SUPPLEMENTAL DATA

S1. MATERIALS AND METHODS

S1.1 Antibodies List

Rabbit polyclonal anti-MIC19/CHCHD3 (1:3,000; #A8584; Abclonal), Rabbit monoclonal anti-MIC60/Mitofilin (1:1,000; #ab137057; Abcam), Rabbit polyclonal anti-MIC10/MINOS1 (1:1,000; #ARP44801; Aviva), Rabbit polyclonal MIC26/APOO (1:1,000; #HPA003187; Atlas), Rabbit polyclonal MIC27/APOOL (1:1,000; #HPA000612, Atlas), Mouse monoclonal anti-GAPDH (1:2,000; #ab8245; Abcam), Mouse monoclonal anti-TUBULIN (1:2,000; #T5201; Sigma), Rat monoclonal anti-HA (1:5,000; #11867423001; Roche), Mouse monoclonal anti-VDAC1/Porin (1:1000, #ab14734; Abcam), Rabbit monoclonal anti-VINCULIN (1:5000, #ab129002; Abcam), Mouse monoclonal anti-NDUFS3 (1:1000, #ab110246; Abcam), Mouse monoclonal anti-UQRC2 (1:1000, #ab14745; Abcam), Mouse monoclonal anti-SDHB (1:1000, #ab14714, Abcam), Mouse monoclonal anti-SDHA (1:1000, #ab14715, Abcam), Mouse monoclonal anti-NDUFB6 (1:1000, #ab110244, Abcam), Mouse monoclonal anti-COXIV (1:1000, #ab14744, Abcam), Mouse monoclonal anti-OXPHOS Blue Native Cocktail (1:1000, #ab110412), Mouse monoclonal anti-ATP5A (1:300; #ab4748; Abcam), Rabbit monoclonal anti-TOMM20 Alexa Fluor 647 (1:500; #ab209606; Abcam), Abberior STAR 488 anti-rat (1:100; #2-0132-006-8; Abberior), Alexa Fluor 568 Goat Anti-mouse (1:300; #A11004; Invitrogen), Alexa Fluor 568 Goat Anti-rabbit (1:300; #A11036; Invitrogen).

S1.2 Cloning and Primers

To clone WT and I117T APOO-HA into pWPXLd-Ires-Puro^R, derived from pWPXLd, a gift from Didier Trono (Addgene plasmid #12258). Forward and reverse primers were designed (SnapGene 4.3.3) with homology adapted ends to enable the use of Gibson Assembly Cloning Kit (New England Biolabs), of the PCR products into the *PmeI* site in the vector. *CG5903* (*dApoO*) cDNA was cloned in pAc5-STABLE2-neo vector to generate a C-terminal HA tag as follows; pAc5-STABLE2-neo was digested *EcoRI* and *HindIII* and the vector backbone gel extracted. *dApoO* cDNA was used as template for Pfu PCR amplification. PCR product was column purified and cloned into pAc5-STABLE2-neo (*EcoRI/HindIII*) by In-Fusion cloning (Takara Clontech).

Yeast *MIC26^{WT}* was cloned in pFL38 and *mic26^{I127T}* mutant allele was constructed through overlap mutagenic PCR.

APOO sequencing Fw: 5'-GTCAGTAGGTTGTTGGACCAAAGG-3' and Rv: 5'-GTCATCACTGACTGAAGGCATGAC-3'.

APOO in pWXLd-Ires Fw: 5'-CTAGCCTCGAGGTTTCGTTTAAACATGTTCAAGGTAATTCAGAGG-3' and Rv: 5'-CGGATCCCGTAGTTTTCAAGCGTAATCTGGAACATCGTATGG-3'

CG5903 (dApoo) cDNA in pAc5-STABLE2-neo Fw: 5'-TCCAGTGTGGTGGAATTCCAAAATGCTGCGCAAAACGGCA-3' and Rv: 5'-TCTGCCCTCAAGCTTTTAAGCGTAATCTGGAACATCGTATGGGTACTTCTTTTTGGGGAAGATTAG-3'

Rp49 Fw: 5'-ATCGGTTACGGATCGAACAA-3' and Rv: 5'-GACAATCTCCTTGCGCTTCT-3'.

dAPOO Fw: 5'-GACAGAACCCAAGCCAGAAC-3' and dAPOO-Rv 5'-GCCTTATATCCAGACTGCACCTC-3'.

Yeast Mic26 Fw: 5'-CGGGCCTGCAGCATGTAAGGGAATTTGTACTTGG-3' and Mic26 Rv: 5'-CGGGCGAGCTCCTTTTATGCAAGCTATGATTGG-3'

Yeast mutagenesis $Mic26^{I127T}$ Fw: 5'-CCTAATACCGGGGTTGTTGTCgAcTTTGGTTGCTTCCATGACAGG-3' and $Mic26^{I127T}$ Rv: 5'-CCTGTCATGGAAGCAACCAAAgTcGACAACAACCCCGGTATTAGG-3'

S1.3 Image acquisition

Image acquisition was performed using either a Zeiss LSM880 confocal system equipped with a Zeiss Plan-Apochromat 100x/1.4 N.A. objective or a N-SIM Microscope system (Nikon) equipped with a SR Apo TIRF 100 x 1.49 N.A. objective and a DU897 Ixon camera (Andor Technologies). 3D-SIM image stacks were acquired with a Z-distance of 0.1 µm and all the raw images were computationally reconstructed using the reconstruction slice system from NIS-Elements software (Nikon) keeping the same parameters.

S1.4 RNA Isolation from flies, Reverse Transcription and qRT-PCR

Total RNA was extracted from 10 adults (1:1 males-females) using TRIzol reagent (Thermo Fischer), according to the manufacturer's instructions. Total RNA was used for first strand cDNA synthesis employing Omniscript RT kit (Qiagen) according to the manufacturer's protocol. qRT-PCRs were performed in triplicate using a QuantStudio™ 3 system (Applied Biosystems) using Power SYBR™ Green chemistry (Thermo Fisher

Scientific). The $2^{-\Delta\Delta Ct}$ (RQ, relative quantification) method was used to calculate the relative expression ratio and Rp49 was used as endogenous control.

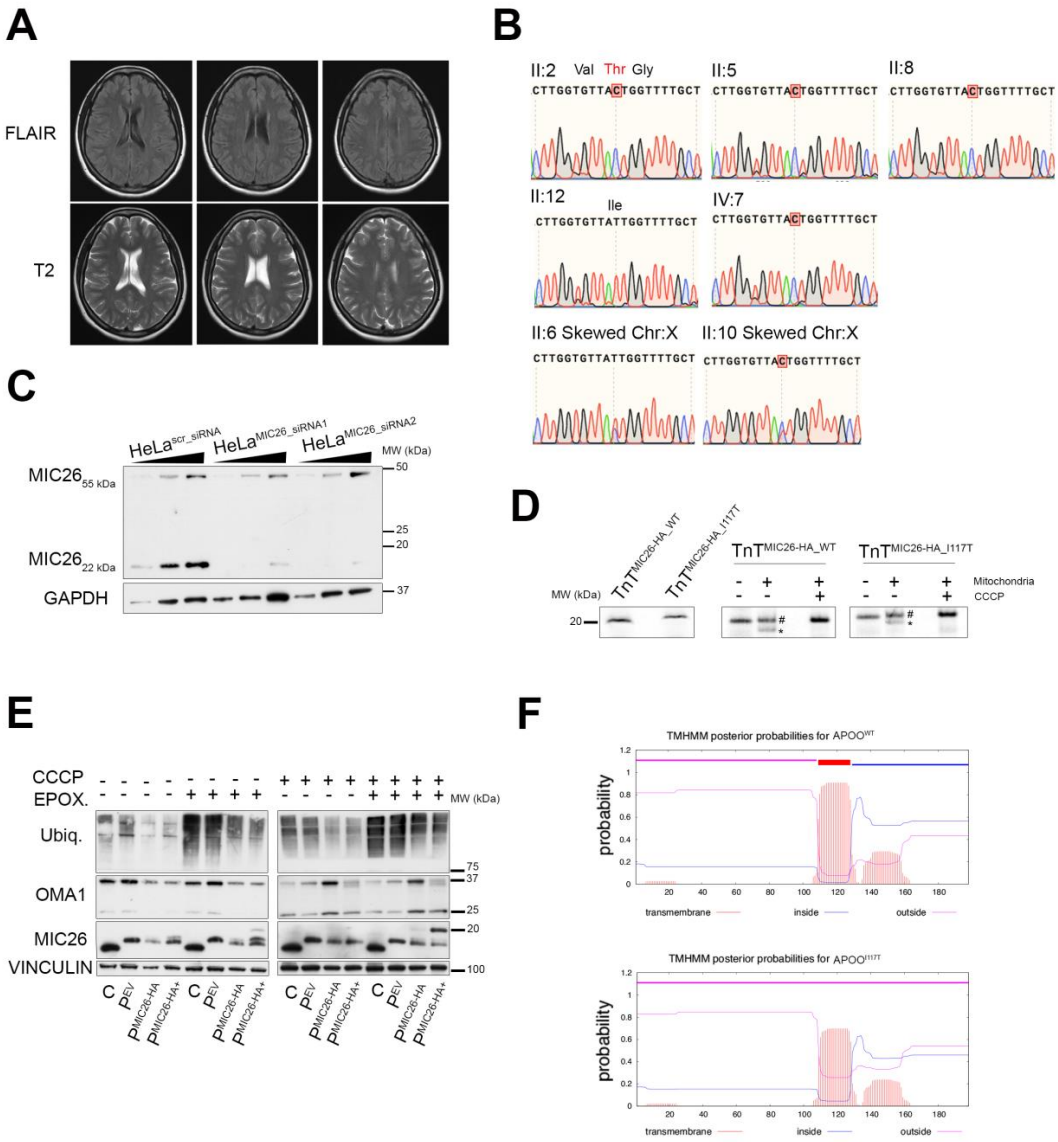


Figure S1: Brain MRI of proband mother showing hyperintense signal in the white matter, female presentation of phenotype is variable depending on skewed X-chromosome. (A) Brain MRI of proband mother showing hyperintense signal in white matter. (B) Sequence chromatograms showing family members of proband with the mutation c.350T>C in males (II:2, II:5, II:8, II:12 and IV:8) and in the females skewed X-chromosome (II:6 and II:10). (C) SDS-PAGE for MIC26 and GAPDH in HeLa

cells after 72 h downregulation of MIC26 by siRNA (HeLa^{MIC26_siRNA1} and HeLa^{MIC26_siRNA2}). Scramble siRNA was used as control (HeLa^{scr_siRNA}). Representative image of three biological replicates. (D) TnT and in vitro organelle import of MIC26-HA WT and mutant showing the non-imported precursor (#) and two imported processed forms (* and **). Representative image of two biological replicates. (E) SDS-PAGE for UBIQUITIN, OMA1, MIC26 and VINCULIN in fibroblasts treated with 20 μM CCCP, 100 nM Epoxomicin, 100 nM Epoximicin or 20 uM CCCP + 100 nM Epoximicin for 4 h prior lysis (DMSO was used as control) Representative image of two biological replicates. (F) Prediction with TMHMM 2.0 using default parameters for MIC26^{WT} and MIC26^{I117T}.

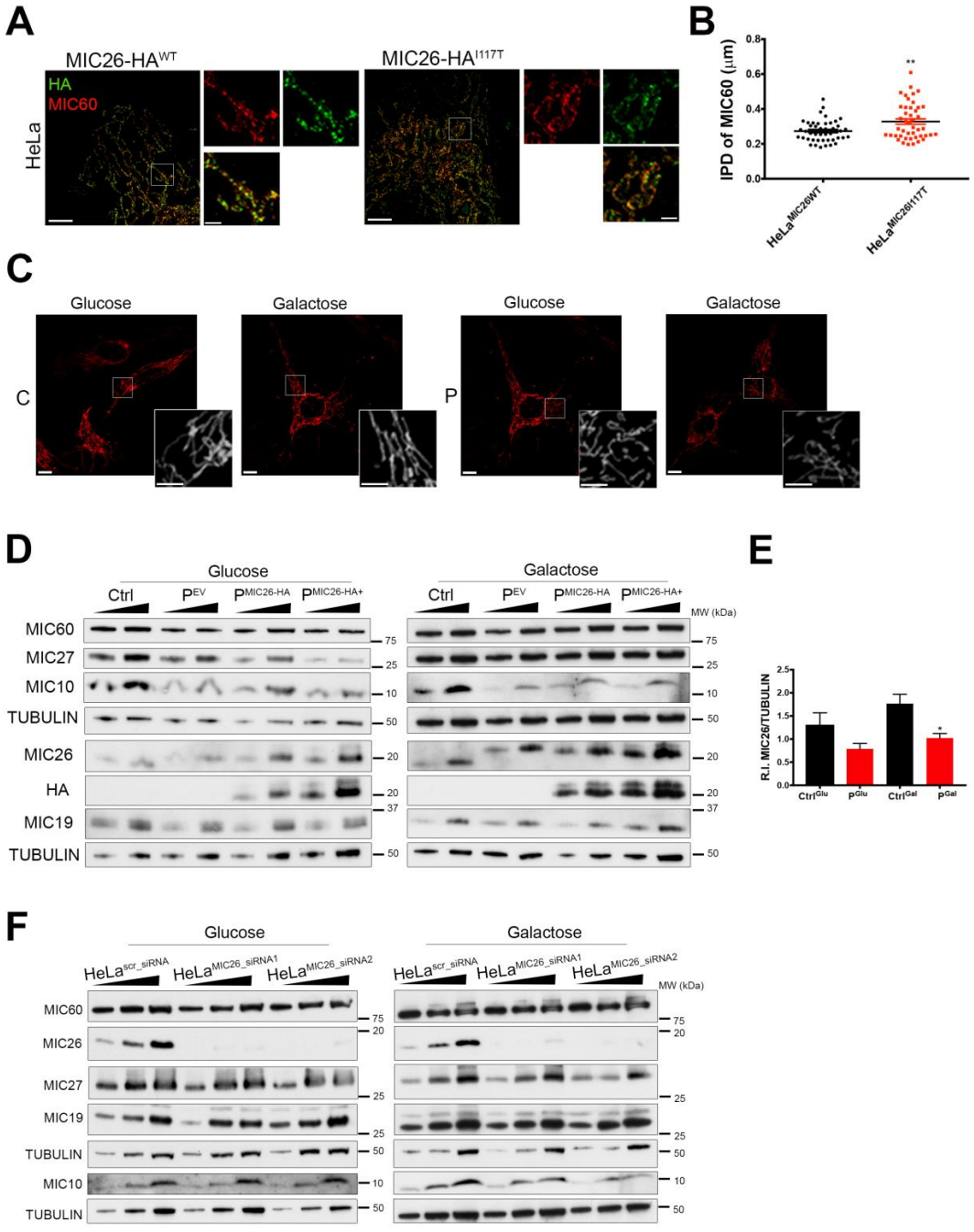


Figure S2: MIC26 in proband (P) fibroblasts and HeLa cells knocked down for MIC26 present no differences in the steady-state levels of MICOS subunits. (A) N-SIM super-resolution micrographs showing HeLa cells expressing MIC26-HA (WT or I117T), labelled with anti-MIC60 and anti-HA antibodies. Maximum intensity projection is shown. Scale bars: 5 µm, inset 1 µm. Representative

images of three biological replicates. (B) Chart shows mean ± S.E.M (n=50 from 5 cells each) of Interpunctae distance (IPD) of MIC60 in the IBM when MIC26^{WT} or MIC26^{I117T} is overexpressed (Unpaired t-test p=0.0201**). (C) Confocal micrographs showing mitochondrial morphology (TOMM20 in red) of control (C) and proband (P) fibroblasts growing in glucose or galactose. Scale bars: 5 µm, inset 1 µm. Representative images of three biological replicates. (D) SDS-PAGE for MIC60, MIC27, MIC26, MIC19, MIC10 and TUBULIN in Ctrl, P fibroblast expressing empty vector (PEV) or MIC26-HA (P$^{MIC26\text{-}HA}$ and P$^{MIC26\text{-}HA+)}$ grown in glucose vs. galactose. Representative image of five biological replicates. (E) Relative densitometric quantification of the bands normalized to the intensity of the loading control marker. Chart shows mean ± S.E.M. of five biological replicates (one-way ANOVA *p=0.0447). (F) SDS-PAGE for MIC60, MIC27, MIC26, MIC19, MIC10 and TUBULIN in HeLa cells grown in glucose vs. galactose (7 days) after 72 h downregulation of MIC26 by siRNA (HeLa^{MIC26_siRNA1} and HeLa^{MIC26_siRNA2}). Scramble siRNA was used as control (HeLa^{scr_siRNA}). Representative image of three biological replicates.

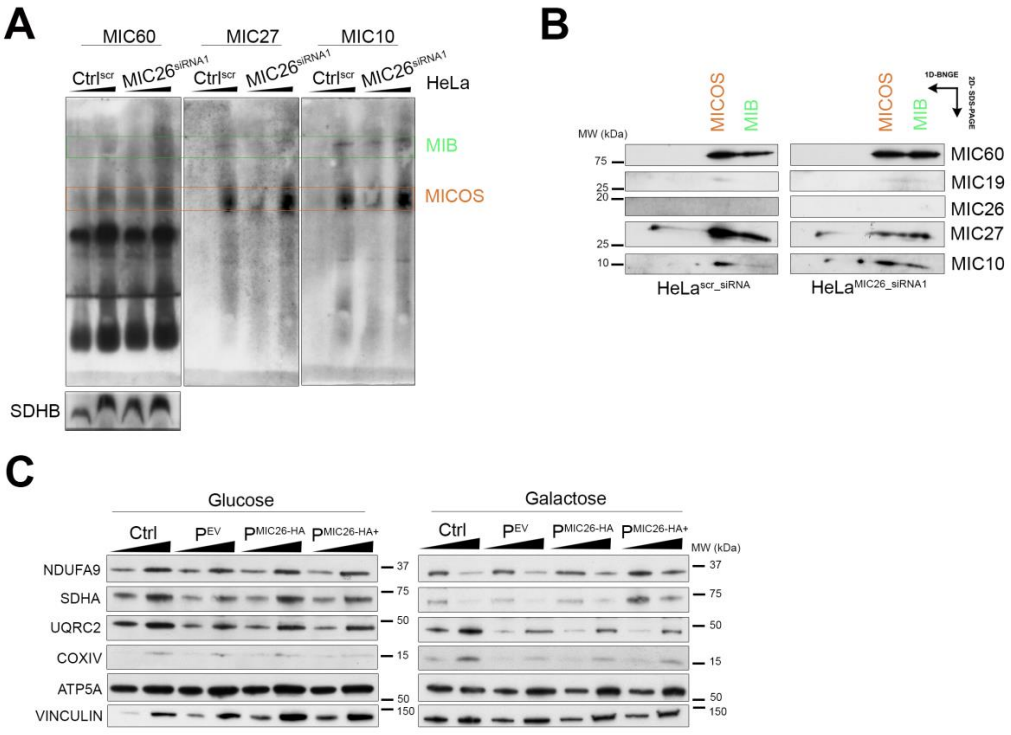
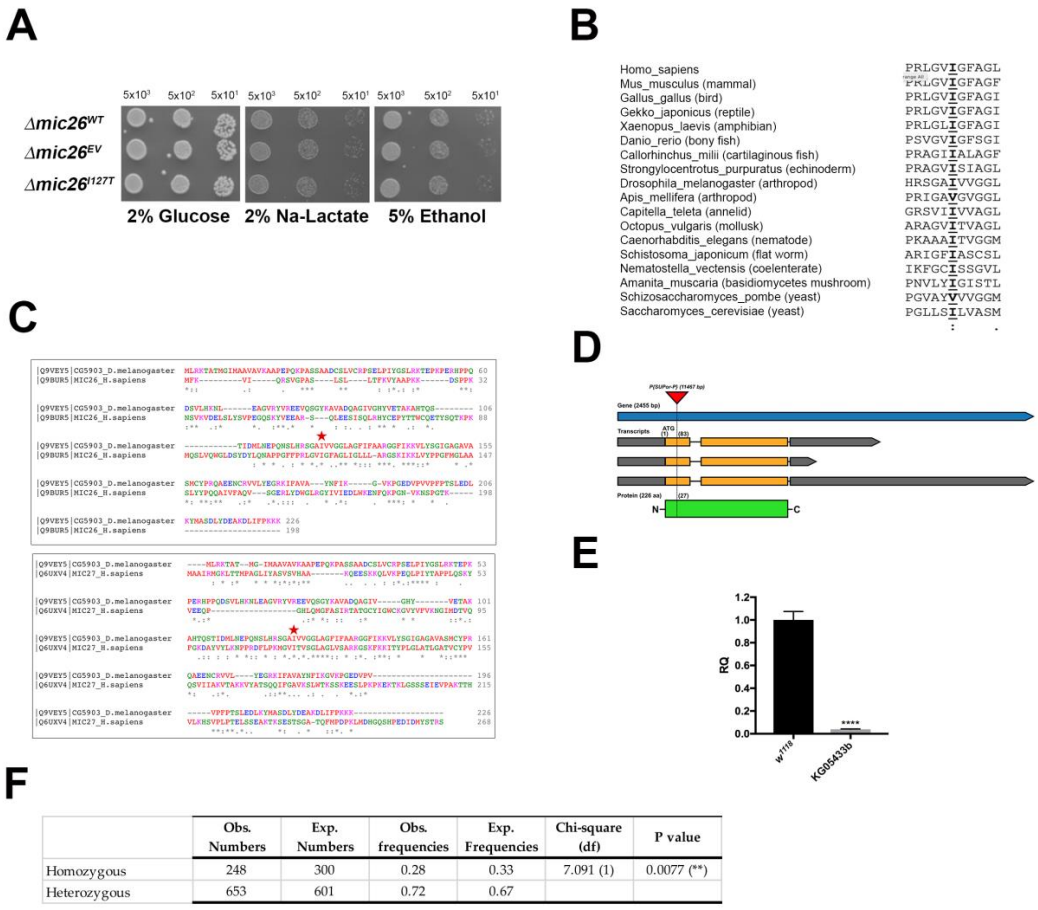


Figure S3: Distribution of MIB and MICOS components is altered in HeLa cells knocked down for MIC26. (A) BN-PAGE showing the distribution of MIB and MICOS complex immunoblotted for MIC60, MIC27 and MIC10 antibodies in HeLa cells treated with control (Ctrl^{scr_siRNA}) and MIC26 (MIC26^{siRNA1}) siRNA for 72 h. Representative image of three biological replicates. (B) Second dimension PAGE from BN immunoblotted for MIC60, MIC26, MIC27and MIC10 antibodies. Representative image of three biological replicates. (C) SDS-PAGE for OXPHOS subunits NDUFA9 (CI), SDHA (CII), UQRC2 (CIII), COXIV (CIV) and ATP5A (CV) and VINCULIN in control (Ctrl) and proband (P) fibroblasts grown in glucose and galactose. Representative image of two biological replicates.



	Obs. Numbers	Exp. Numbers	Obs. frequencies	Exp. Frequencies	Chi-square (df)	P value
Homozygous	248	300	0.28	0.33	7.091 (1)	0.0077 (**)
Heterozygous	653	601	0.72	0.67		

Figure S4: The knockout of the *APOO* ortholog *CG5903* (*dApoO*) in *Drosophila melanogaster* shows partial lethality. (A) Spot assay on SC medium supplemented with 2% glucose (2 days), 2% sodium lactate (2 days) or 5% ethanol (5 days). (B) The proteins were selected from sequences

obtained from a 5-iteration PSI-Blastp with default parameters research. Isoleucine at position 117 is present in all organisms except for few birds, coelenterates, and some arthropods, nematodes and fungi, where valine can be present. (C) Alignment of dMic26 and MIC26 showing identical residues (*) and similar ones (. and :). The mutated residue found in patients p.I117 correspondent to p.I124 in dMic26 has been highlighted with a red star. (D) Scheme showing the deletion of *dApoO* performed in *D. melanogaster* (E) qRT-PCR showing expression levels of *CG5903* in w^{1118} and KO flies. Data plotted are mean ± S.D. of three biological replicates (unpaired t-test, p<0.0001****) (F) Mendelian frequencies observed in progeny obtained crossing *dApoO* heterozygous null flies. A total number of 901 flies has been counted (chi-square 7.091 df(1), p=0.0077**).