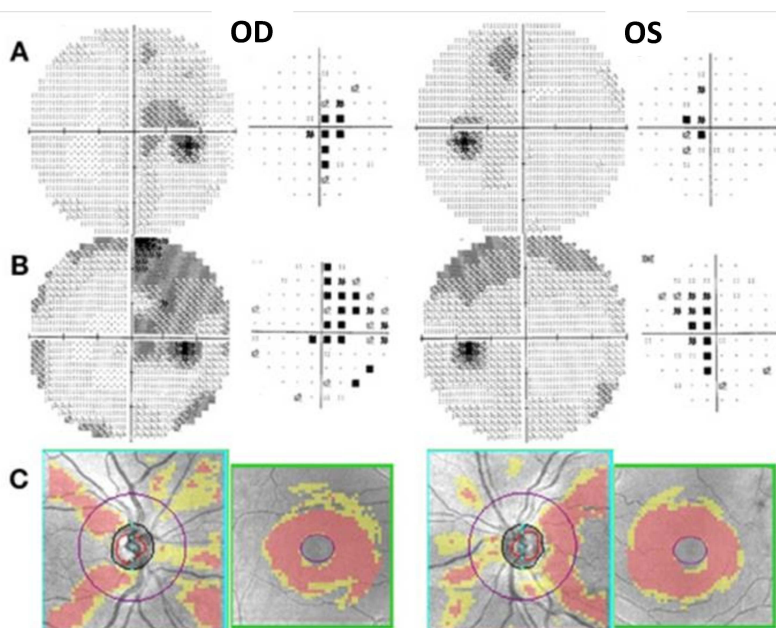
Oligonucleotide	Sequence	Use
ETR1DFw	CCTTAAAAGAGTGGTTCTCC	<i>ETR1</i> disruption
ETR1DRv	CTCCTACAAAGATAGCACTGG	<i>ETR1</i> disruption
ETR1CBamFw	GGCCGGGATCCGATAGATTAAGAGAGAAGGCTCG	<i>ETR1</i> cloning
ETR1CPstRv	GCGCACTGCAGCCATTCTCAGGAAAATTGC	<i>ETR1</i> cloning
MECRCNOTFW	CATGTAGCGGCCGCACACAATGTGGGTCTGCAGTACCCTG	<i>MECR</i> cloning
MECRCNOTRV	CATGAAGCGGCCGCGGATGATCACATGGTGAGAATCTGC	<i>MECR</i> cloning
MECRR258WFw	GGACATGCCCCAGCCATGGCTTGCTCTCAACTGTGTTGGTG	<i>MECR</i> mutagenesis
MECRR258WRv	CACCAACACAGTTGAGAGCAAGCCATGGCTGGGGCATGTCC	<i>MECR</i> mutagenesis

**Online supplemental figures****Online supplemental figure 1**

Visual field of patient 1 showing small central scotoma at 29 years (A) and enlarged central scotoma at 44 years (B). OCT at the time of the latter visual field detecting temporal and inferior RNFL thinning and diffuse macular GCL thinning.

**Online supplemental figure 2**

Visual field of patient 2 showing small central scotoma at 19 years (A) and enlarged central scotoma at 34 years (B). OCT at the time of the latter visual field detecting temporal RNFL thinning and diffuse macular GCL thinning.



## Supplemental references

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