In vitro characterization of mitochondrial function and structure in rat and human cells with a deficiency of the NADH:ubiquinone oxidoreductase Ndufc2 subunit.

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a. Performing the characterization of fibroblast primary cultures from the *Ndufc2* knockout rat model

To analyze the biological characteristics of primary cells, we used all cultures at the second passage (P2) to avoid in vitro differentiation (Leone L. et al; doi: 10.1371/journal.pone.0146365).

The mesenchymal immunophenotype of the primary cultures was verified by immunofluorescence assessment of the expression of vimentin, a component of the intermediate filaments widely used as a fibroblast marker. The analysis showed that all cultures were characterized by a strong staining for vimentin, displaying a signal compatible with the structure and localization of perinuclear cytoplasmic bundles of filaments (Supplementary Fig. 1A). To confirm the purity of cultures, we ruled out the presence of epithelial cells by assessment of the cytokeratins expression, components of the epithelial intermediate filaments; no signal was detected in all isolated primary cultures (Supplementary Fig. 1A).

To evaluate the degree of fibroblasts differentiation, we used a morphological characterization to distinguish in vitro the differentiation stages based on distinct morphological features and we calculated the L:E differentiation index, as defined in Materials and Methods. The quantitative analysis revealed that the L:E ratio was homogeneous, with no appreciable differences among all fibroblast cultures (median of L:E index: 0.83 vs 0.85 for SHRSR and SHRSR_KO *Ndufc2*, respectively; p=NS; Supplementary Fig. 1B).

To better evaluate the differentiated phenotype of all fibroblast cultures, we analyzed by quantitative immunofluorescence the percentage of positive cells for the expression of α-smooth muscle actin, the most commonly used marker for activated and differentiated fibroblasts. Both fibroblast cultures from KO and WT rats displayed a percentage of positive staining for the α-SMA close to 25%, with fluorescent signal localized in the peripheral areas of the cytoplasms, representative of an higher content of actin bundles (median of α-SMA positive staining: 23.2% vs 26.9% for SHRSR and SHRSR_KO *Ndufc2*, respectively; p=NS; Supplementary Fig. 1C).

To verify the typical ultrastructural features of primary fibroblasts, all cultures were analyzed by transmission electron microscopy (TEM). The cells showed a typical spindle-like shape and a cytoplasm with a few rough endoplasmic reticulum cisternae and intracellular filaments; in addition, some fibroblasts were characterized by a cytoplasm with very abundant organelles, numerous cisternae of rough endoplasmic reticulum and evident intracellular filaments, also organized in bundles, localized near the plasma membrane, and consistent with differentiated fibroblasts (Supplementary Figs. 1E-G)

Taken together, these data showed that no appreciable morphological, immunophenotypic and ultrastructural findings were observed between fibroblast cultures from SHRSR_WT and SHRSR_KO *Ndufc2* rats.

b. KCTD21 and NDUFC2 expression study in a larger group of healthy subjects

For this purpose we recruited an overall number of 34 subjects (n=34) homozygous for the major allele (TT/rs11237379; n=9), heterozygous (TC/rs11237379; n=16), or homozygous for the minor allele (CC/rs11237379; n=9). Genotype assessment for the NDUFC2 variant was performed as previously reported (Rubattu S. et al.; doi: 10.1161/JAHA.115.002701). Analysis of NDUFC2 expression was performed by RTPCR of cDNA using specific oligonucleotides following previously published procedures (Rubattu S. et al.; doi: 10.1161/JAHA.115.002701). Primers used for KCTD21 were the following: KCTD21 forward GAG GGC AGG AGG ACT ACT T; KCTD21 reverse GTC GCC AGT GAG GTT GTA TAG. The amount of cDNA target was calculated using GAPDH (primer for: CAAGGCTGTGGGCAAGGT; primer rev: GGAAGGCCATGCCAGTGA) as housekeeping gene.

Legend to Supplementary Figure 1

Characterization of fibroblast primary cultures from the *Ndufc2* knockout rat model

(A) Morphological evaluation (DIC) and immunofluorescence analysis of expression of vimentin and cytokeratins on a representative sample of fibroblast cultures isolated from the mesenchymal tissue of SHRSR and SHRSR_KO *Ndufc2*. In all cultures, the cells showed a strong staining for the mesenchymal marker vimentin (red), which appears as perinuclear cytoplasmic bundles of filaments. No fluorescent signal was detected for cytokeratins (green), ruling out the presence of epithelial cells in the cultures. Nuclei are stained with DAPI (blue).

(B) Morphological evaluation of the differentiation degree of fibroblasts cultures by differential interference contrast microscopy. The quantitative analysis revealed that the L:E differentiation index was homogeneous among both cultures. The scatter dot-plot showed the percent of L:E ratio values obtained from three independent experiments for each culture; the median values and the interquartile ranges of the groups are indicated (Kruskal-Wallis test: ^p=NS versus SHRSR_WT).

(C) Evaluation of α-smooth muscle actin expression. The immunofluorescence analysis of the expression of the differentiation marker α-SMA showed a signal localized in intracellular filaments, also organized in bundles, situated in the peripheral cytoplasmic areas of the cells. The quantitative analysis displayed a similar percentage of positive staining in both fibroblast cultures (scatter dot-plot with median values and interquartile ranges of α-SMA staining; Kruskal-Wallis test: ^p=NS versus SHRSR_WT).

(D-G) Ultrastructural features of the cell cultures. Both the fibroblasts isolated from skin of SHRSR_WT (D-E) and SHRSR_KO *Ndufc2* (F-G) showed a typical spindle-like shape with cytoplasm characterized by high cytoplasmatic complexity, with abundant organelles and intracellular filaments localized in the peripheral areas of the cell, near the plasma membrane, consistent with the intracellular localization of α-smooth muscle actin. (TEM micrographs, uranyl acetate/lead citrate; Legend: Nu, nucleus; NM, nuclear membrane, PM, plasma membrane; Mt, mitochondrion, IFb, intermediate filament bundles; rER, rough endoplasmic reticulum).

Legend to Supplementary Figure 2

NDUFC2 expression study in a larger group of healthy subjects and GTEx analysis

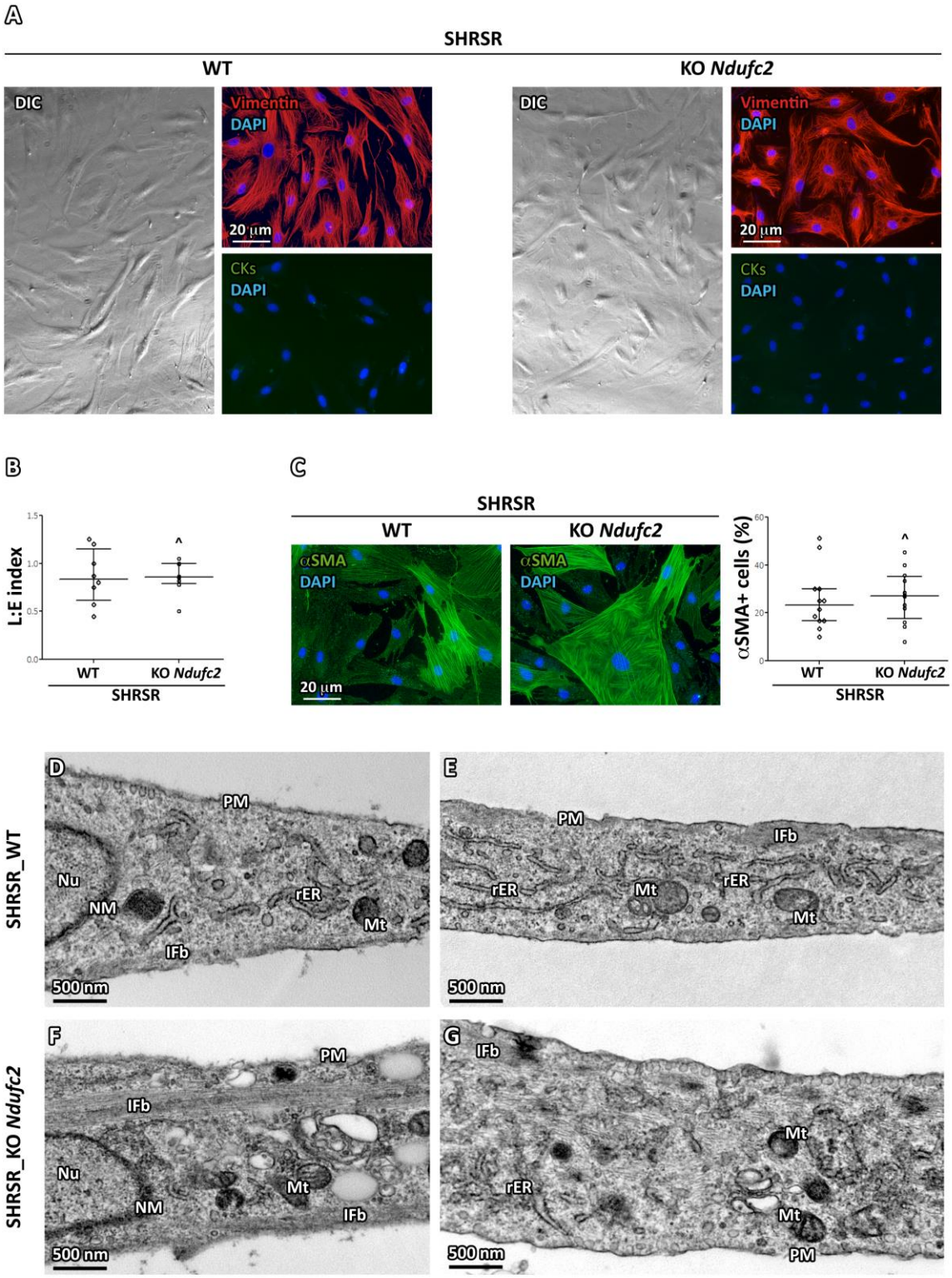
(A) NDUFC2 expression, assessed by RTPCR, in PBMCs of the subjects used for all *in vitro* studies described in the main text (n=6 for each rs11237379/NDUFC2 genotype).

**P<0.001 for both CT and TT versus CC genotype. Values are expressed as means ±SEM.

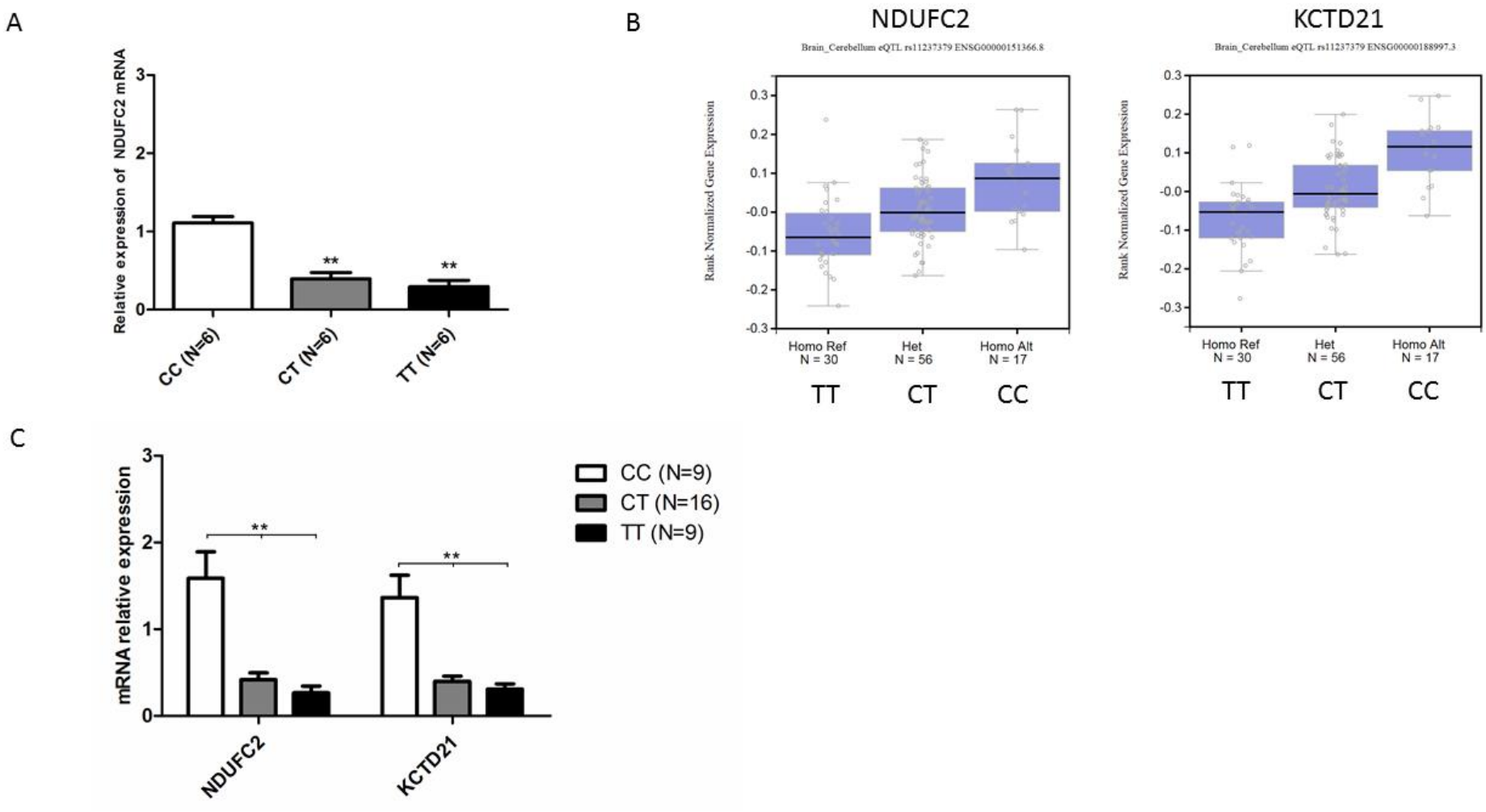
(B) Box plots from Genotype-Tissue Expression GTEx for brain NDUFC2 and KCTD21. The GTEx Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the analyses described in this manuscript were obtained from: the GTEx Portal on 06/20/17

(C) NDUFC2 and KCTD21 expression assessed by RTPCR in PBMCs of a larger group of healthy subjects carrying the three genotypes for rs11237379/NDUFC2.

**P<0.001 for both CT and TT versus CC genotype. Values are expressed as means ±SEM.



Supplementary Fig. 1



Supplementary Fig. 2