DEPARTAMENTO DE BIOLOGÍA CELULAR FISIOLOGÍA E INMUNOLOGÍA

Programa de Doctorado de Biomedicina

Mechanisms of longevity extension and healthy aging by over-expression of cytochrome b5 reductase 3

Mecanismos de extensión de la longevidad y envejecimiento saludable por sobre-expresión de citocromo b5 reductasa 3

TESIS DOCTORAL

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TITULO: *Mechanisms of longevity extension and healthy aging by over-expression of cytochrome b5 reductase 3*

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Mechanisms of longevity extension and healthy aging by over-expression of cytochrome b5 reductase 3

TESIS DOCTORAL

Memoria de Tesis Doctoral presentada por **Sandra Rodríguez López**, Graduada en Bioquímica, para optar al grado de **Doctor en Biomedicina** por la Universidad de Córdoba con la mención de *Doctorado Internacional*

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TÍTULO DE LA TESIS: Mechanisms of longevity extension and healthy aging by over-expression of cytochrome b_5 reductase 3

DOCTORANDO/A: Sandra Rodríguez López

INFORME RAZONADO DE LOS DIRECTOR/ES DE LA TESIS

El objetivo general de la Tesis Doctoral que se presenta es elucidar los mecanismos por los cuales la sobreexpresión de la enzima NADH-citocromo b_5 reductasa 3 (CYB5R3) optimiza las funciones mitocondriales resultando en una mejoría del metabolismo y en una mayor longevidad en ratones transgénicos CYB5R3. CYB5R3 es una flavoproteína que cataliza la transferencia de electrones desde el NADH hasta el citocromo b_5 o hasta aceptores electrónicos alternativos como coenzima Q y muchos compuestos exógenos. La investigación desarrollada en el grupo de investigación durante los últimos años ha permitido demostrar que los ratones que sobre-expresan CYB5R3 presentan una mayor longevidad, manifiestan una mejor sensibilidad a la insulina, menor inflamación, menor daño oxidativo y están parcialmente protegidos frente a la inducción de cáncer. Sin embargo, no son conocidos los mecanismos por los cuales se producen estos cambios, que en algunos casos parecen proceder de manera similar a las alteraciones que acompañan a la restricción calórica - como es el caso de los beneficios indicados -, mientras que existen también observaciones divergentes entre ambos modelos, como son la mayor acumulación de grasa y el aumento del grado de insaturación de los lípidos de membrana en los ratones transgénicos CYB5R3, efectos contrarios a lo encontrado con la restricción calórica. Por tanto, en la tesis se han estudiado marcadores mitocondriales clave a la hora de determinar la pauta de envejecimiento en hígado y corazón de ratones transgénicos que sobre-expresan CYB5R3 y sometidos a diferentes intervenciones nutricionales (restricción calórica y alteraciones en la composición de ácidos grasos de la dieta). También se han estudiado cómo se ven afectados estos factores por el envejecimiento. La hipótesis de trabajo es que la eficiencia mitocondrial estará optimizada en los ratones transgénicos, lo que da lugar a una mejora en la producción de energía y a una mayor preservación de la mitocondria durante el envejecimiento. Por otro lado, se ha indicado que puede existir una interferencia en la sobreexpresión y CYB5R3 y la restricción calórica, dado que, aunque la sobreexpresión de CYB5R3 simula algunos de los efectos beneficiosos para la salud de la restricción calórica, también existen efectos metabólicos antagónicos.

La Introducción ilustra adecuadamente sobre aspectos clave relativos al envejecimiento, los mecanismos asociados al mismo, y las principales teorías que tratan de explicar sus causas, prestando especial interés a la teoría de los radicales libres, a los efectos deletéreos de las especies reactivas y al papel clave desempeñado por la mitocondria en este proceso. El concepto de deficiencias asociadas a la edad en la función mitocondrial, a nivel de bioenergética, biogénesis y dinámica de fusión y fisión, está bien presentado y actualizado. Otra función que

resulta desregulada con el envejecimiento es la autofagia. Tras este apartado, se abordan las intervenciones cuyo objetivo es paliar o retardar los efectos deletéreos del envejecimiento como son la restricción calórica y la preservación de los niveles de NAD^+ mediante sobreexpresión de CYB5R3, una enzima que, además, desempeña importantes funciones metabólicas entre las que se encuentra la regeneración de antioxidantes y la desaturación y elongación de ácidos grasos, aspecto que también es posible modificar mediante intervenciones basadas en el consumo de dietas con una composición lipídica determinada. La doctoranda concluye este capítulo señalando la importancia del hígado y el corazón como modelos – mitótico y postmitótico, respectivamente - para investigar las alteraciones tisulares asociadas al envejecimiento y el efecto de las intervenciones genéticas y nutricionales.

Los métodos utilizados en la Tesis son acordes con la consecución de los objetivos perseguidos. Para ello se han investigado posibles alteraciones relacionadas con la función mitocondrial en los ratones transgénicos CYB5R3 y controles "*wild type*" (WT) en tres contextos distintos:

1) restricción calórica en animales jóvenes/adultos (4 meses de intervención, 7 meses de edad en el momento del sacrificio)
2) efecto de la grasa de la dieta (basada en la formulación AIN93M pero conteniendo manteca, aceite de oliva, aceite de soja o aceite de pescado) en animales jóvenes/adultos (4 meses de intervención, 7 meses de edad en el momento del sacrificio)
3) envejecimiento, en una cohorte de animales alimentados con la dieta AIN93M estándar (24 meses de edad en el momento del sacrificio).

Una vez finalizados los periodos correspondientes de intervención, la actividad ha estado encaminada al análisis de distintos marcadores metabólicos en muestras derivadas de animales jóvenes o viejos, sometidos a las intervenciones indicadas. Los estudios se han desarrollado sobre hígado y músculo cardíaco como modelos de tejido mitótico y postmitótico, respectivamente.

En lo que respecta al primero de los contextos indicados (restricción calórica), se demuestra que el aumento de los niveles de MFN2 (implicado en la fusión mitocondrial), observado en hígado en condición de restricción calórico, no tuvo lugar en los ratones transgénicos, lo que podría estar relacionado con un mayor metabolismo de carbohidratos en estos animales. Sin embargo, tanto en los animales WT como en los transgénicos se produjo aumento en los niveles de complejo III en condiciones de restricción calórica. Unidas estas observaciones a las obtenidas en músculo esquelético (objeto de otra tesis doctoral), los resultados apoyan la idea de que la sobreexpresión de CYB5R3 podría mitigar parcialmente algunas de las adaptaciones metabólicas a la restricción calórica, lo cual era una de las hipótesis iniciales del proyecto de investigación en el cual se encuadra esta tesis. Estos resultados se han publicado en un artículo en la revista *Geroscience* (IF: 8.88; Q1). 2020 Jun;42(3):977-994. doi: 10.1007/s11357-020-00187-z. Epub 2020 Apr 22. PMID: 32323139, en el que la doctoranda es primera firmante.

En lo referente al segundo de los contextos (influencia de la grasa de la dieta), los datos obtenidos han indicado que la sobreexpresión de CYB5R3 puede producir diferentes efectos sobre la función mitocondrial dependiendo de la fuente de grasa utilizada en la dieta. Aunque la sobreexpresión de CYB5R3 no parece conferir un fenotipo marcado por sí mismo, en el caso de los ratones alimentados con la dieta basada en manteca de cerdo, se observa una prevención de la fragmentación mitocondrial, lo que sugiere un estado mitocondrial más funcional en comparación

con sus controles WT. En el caso de los ratones alimentados con una dieta enriquecida con aceite de oliva, la sobreexpresión de CYB5R3 parece promover un fenotipo saludable al aumentar las proteínas de fusión mitocondrial. Sin embargo, también parece existir alguna interferencia entre la sobreexpresión de CYB5R3 y el consumo de una dieta enriquecida con aceite de pescado, ya que algunas de las acciones beneficiosas de cada una de las intervenciones de manera aislada pueden verse obstaculizadas, como se observa en los marcadores de dinámica mitocondrial y en el aumento de peso de los ratones transgénicos con esta dieta. El trabajo de la doctorada también se ha enfocado en profundizar en el mecanismo por el cual los ácidos grasos modulan la síntesis de coenzima Q, uno de los sustratos naturales de CYB5R3. Los resultados obtenidos indican que los ácidos grasos poliinsaturados son moduladores de la síntesis de coenzima Q y determinantes clave de la relación entre las dos isoformas de la coenzima Q (9 y 10) presentes en los tejidos murinos. Estos resultados acaban de ser aceptados en la revista *Redox Biology* (IF: 9.986; Q1), estando aún en fase de producción por la Editorial previa a la publicación. Además, se ha llevado a cabo la publicación de un capítulo de libro en el que la doctoranda es co-autora (Chapter 15. Calorie Restriction, Longevity and Coenzyme Q. Coenzyme Q in Aging pp 311-328. Springer-Nature, 2020. https://doi.org/10.1007/978-3-030-45642-9_15).

Finalmente, respecto al tercero de los contextos indicados (envejecimiento), encontramos que tanto en hígado como en corazón se produce una alteración en los marcadores de dinámica mitocondrial con el envejecimiento que es distinta según su genotipo, con un predominio de marcadores de fusión con el envejecimiento en los ratones WT, mientras que predomina la fisión en los transgénicos. De nuevo, encontramos cambios en los niveles de complejos mitocondriales, que aumentan con la edad, siendo este efecto potenciado por la sobreexpresión de CYB5R3. Estos resultados son consistentes con los encontrados en músculo esquelético de animales de la misma edad, lo cual apoya que un reciclado mitocondrial más eficiente y una mejora en la capacidad respiratoria de los animales podrían estar detrás de los beneficios de la sobreexpresión de CYB5R3 sobre la longevidad.

Las técnicas y la metodología son innovadoras y adecuadas para abordar los objetivos de la investigación. Los resultados se discuten claramente y se colocan adecuadamente en el contexto del estado actual de conocimiento en el campo de la fisiología mitocondrial en hígado y corazón. Así mismo, las conclusiones alcanzadas ofrecen un potencial traslacional de posibles enfoques en tratamientos sobre envejecimiento. En conjunto, este trabajo muestra originalidad, alta calidad en enfoques técnicos y experimentales y proporciona avances significativos en nuestra comprensión de los efectos beneficiosos de CYB5R3 en la promoción de un envejecimiento saludable.

En estrecha relación con el contenido de la Tesis, la doctoranda ha participado en otras investigaciones que enriquecen significativamente su grado de formación científica, producto de las estancias llevadas a cabo en laboratorios internacionales. Así, fruto de su estancia en el grupo de la Dra. Judith Campisi (Buck Institute, San Francisco, CA), ha participado en una investigación sobre el papel de dos isoformas de p21, una proteína clave en la senescencia. A este respecto se ha llevado a cabo otra publicación en la revista Aging (Albany NY) (IF: 4.831; Q1). 2021 May 25;13(10):13380-13392. doi: 10.18632/aging.203110. Epub 2021 May 25, en la que la doctoranda es también primera autora. Son también reseñables las estancias llevadas a cabo en el laboratorio del Dr. Johan Auwerx (Laboratory of Integrative systems Physiology at École polytechnique fédérale de Lausanne, EPFL, Lausanne, Suiza), en las que la doctoranda ha participado en una investigación encaminada a

caracterizar la cepa de ratón más susceptible a la enfermedad del hígado graso inducida por una dieta occidental a través de un extenso estudio fenotípico, con el fin de identificar nuevos objetivos terapéuticos potenciales para evaluar el componente genético de esta enfermedad. Es importante resaltar que una de estas estancias ha estado financiada a través de una convocatoria competitiva de EMBO.

Además de las publicaciones indicadas, la doctoranda ha presentado numerosas comunicaciones (póster y orales) en congresos nacionales e internacionales, tanto de carácter generalista como específicos a nivel internacional en el campo de las especies reactivas y los antioxidantes.

En lo relativo al programa formativo, éste se ha desarrollado también de manera excelente. Cabe destacar su participación en jornadas, congresos (nacionales e internacionales), estancias y cursos formativos, así como en la docencia asignada en el PDD del departamento de Biología Celular, Fisiología e Inmunología, la cual ha desarrollado con total responsabilidad. Por todo lo indicado, nuestra consideración sobre la labor realizada en el período desde el inicio de los estudios de la doctoranda hasta la actualidad, así como sobre el grado de cumplimiento de los planes de investigación y de formación, es óptima.

Por todo lo expuesto, consideramos que la Tesis presentada por Dª Sandra Rodríguez López cumple todos los requisitos necesarios para optar al Grado de Doctor con Mención Internacional por la Universidad de Córdoba, y autorizamos su presentación ante el correspondiente Tribunal.

Córdoba, 29 de junio de 2021

Firma del/de los director/es

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Esta Tesis Doctoral ha sido realizada en el Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba, bajo la dirección de los **Dres. José Manuel Villalba Montoro, José Antonio González Reyes y José Alberto López Domínguez.** El trabajo ha sido financiado por los proyectos de investigación **BFU2015-64630-R del Ministerio de Economía y Competitividad (MINECO),** RTI2018-100695-B-I00 del Ministerio de Ciencia, Innovación y Universidades (MICIU), P18-RT-4264 y 1263735-R de la Junta de Andalucía, por el programa FEDER de la Unión Europea, y por la Universidad de Córdoba dentro del grupo de investigación BIO-276 ("Biomembranas, Antioxidantes y Estrés oxidativo"). El contrato de la doctoranda ha sido financiado por el programa nacional de Formación del Personal Investigador (FPI, BES-2016-078229, MINECO). Esta Tesis Doctoral ha sido realizada en un modelo de ratón sobre-expresante de la proteína CYB5R3, los cuales han sido donados por el Dr. Rafael de Cabo (NIH, Baltimore,USA).

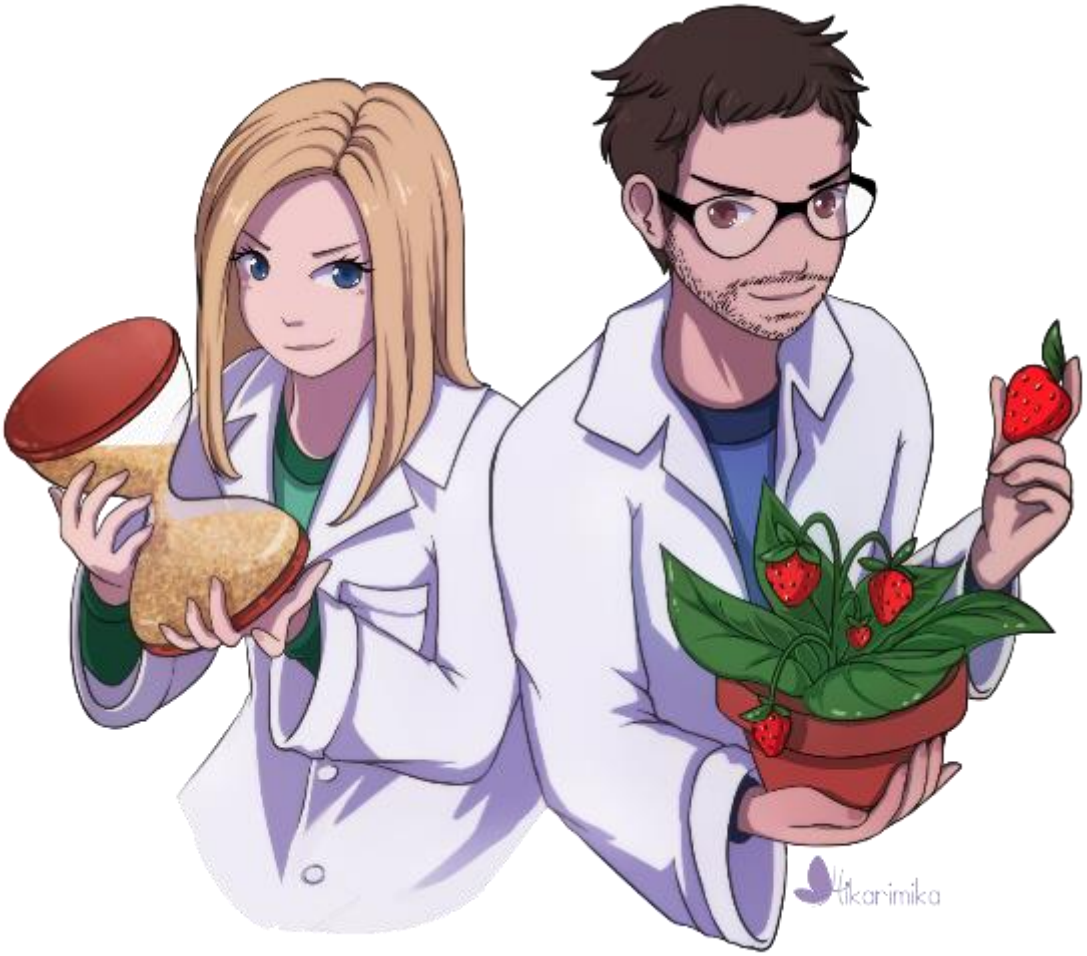
La estancia internacional de 3 meses realizada en el *Buck Institute for research **on Aging*** (Novato, CA, USA) bajo la supervisión de la **Dra. Judith Campisi,** ha sido financiada por el programa **FPI (BES-2016-078229, MINECO)** y de la cual se ha publicado el artículo "***Cdkn1a* transcript variant 2 is a marker of aging and cellular senescence**" (DOI: 10.18632/aging.203110) en el que la doctoranda figura como primera autora.

La estancia internacional de 6 meses realizada en la **École polytechnique fédérale de Lausanne (EPFL)** bajo la supervisión del **Dr. Johan Auwerx** ha sido financiada por el **programa EMBO Short-Term Fellowship (Fellowship number 8521)** durante los tres primeros meses y por el propio laboratorio del Dr. Auwerx durante los 3 siguientes meses.

El acceso a la bibliografía científica de esta tesis no hubiera sido posible sin la inestimable labor de Alexandra Elbakyan, la creadora de Sci-Hub, que ha eliminado las barreras en el acceso al conocimiento científico.

Agradecimientos

Lo mejor de pertenecer al *Dark side* es que siempre hay café y galletas



Félix, ya queda menos para que tú y yo montemos un laboratorio, en el que hagamos geles 10X, tomemos café exprés y nos llamemos a voces y aporreando las paredes

Por fin toca escribir la mejor parte de la tesis, los agradecimientos. Hacer una tesis nunca es fácil, pero escribir este apartado implica que lo difícil ya está hecho. Solo queda echar la vista hacia atrás y ver cuanta gente te ha acompañado, aguantado y ayudado en este largo y tedioso camino que es la ciencia. Una de las cosas que he aprendido en estos últimos años es que no hay que hacer las cosas en el momento y no decir "*que no pasa na' que no se va a acabar el mundo porque esperes unos días*". A la vista está que, si no se ha acabado el mundo en este último año y medio, poco ha faltado, porque vivir una pandemia en el último año de tu tesis no es lo mejor que te puede pasar. Que saquen la revista donde has publicado tu D1 de los índices de impacto tampoco ayuda, y podría seguir diciendo un montón de cosas que han ido pasando a lo largo de este año para justificar que si no se ha acabado el mundo poco ha faltado, al menos para el que se está doctorando.

A pesar de ser una situación complicada, siempre hay personas que te apoyan y te hacen más llevadera esta situación (o lo intentan). Algunos por la experiencia de haber pasado ya por esto, otros porque lo viven a la vez que tú y otros simplemente porque te apoyan a pesar de no entender qué implica para los que nos dedicamos a la ciencia hacer una tesis. En cualquier caso, es de agradecer a todas estas personas que te ayudan a superar las adversidades y te hacen más fuerte. Porque sin esas personas, hacer una tesis sí que sería imposible.

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Abbreviations

-OH	Hydroxide ion
●OCl	Hypochlorite
●OH	Hydroxyl radical
●ONOO	Peroxynitrite
16S rDNA	16S ribosomal RNA
1O2	Singlet oxygen
8-OH-deoxyguanosine	8-hydroxy-2'-deoxyguanosine
AA	Arachidonic acid
ADP	Adenosine diphosphate
AL	Ad libitum
ALA	α-linolenic acid
AMPK	AMP-activated protein kinase
Atg32	Autophagy-related protein 32
ATP	Adenosyl triphosphate
ATP5A	ATP synthase lipid-binding protein
b2M	Beta-2-microglobulin
BCL2	B-cell lymphoma 2
BER	Base excision repair
BH3	BCL-2 homology domains
BNIP3	BCL2 Interacting Protein 3
BNIP3L/NIX	BCL2 Interacting Protein 3 Like
Ca2+	Calcium ions
cADPR	Cyclic ADP-ribose
CD157/ CD38	ADP ribosyl-cyclases
CLAP	Chymostatin, Leupeptin, Antipain and Pepstatin
CoQ	Coenzyme Q
COX	Cytochrome c oxidase
COX17	Cytochrome C Oxidase Copper Chaperone
COX7A	Cytochrome c oxidase subunit 7A
CR	Caloric restriction
ctb-1	Cytochrome b
Cu+	Cuprous ion
CVD	Cardiovascular disease
CYB5A	Cytochrome B5 Type A
CYB5B	Cytochrome B5 Type B
CYB5R1	NADH- Cytochrome b5 reductase 1
CYB5R3	NADH- Cytochrome b5 reductase 3
daf-2	Insulin-like receptor
DEPC	Diethyl pyrocarbonate
DHA	Docosahexaenoic acid
D-loop	Displacement loop
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DRP1	Dinamin-related protein 1
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EFAs	Essential fatty acids
EPA	Eicosapentaenoic acid
ER	Endoplasmic Reticulum
ERR-α	Estrogen-related receptor alpha
ETC	Electron transport chain
F	Fish oil
FA	Fatty acids
FADH2	Flavin adenine dinucleotide (reduced)
FADS1	Fatty Acid Desaturase 1
FADS2	Fatty Acid Desaturase 2
fat-1	Omega-3 fatty acid desaturase fat-1
Fe+2	Iron(II)
Fe+3	Iron(III)
FFA	Free fat acids
FIS1	Mitochondrial fission protein 1
FOXO	Forkhead box
FOXO3a	Forkhead box O3
gDNA	Genomic DNA
GPX	Glutathione peroxidase
GSH	Glutathione
GTP	Guanosine triphosphate
H2O2	Hydrogen peroxide
HDL	High-density lipoprotein
HeLa	Human cervical cancer cells
HEPA	High-efficiency particulate absorbing
Hepa 1.6	Mouse liver hepatoma cells
HepG2	Human liver hepatoma cells
HFD	High fat diet
HK2	Hexokinase 2
HMG	High-mobility-group
HO2	Hydroperoxyl radical
Hsc-70	Heat shock 70 kDa protein
IDL	Intermediate-density lipoprotein
Igf1	Insulin-like growth factor 1
IL2	Interleukin 2
IMF	Intermyofibrillar mitochondria
IMM	Inner mitochondria membrane
INF2	Inverted Formin 2
isp-1	Cytochrome b-c1 complex subunit Rieske, mitochondrial
L	Lard
LA	Linoleic acid
LAMP-2A	Lysosome-associated membrane protein 2
LC3	Microtubule-associated protein light chain 3
LDL	Low-density lipoprotein
LINC00116	long intergenic non-protein coding RNA 116

lncRNA	Long Noncoding RNA
MAMs	mitochondria associated membranes
mev-1	Succinate dehydrogenase cytochrome b560 subunit, mitochondrial
MFF	Mitochondrial fission factor
MFN1	Mitofusin-1
MFN2	Mitofusin-2
MiD49	Mitochondrial elongation factor 2
MiD51	Mitochondrial elongation factor 1
MIEF1	Mitochondrial dynamics protein MID51
MIEF2	Mitochondrial dynamics protein MID49
MIRO1	Mitochondrial Rho GTPase 1
MPP	Mitochondrial processing peptidase
mRNA	Messenger RNA
MTCO1	Mitochondrially encoded cytochrome c oxidase I
mtDNA	Mitochondrial DNA
mTERF	Mitochondrial transcription termination factor
mth	(Methuselah) G-protein coupled receptor
Mtln	Mitoregulin
mTOR	Mammalian target of rapamycin
MUFA	Monounsaturated fatty acids
Na	number of figures per area
NaCl	Sodium chloride
NAD+	Nicotinamide adenine dinucleotide (oxidized)
NADase	NAD+ nucleosidase
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NAFLD	Non-alcoholic fatty liver disease
NaOH	Sodium hydroxide
NASH	Non-alcoholic steatohepatitis
ND	NADH-ubiquinone oxidoreductase chain
ND4L	ubiquinone oxidoreductase core subunit 4L
NDUFA11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 11
NDUFB4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4
NDUFB8	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8
NDUFB9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 9
NFE2L2	Nuclear Factor, Erythroid 2 Like 2)
NIA	National Institute of Aging
NOXs	NADPH oxidases
NQO1	NAD(P)H-quinone oxidoreductase 1
NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
nuDNA	Nuclear DNA
Nv	Numerical density
O	Olive oil
O2	Oxygen
O2^●-	Superoxide ion radical
O2^2-	Peroxide ion

O3	Ozone
OMM	Outer mitochondria membrane
OPA1	Optic Atrophy 1/ Mitochondrial Dynamin Like GTPase
OXPHOS	Oxidative phosphorylation
p62/SQSTM1	Sequestosome-1
PARL	Presenilin associated rhomboid like
PARP	poly(ADP-ribose) polymerase
PCR	Polymerase chain reaction
PGAM5	Phosphoglycerate mutase 5
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PINK1	PTEN-induced kinase
PMSF	Phenylmethylsulphonyl fluoride
PNPLA3	Patatin-like phospholipase domain-containing protein 3
Polγ	DNA polymerase gamma
PPAR	Peroxisome proliferator-activated receptor
PRX	Peroxiredoxin
PTEN	Phosphatase and tensin homolog
PUFA	Polyunsaturated fatty acid
qPCR	Quantitative polymerase chain reaction
RET	Reverse electron transfer
RHM	Recessive hereditary methemoglobinaemia
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
Rnase	Ribonuclease
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
S	Soybean oil
SAEX	Servicio de Animales de Experimentación
SCs	Supercomplexes
SD	Standard deviation
SDHB	Succinate dehydrogenase (ubiquinone) iron-sulfur subunit
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFA	Saturated fatty acids
SH-SY5Y	Human neuroblastoma cells
SIRT	Sirtuins
SOD	Superoxide dismutase
SP1	Specificity protein 1
SSM	Subsarcolemmal mitochondria
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TFAM	Mitochondrial transcription factor A
TFB1M	Mitochondrial transcription factor B1
TFB2	Mitochondrial transcription factor B2
TG	Triglycerides
Tg	Transgenic mice
TM6SF2	Transmembrane 6 superfamily 2

TOM20	Translocase of the outer mitochondrial membrane 20
tRNA	Transfer RNA
TTBSL	Tween-Tris-buffered saline
UCP2	Mitochondrial uncoupling protein 2
UQCRC1	Ubiquinol-Cytochrome C Reductase Core Protein 2
UQCRC2	Cytochrome b-c1 complex subunit
UQCRFS1	Ubiquinol-Cytochrome C Reductase, Rieske Iron-Sulfur Polypeptide 1
UQCRQ	Ubiquinol-Cytochrome C Reductase Complex III Subunit VII
UV	Ultraviolet
VDAC1	Voltage dependent anion channel 1
VLDL	Very low density lipoprotein
Vv	Volume density
WNPRC	Wisconsin National Primate Research Center
WT	Wild type

Abstract

Introduction

Aging is a natural time-dependent process characterized by the accumulation of damage in cellular structures that compromises cellular and tissue function leading ultimately to the death of the organism. Furthermore, aging is the most significant risk factor for chronic diseases including cancer, cardiovascular disease (CVDs) and neurodegeneration. Nine candidates have been proposed as the main features of aging. These candidates which have been regarded as "*The Hallmarks of Aging*" are: telomere attrition, genomic instability, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, deregulated nutrient sensing, cellular senescence, stem cell exhaustion, and altered intercellular communication.

Given the great complexity of the changes that participate in the establishment of the aging phenotype, numerous theories have been proposed to explain its aetiology, but neither of them appears to be capable of fully explaining the origin of this process, and they often contradict each other. Among them, the *Free Radical Theory of Aging* proposes that oxidative stress and redox imbalance are factors directly related with aging. The general idea of this theory is to consider reactive oxygen species (ROS) and other reactive species, that are continuously generated in the organism, as the main cause of aging. Therefore, cellular aging is associated with chronic oxidative stress. ROS are products of normal cellular metabolism in mitochondrial respiration and can be also produced in defensive responses of cells against infectious agents, upon exposure to xenobiotics, cytokines, redox stress, and in a several of intracellular signaling pathways.

Mitochondria are both the main source and a major target of ROS. Mitochondria are one of the most important organelles in aerobic organisms, as the sites where aerobic respiration takes place to produce the energy that is necessary to sustain most cellular processes. Mitochondria also participate in additional cellular processes of great relevance, including β-oxidation of fatty acids, calcium signaling, and apoptosis. However, ROS and free radicals are generated as by-products of mitochondrial metabolism that attack nucleic acids, proteins, and membrane phospholipids. Among phospholipids, those containing polyunsaturated fatty acids (PUFAs) are more susceptible to oxidation by free radicals, leading to lipid peroxidation that causes a loss of membrane fluidity and impairs many cell functions. Additionally, with age,

mitochondrial enzymes, ATP production, and respiratory capacity decline and, at the same time, ROS and mitochondrial mutations increase, being these changes accompanied by alterations in morphology and abundance of these organelles. Mutations in mtDNA result in a loss of mitochondrial function and turnover. This leads to the autophagic digestion of defective mitochondria which, if not accompanied by efficient mitochondrial biogenesis, may lead to the consequent decrease in the synthesis of ATP and bioenergetic dysfunction.

Mitochondria are generated by growth and division of pre-existing organelles. In addition, they are highly dynamic structures that undergo continuous changes in size, shape, number, and distribution, which is highly controlled by a balance between fusion and fission. Their dynamics is determined by mitochondrial turnover, biogenesis, and a balance between the frequency of fusion and fission events. These events are critical for the normal function of cells, allowing the replacement of old or damaged mitochondria which are continuously removed from the cell. All these mechanisms allow the maintenance and distribution of mtDNA, the quality control of the organelle, and the regulation of apoptosis. Imbalanced mitochondria dynamics contributes to the loss of mitochondrial homeostasis, leading to alterations in mitochondrial function, metabolism, and signaling. Progressive mitochondrial dysfunction is considered a hallmark of aging, and it has been involved in a wide range of pathological conditions whose incidence increases with aging, as neurodegenerative diseases, diabetes, CVDs, muscle atrophy and cancer, and it is also related with several processes of embryonic development. Mitochondria are eliminated when they are damaged or dysfunctional through a process regulated called mitophagy.

Nutritional and genetic interventions are among the strategies currently followed by researchers to delay or even reverse the deleterious consequences of aging. Caloric restriction (CR) without malnutrition is the most effective nongenetic intervention that delays aging and increases lifespan in numerous organisms. Beneficial effects of CR on longevity are mainly due to an improvement in mitochondrial function. Furthermore, there is an important metabolic adaptation to CR, causing substantial changes in numerous signaling pathways that regulate growth, metabolism, repair of damage, oxidative stress, autophagy and inflammatory processes. Among these changes, CR increases the amount of monounsaturated fatty acids (MUFAs) in

membranes while decreasing PUFAs without any observed changes in saturated fatty acids (SFA), which is in accordance with the *Theory of Membranes in Aging* that proposes that lifespan is inversely related to the level of unsaturation, particularly the level of n-3 PUFA, in membrane phospholipids. Moreover, CR modulates key molecules involved in the regulation of autophagy and increases NAD^+ levels activating enzymatic activity of the sirtuins (SIRTs). Homeostasis of NAD^+ is important for the maintenance of many functions and protection against aging and age-related diseases. NAD^+ is a metabolic regulator and its efficacy has been reported in metabolic diseases such as insulin resistance, fatty liver or hypertension. The overexpression of enzymes capable of generating NAD^+ from the oxidation of NADH, as NADH-cytochrome b_5 reductase-3 (CYB5R3), is another strategy that is currently under evaluation.

CYB5R3 is a flavoprotein that participates in the elongation and desaturation of fatty acids, which seems to be an excellent candidate as a novel effector for the regulation of metabolism, mainly at mitochondrial level. CYB5R3 plays an important role in the regulation of metabolic pathways associated with healthspan and aging through mechanisms that appear to be related with protection against oxidative stress, production of cellular NAD^+ and alterations in fatty acids composition. Although CYB5R3 shows a constitutive basal expression in many tissues, its expression can be also induced in response to environmental stress and nutrient deprivation. Constitutive expression of the *Cyb5r3* gene is controlled by SP1 transcription factor, which can be further upregulated through pathways involving forkhead box O3 (FOXO3a) and nuclear factor erythroid 2-related factor 2 (NFE2L2) transcription factors. Mice overexpressing this protein showed increased longevity and displayed greater protection against diseases associated with aging. At the metabolic level, CYB5R3 overexpression increases insulin sensitivity, decreases inflammation and oxidative stress, and protects mice against cancer induction. Therefore, this enzyme plays a fundamental role in the regulation of lipid metabolism that allows to increase lifespan, suggesting that strategies aimed at incrementing CYB5R3 expression and/or activity may constitute viable interventions to confer protection against metabolic pathologies and to improve healthspan.

Dietary fats are essential components to generate energy. Fatty acids (FAs) can be categorized as saturated (SFAs), monounsaturated (MUFAs), or polyunsaturated

(PUFAs) and most of them can be synthesized by the organisms excepting the so-called essential fatty acids that must be ingested through the diet. In addition, FAs are an important source of energy due to their ability to be efficiently stored as triglycerides (TGs). Several studies have reported the relationship between dietary fat and the risk of metabolic and CVDs, among other disorders. Diets rich in MUFAs and PUFAs have cardioprotective effects, while the relationship between SFA and the risk of coronary heart disease is still controversial. Fatty acids can undergo autoxidation reactions generating lipid peroxides that affect the properties of biological membranes. Additionally, the toxic products derived from peroxidation can damage proteins, DNA, and other biomolecules. Since lipids are prone to oxidation, which exerts negative effects in health, the use of antioxidants in foods has become extensive to prevent lipid oxidation.

The liver and heart are closely related in health and in disease. Deregulated hepatic lipid metabolism and many metabolic diseases, such as diabetes and obesity, are ultimately detrimental to cardiac function.

The liver is the main organ in the regulation of energy metabolism. It performs three essential functions: the metabolic processing of proteins, carbohydrates and lipids after their absorption in the intestine, the detoxification of toxic agents and substances, and the storage of energy in form of glycogen. In spite of some changes occurring during aging (see below), the liver ages fairly well. Although in normal conditions hepatocyte turnover rate is low, the liver shows a relatively high rate of regeneration. That is why this organ has been widely used as a model to study the effects of diets, CR and other anti-aging interventions. Aging-related changes in liver include a reduction of weight and volume, a decline in the rate of hepatic regeneration, increased size of hepatocytes, polyploid nuclei, decreased area of smooth endoplasmic reticulum, decreased clearance of free radicals, and a decrease in the number and function of mitochondria, resulting in a decline in the ATP production. Aging is also associated with redistribution of fat in the body characterized by increased fat deposition in non-adipose tissues, including liver. All these structural and functional alterations could contribute to the development of liver disease with age.

The heart is the main organ of the circulatory system and one of the first organs to be formed during the embryonic development. The heart is a complex organ containing different cell types that contribute to the structural, biochemical, mechanical, and electrical properties of the organ. Among the cell types, cardiomyocytes, responsible for the contractile function of the myocardium, are well differentiated cells without regeneration capacity. The most drastic changes in heart function occurring with age are: left ventricular hypertrophy, alterations in the diastolic function, aortic valve calcification, arrhythmia and heart failure. These alterations are, at least in part, due to a decrease in the number of cardiomyocytes, as well to hypertrophy and fibrosis due to an increased collagen and alterations in the extracellular matrix. As postmitotic cells, cardiomyocytes are highly susceptible to age-related mitochondrial damage and ROS production which results in an overall enhancement in the rate of cardiomyocyte death with age. The progressive decline of cardiomyocyte mitochondrial function is considered the major mechanism underlying heart aging, in addition to the accumulation of cholesterol and fatty acids in tissues which lead to the production of inflammatory cytokines as well as ROS that further contribute to CVDs prevalence and mortality.

Starting hypothesis and objectives

Previous studies developed in our group were focused on NADH-cytochrome b_5 reductase-3 (*Cyb5r3*) as a new pro-longevity gene. Mice overexpressing CYB5R3 contain higher levels of polyunsaturated fatty acids in hepatic membranes, which resembles, at least partially, the effects of the consumption of a diet containing fish oil or the overexpression of the *Fat-1* gene of *C. elegans*. Unsaturated fatty acids show high susceptibility to peroxidation. However, CYB5R3 transgenic mice exhibited a significant reduction in lipid peroxidation. The specific changes in FA composition that take place in mice overexpressing CYB5R3 may be the result of a metabolic reprogramming, and could be indicative of an improvement in mitochondrial function, leading to a lower generation of ROS and the prevention of the oxidative damage accumulation, which partially mimics the effects observed in animals subjected to CR. These mice showed increased insulin sensitivity and improved regulation of glucose homeostasis, less inflammation and decreased oxidative damage, and were protected against induced cancer. However, mice over-

expressing CYB5R3 fed a standard diet *ad libitum* were fatter than wild-type controls, and preferentially use carbohydrate to meet their energy needs. This differs from studies with CR as antiaging intervention that showed an improvement in healthspan and lifespan due to the enhancement of β-oxidation as a preferential source of energy. Thus, CYB5R3 overexpression could contribute to extend lifespan in mice by mechanisms that may be independent of those described for CR. Since CYB5R3 increase has been reported in long-term CR intervention, it has been hypothesized that the beneficial effects of CR could be mediated, at least partially, by a such increase of CYB5R3. However, it has not yet been studied how the CYB5R3 overexpression affects mitochondrial processes that are relevant to determine the aging pattern, and how the alterations in fatty acid composition and CR can interact with the changes elicited by CYB5R3 overexpression in transgenic mice. The working hypothesis of this PhD is that mitochondrial efficiency is optimized in CYB5R3 transgenic mice, which leads to an improvement in energy production and better preservation of mitochondria during aging. For this reason, our main objective has been to study several mitochondrial function markers in liver and heart tissues of CYB5R3-overexpressing mice submitted to nutritional interventions (CR and alterations in fat composition of the diet) and aging.

To this end, we have pursued the following 5 specific aims:

1. To study the physiological effects of CYB5R3 overexpression, dietary fats, CR and aging on body composition and biochemical profile.
2. To characterize the CYB5R3 overexpression in liver and heart in the three proposed interventions.
3. To determine the combined effect of CYB5R3 overexpression and CR on key markers of mitochondrial function.
4. To determine dietary conditions that maximize mitochondrial preservation in mice overexpressing CYB5R3.
5. To determine the effects of aging on the mitochondria parameters studied in the previously indicated aims in CYB5R3 overexpressing mice.

The results of this PhD can help identify healthy lifestyles and nutritional patterns that can modulate healthy aging and extend longevity, as well as to identify new

mitochondrial targets that allow the preservation of mitochondrial physiology and aid in the prevention of metabolic diseases associated with age.

Material and Methods

For this study, male mice of the C57BL/6J strain and overexpressing the cytochrome b_5 reductase 3 gene (*Cyb5r3*) were used. Three cohorts of mice were established. All cohorts consisted of transgenic mice overexpressing CYB5R3 (Tg mice) and wild-type mice of the same genetic background (WT mice). These WT mice were obtained from the same litters of Tg mice and used as controls. The starting male Tg mice that allowed us to establish our colony at the Servicio de Animales de Experimentación (SAEX) of the University of Córdoba were kindly provided by Dr. Rafael de Cabo (Translational Gerontology Branch, National Institute on Aging, Baltimore, Maryland, USA).

All mice were housed in sterile filter-capped cages in a fully controlled environment with 12 hours light/dark cycle at 22 °C with free access to water and were fed a standard chow until they reached the age of three months. Mice were then transferred to the experimental diets. During intervention, the animals were group-housed, excepting those mice belonging to caloric-restricted (CR) groups, that were housed individually to have an exhaustive control of the intake.

A first cohort, called ***the aging intervention groups,*** consisted of two experimental groups as a function of age. These mice, were fed *ad libitum* for 4 or 21 months respectively (reaching 7 or 24 months of age at the end of the intervention) with standard AIN93M diet (containing soybean oil as main source of fat).

A second cohort, called ***the dietary fat intervention groups*** consisted of four experimental groups that differed in the source of fat used for diets formulation. These mice were fed ad libitum for 4 months (7 months of age at the end of the intervention) with an AIM93M-based diet containing either soybean, lard, olive oil or fish oil as the main source of fat.

A third cohort called ***the caloric restriction intervention groups*** consisted of two experimental groups that differed in calory intake. Both groups of mice were fed for 4 months (reaching 7 months of age at the end of the intervention) with standard

AIM93M diet (containing soybean oil). The two dietary groups of this cohort were *ad libitum* and caloric restriction (CR) with a 40% reduction of the *ad libitum* intake.

The 7-months old mice fed *ad libitum* with the standard AIM93M diet were the same in all the cohorts, and used as controls. All diets were prepared monthly by the Animal Experimentation Service of the University of Granada (Spain) and stored at −20 °C to minimize fatty acid oxidation.

At the end of each intervention, mice were fasted for 24 hours and anesthetized with isoflurane. Blood was then withdrawn by cardiac puncture to allow the measurement of several biochemical parameters, and mice were finally euthanized by cervical dislocation. The liver and heart were quickly dissected and weighed for subsequent biochemical and ultrastructural analysis. The biochemical analysis included blood biochemicals measurement that were determined by the personnel of the SAEX, determinations of protein expression markers representing key mitochondrial pathways (performed though immunoblotting), measurement of the mitochondrial-to-nuclear DNA ratio (mt/nuDNA) and ATP determinations. Structural analysis were carried out by transmission electron microscopy:

For immunoblotting, a thin slice of liver and heart were cut off and homogenized in radioimmunoprecipitation assay (RIPA) buffer at 4ºC, which contains protease and phosphatase inhibitors. The tissues were homogenized using Qiagen TissueLyser II Retsch MM400 (Qiagen) at 2.5 Hz during 1.5 min. Then, the homogenates were centrifuged to separate supernatants containing the protein extracts. Afterwards, the samples were sonicated and stored frozen at −80 °C until subsequent use.

The total amount of protein in the extracts was determined by using the Stoscheck modification of the dye-binding method of Bradford, and 50 μg of protein extracts were then used for Western blot immunodetection. Proteins were loaded and separated by electrophoresis in Criterion TGX 4-20% polyacrylamide gradient Precast Gels. The proteins were transferred to nitrocellulose membranes and, after blocking unspecific binding sites, they were incubated with the corresponding primary antibody overnight at 4 ºC with shaking. After washing, the membranes were incubated during 1h with the corresponding species-specific secondary antibody at room temperature. Finally, chemiluminescent signal was recorded using a ChemiDoc Imaging System (Bio-Rad).

For quantitative polymerase chain reaction (qPCR), tissue genomic DNA (gDNA) was isolated using the kit NucleoSpin Tissue (Macherey-Nagel) according to the manufacturer's instructions. The gDNA concentration was measured using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) and a final concentration of 10 ng/µl was used for qPCR. A set of genes were measured to evaluate the relative copy number of mtDNA and nDNA and the mtDNA/nDNA ratio was calculated using the ΔΔCt method.

For measurement of ATP levels, 15-20 mg of tissue sample were homogenized in RIPA buffer and the ATP concentration was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Tissue processing for electron microscopy was performed by a standard embedding protocol for electron microscopy. Thin sections of liver and heart tissues were obtained and mounted on nickel grids and stained with uranyl acetate and lead citrate. The sections were viewed and photographed using a Jeol Jem 1400 electron microscope at the Servicio Centralizado de Apoyo a la Investigación (SCAI, University of Córdoba). Planimetric data of mitochondria were obtained using ImageJ software (NIH, USA). Stereological data were obtained from the micrographs by a pint counting method.

Results and discussion

Aging is associated with increased fat deposition and distribution, mainly affecting visceral fat, which may result in negative health consequences. In our cohort, CYB5R3- Tg mice had similar body weights to WT animals after 4 months of *ad libitum* feeding with diets containing different dietary fats. However, we found striking variations depending on the source fat: i.e., a lard-rich diet increased the weight of WT mice compared to a soybean oil-enriched diet, and an increase in the body weight of Tg mice fed with fish oil-based diet was also observed.

Interventions leading to a reduction in body fat have been associated with life span extension. As expected, CR produced a significant decrease of body weight and this effect was observed in both genotypes. However, CYB5R3 overexpression led to a greater preservation of fat. According with previous reports, the maintenance of body weight in Tg animals on CR may be due to a higher accumulation of fat in these

animals in comparison with WT mice, due to the preferential use of carbohydrate in mice overexpressing CYB5R3 to meet their energy demands. This effect could also account for the beneficial effect of CYB5R3 overexpression on lifespan. In agreement with our observations, a recent study showed a positive correlation between mouse longevity and the preservation of body fat with aging.

Previous studies have reported that organs weight decrease with CR regardless the fat source in the diet. Although a CR intervention indeed produced a significant decrease of body weight in both genotypes, CYB5R3 overexpression led to a greater preservation of body weight which was also reflected in all the tissues. Thus, CYB5R3 emerges for the first time as a key determinant in the control of both organ and body weight in mice under CR.

Despite the fact that in young mice there were no significant changes in body weight regardless of the genotype, our longitudinal study revealed that Tg mice gained more weight as they became aged. The increase of unsaturated fatty acids achieved in CYB5R3 Tg mice due to a higher desaturase activity might inhibit β-oxidation, which could explain the observed changes in body weight of Tg mice with aging.

We next analysed the levels of CYB5R3 polypeptide in the two tissues studied here, and in the three contexts (alteration of dietary fat, CR, and aging). We found that CYB5R3 polypeptide levels were highly influenced by dietary fat in mitotic organs such as liver, probably related with the desaturase activity of this enzyme. In the liver, PUFAs decreased the abundance of this enzyme, as it was observed with soybean oil and, particularly, with fish oil where we found that the lowest levels of CYB5R3 regardless of genotype. No differences were detected when comparing WT and Tg mice within each dietary group. In heart, dietary fat did not affect CYB5R3 levels and, contrary to what was found in liver, a clear overexpression of CYB5R3 was found in Tg mice in all dietary conditions. On the other hand, CR did not affect CYB5R3 levels in heart but a decrease was found in liver, particularly in the case of Tg mice. A totally different pattern was observed with age, where an increase of CYB5R3 content was observed both in liver and heart from old animals. These observations point out the existence of tissue- and age-specific mechanisms regulating CYB5R3 levels by CR or diet, being post-mitotic tissues (as heart) the

most appropriate models to study the direct effects of CYB5R3 overexpression on mitochondrial physiology.

Regarding the effects of CR on different markers of mitochondrial dynamics and biogenesis in WT and Tg mice, we found that this intervention did not modify mitochondrial abundance in liver, but MFN2, TFAM, and the relative mt/nu DNA ratio were dramatically increased, and NRF1 also tended to increase in WT mice. These observations are partially in accordance with previous research documenting the upregulation of hepatic mitochondrial biogenesis parameters in CR fed mice. Of note, these changes were prevented by CYB5R3 overexpression, that produced a decrease of the mitochondrial biogenesis-related transcription factor NRF1. CYB5R3 overexpression also induced an increase in the average mitochondrial area in the two dietary conditions, which might be related with an improved mitochondrial function if, as previous studies have proposed, an increased size is accompanied by increased number of mitochondrial cristae. The observations reported here are, however, in contrast with other studies carried out by our group based on a quantitative approach using electron microscopy, which documented that a 6-month CR intervention based on the AIN93G diet was sufficient to increase mitochondrial abundance in mouse liver. The differences in mitochondrial abundance between these studies could be due to various factors as the age of the animals and the amount of lipids included in the diet (AIN93M vs. AIN93G diets). Moreover, controversial results regarding changes of mitochondrial mass with CR and aging may also arise from the heterogeneity in the methods used to assess mitochondrial content.

Regarding the effect of the interventions on the abundance of mitochondrial complexes of the electron transport chain, we observed an increase of complex III by CR both in WT and Tg mice, with a much larger effect seen in the case of mice overexpressing CYB5R3. Additionally, a general effect of CR increasing the abundance of mitochondrial complexes independently of the genotype was observed for complex IV (with significant differences between the two groups of Tg mice) and complex V. Together, these data suggest that mitochondrial function, rather than its structure or abundance, may be primarily affected by CYB5R3 overexpression in liver.

In heart, CR strongly increased mitochondrial mass, as well as the levels of the mitofusin MFN1 and the mitochondrial biogenesis-related transcription factors TFAM and NRF1. Once again, overexpression of CYB5R3 appeared to counteract the effect of CR on these parameters. These data contrast however with those obtained from electron microscopy studies, showing that mitochondrial mass was similar in all the experimental groups. However, due to the existence of two different populations of mitochondria in cardiomyocytes, the possibility exists that aging and/or CR, or even CYB5R3 overexpression, differentially affect these populations. Electron microscopy can discriminate both populations allowing separate quantitative studies, whereas protein quantification was performed on heart total homogenates, and the results are therefore referred to both mitochondrial subpopulations considered together.

In the heart, we did not detect any change in protein levels of mitochondrial complexes, except for complex IV in WT mice fed under CR. Our results support that CYB5R3 overexpression significantly modifies the metabolic adaptations of mice to CR. In other studies, CR has been shown to enhance efficiency of complex IV in skeletal muscle, a phenomenon also reported to occur in heart. These results are likely related to a higher affinity and an increase in the binding sites of O_2. Under these conditions, both electrons flow and redox state of the complexes are improved resulting in decreased generation of mitochondrial ROS.

Our data support the idea that many beneficial effects of CR are determined by a better preservation of the physiology of the mitochondria and its ultrastructural features which results, not only in lower production of ROS, but also in the protection of this organelle from mitophagy. These outcomes are partially mimicked by CYB5R3 overexpression in mice fed under *ad libitum* conditions. Nevertheless, as we stated in our initial hypothesis, the accrual of beneficial effects produced by CR and CYB5R3 overexpression appear to operate by different metabolic pathways when studied separately and, when both interventions are applied simultaneously, their effects become partially blunted.

Across different species, those animals with greater longevity show lower degree of fatty acid unsaturation in tissues. Decreased PUFAs in mitochondrial membrane phospholipids results in lower susceptibility to peroxidation, potentially contributing

to the increased lifespan and healthspan of CR mice. We thus investigated in liver and heart the role of different dietary fats and CYB5R3 overexpression on several markers related with mitochondrial metabolism.

In liver, diets rich in MUFA lead to increased mitochondrial fusion markers, especially in CYB5R3 Tg mice, meanwhile a diet rich in SFA exerted the opposite effect, which might be related with an accumulation of free FA, and with the development of obesity and insulin resistance in WT mice fed on the lard-based diet. On the other hand, we found a positive effect of a lard-based diet in Tg mice, since increased VDAC1 and decreased MFF could be indicative of a change in mitochondrial function towards a healthier phenotype and the enhancement of mitophagy pathway. In contrast with the data obtained in the lard-enriched diet, we observed a significantly decrease in the mitochondrial fission marker MFF, compatible with an improvement of mitochondrial function in the olive oil-, soybean oil- and fish oil-based diets. A shift towards mitochondrial fusion has been associated with enhanced mitochondrial function and ATP production. In our experimental groups, in general, we did not observe changes in ETC protein levels. Based on our results and the current literature, we propose that dietary fats target respiratory enzyme activity, supercomplexes formation, or both, rather than modulate protein levels of ETC complexes in liver tissue.

In heart, a postmitotic tissue, we did not observe pronounced changes regarding mitochondrial dynamics and biogenesis markers, except in the case of mice fed on the fish oil-enriched diet. Heart presents abundant large mitochondria and highly expressed fusion proteins that improve mitochondrial metabolism and compensate the appearance of mtDNA mutations in heteroplasmic cells. An increase in proteins involved in mitochondrial fusion was observed in the case of mice fed on PUFA-rich diets, which could be related with the protective beneficial effects of these edible oils for the maintenance of heart function. However, we found that CYB5R3 overexpression in Tg mice apparently eliminated this effect in a context the fish oil-enriched diet consumption, but enhanced fusion in a context of the olive oil-based diet. Regarding mitochondrial respiratory complexes, no major changes have been found except for the increment in the levels of some ETC markers in heart from mice fed the fish oil-based diet, which could be related with the reported benefits of these fatty acids against cardiovascular diseases

We suggest that CYB5R3 can produce different effects on mitochondrial function depending on the dietary fat source used. Although overexpression of CYB5R3 does not appear to confer a marked phenotype by itself, in the case of mice fed the lard-based diet it prevents mitochondrial fragmentation, which could be indicative of a healthier mitochondria state compared to their WT counterparts. In the case of mice fed the olive oil-enriched diet, CYB5R3 overexpression appears to promote a healthy phenotype by increasing mitochondrial fusion proteins. However, some interference between CYB5R3 overexpression and the consumption of a fish oil-enriched diet appears to exist, and some of the beneficial actions of each isolated intervention may be hampered, as evidenced by a decrease in mitochondrial dynamics markers and greater weight gain.

The liver and heart are organs that are closely related in health and in disease, and mitochondria of both tissues are deeply affected in aging. There are two mechanisms by which the cell can compensate mitochondrial damage. On the one hand, mitofusins are highly expressed in tissues with high energetic requirements and rich in mitochondria, such as the heart and skeletal muscle, and tend to maintain a fused mitochondrial network that compensates the appearance of mtDNA mutations in heteroplasmic cells (through a mixture of content that dilutes mitochondrial dysfunction), contributing to the energy dissipation in cells, and improving the tethering of the ER to the mitochondria. However, other tissues, tend to have a more fragmented network which serves to eliminate damaged mitochondria by mitophagy in order to maintain their bioenergetic capacity. Both mechanisms coexist and are regulated by the energy demand of the cell. We thus studied the effect of CYB5R3 overexpression on markers of hepatic and cardiac mitochondrial dynamics and metabolism in a context of aging,

Our data in liver yielded mixed results since aging induced a trend towards increased levels of MFN2 (a change that was not observed in Tg mice) without changes in MFN1, suggesting different adaptation mechanisms to aging in WT compared with Tg mice. In this regard, we suggest that the hepatic mitochondrial network of old Tg mice is balanced towards enhanced fission, which would then indicate the occurrence of greater mitochondria fragmentation to facilitate its autophagic elimination. However, in WT animals, our results are compatible with a response against aging-induced stress, promoting fusion mechanisms to improve mitochondrial function.

Several studies have shown an age-related decrease in mitochondrial complexes activity, being complexes I and IV the most affected. This effect may be due to several factors: modification of proteins by ROS-driven damage, inhibition of enzymatic activities by products generated during aging, or even to decreased expression of proteins. Our analysis of protein levels of mitochondrial complexes subunits in liver indicated an overall increase with age in mice of both genotypes, although the changes were more pronounced in Tg mice. However, it is important to acknowledge that the activity of complexes was not analysed, and the possibility exists that the increase in the protein levels of the complexes observed in these mice with to aging could be related with a need to relieve a dysfunctional enzymatic activity.

Some studies have shown that age-related changes are more evident in post-mitotic tissues. We found a dramatic increase in MFN2 in heart from old WT mice whereas, conversely, a decrease was observed in mice overexpressing CYB5R3, an effect that could have a positive outcome in these animals. Although mitochondria from post-mitotic organs tend to be in a more fused state to improve their function, the persistence of the fusion/fission balance in favour of fusion may cause a "hyper-fused" state and that can produce a pathological effect. On the other hand, we evidenced that most of the complexes were increased in aged heart, and their levels were even higher in Tg mice, which depicts a pattern similar to that observed in liver, excepting for complex IV, which was decreased in CYB5R3-overexpressing mice. There is evidence that complexes I, III, and IV (but not complex II), together with the electron carriers, coenzyme Q and cytochrome *c*, can be arranged in different combinations at the inner mitochondrial membrane to produce supercomplexes, which provide kinetic advantages to the electron transfer. Previous studies have shown that murine hearts showed a loss of supercomplexes with age while electron transport chain complex subunits protein levels remained stable. However, other studies have shown that the loss of complexes activities is more relevant than the variation in the amount of protein. Thus, alterations in complex IV function, which is the last component to be incorporated into supercomplexes, could destabilize the assembling of supercomplexes. According to this hypothesis, the decrease in complex IV levels found in Tg mice could imply the stabilisation of these structures.

As a whole, the results presented in this Thesis constitute the first study that has been set up to analyse the effect of dietary fat, CR and aging on the outcome of CYB5R3 overexpression on key markers related with mitochondrial function and ultrastructure. The data so obtained have allowed us to demonstrate the existence of tissue-dependent mechanisms for the modulation of CYB5R3 levels, which is also influenced by dietary interventions and by aging. However, the results presented in this PhD Thesis will require a more in-depth study focused on physiological parameters related with mitochondrial function in order to verify the hypothesis that CYB5R3 overexpression leads to an improved energy production with less generation of ROS, less apoptotic signaling, and better mitochondrial preservation with aging. It is planned to continue the analysis of the effects of CYB5R3 overexpression on other aging and mitochondria related parameters, as mitophagy and apoptosis processes, as well as to extend our studies to other tissues as skeletal muscle and kidney (in process). On the other hand, it is also important to acknowledge that this study has been focused exclusively on male mice. It is thus expected to expand our studies to female mice in order to elucidate the possible existence of sexual dimorphism in the outcomes of CYB5R3 overexpression.

Resumen

Introducción

El envejecimiento se define como el declive funcional dependiente del tiempo que se caracteriza por la acumulación de daños en las estructuras celulares, comprometiendo la función celular y tisular dando lugar, en último término, a la muerte del organismo. El envejecimiento es el principal factor de riesgo de las enfermedades crónicas incluyendo cáncer, enfermedades cardiovasculares (CVDs) y neurodegenerativas. Se han propuesto nueve causas principales por las que se produce el envejecimiento: las denominadas "marcas distintivas del envejecimiento", que son las siguientes: acortamiento telomérico, inestabilidad genómica, modificaciones epigenéticas, pérdida de la proteostasis, disfunción mitocondrial, desregulación de la detección del estado nutricional, senescencia celular, alteración de la comunicación intercelular y perdida de células madre.

Para tratar de explicar los cambios celulares que dan lugar el fenotipo del envejecimiento se han propuesto numerosas teorías, pero, debido a la complejidad de este proceso, ninguna de ellas puede explicar completamente el origen del envejecimiento. De estas teorías, la llamada *Teoría de los Radicales Libres* propone que el estrés oxidativo y el desajuste en el estado redox del organismo dan lugar al envejecimiento. Esta teoría considera que las especies reactivas de oxígeno (ROS) y otras especies reactivas (que están continuamente generándose en el organismo) son la principal causa del envejecimiento. Por lo tanto, el envejecimiento celular estaría provocado por un estrés oxidativo crónico. Las ROS son productos del metabolismo celular producidos por la respiración mitocondrial, respuesta defensiva de las células frente a agentes infecciosos, exposición a xenobióticos, respuesta a citoquinas y estrés redox. Además, actúan como moléculas de señalización celular.

Las mitocondrias son la mayor fuente de ROS y las principales afectadas por el daño oxidativo que estas moléculas pueden llegar a causar. Además, son uno de los orgánulos más importantes en los organismos aeróbicos, que se encargan de la respiración celular y producen la energía necesaria para las funciones vitales de la célula. Las mitocondrias también participan en otros procesos celulares de gran importancia como la β-oxidación de ácidos grasos, homeostasis de calcio y apoptosis. Sin embargo, las ROS y los radicales libres son productos del metabolismo mitocondrial que, debido a su alta reactividad, interaccionan con los ácidos nucleicos,

proteínas y fosfolípidos de membranas, dañando sus estructuras. Los fosfolípidos de membrana que contienen ácidos grasos poliinsaturados (PUFA) son los más susceptibles de ser oxidados por los radicales libres, dando lugar a la peroxidación lipídica, que altera las características de las membranas biológicas y afecta considerablemente a la función celular.

Por otro lado, con el envejecimiento, las enzimas mitocondriales, la producción de ATP y la capacidad respiratoria de la célula disminuyen, a la vez que aumenta la producción de ROS. Los daños producidos en las mitocondrias dan lugar a mutaciones y alteraciones en la morfología y abundancia de estos orgánulos. Las mutaciones en el DNA mitocondrial (mtDNA) dan lugar a una pérdida de la función mitocondrial y a una desregulación de su recambio en la célula. El recambio mitocondrial se produce mediante la eliminación de mitocondrias dañadas por el proceso de autofagia, y la síntesis de nuevas mitocondrias a través de la biogénesis mitocondrial. Alteraciones en alguno de esos procesos dan lugar a una disminución de la síntesis de ATP y disfunción bioenergética.

La biogénesis mitocondrial se produce por el crecimiento y división de mitocondrias preexistentes. Además, estos orgánulos son altamente dinámicos y están constantemente cambiando de forma, tamaño, número y distribución en la célula. Esta dinámica está controlada por un balance entre los procesos fusión y fisión mitocondrial, siendo estos eventos críticos para el correcto funcionamiento de las células que, a su vez, permiten el reemplazo de las mitocondrias viejas o dañadas. Todos esos mecanismos, además, participan en la distribución del DNA mitocondrial, en el mantenimiento del orgánulo en condiciones óptimas y en la regulación de la apoptosis. Un desajuste en la dinámica mitocondrial da lugar a la perdida de la homeostasis del orgánulo, provocando alteraciones en su funcionamiento, metabolismo y señalización. La disfunción mitocondrial progresiva está considerada una marca distintiva del envejecimiento e involucra una gran variedad de condiciones patológicas que aumentan con el envejecimiento como son las enfermedades neurodegenerativas, diabetes, CVDs, atrofia muscular y cáncer. También están implicadas en numerosos procesos del desarrollo embrionario. La eliminación mitocondrial por autofagia es un proceso altamente regulado llamado mitofagia.

Con el fin de retrasar o revertir los efectos deletéreos del envejecimiento, numerosas intervenciones nutricionales y genéticas están siendo estudiadas. Entre ellas, la restricción calórica (CR) sin malnutrición es la intervención no genética más efectiva que retrasa el envejecimiento e incrementa la vida máxima en numerosos organismos. Los efectos beneficiosos de la CR sobre la longevidad se deben en gran parte a una mejora en la función mitocondrial. Además, la CR produce adaptaciones metabólicas muy importantes dando lugar a cambios en numerosas rutas de señalización que participan en el crecimiento, metabolismo, mecanismos de reparación de daños, estrés oxidativo, autofagia y procesos inflamatorios. Entre esos cambios, la CR aumenta el contenido de ácidos grasos monoinsaturados (MUFA), disminuye el contenido de PUFA y no provoca cambios en la cantidad de ácidos grasos saturados (SFA) en las membranas biológicas, lo que apoya la *Teoría de las Membranas* en el envejecimiento que establece que la longevidad máxima esta inversamente relacionada con el nivel de insaturación de las membranas biológicas, particularmente con los niveles de PUFA n-3.

Por otro lado, la CR modula moléculas clave que participan en la regulación de la autofagia e incrementa los niveles de NAD^+ resultando en la activación de enzimas como las sirtuinas. La homeostasis de NAD^+ es importante para el mantenimiento de muchas funciones y protección frente al envejecimiento y de las enfermedades relacionadas con éste. El NAD^+ es un regulador metabólico y su eficacia ha sido probada en enfermedades metabólicas como la resistencia a insulina, hígado graso o hipertensión. La sobreexpresión de enzimas capaces de generar NAD^+, como la NADH citocromo b_5 reductasa 3 (CYB5R3), es una estrategia que está siendo estudiada actualmente.

La CYB5R3 es una flavoproteína que participa en la elongación y desaturación de ácidos grasos, lo que la hace una excelente candidata para la regulación del metabolismo, principalmente a nivel mitocondrial. La CYB5R3 tiene un rol fundamental en la regulación de rutas metabólicas asociadas con la prolongación de la vida saludable y el envejecimiento a través de mecanismos que parecen estar relacionados con la protección frente a estrés oxidativo, la producción de NAD^+ celular y las alteraciones en la composición de ácidos grasos. A pesar de que esta proteína tiene una expresión basal constitutiva en muchos tejidos, puede ser también inducida en respuesta a estrés ambiental y deprivación de nutrientes. La expresión

constitutiva del gen *Cyb5r3* está controlada por el factor de transcripción SP1, siendo además regulada por los factores de transcripción FOXO3a y NFE2L2. Los ratones que sobrexpresan CYB5R3 muestran una mayor longevidad y una mayor protección frente enfermedades asociadas con el envejecimiento. A nivel metabólico, la sobreexpresión de esta enzima aumenta la sensibilidad a insulina, disminuye la inflamación y el estrés oxidativo y protege a los ratones frente a la inducción de cáncer. Además, esta enzima tiene un papel fundamental en la regulación del metabolismo lipídico, lo que permite aumentar la esperanza de vida, sugiriendo que aquellas estrategias centradas en aumentar su expresión y/o actividad pueden constituir intervenciones viables que confieran protección frente a patologías metabólicas y prolonguen la vida saludable.

Los ácidos grasos son componentes de la dieta esenciales para generar energía. Los ácidos grasos pueden ser categorizados como saturados (SFA), monoinsaturados (MUFA) y poliinsaturados (PUFA) y la mayoría de ellos pueden ser sintetizados por el organismo, a excepción de los llamados ácidos grasos esenciales, que solamente pueden ser obtenidos a través de la ingesta. Además, los ácidos grasos son fuentes importantes de energía que pueden ser almacenados en forma de triglicéridos. Numerosos estudios han informado de la relación de los ácidos grasos de la dieta con el riesgo de enfermedades metabólicas y cardiovasculares, así como con otros trastornos. Las dietas ricas en MUFA y PUFA tienen efectos cardioprotectores, mientras que la relación entre SFA y el riesgo de sufrir enfermedades cardiovasculares sigue en debate. Los ácidos grasos pueden dar lugar a reacciones de autooxidación provocando peroxidación lipídica, lo que afecta a las propiedades de las membranas biológicas. Además, los productos tóxicos derivados de la peroxidación lipídica pueden dañar proteínas, DNA y otras biomoléculas. Debido a que los lípidos son propensos a la oxidación y causar efectos negativos en la salud, se ha extendido el uso de antioxidantes en los alimentos para prevenir su oxidación.

El hígado y el corazón son dos órganos estrechamente relacionados en la salud y enfermedad. La desregulación del metabolismo lipídico hepático y muchas enfermedades metabólicas como la diabetes y la obesidad son, en última instancia, perjudiciales para la función cardíaca.

El hígado es el principal órgano que regula el metabolismo energético desempeñando tres funciones fundamentales: el procesamiento metabólico de proteínas, carbohidratos y lípidos tras su absorción en el intestino, la detoxificación de agentes tóxicos y fármacos, y el almacenamiento de energía en forma de glucógeno. El hígado envejece relativamente bien a pesar de algunos cambios que ocurren con el envejecimiento. En condiciones normales el hígado tiene una tasa de regeneración bastante alta, a pesar de que el recambio de sus hepatocitos es relativamente bajo. Es por ello que el hígado ha sido muy utilizado por los investigadores como modelo de estudio del efecto de las dietas, la CR y otras intervenciones antienvejecimiento. Los cambios relacionados con la edad que experimenta el hígado incluyen una reducción del peso y volumen del órgano, el aumento del tamaño de los hepatocitos, la aparición de núcleos poliploides, una disminución del área del retículo endoplásmico liso, una menor capacidad de eliminación de ROS y la disminución en el número y función de las mitocondrias, procesos que tienen como resultado un declive de la producción de ATP. El envejecimiento ha sido asociado con la redistribución de la grasa del cuerpo, caracterizado por un incremento en la deposición de grasa visceral, incluido el hígado. Todos esos cambios estructurales y funcionales pueden contribuir a desarrollar enfermedades hepáticas con la edad.

El corazón es el órgano principal del sistema circulatorio y, debido a su importancia, es uno de los órganos que se forman en primer lugar durante el desarrollo embrionario. El corazón es un órgano complejo formado por diferentes tipos celulares que dan lugar a sus distintivas propiedades estructurales, bioquímicas, mecánicas y eléctricas. Los cardiomiocitos son las células responsables de la función contráctil del miocardio y son células altamente diferenciadas sin capacidad de regeneración. Los cambios más drásticos que se producen en la función cardiaca con el envejecimiento son: hipertrofia del ventrículo izquierdo, alteraciones de la función diastólica, calcificación de la válvula aórtica, arritmia y fallo cardiaco. Esas alteraciones son, en parte, debido a una disminución en el número de cardiomiocitos, así como la hipertrofia y fibrosis provocadas por un incremento en el colágeno y por las alteraciones de la matriz extracelular. Como células postmitóticas, los cardiomiocitos son altamente susceptibles a los daños mitocondriales relacionados con el envejecimiento y producción de ROS, lo que potencia su muerte con la edad. El declive progresivo de la función mitocondrial de los cardiomiocitos es considerado

la señal más clara de envejecimiento en el corazón. Por otra parte, la acumulación de colesterol y ácidos grasos en los tejidos da lugar a la producción de citoquinas inflamatorias, así como a ROS, que contribuyen a la prevalencia y mortalidad de las CVDs.

Hipótesis inicial y objetivos

Estudios previos desarrollados en nuestro grupo se han centrado en como un nuevo gen con efectos pro-longevidad. Los ratones que sobre-expresan CYB5R3 presentan un número elevado de PUFA en las membranas hepáticas, efecto que se asemeja (al menos parcialmente) a los producidos por el consumo de una dieta rica en aceite de pescado o por sobreexpresión de la desaturasa *Fat-1* de *C.elegans*. Los ácidos grasos insaturados son altamente susceptibles a la peroxidación. Sin embargo, los ratones transgénicos que sobre-expresan CYB5R3 muestran una reducción significativa en la peroxidación lipídica. Los cambios específicos en la composición de ácidos grasos que tiene lugar con la sobreexpresión de esta proteína pueden ser el resultado de un reajuste en el metabolismo, lo que además podría indicar una mejora en la función mitocondrial dando lugar a menos generación de ROS y la prevención de acumulación de daño oxidativo, efectos que mimetizan parcialmente a los observados en los animales sometidos a CR. Esos ratones transgénicos muestran una mayor sensibilidad a la insulina y una mejora en la homeostasis de la glucosa, menor inflamación, disminución del daño por estrés oxidativo y mayor prevención frente a la inducción de cáncer. Sin embargo, los ratones que sobre-expresan CYB5R3 alimentados *ad libitum* con una dieta estándar presentaron mayor peso corporal que sus controles y preferentemente obtenían energía del metabolismo de los carbohidratos. Por el contrario, los estudios centrados en la CR como intervención antienvejecimiento han mostrado una mejora en la prolongación de la vida saludable y de la longevidad máxima debido a una mayor utilización de la β-oxidación de ácidos grasos como fuente principal de energía, lo que difiere de los mecanismos por los que actúa la sobreexpresión de CYB5R3. Por lo tanto, la sobreexpresión de CYB5R3 puede contribuir a prolongar la vida máxima en ratones por mecanismos independientes de los descritos por la CR. Por otra parte, se ha informado que una intervención de CR a largo plazo induce la sobreexpresión de CYB5R3, por lo que se piensa que el efecto beneficioso de la CR podría estar mediado, al menos en parte,

por el incremento de CYB5R3. Sin embargo, no ha sido estudiado aún cómo la sobreexpresión de CYB5R3 afecta a los procesos mitocondriales que son relevantes para determinar la pauta de envejecimiento. Tampoco se ha descrito cómo las alteraciones en la composición de ácidos grasos y la CR podrían interaccionar con los cambios provocados por la sobreexpresión de CYB5R3 en ratones transgénicos.

La hipótesis de trabajo de esta Tesis Doctoral es que la eficiencia mitocondrial está optimizada en ratones transgénicos que sobre-expresan CYB5R3, lo que da lugar a una mejora en la producción de energía y una mayor preservación de la mitocondria durante el envejecimiento. Por esto mismo, nuestro principal objetivo ha sido estudiar marcadores mitocondriales en hígado y corazón de ratones que sobre-expresan esta proteína, sometidos a diferentes intervenciones nutricionales (CR y alteraciones en la composición de ácidos grasos de la dieta), así como estudiar cómo se ven afectados estos factores por el envejecimiento.

Para lograr este fin, hemos planteado 5 objetivos específicos:

1. Estudiar el efecto fisiológico de la sobreexpresión de CYB5R3, los ácidos grasos de la dieta, la CR y el envejecimiento sobre el peso corporal y el perfil bioquímico.
2. Caracterizar la sobreexpresión de CYB5R3 en hígado y corazón en las tres intervenciones propuestas.
3. Determinar los efectos combinados de la sobrexpresión de CYB5R3 y de la CR sobre marcadores clave de la función mitocondrial.
4. Determinar los patrones nutricionales que maximizan la preservación mitocondrial en ratones que sobreexpresan CYB5R3.
5. Determinar los efectos del envejecimiento sobre los parámetros mitocondriales estudiados en los objetivos previamente citados, en ratones sobre-expresantes de CYB5R3.

Los resultados de esta Tesis Doctoral podrían ayudar a identificar estilos de vida saludables y patrones nutricionales que pueden modular el envejecimiento saludable y la extensión de la vida máxima. También servirán para identificar nuevas dianas que permitan la preservación de la fisiología mitocondrial con el objetivo de prevenir enfermedades metabólicas asociadas con el envejecimiento.

Materiales y métodos

Para este estudio se emplearon ratones macho de la cepa C57BL/6J que sobre-expresan el gen de la *Cyb5r3*, habiéndose establecido tres cohortes dependiendo de la intervención. En todos los grupos experimentales, han sido usados como controles ratones de estirpe silvestre (WT) que poseen el mismo fondo genético que los ratones sobre-expresantes de CYB5R3 (Tg). Los animales WT se obtuvieron de las mismas camadas que los ratones Tg. Los ratones Tg macho iniciales fueron donados amablemente por el Dr. Rafael de Cabo (*Translational Gerontology Branch, National Institute on Aging, Baltimore, Maryland, USA*), lo cual nos permitió establecer una colonia propia en el Servicio de Animales de Experimentación (SAEX) de la Universidad de Córdoba.

Todos los ratones fueron mantenidos en jaulas con filtro HEPA conectadas a un sistema de ventilación. Además, el ambiente fue totalmente controlado bajo ciclos de luz y oscuridad de doce horas, una temperatura de 22ºC y con acceso continuo al agua y comida. A partir de los tres meses de edad, los ratones fueron asignados aleatoriamente a uno de los diferentes grupos experimentales. Durante la intervención nutricional, los ratones fueron mantenidos en grupos de cuatro, exceptuando aquellos sometidos a CR, que fueron alojados en jaulas individuales para garantizar el control exacto de la ingesta.

La primera cohorte, llamada **intervención de envejecimiento**, consistió en dos grupos experimentales en función de la edad: grupo de ratones jóvenes y grupo de ratones viejos, respectivamente. Los animales fueron alimentados *ad libitum* con dieta AIN93M (cuya fuente principal grasa proviene del aceite de soja) durante 4 o 21 meses (7 o 24 meses de edad al final de la intervención).

La segunda cohorte experimental, llamada **intervención de grasa de la dieta**, consistió en cuatro grupos experimentales en función de la dieta administrada. Todas las dietas fueron formuladas de acuerdo con la composición AIN93M, pero modificando la fuente grasa principal: aceite de soja, aceite de oliva, aceite de pescado y manteca de cerdo, respectivamente. Los ratones fueron alimentados *ad libitum* durante 4 meses (7 meses de edad al final de la intervención).

La tercera cohorte experimental, llamada **intervención de restricción calórica**, consistió en dos grupos experimentales que difirieron en la ingesta calórica. Ambos

grupos fueron alimentados durante 4 meses (7 meses de edad al final de la intervención) con la dieta AIN93M formulada con aceite de soja. Uno de los grupos fue alimentado *ad libitum* y el otro fue alimentado con una reducción del 40% de la ingesta calórica respecto a los anteriores.

Los ratones de 7 meses de edad alimentados de manera *ad libitum* con la dieta AIM93M formulada con aceite de soja constituyó el grupo control, que fue el mismo para las tres intervenciones. Las dietas fueron preparadas por el Servicio de Experimentación Animal de la Universidad de Granada, y almacenadas a -20ºC para minimizar la oxidación de los ácidos grasos.

Al final de cada intervención, los ratones se mantuvieron en ayuno el día previo al sacrificio. Durante el sacrificio, los ratones fueron anestesiados con isoflurano y se les realizó una punción cardiaca con el fin de extraer la sangre para determinar posteriormente diversos parámetros bioquímicos. Finalmente, los ratones fueron sacrificados mediante dislocación cervical. El hígado y el corazón fueron rápidamente diseccionados y pesados para los subsiguientes análisis bioquímicos y ultraestructurales. Los análisis bioquímicos sobre muestras de sangre fueron llevados a cabo por el personal del SAEX. En las muestras de tejido se determinó (mediante inmunotransferencia) la expresión de diversas proteínas marcadoras de procesos mitocondriales clave. Además, se obtuvo DNA genómico para calcular la ratio DNA mitocondrial / nuclear y se determinó la producción de ATP. Los análisis ultraestructurales se llevaron a cabo mediante microscopia electrónica de transmisión (TEM).

Para la inmunotransferencia, una pequeña porción del hígado y del corazón se separó y homogenizó con tampón RIPA a 4ºC que contenía inhibidores de proteasas y fosfatasas. Los tejidos fueron homogenizados usando el sistema TissueLyser II MM400 (Qiagen) a 2,5 Hz durante 1,5 min. Posteriormente, los homogenados fueron centrifugados para separar el sobrenadante que contenía el extracto proteico. Las muestras fueron además sometidas a sonicación y almacenadas a -80 ºC hasta su uso.

La concentración total de proteínas fue determinada por la modificación del método de Bradford llevada a cabo por Stoscheck, usando una cantidad de 50 µg de extracto de proteína para la inmunodetección mediante Western blot. Las proteínas fueron introducidas en los pocillos de geles en gradiente de poliacrilamida Criterion TGX 4-

20% para su separación electroforética. Posteriormente, fueron transferidas a membranas de nitrocelulosa, bloqueadas con leche, e incubadas toda la noche a 4ºC en agitación con el anticuerpo primario específico para cada proteína sometida a estudio. Después de retirar el anticuerpo primario, las membranas fueron incubadas a temperatura ambiente con el correspondiente anticuerpo secundario específico de especie. Finalmente, la señal quimio-luminiscente fue tomada usando el sistema de imágenes ChemiDoc (Bio-Rad).

Para la realización de la PCR (reacción en cadena de la polimerasa) cuantitativa, el DNA genómico (ADNDNAg) fue aislado usando el kit comercial NucleoSpin Tissue (Macherey-Nagel) de acuerdo con las instrucciones del fabricante. La concentración de DNAg fue medida usando un espectrofotómetro NanoDrop 2000 UV-Vis (Thermo Scientific) usándose una concentración de 10 ng/µl en la PCR. Un grupo de genes nucleares y mitocondriales fue medido para evaluar el número de copias relativas y la ratio DNA mitocondrial y nuclear. La cuantificación se realizó aplicando el método ΔΔCt.

El procesamiento de los tejidos para TEM se realizó mediante un protocolo estándar de inclusión en resina. Se obtuvieron secciones ultrafinas de hígado y corazón que fueron montadas en rejillas de níquel y posteriormente contrastadas con acetato de uranilo y citrato de plomo. Los cortes fueron visualizados y fotografiados usando un microscopio electrónico de transmisión Jeol Jem 1400 en el Servicio Centralizado de Apoyo a la Investigación (SCAI, Universidad de Córdoba). Los datos planimétricos y estereológicos de las mitocondrias fueron obtenidos mediante el uso de un software de análisis de imagen (Image J; NIH; USA) y aplicando diversas técnicas de cuantificación.

Resultados y discusión

El envejecimiento se asocia con una mayor deposición y distinta distribución de grasa, que afecta principalmente a la grasa visceral, lo que puede tener consecuencias negativas para la salud. En nuestra cohorte, los ratones CYB5R3-Tg presentaron pesos corporales similares a los WT después de 4 meses de intervención alimentados *ad libitum* con dietas que contenían diferentes fuentes grasas. Sin embargo, sí que encontramos variaciones según la fuente de grasa usada: es decir, la dieta rica en manteca de cerdo aumentó el peso de los ratones WT en comparación con la dieta

enriquecida con aceite de soja. También se observó un aumento en el peso corporal de los ratones Tg alimentados con aceite de pescado.

Las intervenciones que dan lugar a una reducción de la grasa corporal han sido asociadas con una extensión en la longevidad. En nuestro caso, la CR produjo una disminución significativa del peso corporal, efecto que fue observado en ambos genotipos. Sin embargo, la sobreexpresión de CYB5R3 dio lugar a una mayor preservación de la grasa. En estudios previos se ha mostrado que el mantenimiento del peso corporal en animales Tg para CYB5R3 sometidos a CR puede estar relacionado con una mayor acumulación de grasa abdominal en comparación con sus controles WT, lo cual puede deberse a la obtención preferencial de energía a través de los carbohidratos en los ratones que sobreexpresan CYB5R3. Los efectos sobre el peso y la grasa observados en nuestros ratones Tg, podrían explicar el efecto beneficioso de la sobreexpresión de CYB5R3 en la esperanza de vida. De acuerdo con nuestras observaciones, un estudio reciente mostró una correlación positiva entre la longevidad de los ratones sometidos a CR y la preservación de su grasa corporal durante el envejecimiento.

Estudios anteriores han informado que el peso de los órganos disminuye con la CR independientemente de la fuente de grasa en la dieta. Aunque la intervención de CR dio lugar a una disminución significativa del peso corporal en ambos genotipos, la sobreexpresión de CYB5R3 condujo a una mayor preservación del peso corporal que también se reflejó en el peso de todos los órganos analizados. Por lo tanto, CYB5R3 surge por primera vez como un determinante clave en el control del peso corporal y de los órganos en ratones sometidos a CR.

A pesar de que en los ratones jóvenes no hubo cambios significativos en el peso corporal independientemente del genotipo, nuestro estudio de longevidad reveló que los ratones Tg para CYB5R3 aumentaron más de peso a medida que envejecían. El aumento de ácidos grasos insaturados, que ha sido informado en ratones Tg sobrexpresantes de CYB5R3, es debido a una mayor actividad desaturasa que podría inhibir la β-oxidación, lo cual podría explicar los cambios observados con el envejecimiento en el peso corporal de los ratones Tg respecto a los WT.

A continuación, analizamos los niveles del polipéptido de CYB5R3 en hígado y corazón y en los tres contextos de estudio (alteración de la grasa de la dieta, CR y

envejecimiento). Encontramos que los niveles de polipéptido de CYB5R3 estaban altamente influenciados por la grasa de la dieta en un órgano mitótico como es el hígado, lo que probablemente esté relacionado con la actividad desaturasa de esta enzima. Asimismo, en hígado las dietas ricas en PUFA disminuyeron la cantidad de proteína, como se observó con la dieta basada en aceite de soja y, especialmente, con la rica en aceite de pescado donde encontramos los niveles más bajos de CYB5R3 con independencia del genotipo. No se detectaron diferencias al comparar ratones WT y Tg dentro de cada grupo nutricional. En el corazón, órgano postmitótico, vimos que, al contrario que lo encontrado en hígado, la fuente grasa de la dieta no afectó los niveles de CYB5R3, pero si se encontró una sobreexpresión clara de CYB5R3 en los ratones Tg en todos los grupos experimentales. Por otro lado, la CR no afectó los niveles de CYB5R3 en este órgano, aunque se detectó una disminución de la cantidad de proteína en el hígado, muy acentuado en el caso de los ratones Tg. Por su parte, en el grupo experimental de envejecimiento, se encontró un patrón totalmente diferente, observándose un aumento del contenido de CYB5R3 tanto en hígado como en corazón de animales envejecidos. Estas observaciones indican la existencia de mecanismos específicos del tejido y de la edad que regulan los niveles de CYB5R3 mediante CR o la fuente grasa de la dieta, siendo los tejidos postmitóticos (como el corazón) modelos de estudio adecuados para evaluar los efectos directos de la sobreexpresión de CYB5R3 sobre la fisiología mitocondrial.

Con respecto a los efectos de la CR sobre diferentes marcadores de dinámica y biogénesis mitocondrial, encontramos que esta intervención no modificó el contenido mitocondrial en el hígado ni en ratones WT ni en los Tg. Sin embargo, se observó un aumento en los niveles de MFN2, TFAM y en la ratio mt/nu DNA, así como una tendencia a aumentar NRF1 en los ratones WT. Estos resultados concuerdan parcialmente con estudios previos que han documentado que la CR da lugar a una regulación positiva de los parámetros de biogénesis mitocondrial en hígado de ratón. Sin embargo, estos cambios fueron contrarrestados por la sobreexpresión de CYB5R3, que produjo una disminución del factor de transcripción NRF1.

La sobreexpresión de CYB5R3 también dio lugar a un aumento en el área mitocondrial en ambas intervenciones, lo que podría estar relacionado con una función mitocondrial mejorada si, como han propuesto estudios previos, un aumento de tamaño mitocondrial se acompaña de un mayor número de crestas. Sin embargo,

las observaciones realizadas en nuestros ratones contrastan con otros estudios previos de nuestro grupo donde, usando microscopía electrónica, se documentó que una intervención de CR de 6 meses basada en la dieta AIN93G fue suficiente para aumentar la masa mitocondrial en hígado de ratón. Las diferencias en el contenido mitocondrial entre ambos estudios podrían deberse a factores como la edad y el porcentaje de grasa usado en la dieta (dietas AIN93M *vs*. AIN93G). Además, las diferencias en los resultados respecto a los cambios de la masa mitocondrial con la CR y el envejecimiento también se pueden explicar en base a la heterogeneidad en los métodos utilizados para evaluar este parámetro.

En cuanto al efecto de las intervenciones sobre la expresión de complejos mitocondriales de la cadena de transporte de electrones, se observó un aumento del complejo III por la CR tanto en ratones WT como en los Tg, aunque el efecto fue más acusado en éstos últimos. Además, se observó un aumento en la abundancia de los complejos mitocondriales IV (con diferencias significativas entre los dos grupos de ratones Tg) y V con la CR independientemente del genotipo. Estos datos sugieren que la función mitocondrial, más que su estructura o abundancia, puede verse afectada principalmente por la sobreexpresión de CYB5R3 en el hígado.

En el corazón, la CR aumentó drásticamente los niveles de la mitofusina MFN1 y de los factores de transcripción relacionados con la biogénesis mitocondrial, TFAM y NRF1. Una vez más, se observó que la sobreexpresión de CYB5R3 contrarrestó el efecto producido por la CR sobre estos parámetros. Sin embargo, estos resultados contrastan con los obtenidos a partir de las micrografías obtenidas por microscopía electrónica, que mostraron que la masa mitocondrial fue similar en todos los grupos experimentales. Debido a la existencia de dos poblaciones diferentes de mitocondrias en los cardiomiocitos (mitocondrias subsarcolémicas e intermiofibrilares), existe la posibilidad de que el envejecimiento y/o la CR, o incluso la sobreexpresión de CYB5R3, afecten de manera diferencial a estas poblaciones. La microscopía electrónica puede discriminar entre ellas permitiendo estudios cuantitativos separados, mientras que la cuantificación de proteínas se realizó en homogeneizados totales de corazón, por lo que los resultados se corresponden con ambas subpoblaciones tomadas conjuntamente.

En el corazón no se detectó ningún cambio en los niveles de proteínas de los complejos mitocondriales, a excepción del IV en ratones WT sometidos a CR. Estos resultados apoyan la hipótesis de que la sobreexpresión de CYB5R3 modifica significativamente las adaptaciones metabólicas de los ratones sometidos a CR. Otros estudios han demostrado que la CR mejora la eficiencia del complejo IV en el músculo esquelético, un fenómeno que también se ha informado ocurre en el corazón. Es probable que estos resultados estén relacionados con una mayor afinidad y un aumento en los sitios de unión del O_2. En estas condiciones, tanto el flujo de electrones como el estado redox de los complejos mitocondriales son más eficientes, lo que da lugar a una menor generación de ROS por la respiración mitocondrial.

Nuestros datos apoyan la idea de que muchos efectos beneficiosos de la CR están determinados por la mayor preservación de la fisiología mitocondrial y de sus características ultraestructurales, lo que da como resultado no solo una menor producción de ROS, sino también una mayor protección de este orgánulo frente a la mitofagia. Estos resultados son parcialmente mimetizados por la sobreexpresión de CYB5R3 en ratones alimentados *ad libitum*. Sin embargo, como se planteó en nuestra hipótesis inicial, los efectos beneficiosos producidos por un lado por la CR y por otro por la sobreexpresión de CYB5R3 parecen operar a través de vías metabólicas diferentes, ya que cuando se aplican por separado sí se detectan dichos efectos beneficiosos, pero cuando ambas intervenciones son aplicadas de manera simultánea, sus efectos se atenúan parcialmente.

En diferentes especies se ha constatado que los animales con mayor longevidad muestran un menor grado de insaturación de ácidos grasos en los tejidos. La disminución de PUFA en los fosfolípidos de la membrana mitocondrial da como resultado una menor susceptibilidad a la peroxidación, lo que potencialmente contribuye a una mayor esperanza de vida y un envejecimiento saludable en ratones sometidos a CR. Por lo tanto, uno de nuestros objetivos fue investigar el papel de las diferentes fuentes grasas de la dieta en marcadores relacionados con el metabolismo mitocondrial en hígado y corazón de ratones que sobreexpresaban CYB5R3.

En el hígado, las dietas ricas en MUFA condujeron a un aumento de los marcadores de fusión mitocondrial, especialmente en los ratones Tg, mientras que una dieta rica en SFA ejerce el efecto contrario, lo que podría estar relacionado con una

acumulación de ácidos grasos libres y con el posible desarrollo de obesidad y resistencia a la insulina en los ratones WT alimentados con la dieta rica en manteca de cerdo. Por otro lado, se encontró un efecto positivo en ratones Tg alimentados con una dieta rica en manteca de cerdo, ya que se observó un aumento de VDAC1 y una disminución en MFF, lo que podría ser indicativo de un cambio en la función mitocondrial hacia un fenotipo más saludable y una mejora de la mitofagia. Se observó asimismo una disminución significativa del marcador de fisión mitocondrial MFF, lo que podría ser compatible con una mejora de la función mitocondrial en las dietas enriquecidas con aceite de oliva, aceite de soja y aceite de pescado. Un cambio hacia la fusión mitocondrial se ha asociado con una mejor función mitocondrial y de producción de ATP. En nuestros grupos experimentales, en general, no observamos cambios en los niveles de proteína de los complejos mitocondriales. Dados los resultados obtenidos, es posible que las grasas de la dieta modulen de manera preferencial la actividad enzimática de las proteínas implicadas en la respiración mitocondrial, o la formación de supercomplejos, o ambas cosas, en lugar de modular los niveles de proteínas de los complejos mitocondriales en el tejido hepático.

En el corazón, un tejido postmitótico, no se observaron cambios muy pronunciados en cuanto a marcadores de dinámica y biogénesis mitocondrial, excepto en el caso de los ratones alimentados con una dieta enriquecida con aceite de pescado. El corazón presenta un gran número de mitocondrias de gran tamaño, así como una gran cantidad de proteínas de fusión que mejoran el metabolismo mitocondrial y compensan la aparición de mutaciones del mtDNA en células heteroplásmicas. En el caso de ratones alimentados con dietas ricas en PUFA, se observó un aumento de proteínas involucradas en la fusión mitocondrial, lo que podría estar relacionado con los efectos beneficiosos sobre el mantenimiento de la función cardiaca que diversos estudios han informado respecto al consumo de este aceite. Sin embargo, en los ratones que sobre-expresan CYB5R3 se observó una disminución de los niveles de proteínas relacionadas con la fusión mitocondrial con el consumo de una dieta enriquecida con aceite de pescado mientras que, por el contrario, se observó un aumento de estas proteínas en los animales alimentados con dietas basadas en aceite de oliva. En relación a los complejos respiratorios mitocondriales, no se encontraron cambios importantes, exceptuando el incremento en los niveles de algunos marcadores en ratones alimentados con la dieta enriquecida en aceite de pescado, lo que podría estar

relacionado con los beneficios reportados de estos ácidos grasos contra las enfermedades cardiovasculares.

Los datos obtenidos sugieren que la sobreexpresión de CYB5R3 puede producir diferentes efectos sobre la función mitocondrial dependiendo de la fuente de grasa utilizada en la dieta. Aunque la sobreexpresión de CYB5R3 no parece conferir un fenotipo marcado por sí mismo, en el caso de los ratones alimentados con la dieta basada en manteca de cerdo, se observa una prevención de la fragmentación mitocondrial, lo que sugiere un estado mitocondrial más funcional en comparación con sus controles WT. En el caso de los ratones alimentados con una dieta enriquecida con aceite de oliva, la sobreexpresión de CYB5R3 parece promover un fenotipo saludable al aumentar las proteínas de fusión mitocondrial. Sin embargo, parece existir también alguna interferencia entre la sobreexpresión de CYB5R3 y el consumo de una dieta enriquecida con aceite de pescado, ya que algunas de las acciones beneficiosas de cada una de las intervenciones de manera aislada pueden verse obstaculizadas, como se observa en los marcadores de dinámica mitocondrial y en el aumento de peso de los ratones Tg con esta dieta.

En hígado y corazón las mitocondrias se ven altamente afectadas por el envejecimiento. Existen dos mecanismos mediante los cuales la célula puede compensar el daño mitocondrial. Por un lado, la fusión mitocondrial controlada por mitofusinas, que se expresan especialmente en tejidos con altos requerimientos energéticos y ricos en mitocondrias, como el corazón y el músculo esquelético. De esta forma se tiende a mantener una red mitocondrial fusionada que compensa la aparición de mutaciones de mtDNA en células heteroplásmicas (a través de una mezcla de contenido que diluye las mutaciones mitocondriales), lo que contribuye a la disipación de energía en las células y a mejorar la unión del retículo endoplásmico a las mitocondrias. Sin embargo, otros tejidos, tienden a tener una red más fragmentada que sirve para eliminar por mitofagia las mitocondrias dañadas, manteniéndose integra su capacidad bioenergética. Ambos mecanismos coexisten y están regulados por la demanda energética de la célula. Por esta razón uno de los objetivos fue el estudio del efecto de la sobreexpresión de CYB5R3 sobre los marcadores de la dinámica y el metabolismo mitocondrial hepático y cardíaco en un contexto de envejecimiento.

Nuestros datos en hígado mostraron resultados mixtos, ya que con el envejecimiento se observó una tendencia al alza de los niveles de proteína de MFN2 (un cambio que no se observó en los ratones Tg) sin que se apreciaran cambios en MFN1, lo que sugiere que existen diferentes mecanismos de adaptación al envejecimiento en ratones WT y Tg. En este sentido, es posible que la red mitocondrial hepática de los ratones Tg viejos esté equilibrada hacia una fisión más eficiente, lo que indicaría la aparición de mayor fragmentación que facilitaría la eliminación mitofágica de mitocondrias dañadas. Sin embargo, los resultados observados en animales WT son compatibles con una respuesta contra el estrés inducido por el envejecimiento promoviendo mecanismos de fusión para diluir las mutaciones en el DNA mitocondrial y mejorar la función de estos orgánulos.

Numerosos estudios han informado de una disminución en la actividad de los complejos mitocondriales con la edad, siendo los complejos I y IV los más afectados. Este efecto puede deberse a varios factores: a la modificación de proteínas por daño inducidos por ROS, a la inhibición de las actividades enzimáticas por productos generados durante el envejecimiento, o incluso a la disminución de la expresión de proteínas. En hígado de ratones de ambos genotipos se observó un aumento con la edad de los niveles de proteínas de los complejos mitocondriales, siendo estos cambios más pronunciados en los ratones Tg. Sin embargo, cabe destacar que la actividad de los complejos no se analizó, existiendo la posibilidad de que el aumento en los niveles de proteínas de los complejos observado con el envejecimiento pudiera estar relacionado con la necesidad de aliviar una actividad enzimática disfuncional.

Algunos estudios han demostrado que los cambios relacionados con la edad son más evidentes en los tejidos postmitóticos. Así, se observó un aumento drástico de MFN2 con la edad en el corazón de ratones WT mientras que, por el contrario, se encontró una disminución en los ratones que sobre-expresan CYB5R3, un efecto que podría significar una mejora funcional de la mitocondria a medida que se alcanza una edad más avanzada. Aunque las mitocondrias de los órganos postmitóticos tienden a estar en un estado más fusionado, la persistencia del balance a favor de la fusión puede provocar un estado "hiperfusionado" y dar lugar a efectos patológicos. Por otro lado, se observó que la mayoría de los complejos mitocondriales estaban aumentados en el corazón de ratones envejecidos, siendo este efecto especialmente pronunciado en los ratones Tg, de manera similar al patrón observado en el hígado, a excepción del

complejo IV, que estaba disminuido en ratones que sobreexpresaban CYB5R3. Existen evidencias de que los complejos I, III y IV (pero no el II), junto con los transportadores móviles de electrones coenzima Q y citocromo c, se pueden organizar en estructuras llamadas supercomplejos, que proporcionan ventajas cinéticas durante la transferencia de electrones. Estudios previos en modelos murinos han señalado que el corazón experimenta con la edad una pérdida de supercomplejos, mientras que los niveles de proteínas de las subunidades que forman los complejos de la cadena de transporte de electrones no se modifican. Sin embargo, en otros estudios se ha mostrado que es más relevante la pérdida de actividad de complejos que la variación en la cantidad de proteína. Así, las alteraciones en la función del complejo IV, que es el último componente que se incorpora a los supercomplejos, podrían desestabilizar el ensamblaje los mismos. En base a esta hipótesis, se podría sugerir que la disminución de los niveles del complejo IV que se observa en los ratones Tg podría implicar la estabilización de estas estructuras.

En su conjunto, los resultados presentados en esta Tesis constituyen el primer estudio en el que se analiza y correlaciona el efecto de distintas fuentes grasas en la dieta, la CR y el envejecimiento junto a la sobreexpresión de CYB5R3 en marcadores clave implicados en la función y la ultraestructura mitocondrial. Los datos obtenidos nos han permitido señalar la existencia de mecanismos dependientes de tejido que modulan los niveles de CYB5R3. Además, estos niveles también se afectan por las diferentes intervenciones nutricionales y por el envejecimiento. No obstante, la consolidación de los resultados presentados en esta Tesis Doctoral requerirá de estudios adicionales centrados en parámetros fisiológicos relacionados con la función mitocondrial. De esta forma, se podría verificar la hipótesis de que la sobreexpresión de CYB5R3 conduce a una mayor producción de energía, menor generación de ROS, menor señalización apoptótica, y mejor conservación de la función mitocondrial durante el envejecimiento. En este sentido, está previsto continuar con el análisis de los efectos de la sobreexpresión de CYB5R3 sobre otros parámetros relacionados con el envejecimiento y la función mitocondrial, como son la mitofagia y la apoptosis. Así mismo, está previsto ampliar el estudio a otros tejidos como músculo esquelético y riñón (actualmente en desarrollo). Por otro lado, es importante señalar que este estudio se ha llevado a cabo exclusivamente en ratones macho, lo cual supone una limitación sustancial. Por tanto, se prevé ampliar nuestros estudios a ratones hembra

con el fin de establecer la posible existencia de dimorfismo sexual en los animales en cuanto al efecto de la sobreexpresión de CYB5R3.

Introduction

The Hallmarks of Aging

Perhaps one of the main questions we have asked ourselves over the centuries is why we age. Throughout history, humans have sought to avoid aging and have tirelessly searched the for “Fountain of Youth,” and the elixir of immortality. Aging is one of the most interesting processes in nature that leads to declining health and inevitably to mortality. Reversing the aging process has always been in the sights of mankind, and that is why it is not surprising that more and more effort is being dedicated to research to achieve what we have always longed for [1].

Aging can be defined as a natural time-dependent degenerative process suffered by living beings. It is characterized by alterations and injuries that compromise cell and tissue function leading ultimately to the death of the organism. Furthermore, aging is the most significant risk factor for a wide range of diseases including cancer, cardiovascular disease and neurodegeneration [2]. To understand how this process works and how we can avoid and/or even reverse it, nine features have been proposed that contribute to the aging process. They have been called " The Hallmarks of Aging" and together they characterize the aged phenotype [3].

These hallmarks ideally meet a series of criteria such as that they should occur during normal aging, their experimental exacerbation must speed up the aging process and their experimental rectification must attenuate it. Although they are all interconnected, these hallmarks are divided into three groups: 1) those causing harm, also called primary hallmarks, including: telomere attrition, genomic instability and epigenetic alterations; 2) those that respond to damage, also called antagonistic hallmarks, are: loss of proteostasis, mitochondrial dysfunction, deregulated nutrient sensing, and cellular senescence; 3) finally, those causing the aging phenotype, also called integrative hallmarks, are: stem cell exhaustion and altered intercellular communication (see Fig. 1) [3].

The aging process and the molecular mechanisms that control longevity are complex. That is why, knowing these nine candidates allows us to design effective intervention strategies to improve lifespan and healthspan of individuals. However, to better understand how these hallmarks contribute to extending the knowledge of the mechanisms of aging, it is necessary to know what theories are currently trying to explain why and how we age.

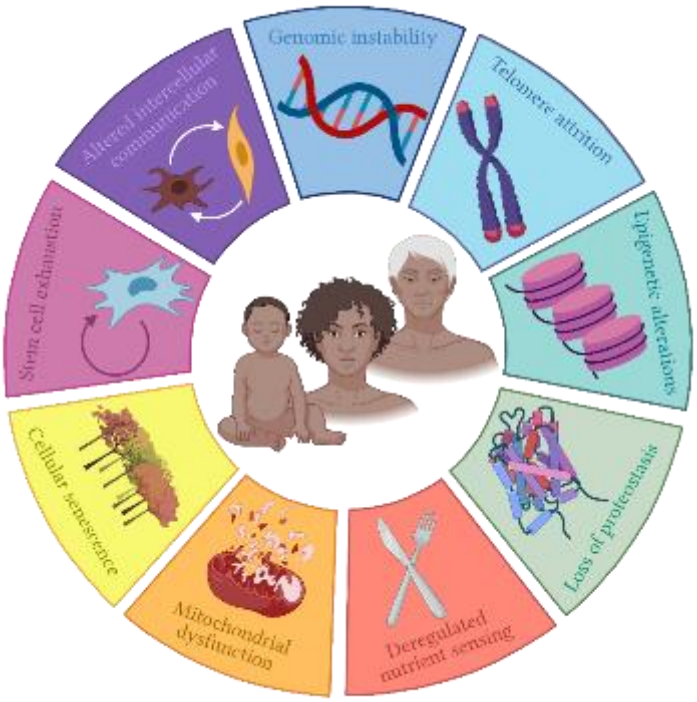


Figure 1. Hallmarks of aging. Adapted from Lopez-Otín et al.[3]

Theories of Aging

The Theories of Aging try to answer the ultimate question of what is the cause of aging. To explain the aging process, different theories have been proposed, although neither of them appears to be capable of fully explaining it and they often contradict each other. The proposed theories are divided into two main categories: programmed and damage aging theories [4].

The programmed aging theories postulate that maximum lifespan is genetically imposed and its existence is related with the evolutionary purpose of limiting the organisms beyond a species-specific age [5]. The attempt to explain the evolution of aging was first articulated by Weismann [6], and current defenders of these theories have suggested that programmed aging may constitute an altruistic plan that tries to avoid over-population by eliminating individuals after the reproductive stage and promoting succession of generations [7]. Programmed aging implies the existence of an internal hormone-regulated clock, controlling the expression of a gene set, likely regulated by the decline in the immune system function [8]. The programme theory is further supported by studies in the nematode *Caenorhabditis elegans* (*C. elegans*) where has been reported a large number of genes whose mutation results in increased

maximum lifespan [9, 10], or in mutant variations of *Drosophila* producing longer-lived descendants [11]. There is also the fact that every organism has a physiological lifespan that is characteristic for its species. Nevertheless, these theories generate controversy in the scientific community. There are still many questions that cannot be answered by the idea that aging is programmed, and none of the mutations that have been described to extend lifespan have been reported to abolish the aging process [5, 12].

The damage theories propose that environmental factors can induce mutations and injury at different levels in the cells. Despite the action of multiple, sophisticated repair mechanisms, an imbalance between the accumulation of damage and the ability of cells to repair them, inevitably leads to tissue dysfunction and cell death. Among the damage theories, the so-called Free Radical Theory of Aging proposes that oxidative stress and redox imbalance are factors directly related with macromolecular damage leading to aging [13]. Although it remains one of the most cited theories to explain aging, there is a great controversy among researchers that consider reactive oxygen species (ROS) and other reactive species as the main cause of aging [14]. However, there is a consensus to identify the alterations that the ROS generation entails.

Many interventions and mutations extend lifespan in different animal models, but none of them abolish or reverse the progression of aging. Thus, aging emerges as a multifactorial and complex process not due to a single cause, where the proposed theories seem to interact with each other in a highly interconnected and regulated process [15].

Reactive oxygen species

ROS are partially reduced or excited forms of oxygen and are divided in nonradical species, as hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) and free radicals as superoxide anion ($O_2^{\bullet -}$), and hydroxyl radical ($^{\bullet}OH$). They can be spontaneously or enzymatically interconverted from one to another [16]. ROS are products of normal cellular metabolism by mitochondrial respiration or by cell responses in defence against infectious agents, xenobiotics, redox stress, cytokines; they also intervene in a number of intracellular signaling pathways [17, 18].

ROS can be either generated exogenously or intracellularly from numerous sources. The main sources of ROS that we can find at the cellular level are the following (see Fig. 2) [16, 19]:

Superoxide ion radical ($O_2^{\bullet-}$): It can exist in two forms; as $O_2^{\bullet-}$ or its protonated form, hydroperoxyl radical (HO_2) at low pH. HO_2 is even more reactive than the charged form ($O_2^{\bullet-}$), and can easily enter the phospholipid bilayer. $O_2^{\bullet-}$ is the most important ROS species produced mainly by mitochondrial electron transport chain. However, other potential sources of $O_2^{\bullet-}$ as a by-product of their normal enzymatic function include NADPH oxidases (NOXs), xanthine oxidase, cyclo-oxygenases, and cytochrome p450 enzymes, among others. Under physiological conditions, $O_2^{\bullet-}$ can act as a reducing agent of free copper ions or iron complexes such as cytochrome *c* reducing Fe^{+3} to Fe^{+2}. Conversely, Fe^{+2} auto-oxidation is also another important source of superoxide.

$$O_2 + Fe^{+2} \rightarrow Fe^{+3} + O_2^{\bullet-} \text{ (auto-oxidation)}$$

Hydroxyl Radical ($^{\bullet}OH$): It is considered the most reactive oxygen-derived species and can cause more serious damage to cells than any other ROS. $^{\bullet}OH$ can be formed in the Fenton reaction, in which H_2O_2 reacts with metal ions (Fe^{+2} or Cu^{+}) and by the Haber–Weiss reaction between $O_2^{\bullet-}$ and H_2O_2.

$$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + {}^{\bullet}OH + {}^{-}OH \text{ (Fenton reaction)}$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + {}^{-}OH \text{ (Haber-Weiss reaction)}$$

Hydrogen Peroxide (H_2O_2): This non-radical reactive oxygen species is formed by a dismutation reaction catalysed by superoxide dismutase (SOD). H_2O_2 can easily cross cell membranes causing direct damage, such as inactivation of the enzyme glyceraldehhyde-3-phosphate dehydrogenase. In the presence of metal ions, it can react and produce hydroxyl radical ($^{\bullet}OH$) through Fenton chemistry (see above).

Singlet Oxygen ($^1O_2$): It is a highly toxic reactive oxygen species that can produce DNA and tissue damage. It is generated by the activation of neutrophils and eosinophils, and by some enzymes such as lipoxygenases, dioxygenases, and lactoperoxidase.

The presence of unpaired electrons makes ROS very unstable and highly interacting with membrane lipids, nucleic acids, proteins, and other small molecules, in order to

extract electrons and achieve their stability. The attacked molecule loses an electron and becomes a free radical itself, starting a chain reaction cascade resulting in cellular harm [19]. Usually, the organism can handle most of the produced ROS by small-molecule antioxidants such as glutathione (GSH) and coenzyme Q (CoQ), among many others, and free radical scavenging enzymes as SOD, catalase, glutathione peroxidase (GPX) and peroxiredoxin (PRX). Through these mechanisms, a balance is achieved between the production and removal of ROS [16].

ROS homeostasis is essential for the cell. Under normal physiological conditions, it generally holds slightly in favour of cell-tolerated pro-oxidant forms that can be used for other purposes. However, higher levels of ROS may cause oxidative damage or induce apoptosis during activation of immune cells or pathological conditions [20].

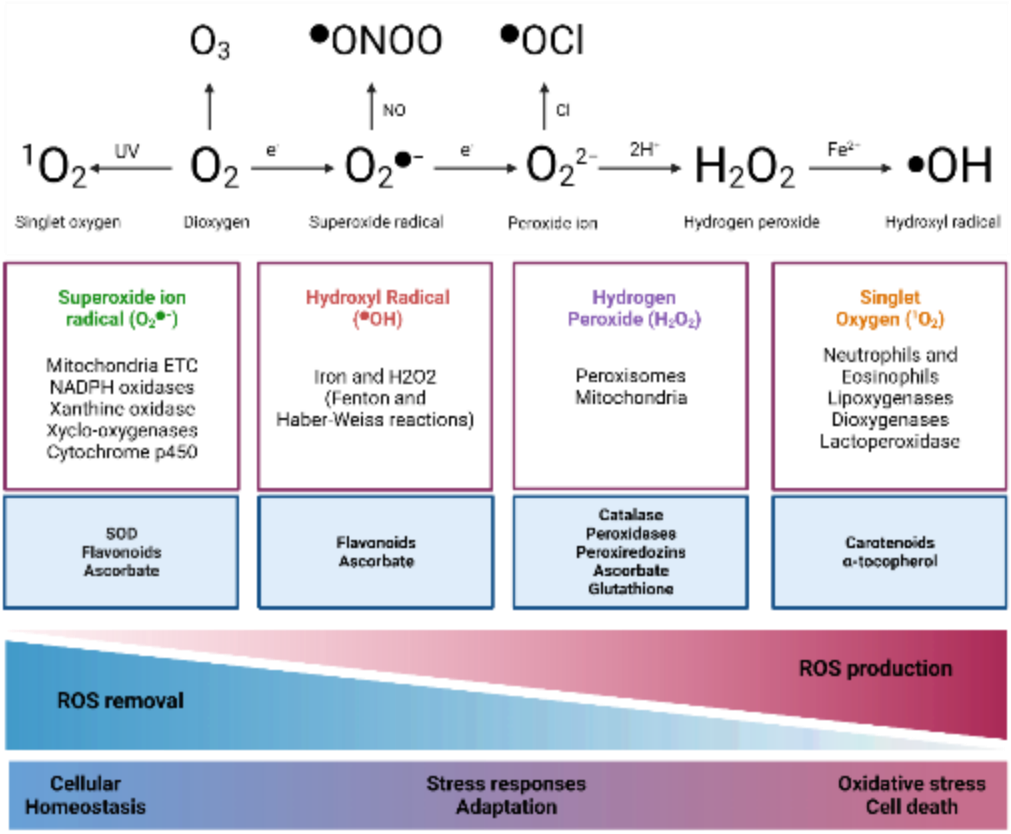


Figure 2. Reactivity, formation of different ROS, and antioxidant defences

Oxidative stress and aging

Oxidative stress can be defined as the imbalance generated by the excessive production of ROS that cannot be counteracted by the antioxidative defences of the

cells, resulting in the disruption of redox signaling and causing toxic effects that harm macromolecules and cell components [21, 22]. This imbalance can be due to different causes: inflammation, increased mitochondrial respiration or aging, among others. Also, environmental conditions, such as nutrition and abiotic stress account to generate excess of ROS in the tissues. Furthermore, a decrease in antioxidant enzyme activities and/or antioxidant molecules, mainly acquired through the diet, also leads to the generation of oxidative stress.

The main targets of ROS during oxidative stress are nucleic acids, proteins, lipids, and carbohydrates, and the damage induced in these components results in structural changes which affect their biological functions thus promoting accelerated aging or multiple diseases. In this way, oxidative stress considerably affects the cell, altering different biochemical processes that lead to activation of the apoptotic cell death program [19]. The most significant adverse effects are:

Lipid peroxidation: membrane phospholipids and lipoproteins are especially vulnerable to ROS through a reaction called lipid peroxidation. Due to the high number of double bonds, polyunsaturated fatty acids (PUFAs) are the most susceptible lipids to peroxidation. Once this process has started, it continues as a chain reaction, involving the stages of initiation, propagation and termination. Lipid peroxidation causes decreased membrane fluidity and permeability, inactivation of membrane bound enzymes and receptors, membrane potential failure and even the rupture of the membrane resulting in cell apoptosis. Malondialdehyde is an end cytotoxic product derived from lipid peroxidation that can cause damage to DNA and proteins.

Protein oxidation: amino acids are prone to free radical attack, usually by $^{\bullet}OH$, which leads to broken chains, generation of protein carbonyl derivatives and formation of aggregates. The consequences are loss or modification of the biological function, enzyme activity, function of receptors and transport protein.

DNA oxidation: at this level, oxidative damage can lead to mutations, loss of DNA expression and protein synthesis, strand breaking, DNA-protein cross-links, and deterioration and demethylation of "CpG islands" that can activate the expression of certain genes, among other actions in both mitochondrial and nuclear DNA. Since mitochondrial DNA is located close to the main ROS generation site, it is more

susceptible to ROS attack than nuclear DNA. The levels of 8-OH-deoxyguanosine, a molecule generated during oxidative stress on nucleic acids, constitute a reliable biomarker of DNA oxidation, and it is also considered a predictive marker of aging and multiple diseases.

The progressive accumulation of oxidative damage to macromolecules, alteration in its signaling function and a decrease in the endogenous defence mechanisms over time inevitably lead to functional decline, accelerated aging and multiple age-related diseases such as diabetes, neurodegenerative, and cardiovascular diseases (CVDs), cancer, inflammation and other pathologies [19].

Lifespan is dependent on alterations in oxidative stress which is related to the level of cellular ROS. Since the oxidative stress theory was proposed [23], different studies have been carried out with two different approaches. The first one is focused on altering the mitochondrial antioxidant capacity by inducing genetic alterations or through dietary supplements, and the second is based on reducing oxidative stress. In this case, dietary interventions have been extensively used. Among them, caloric restriction (CR) and CR mimetics are by far the most widely used interventions.

By mean of genetic alterations, a relationship has been found between life span and ROS levels in different animal models. Thus, *C. elegans age-1* and *daf-2* mutants show extended lifespan (65% and 100% respectively) and are resistant to a wide variety of stresses including oxidative stress, heat, UV, and the presence of heavy metals. This resistance appears to be mediated by the positive regulation of ROS detoxifying enzymatic activities increasing the resistance to oxidative stress [24-26]. In addition, the *isp-1* and *ctb-1* double mutants carry a nonsense mutation in two proteins of the catalytic subunit of electron transport complex III. These double mutants show decreased respiration, increased longevity and are resistant to paraquat-induced oxidative stress, a feature also found in *daf-2* mutants [27]. In contrast, short-lived *mev-1* mutants are more susceptible to oxidative stress and have 30% reduced lifespan. These animals show decreased antioxidant enzymatic activity, reduced glutathione content, and increased mitochondrial superoxide anion production [25]. All these data strongly suggest that low ROS production and high capacity to its removal, underlies the extended lifespan in *C. elegans*.

A similar link between longevity and stress resistance has been reported in *Drosophila*. For example, *mth* mutants have extended lifespan and increased resistance to heat stress and paraquat [28]. In addition, in flies it is possible to increase lifespan by overexpression of enzymes such as glutathione reductase, SOD and catalase [29]. Longevity can be extended not only in invertebrates. Catalase overexpressing mice, $Igf1r^{+/-}$ and $p66^{sh}$ mutants showed direct effect of resistance to oxidative stress and lifespan in mice [30]. Other study also showed that overexpressing in mice of human catalase can extend lifespan [31].

On the other hand, numerous studies have examined the effect of antioxidant supplementations (specially vitamins C and E), and their role in the prevention and treatment of aging and aging-related disorders such as CVDs and hepatic steatosis. However, regarding antioxidant supplementation, there is an ongoing controversy, since a number of studies carried out in different organisms have yielded divergent results: while some reported a prolonged lifespan, in others no effect was observed [32, 33]. In addition, the role of ROS in aging has been widely questioned as other studies did not show longevity extension in animals overexpressing SOD nor accelerated aging in $Gpx1^{-/-}$ knocked-out mice despite their high concentrations of ROS [34-36]. Furthermore, some studies even found a large extension of lifespan in models with elevated ROS [37, 38].

Although there is quite convincing evidence that ROS are important in aging, there is still debate in this field, particularly in considering ROS as the cause of aging [39]. In part this is because there is no consensus on the optimal biomarkers reflecting the actual overall cellular redox status. However, most researchers do agree that mitochondrial metabolism and mitochondria-derived ROS play a critical role in the aging process [40].

The role of mitochondria in aging

In 1972 Harman extended his *Free Radical Theory of Aging* suggesting that mitochondria are both the main source and a major target of ROS. Subsequently, in 1980, Miquel formulated the *Mitochondrial Theory of Aging* postulating that damage and increased levels of mitochondrial mutations that contribute to aging and age-related diseases [23, 41, 42]. Closely related with the Free Radical Theory of Aging, the *Membrane Theory of Aging* proposes that lifespan is related to the level of

unsaturation, particularly the level of n-3 polyunsaturated fatty acids (PUFA), in membrane phospholipids [43-46].

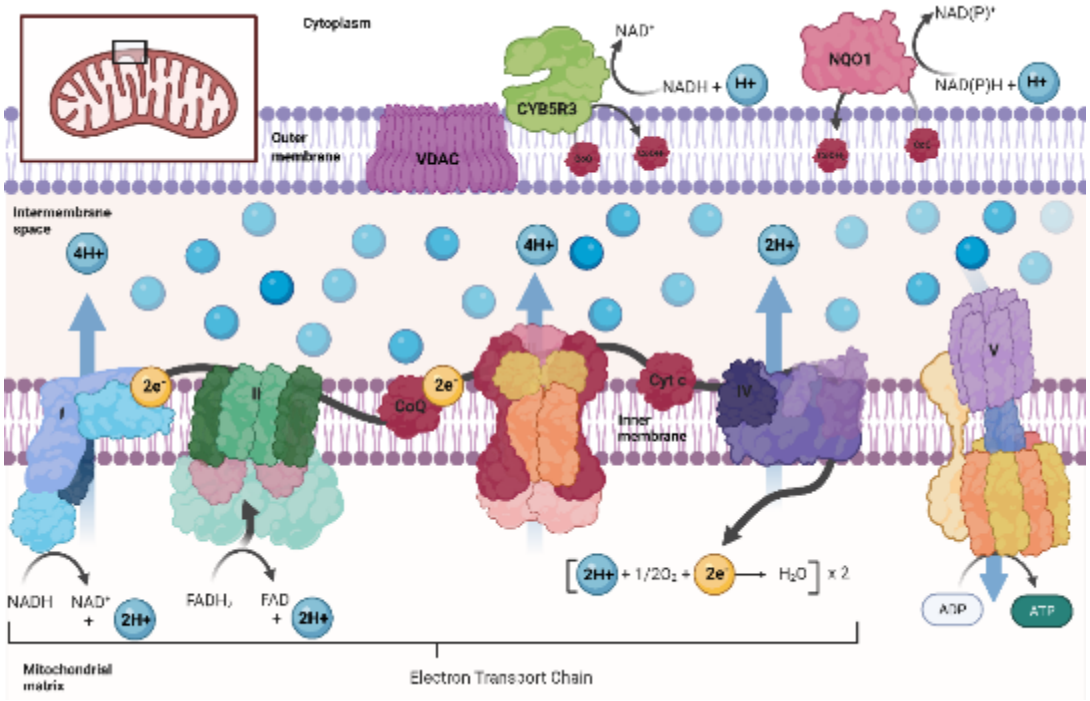
The mitochondria are one of the most important organelles in aerobic organisms. There are located at the cytoplasm of eukaryotic cell, and they are responsible of aerobic respiration, which produces most of the necessary energy for all cellular processes. At a structural level, this organelle is primarily characterized by the presence of a double membrane. These membranes, which are structurally and functionally different, are the outer (OMM) and the inner (IMM) mitochondrial membrane. Between them, there is a watery space called the intermembrane space.

The generation of ATP is carried out at the IMM through a series of protein complexes involved in oxidative phosphorylation (OXPHOS). Mitochondria also participate in numerous cellular processes, including β-oxidation of fatty acids, calcium signaling, and apoptosis. However, respiration also has deleterious consequences, since ROS and free radicals are generated as by-products of aerobic respiration [47].

Cellular respiration can be divided into three interconnected pathways: glycolysis, tricarboxylic acid (TCA) cycle, and mitochondrial electron transport. The glycolytic pathway takes place in the cytosol where glucose oxidization to pyruvate occurs under physiological conditions. The respiratory process continues with the entry of pyruvate into the mitochondrial matrix, where it is decarboxylated to acetyl CoA, a molecule that enters to mitochondrial TCA cycle. In the TCA cycle, acetyl CoA is oxidized in a series of reactions yielding three molecules of NADH and two of $FADH_2$ among other products. The last part of the pathway regenerates oxalacetate, the molecule used in the first step, allowing the cycle to start again. Electrons from NADH and $FADH_2$ are transferred to the electron transport chain (ETC) with a chemiosmotically coupled synthesis of ATP [48].

Mitochondrial ETC consists of four membrane-bound protein complexes responsible for transporting electrons from oxidative substrates to oxygen, the final acceptor, through a series of redox reactions that generate water (see Fig. 3). During this process, NADH and $FADH_2$ are oxidized in mitochondrial complexes I and II, respectively. The electrons released are transported to complex III by coenzyme Q (CoQ), and later to complex IV by cytochrome *c*, being then transferred to molecular

oxygen to produce water. During the electron transport, protons are pumped from the matrix to the intermembrane space through complexes I, III, and IV creating an electrochemical gradient by imbalanced hydrogen ion concentration between the mitochondrial matrix and the intermembrane space. IMM is impermeable to ions and, therefore, protons can only cross the membrane through protein channels as complex V. Thus, the proton gradient generated by the ETC is coupled to the oxidative phosphorylation process through complex V (ATP synthase). ATP synthase drives protons into the matrix and uses the liberation of energy to phosphorylate ADP, thus synthesizing ATP, and providing the driving force for the transport of small molecules into and out of mitochondria. During respiration, ROS are generated



mainly by complexes I and III in the form of $O_2^{\bullet -}$, which can be further converted to H_2O_2 and $^{\bullet}OH$ [49].

Figure 3. Mitochondria electron transport chain. The electron transport chain is constituted by a series of complexes in the inner mitochondrial membrane that shuttles electrons from NADH and FADH2 to molecular oxygen and make ATP. In the process, protons are pumped out of the mitochondrial matrix, and oxygen is reduced to form water. Protons come back into the matrix by electrochemical gradient through ATP synthase channels which capture their energy to convert ADP to ATP.

Two models have been proposed in an attempt to explain the arrangement of the complexes into the IMM. The “fluid model” proposes that the complexes freely diffuse in the IMM and that the electron transfer takes place on the basis of random collisions. The second one, the "solid model", proposes that free respiratory

complexes can be found independently in the membrane, but also organized in larger structures that allow more efficient transport of electrons. However, a new model, hybrid of fluid and solid models has been proposed, and it is known as the "plasticity model" [50, 51].

Although the association of the ETC complexes into higher-order supramolecular structure known as supercomplexes or respirasomes (SCs) was initially controversial, more recently they have been detected in a wide variety of organisms, from bacteria to humans, using a multitude of approaches [52].

In mammalian mitochondria, complexes I, III and IV form SCs. However, not all the subunits of the complexes participate in assembling of the SCs. The most stable interactions are observed between the subunits NDUFA11, NDUFB4, NDUFB9 of complex I and UQCRQ, UQCRC1, UQCRFS1 of complex III. COX7A subunit of complex IV interacts with complex I and III but in a less tight way and varies between the different respirasome structures [53, 54]. Complex II has not been detected in SCs, although recent studies suggested that the complex II can be involved in SCs forming a megacomplex containing all four complexes [55].

The role of the supercomplexes is not clear. It has been proposed that as highly dynamic structures, SCs may confer to cells some advantage allowing their physiological adaptation during metabolic fluctuations. SCs may also improve the stability of the complexes, specially of complex I. Finally, SCs facilitates the efficient electron transfer from complexes I and II to complex IV increasing the prevention of ROS which, in turn, constitutes a kinetic advantage [52, 56].

In 1980, Miquel *et al*. suggested that the progressive damage in mitochondria by ROS leads to mitochondria dysfunction [42]. With age, mitochondrial enzymes, ATP production, and respiratory capacity decline and, at the same time ROS and mitochondrial mutations increase. These changes are accompanied by alterations in morphology and abundance of these organelles. Linnane *et al*. proposed that the accumulation of mutations in mitochondrial DNA (mtDNA) is a major contributor to aging and aged-related diseases [41].

In animal tissues, mitochondria are the only organelles having their own genome with a modified genetic code. The mammalian mtDNA is transmitted exclusively through the maternal germ line and is essential for the maintenance and function of the

organelle. mtDNA is a closed-circular double-stranded DNA that encodes thirteen peptides involved in OXPHOS: seven subunits of complex I, one of complex III, three of complex IV, and two of complex V. Most information is encoded on the heavy (H) strand, with genes for two rRNAs, 14 tRNAs, and 12 polypeptides. The light (L) strand codes a single polypeptide and eight tRNAs. The major control site for mtDNA expression is a short triple strand structure called D-loop. This is a non-coding region and acts as a promoter for both the heavy and light strands (see Fig. 4). All the other mitochondrial proteins are encoded by nuclear DNA (nuDNA) and are imported to the mitochondria through translocation systems located in both OMM and IMM [57].

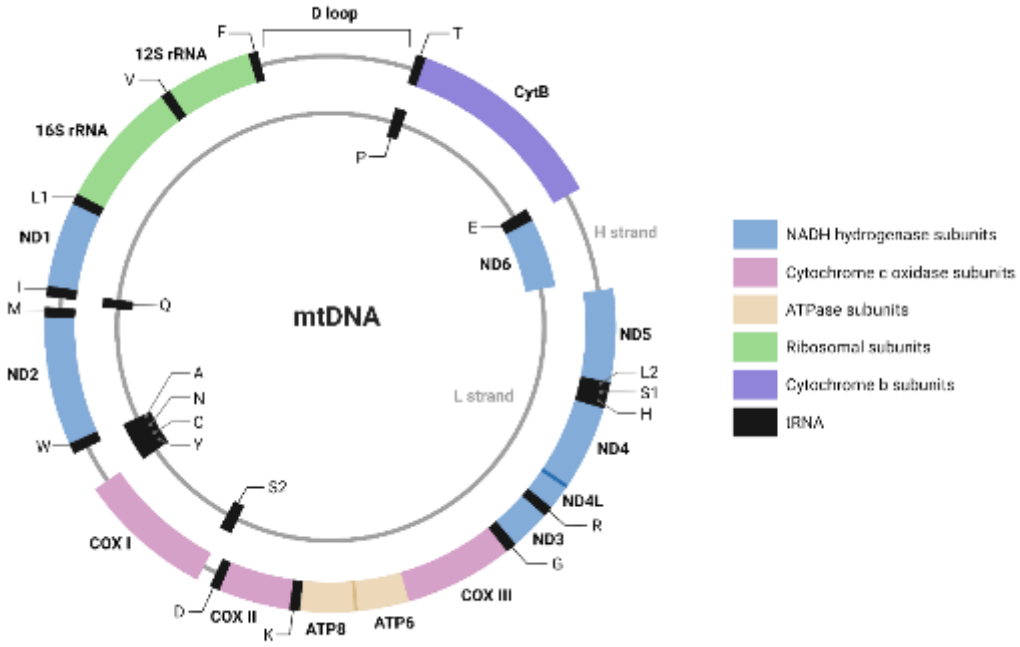


Figure 4. Mitochondrial DNA map. The outer circle denotes the heavy (H) strand of the mtDNA and the inner circle denotes the light (L) strand**.** The genes encoding the subunits of complex I or NADH dehydrogenase (ND1-ND6 and ND4L) are shown in blue; the subunits of complex IV or cytochrome *c* oxidase (COX I-COX III) are shown in pink; cytochrome b of complex III is shown in purple; and the subunits of the complex V or ATP synthase (ATPase 6 and 8) are shown in orange. The genes encoding 12S and 16S rRNAs are shown in green. Finally, 22 tRNAs F, V, L1, I, M, W, D, K, G, R, H, S1, L2, T, P, E, S2, Y, C, N, A are indicated in black boxes. Adapted from "*Human mtDNA Sequence Map*", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates**.**

Mutations in the mtDNA, as well as the reduction of mtDNA content, result in alterations in the stoichiometry of respiratory complexes that consequently cause

lower energy production, contributing to aging phenotypes [58]. The mtDNA mutator mice carrying a deficient mtDNA polymerase (Polγ) demonstrated that mtDNA mutations and deletions are responsible for a progressive decline in respiratory function. These mice have high levels of point mutations and deleted mtDNA, and they recapitulate several signs of premature aging such as increase levels of apoptotic markers, severe respiratory chain dysfunction, reduced lifespan and reduced fertility [59, 60]. In addition, several studies revealed changes in mtDNA especially in post mitotic tissues with active respiratory metabolism, such as skeletal muscle, heart and brain. Most of these changes consisted on altered mtDNA copy number, multiple deletions, tandem duplications in the D-loop region, point mutations in the tRNA genes and in the D-loop region [61, 62]. Somatic mutations in mtDNA and decrease mtDNA abundance progressively accumulate in an age and tissue-specific manner [58].

Mitochondrial biogenesis

Mitochondria are continuously produced in the cell, and therefore their mass and activity must be adapted to tissue function, cellular growth and nutrient availability. However, mitochondria are not synthesized “de novo” but arise by growth and division of pre-existing mitochondria. Mitochondrial biogenesis is thus critical for the normal function of cells, allowing the replacement of old or damaged mitochondria which are removed from the cell by mitophagy.

The synthesis of the necessary proteins for mitochondrial biogenesis and function requires the expression of gene products encoded by both nuclear and mitochondrial genomes. The correct expression of mitochondrial and nuclear genes is regulated by a series of transcription factors, encoded by nuclear genes. Among these factors, there are those that direct the expression of nuclear respiratory genes: the transcription factors nuclear respiratory factor-1 and 2 (NRF1 and NRF2) and the estrogen-related receptor (ERR-α). Regulation is also made up by coactivators, which form complexes with transcription factors and facilitate the chromatin remodelling. In this group the most important are those belonging to the family of coactivators of receptor activated by peroxisome proliferator (PPAR) and PPAR-γ coactivator α (PGC-1α). Finally, mitochondrial biogenesis is controlled by nuclear-encoded transcription factors which promote the expression of mitochondrial genes. These

include mitochondrial transcription factor A (TFAM), B1, B2 (TFB1M and TFB2) and a transcription termination factor (mTERF) [63].

PGC-1α is highly expressed in tissues where mitochondria are abundant and have an active respiration, such as brown adipose tissue, skeletal muscle and heart [64]. PGC-1α is strongly induced by cold exposure and also can be activated in response to growth signals, exercise, energy deprivation or fasting (see Fig. 5) to produce energy in the form of ATP or heat, and is directly modulated by AMPK and SIRT1 through phosphorylation and deacetylation, respectively [65]. Mitochondrial biogenesis is mediated primarily by the activation of PGC-1α. The increased levels of PGC-1α induce the formation of heterodimer complexes with transcription factors, NRF1 and NRF2, and the nuclear receptors, PPARα, PPARδ, PPARγ, that regulate the expression of many nuclear-encoded mitochondrial genes, such as cytochrome *c*, some OXPHOS subunits and TFAM. PGC-1α is post-transcriptionally regulated by modifications including phosphorylation, acetylation, and ubiquitination [66].

The critical role of PGC-1α in mitochondrial biogenesis was studied in PGC-1α-null mice that showed decreased mitochondrial mass and impaired respiratory capacity in muscle and liver [67, 68]. Mutant flies lacking the PGC-1α homolog (*spargel*) also showed defective mitochondrial respiration, and other studies showed that ectopic expression of PGC-1α in cultured myoblasts stimulates mitochondrial biogenesis [69, 70].

NRF1 and NRF2 are essential for life. *Knockout* mice for the *nrf-1* gene show deletions in mtDNA which result in early embryonic lethality, and homozygous for the null *Gabpα* gene, die prior to implantation [71, 72]. NRF1 is transcriptionally and post- translationally (phosphorylation enhances NRF1 transcriptional activity) regulated, increasing its expression in response to energy deprivation, caloric restriction, oxidative stress and exercise [73, 74]. NRF1 and NRF2 act on a number of nuclear genes required for mitochondrial respiratory function including most of those encoding subunits of the OXPHOS, and are positive regulators of the transcription of the gene encoding cytochrome *c* [75]. They are also involved in other functions acting on genes encoding key enzymes of the heme biosynthetic pathway, regulating cell cycle progression, and participating in ATP synthesis through their role in the regulation of γ-ATP synthase expression. They also participate in

mitochondrial proteins import by regulating the expression of the mitochondrial receptor TOMM20 and in the assembly, translation and transport of different mitochondrial proteins [75-78]. NRF1 and NRF2 act downstream from PGC-1α in mediating respiratory gene expression and activating the expression of factors involved in the initiation of mtDNA transcription by TFAM [79].

TFAM is a member of the high-mobility-group (HMG) involved in transcription, replication, repair, maintenance of mtDNA and mtDNA nucleoid constitution [80]. TFAM is one of the primary factors controlling the copy number of mtDNA in cells. In this sense, it has been demonstrated that animal embryos lacking mtDNA (homozygous knockouts $Tfam^{-/-}$) die during gestation. On the other hand, $Tfam^{+/-}$ knockouts exhibit reduced mtDNA copy number and severe respiratory chain deficiency [81]. PGC-1α can control mtDNA-encoded proteins expression through the regulation of TFAM levels. This is because the relative expression of TFAM can alter its affinity for different mtDNA transcription promoters located in the D-loop. Thus, low concentration of TFAM preferentially activates the promoters of the light strand, while high concentration activates the promoters of the heavy strand [80]. However, there is still some controversy about the role of TFAM in mitochondrial physiology as some *in vitro* experiments showed that mitochondrial transcription can occur in the absence of TFAM while others showed that it is necessary. This fact may indicate that under certain conditions, TFAM-independent transcription can be promoted [82, 83].

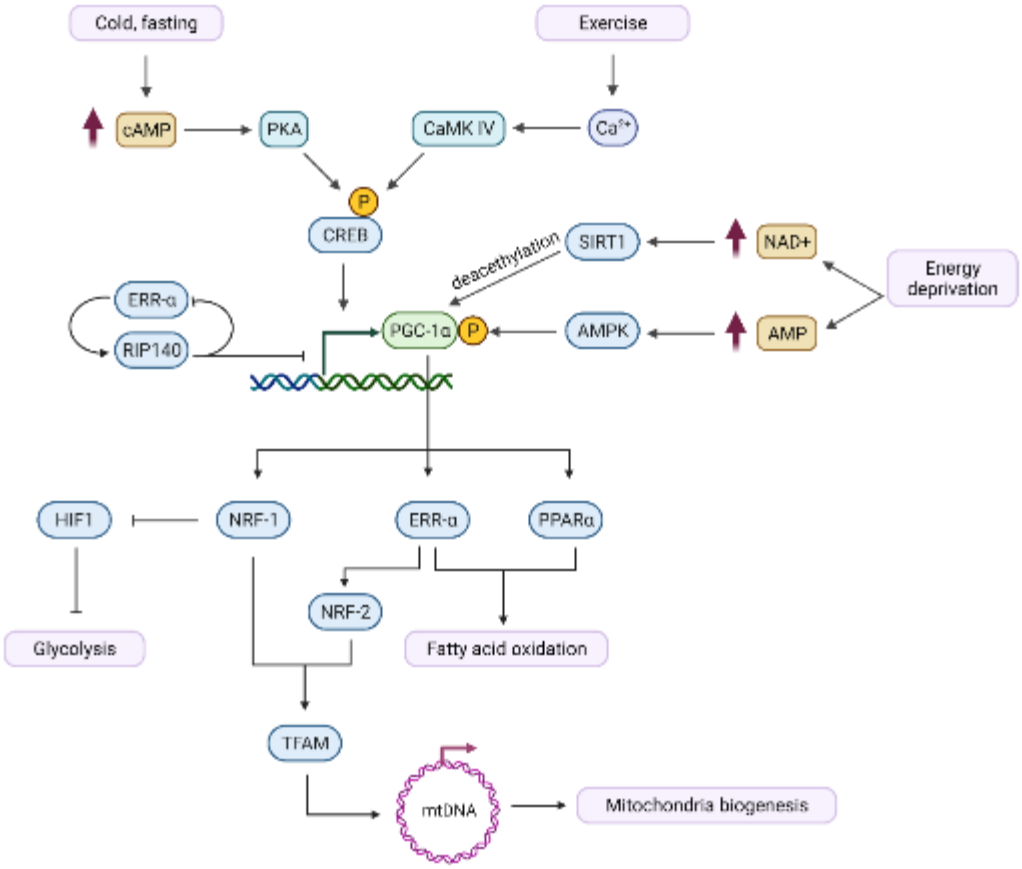


Figure 5. Summarizing scheme of the pathways implicated in mitochondrial biogenesis. Mitochondrial mass can increase in response to different stimuli such as exercise, hormones, cell differentiation and division, oxidative stress, energy deprivation, cold, etc. The generation of mitochondria requires the orchestrated expression of two genomes: nuclear and mitochondrial, so the biogenesis process depends on the import and assembly of proteins in mitochondrial compartments. The expression of the nuclear and mitochondria genes involved in mitochondria function is coordinated by the transcriptional coactivator PGC-1α, considered as the master regulator of mitochondrial biogenesis. PGC-1α lacks DNA-binding domains, thus it works through the activation of multiple transcription factors (as nuclear respiratory factors), receptors activated by peroxisome proliferators and estrogen-related receptors, which control genes involved in oxidative phosphorylation, fatty acid oxidation and glycolysis.

Mitochondrial dynamics

Mitochondria are highly dynamic organelles that undergo continuous changes in size, shape, number and distribution within the cell. Their dynamics are determined by mitochondrial turnover, biogenesis and a balance between the frequency of fusion and fission events that allow mitochondria to adapt to different situations [84]. Mitochondrial fusion and fission are essential for the maintenance and distribution of mitochondrial DNA, the quality control of the organelle and the regulation of the apoptosis. Imbalanced mitochondrial dynamics contribute to the loss of

mitochondrial homeostasis and lead to alterations in mitochondrial function, metabolism and signaling. Progressive mitochondrial dysfunction is considered a hallmark of aging, and has been associated with processes of embryonic development, aging and a wide range of pathological conditions, including diabetes, neurodegenerative and cardiovascular diseases, muscle atrophy and cancer (see Fig. 6) [85].

The core proteins that carry out the fusion and fission events are highly conserved GTPases related to the family of dynamins called dynamin related proteins (DRP). The mitochondrial dynamins include DRP1, mitofusins 1 and 2 (MFN1 and MFN2), and optic atrophy 1 (OPA1) [86].

Mitochondrial fission is mainly controlled by the cytosolic protein DRP1, although the process is not fully understood. The fission begins with the recruitment of DRP1, which is translocated to the mitochondria where it is assumed to anchor to the OMM through several transmembrane proteins such as FIS1, mitochondrial fission factor (MFF), mitochondrial elongation factor 1 (MIEF1/MiD51) and mitochondrial elongation factor 2 (MIEF2/MiD49). DRP1 is assembled to form a spiral composed of hundreds of monomers of DRP1 to wrap the mitochondria, triggering mitochondrial fission through its GTPase activity [86].

The role of mammalian FIS1 in mitochondrial fission is unclear. Some studies have shown that the overexpression of FIS1 accelerates mitochondrial fragmentation, whereas knockdown blocks fission, resulting in fused and elongated mitochondria [87, 88]. However other studies showed that changes in levels of FIS1 have no effect in the subcellular distribution of DRP1 [89]. Moreover, it has been proposed that MFF and MiDs are the genuine receptors of DRP1, whereas FIS1 participates in autophagic removal of mitochondria (mitophagy) and has no role in DRP1 recruitment or plays specific roles under some physiological processes or certain types of cells [90]. The binding of DRP1 to MFF promotes mitochondrial fission, while binding to MiD51 inhibits its activity and promotes fusion. These opposite roles may provide a fine regulation of DRP1 activity during fission progress. First, DRP1 bound to MiDs would facilitate the productive assembling of DRP1 monomers maintaining the GTP-bound state of DRP1, then DRP1 bound to MFF would activate GTP hydrolysis for membrane constriction and scission [91]. By the moment that

DRP1 is recruited in order to constrict the mitochondrion, the diameter of the collar is already much smaller than the mitochondrion itself, thus studies propose that there could be another mechanism that governs an initial step of constriction.

In yeast and mammalian cells, electron microscopy and tomography imaging revealed that the ER wraps and constricts the mitochondria before DRP1 had been recruited. This ER-mediated mitochondrial constriction marks fission sites and possibly assists in the assembly of the DRP1 collar for mitochondria constriction [92]. In this sense, it has been reported that an ER-associated actin modulator, the so-called inverted formin 2 (INF2), could contribute to mitochondrial fission by promoting the formation of actin filaments and myosin II binding to actin filaments generated by INF2, therefore driving an initial mitochondrial constriction that would allow DRP1 to lead a secondary constriction [93, 94]. On the other hand, it has been shown that mitochondrial constriction carried out by the ER can occur in the absence of DRP1 and MFF, which suggests that the selection of fission points by the ER occurs before the assembly of the rest of the fission machinery [92] .

The mitochondrial fusion process requires coordinated actions of outer and inner membranes to ensure the maintenance of compartmentalisation. Mitochondrial fusion is mediated by mitofusin proteins and occurs in two steps. First, the OMMs of two mitochondria fuse through the interaction of MFN1 and MFN2 anchored at the OMM. Later OPA1 fuses the IMMs. These proteins are subjected to very strict control by ubiquitination and proteasomal degradation [86]. Mice deficient in either MFN1 or MFN2 present midgestational lethality whereas the overexpression of any of them can induce perinuclear mitochondrial clustering and hyperfused state. In addition, MFN-1/2-deficient cells, or loss of OPA1 result in severe defects as accumulation of highly fragmented mitochondria [95].

Although MFN1 and MFN2 participate in the fusion process, MFN1 shows higher GTPase activity but lower affinity for GTP. In addition, MFN2 has many other roles:

it is involved in ER-mitochondria connections, energy metabolism and insulin signaling, mitophagy and apoptosis [96].

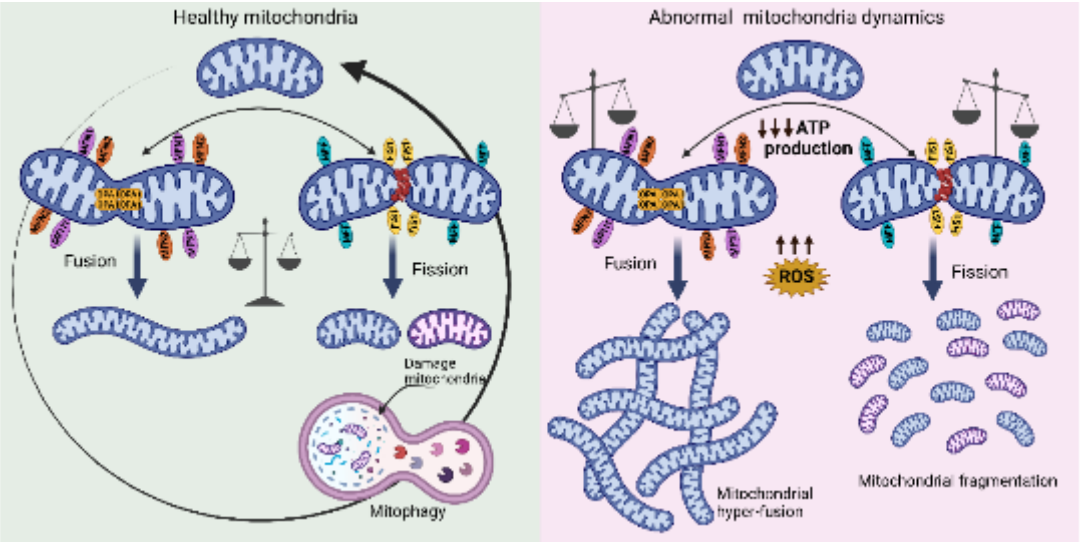


Figure 6. Mitochondria dynamics. Mitochondria continuously undergo fusion and fission processes. Mitochondrial fusion is regulated by MFN1, MFN2, and OPA1. The MFN1 and MFN2 proteins are involved in mitochondrial outer membrane fusion and OPA1 couples the fusion of the outer membrane to that of the inner membrane. Mitochondrial fission is regulated by DRP1 that is recruited from the cytosol to the OMM by various receptors: MFF, FIS1, MiD49 and MiD51, and oligomerizes to mediate mitochondrial constriction. When the fission/ fusion equilibrium is altered, the balance leans towards one of these two processes. When the balance is in favour of fusion, mitochondria form an elongated and interconnected network resistant to mitophagy. Conversely, when the balance shifts towards fission, mitochondria become fragmented. Altered homeostasis due to failures of these pathways results in a wide range of pathological conditions.

Autophagy

Autophagy is a highly conserved catabolic process that eliminates unwanted intracellular materials such as parts of cytoplasm, organelles, protein aggregates and infectious agents, through the action of lysosomes in animal cells. Autophagy is often activated in cells to respond to a large number of intra and extracellular stresses, including nutrient starvation, presence or absence of insulin, pathogen infection, hypoxia, and endoplasmic reticulum stress, among others [97]. The ability of the cell to achieve autophagy decreases with aging. Alterations in the regulation of autophagy underlay numerous pathologies, such as cancer, metabolic dysfunction, neurodegenerative disorders and liver disease [98]. Three types of autophagy have been categorized into macroautophagy, microautophagy and chaperone-mediated-autophagy:

Macroautophagy: usually referred to as "autophagy", is the mechanism by which different components of the cell are recycled, thus providing nutrients and allowing the cells to maintain macromolecular synthesis and energy homeostasis during periods of starvation and other stressful conditions. A portion of the cytoplasm is included in a compartment delimited by a double membrane called an autophagosome. Then, the autophagosome fuses with a lysosome and its content is degraded together with the inner membrane of the autophagosome. Macroautophagy is the best characterized type of autophagy.

Chaperone-mediated autophagy: it is selective for a group of cytosolic proteins marked for degradation and translocated through of the lysosome membrane forming complexes with chaperones, as is the case of Hsc-70, which are recognized through the lysosomal membrane receptor LAMP-2A, and finally translocated to the interior for degradation.

Microautophagy: it is referred to the degradation of cytoplasmic components by direct invagination of the lysosome membrane. Unlike macroautophagy, there is no formation of a double membrane to sequester the material to be degraded.

Mitophagy

Mitochondria are eliminated when they are damaged or dysfunctional through a regulated process called mitophagy, a form of selective macroautophagy. Mitophagy can be classified into three types: basal mitophagy, that routinely regulates mitochondrial regeneration; programmed mitophagy, that removes functional mitochondria (especially important in removal of parental mtDNA inheritance), and stress-induced mitophagy, responsible for the clearance of dysfunctional and damaged mitochondria [99].

The most studied mitophagy mechanism is mediated by PTEN-induced putative kinase 1 (PINK1) and parkin proteins (Fig. 7). Under basal conditions, PINK1 is immediately translocated into the mitochondria where it is processed by the matrix processing peptidase (MPP) and the inner membrane protease PINK1/PGAM5-associated rhomboid-like protease (PARL), and the resulting cleavage product is released into the cytosol to be degraded by the proteasome. When damaged, mitochondrial membrane depolarizes inhibiting PINK1 cleavage and degradation

leading to its accumulation in the OMM. Then, PINK1 recruits and phosphorylates parkin which ubiquitinates various proteins at the OMM including MFN1 and MFN2 blocking the fusion capability of these mitochondria. PINK1 also phosphorylates ubiquitin. Other substrates of parkin have been identified such as voltage-dependent anion channel 1 (VDAC1), translocase of outer membrane 20 (TOM20) and mitochondrial Rho GTPase1 (MIRO1) [100, 101]. To avoid re-fusion with neighbouring healthy populations, myosin forms a complex with parkin and induce the assembly of a F-actin cage that encapsulates the damaged mitochondria forming a physical barrier [102]. Then, the ubiquitination labelling of parkin serves as a signal to recruit the autophagy machinery to eliminate damaged mitochondria. The presence of ubiquitin residues in the resident proteins of the OMM allows the protein p62/sequestosome 1 (p62/SQSTM1) to interact with both ubiquitin-labelled proteins residues and the microtubule-associated light chain 3 (LC3) protein, located in the autophagosome membrane [100, 101].

The second pathway of mitophagy involves proteins such as autophagy-related protein 32 (Atg32), BCL2 interacting protein 3 (BNIP3) or BNIP3-like (BNIP3L or NIX). Atg32, NIX and BNIP3 are OMM-bound proteins that act as mitophagy receptors. Since they can directly interact with LC3, ubiquitin signaling is not required. BNIP3 and NIX members of the BH3-only protein subgroup within the BCL2 family, also involved in cell death. When the mitophagy flow is blocked or enhanced, its role in inducing cell death becomes predominant [103].

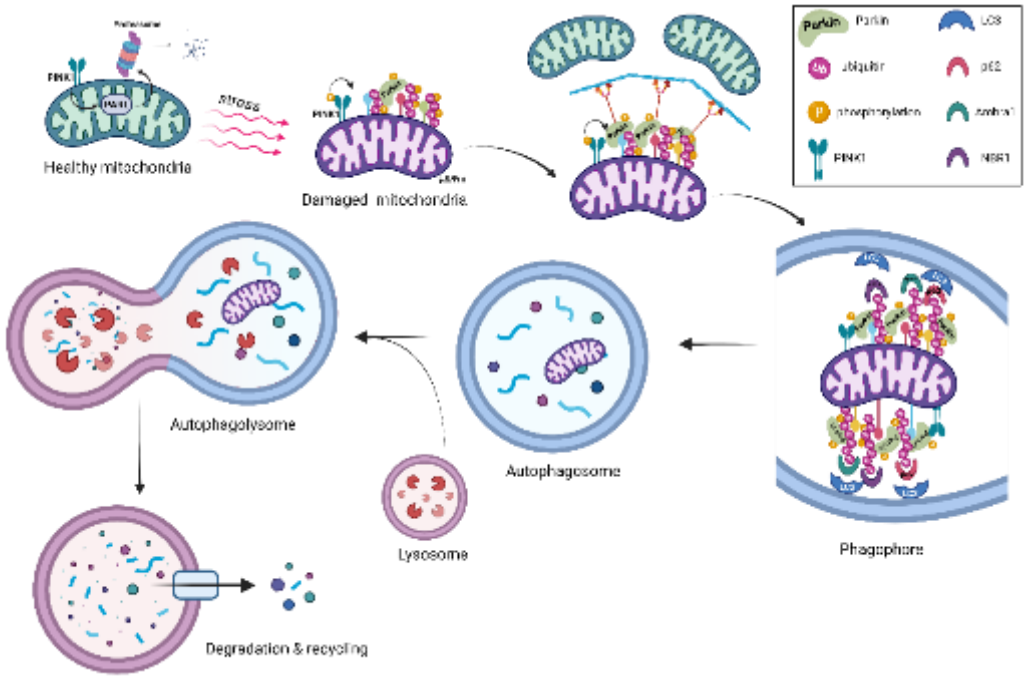


Figure 7. PINK1/parkin dependent mitophagy. In healthy mitochondria (blue), PINK1 is translocated into the mitochondria where it is processed by the protease PARL and released to the cytosol being then degraded by the proteasome. Damaged mitochondria lose their membrane potential, leading to PINK1 stabilization and parkin recruitment to initiate mitophagy. Once activated, parkin ubiquitinates several mitochondrial substrates. Damaged mitochondria are isolated by an F-actin cage in order to prevent re-fusion with healthy mitochondria. Ubiquitinated mitochondrial substrates recruit autophagy receptors p62, NBR1 and Ambra1 that interact with LC3 to enrol the autophagic machinery. Then, the mature autophagosomes fused with lysosomes to form autophagolysosomes, where the mitochondria are subsequently degraded.

Caloric restriction

For decades, several interventions have been reported to delay aging and attenuate a wide variety of diseases associated with this process. Among them, the nutritional and genetic interventions are currently the most used and studied. Other interventions (the so-called "pharmacological") consist of supplementing the diet with some types of compounds, as those acting as CR mimetics (metformin or rapamycin, for example) and others targeting genes that regulate epigenetics, such as resveratrol [104, 105]. Among all, the most used intervention that offers the most consistent results is caloric restriction (CR) [106].

Thus, CR without malnutrition, is the most effective nongenetic intervention that promotes a healthy aging phenotype and extends lifespan. The anti-aging potential of CR was first reported by McCay *et al.* in 1935 demonstrating that rats subjected to

CR had a longer lifespans than the *ad libitum* group [107]. Subsequent studies reported the beneficial effects of CR in a wide range of species including *C. elegans* worms [108], mice [109] or flies [110].

Due to the extraordinary similarity to humans, the rhesus monkey (*Macaca mulatta*) is an excellent model for the study of human aging. To date, three independent studies in this species have been carried out to investigate the effect of CR. The first one was the study developed at the University of Maryland, where the animals were subjected to 30% CR. This study reported a positive association of CR with survival showing an average life expectancy of 32 years of age in the CR group in contrast with *ad libitum* group where the average life expectancy was 25 years of age. However, this study should be considered as preliminary since it was restricted only to eight male specimens [111].

The second study started in 1987 at the National Institute on Aging (NIA) [112] and the third, in 1989, at the Wisconsin National Primate Research Center (WNPRC) [113, 114]. In both studies the monkeys were tracked for more than 20 years with an exhaustive follow-up of different health indicators, as well as the incidence of disease and death. Although the experimental design of both studies was quite similar (30% CR, environmental conditions, etc.), contradictory results were reported.

The NIA study did not report significant improvements in lifespan in CR monkeys, although they found numerous health benefits, including low cholesterol, TGs and glucose levels. In addition, inflammation and immune response were improved in CR and the incidence of diseases such as diabetes, CVDs and cancer were also lower in this group [112]. In the WNPRC, the incidence of age-related deaths was also reduced with CR and other positive effects of this intervention were similar to those described in the NIA study (reduction of diabetes, cancer, CVDs, absence of sarcopenia, etc.). However, this study did reflect the increased longevity of CR monkeys [113, 114]. The discrepancies between both studies were attributed to the different composition of the diets: in the NIA study a naturally sourced diet was employed, in contrast with a semi-purified diet used in WNPRC research. On the other hand, the ages at the beginning of the intervention were different in both studies and there were also sex-related differences in the results [115].

The extension of life- and health span driven by CR is inversely proportional to the intensity and the length of the intervention. In several studies in rodents, it has been shown that the best results are obtained with 20-40% CR (up to 60% in mice). Above these limits, undesirable effects such as premature death begin to appear [116-119]. The complex mechanism of metabolic adaptation to CR (which include changes in signaling pathways regulating growth, oxidative stress, autophagy and inflammatory processes among others) underlie both the modulation of the aging process and the CR outcomes [106].

At the cellular level, an essential effect of CR is the reduction of oxidative stress, since this intervention decreases ROS generation and, at the same time, increases detoxification and repair mechanisms The main cellular sources of ROS are the mitochondria, and the effects of CR on ROS production appears to be mediated by changes in the mitochondrial membrane potential, being this fact probably a consequence of lower oxygen consumption in CR animals [22, 47, 106].

Mitochondrial membrane permeability is also crucial for its proper function and largely depends on its fatty acid composition. CR increases monounsaturated fatty acids (MUFAs) content in membranes while decreasing PUFAs without any observed change in saturated fatty acids (SFA), which is in accordance with the Theory of Membranes in Aging [120, 121]. The mechanism by which CR alters the saturation degree of membrane lipids is not fully understood, but it appears to be related with certain hormones such as insulin and thyroid T3 and T4, that regulate the activity of elongases and desaturases [116]. A lower degree of unsaturation in the membranes implies less damage and greater stability, since the double bond of unsaturated fatty acids is prone to peroxidation by free radicals [116, 120].

Another line of defence against oxidative stress consists of improving the ability to repair damaged DNA by base excision repair (BER), or by increasing the elimination of macromolecules damaged by autophagy [122, 123]. In this sense, CR modulates key molecules involved in the regulation of autophagy through the stimulation of the energy sensor AMP-activated protein kinase (AMPK), which in turn inhibits the kinase activity of the mammalian target of rapamycin (mTOR), an autophagy repressor [124]. Thus, by inhibiting mTOR, autophagic processes are stimulated, resulting in greater renewal of organelles and delaying the process of cellular aging.

Furthermore, NAD^+ homeostasis is important for the maintenance of many functions and protection against aging and age-related diseases [125]. NAD^+ is a coenzyme used as a substrate for several regulatory proteins. These enzymes can be ADP-ribosyltransferases including poly (ADP-ribose) polymerases (PARP), cADPR synthases, the NADase/cADPR hydrolase/cyclase CD38 and its homologue CD157 and deacetylases belonging to the SIRTs family [126]. Increases in cellular levels of NAD^+ and the activation of SIRTs extend longevity in several species such as yeast, mice, flies and worms [127-130]. This places NAD^+ as a key regulator of aging and longevity. Therefore, the exhaustive study of NAD^+ metabolism, can offer many keys to understand aging and how to slow it down or even reverse it.

Under CR conditions, energetic substrates are oxidized at low rate, which results in increased levels of NAD^+ which, in turn, stimulates enzymatic activity of the SIRT family. SIRTs are divided into four classes: SIRT1-SIRT3 belong to class I, SIRT4 to class II, SIRT5 to class III, and SIRT6 and SIRT7 to class IV [131]. The role of SIRTs proteins in survival processes involves the regulation of key genes products as p53, cMyc, FOXO and are also involved in protein repair allowing the cell to tolerate some degree of CR and withstand oxidative stress. CR especially enhances the expression of SIRT1, a regulator of mitochondrial biogenesis in multiple tissues [132, 133].

SIRT1 functions include deacetylation of FOXO and PGC-1 α. FOXO is required for regular response to oxidative stress by increasing the transcription of antioxidant enzymes and consequently increasing resistance to oxidative stress. On the other hand, deacetylated PGC-1α moves to the nucleus and binds to NRF1 activating the transcription of mitochondrial genes resulting in mitochondrial biogenesis [134].

In summary, CR is an effective intervention that delays aging and /or aged-related diseases. Cells respond to stressful conditions due to deficiency of energy intake by controlling processes related to oxidative stress, ATP production, DNA reparation, cell death, senescence and, cancer among others. However, several factors must be considered to obtain all the possible benefits of CR when used as nutritional intervention. Among these factors are the age and sex of the animals as well as the composition of the diet and the degree of CR.

NADH-cytochrome *b5* reductase-3

NAD^+ is a metabolic regulator and its therapeutic efficacy, demonstrated by different approaches aiming to increase NAD^+, has been reported in metabolic diseases such as insulin resistance, fatty liver or hypertension [135]. Likewise, the increase in intracellular NAD^+ improves skeletal muscle, cardiac, and renal function, and has a neuroprotective effect. This places NAD^+ as a key regulator of longevity, aging and disease [126]. Accordingly, great attention has been paid towards those interventions aimed to increase NAD^+/NADH ratio and/or boosting intracellular NAD^+. Overexpression of enzymes capable of increasing NAD^+ levels from the oxidation of NADH, as NADH-cytochrome *b5* reductase-3 (CYB5R3), is a strategy that is being currently analysed [136-138].

CYB5R3 is a flavoprotein included in a larger family of four members in mammals encoded by different genes (*Cyb5r1* through *4*). CYB5R3 catalyses the reduction of cytochrome *b5* and additional alternative acceptors as CoQ using NADH as an electron donor, thus generating NAD^+ [139, 140]. CYB5R3 plays an important role in the regulation of metabolic pathways associated with healthspan and aging through mechanisms related with protection against oxidative stress, production of cellular NAD^+ and alterations in membrane fatty acids composition [141, 142]. Although CYB5R3 shows a constitutive basal expression in many tissues, it can be also induced in response to environmental stress and nutrient deprivation. Endogenous *Cyb5r3* gene expression is controlled by the SP1 transcription factor [143], which can be further upregulated through FOXO3a- and NFE2L2-dependent pathways [144].

The CYB5R3 enzyme has two isoforms differentially distributed in the organism. The differences between the isoforms are due to post-transcriptional modifications by alternative splicing. One of the isoforms is soluble and it is found exclusively in the erythroid lineage. Its main function in erythrocytes is to reduce methaemoglobin to haemoglobin. The methaemoglobin molecule contains iron in the ferric state (Fe3+), a form in which oxygen has a high affinity for the heme group, preventing it to be released into the tissues. The redox activity of soluble CYB5R3 regenerates the ferrous (Fe2+) form (hemoglobin) using the reducing power of NADH. For this reason, this enzyme is also regarded as methemoglobin reductase [145-147].

The other isoform of CYB5R3 is membrane bound, and is mainly found in the OMM and the endoplasmic reticulum, although its association with other cell membranes, as the plasma membrane, has been also reported [140, 141, 148]. At the OMM, this isoform of CYB5R3 plays an important role in the regeneration of cytosolic ascorbate with the participation of a high-potential and OMM-specific isoform of cytochrome b_5 (CYB5B) [149]. On the other hand, endoplasmic reticulum-associated CYB5R3 acts as an oxidoreductase participating in the elongation and desaturation of fatty acids, biosynthesis and metabolism of cholesterol and steroids, inhibition of chronic pro-inflammatory pathways and mono-oxygenations through the cytochrome P450 system, with the participation of a low-potential isoform of cytochrome b_5 (CYB5A) [150, 151]. In addition, CYB5R3, together with NAD(P)H:quinonoe oxidoreductase 1 (NQO1, also known classically as DT-diaphorase), are oxidoreductases belonging to the plasma membrane redox system, that act transferring electrons from cytosolic NADH to CoQ, regenerating its antioxidant capacity [138].

The lack of CYB5R3 causes the accumulation of methemoglobin and a rare recessive hereditary disease called methemoglobinaemia (RHM). Type I RHM is benign, and is limited to the soluble isoform of the enzyme present in erythrocytes, while type II RHM affects all cells and is characterized by the appearance of severe neurological disorders besides being incurable [145-147], which is a reflection of the important functions in which CYB5R3 participates.

It has been reported that some of the antiaging nutritional interventions as CR induce CYB5R3 expression, indicating that some of the salutary effects of this intervention could be mediated, at least partially, by CYB5R3 induction. Moreover, transgenic mice overexpressing this enzyme exhibits longevity extension. At the metabolic level, CYB5R3 increases insulin sensitivity, decreases inflammation and oxidative stress, and protects mice against cancer induction [138, 140, 141]. Therefore, this enzyme plays an essential role in the regulation of lipid metabolism that allows increased life expectancy. Thus, overexpression of CYB5R3 in transgenic mice, as well as NQO1 plus CYB5R3 in double transgenics, mimic some aspects of CR, improving health and prolonging lifespan in mice [137, 138]. These observations suggest that strategies aimed to boost CYB5R3 activity may constitute viable interventions to confer protection against metabolic pathologies and improve healthspan [138].

Lipid metabolism

There are five major sources of metabolic fuel used by animal tissues. These include glucose, fatty acids, ketones, amino acids, and lactate. Due to their ability to efficient storage as triglycerides (TGs), fatty acids (FAs) are a major source of energy for animals. Cells can obtain FA as metabolic fuels from two main sources: diet intake and from adipocyte-stored fats.

In general, dietary lipids are supplied in the form of TGs. Since they are totally water-insoluble, TGs must be hydrolysed to free fatty acids or to monoacylglycerols to be absorbed in the small intestine. However, this process requires a previous solubilization to form absorbable mixed micelles of bile acids and TGs. By this mechanism, TGs become accessible to pancreatic lipases yielding monoacylglycerols and diacylglycerols, which are absorbed by the enterocyte. After translocation through the enterocyte and re-esterification, the new TGs are packed together with cholesterol and specific proteins into lipoprotein particles called chylomicrons, which are sorted to the tissues by the lymphatic and cardiovascular systems. In muscle and adipose tissue capillaries, the enzyme lipoprotein lipase hydrolyses TGs to fatty acids and glycerol that are taken up by the cells. In muscle, fatty acids are oxidized to get energy and in adipose tissue they are re-esterified for storage in lipid droplets in the form of TGs. The remaining blood chylomicrons (relatively free from TGs but still containing cholesterol and apolipoproteins) are then endocytosed by hepatocytes. The TGs entering the liver are used as a local energy supply and as substrates for ketone bodies production. When the diet contains more FAs than necessary to be immediately used for fuel, the liver converts them into TGs which are packaged as specific apolipoproteins to form very low-density lipoproteins (VLDL) with TGs as the main lipidic component. VLDL are transported to the adipose tissue for storage in lipid droplets. The remnants of those VLDL are hydrolysed by lipoprotein lipase resulting in intermediate-density lipoprotein (IDL), that can be further catabolized by hepatic lipase to produce cholesterol-rich low-density lipoproteins (LDL) (Fig. 8) [152].

Dietary fats

Dietary fats are essential components necessary to generate energy. Fats are also required in other relevant functions such as acting as carriers of fat-soluble vitamins (A, D, E, and K) and to protect the body and vital organs from physical trauma and changes in temperature by their storage in fat deposits [153].

Fatty acids can be categorized as saturated (SFAs), monounsaturated (MUFAs), or polyunsaturated (PUFAs) and most of them can be synthesized by the cells. However, the essential fatty acids (EFAs) must be ingested through the diet. EFAs were established from the studies by Barr and Burr [154, 155] who reported several disorders in growth, reproductive function, and dermatitis in rats fed a fat-free diet. EFAs are PUFAs, and they are divided into two groups: the n-6 series PUFAs derived from cis-linoleic acid, and the n-3 series PUFAs derived from α-linolenic acid [154, 155]. Most plant oils are rich sources of n-6 PUFAs. Among the most widely used are soybean, corn, and sunflower oils. On the other hand, n-3 PUFAs in the form eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are found in diets based on seafood or fish oil [156]. There is another non-essential fatty acid from the n-9 series that has special importance in a typical Mediterranean diet: oleic acid. It is mainly obtained from olive oil and is a rich source of n-9 MUFAs [157].

Several studies have reported the relationship between dietary fat and the risk of metabolic and cardiovascular diseases and others disorders [158]. Diets rich in MUFAs and PUFAs have cardioprotective effects [157, 159], while the relationship between SFA and the risk of coronary heart disease is still controversial [160]. Negative effects associated with SFA come from the ability of these fats to increase the levels of low-density lipoprotein (LDL) and cholesterol in blood, while PUFAs have the opposite effect. PUFA-enriched diets, especially in those of the n-3 series, are well known to exert anti-inflammatory effects and to promote protection against several disorders such as cardiovascular disease [161], cancer [162], neurodegenerative diseases[163], diabetes and obesity [164].

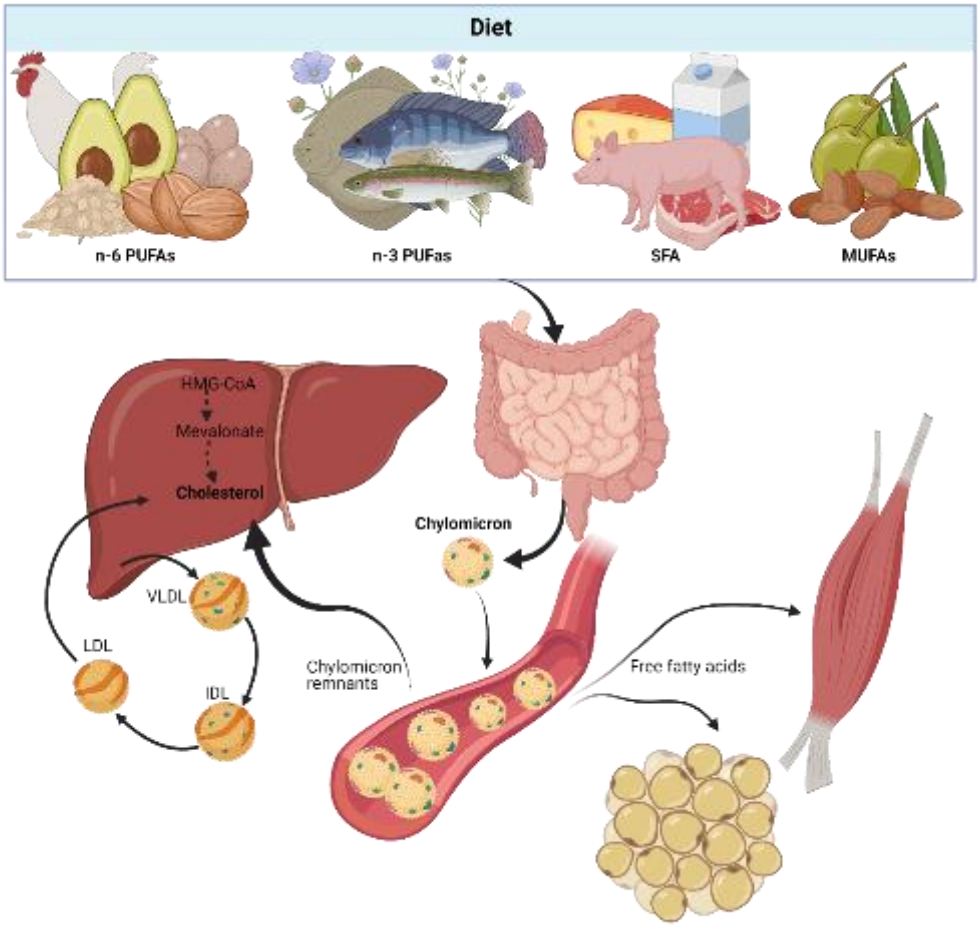


Figure 8. Overview of lipid metabolism. Ingested lipids are hydrolysed in the intestinal lumen and absorbed by enterocytes of the small intestine. TGs and cholesterol are packaged into chylomicrons and travel through the lymphatic system and the blood to the tissues. In the capillaries, TGs are hydrolysed releasing free fatty acids, that are taken up by adipose tissue and skeletal muscle. The chylomicron remnants are cleared by the liver where they are used as fuel or repackaged into VLDL. VLDL particles are released into the circulation and the VLDL remnants are transformed into LDL.

Membranes are plastic structures that can adapt their lipid composition according to the main dietary fat [165, 166]. Changes in the structure of membrane lipids can influence the functions of membrane proteins and, therefore, cause alterations in molecular and metabolic activities in cells, tissues and, specifically, in mitochondrial membranes [167]. PUFA-rich diets lead to more unsaturated membranes with higher susceptibility to free radical oxidation than SFA or MUFA-based diets [168]. Fatty acids can undergo autoxidation generating lipid peroxides that compromises the properties of biological membranes [169]. Furthermore, the toxic products derived from peroxidation can harm proteins, DNA and other biomolecules [170-172]. Since

lipids are prone to oxidation with the consequent negative effects in health, the use of antioxidants in foods has become widespread to prevent lipid oxidation [173].

Liver and aging

Most of the nutrients absorbed in the digestive tract are processed in the liver, which is the largest gland and the second largest organ in the body. Liver secretes bile, which contains bile salts needed for the digestion, synthesizes glucose from glycogen, produces blood serum proteins including albumin, globulin, and several coagulation factors, and release them into the bloodstream. The liver also plays a key role in the metabolism by processing molecules such as lipids, proteins, carbohydrates and hormones, and it is also important for detoxifying alcohols and drugs [174].

The functional unit of the liver is the hepatocyte, the parenchymal cell where most of the hepatic function take place. In the hepatic lobules, the hepatocytes are arranged in cords alternating with the sinusoid vessels. On the other hand, in the "portal spaces" (located at the angles of the lobules) are the portal triads, consisting of an arteriole, a venule and a biliary duct. The liver receives blood from two different sources: the majority arrives through the portal vein and contains low-oxygenated blood and nutrients, allowing the adequate regulation of the nutrients supply from the diet, the rest of the blood comes from the hepatic artery that supplies oxygen to hepatocytes [174].

Despite the reported changes (see below), the liver ages relatively well. Although in normal conditions hepatocyte turnover rate is low, the liver shows a relatively high rate of regeneration. That is why liver has been widely used as a tissue model to study the effects of different diets, CR and other anti-aging interventions [175-177]

Age-related changes in liver include a reduction of weight and volume, a decline in the rate of hepatic regeneration, increased hepatocyte size and polyploid nuclei, decreased area of smooth endoplasmic reticulum, decreased clearance of free radicals and a reduction in the number and function of mitochondria resulting in a decreased in the ATP production. The liver also undergoes functional alterations such as reduced hepatic blood flow, decreased phagocytic capacity of Kupffer cells, and reduced activity of cytochrome P450. Aging is also associated with abnormal redistribution of fat in the body, with increased ectopic fat deposition in non-adipose

tissues, including liver [178, 179]. All these structural and functional alterations contribute to the development of liver disease with age.

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in young and old people affecting up to one quarter of the human population worldwide [180]. NAFLD is characterized by the abnormal accumulation of lipids in the hepatocytes, accounting for more than 5% of the liver weight [181]. This hepatocellular fat deposition can progress and trigger a range of pathologies from steatosis, non-alcoholic steatohepatitis (NASH), increasing inflammation, hepatic fibrosis, cirrhosis and eventually hepatic failure including the potential risk of developing hepatocellular carcinoma [182]. The processes leading to NAFLD are multifactorial. A high- fat diet, circulating lipid levels, *de novo* lipid synthesis, changes in fatty acid oxidation, obesity and insulin resistance cause FAs and TGs accumulation in hepatocytes. On the other hand, in genetically predisposed individuals the progression of NAFLD to NASH can be triggered faster. Among the genetic factor involved, polymorphisms in genes such as PNPLA3 and TM6SF2 have been identified as determinants of steatosis [183, 184]. However, the molecular mechanisms involved in the development of NAFLD and the progression of steatosis to steatohepatitis are not well understood.

Several studies reported alterations in mitochondria morphology and function in the liver, and NASH has been considered a mitochondrial disease [185-188]. As the mitochondria are the main source of ROS inside the cell, one proposed mechanism for NAFLD is the increased oxidative damage by mitochondrial ROS that leads to mitochondrial dysfunction, causing also an abnormal activity of the ETC, inflammation and fibrosis [187]. Oxidative stress, lipotoxicity, and inflammation are factors directly related to aging, which is the most common cause for the progression of NAFLD [189].

Heart and aging

The heart is the main organ of the circulatory system and is one of the first organs to form during embryonic development. It is in charge of driving the blood that carries oxygen and nutrients to every cell of the body through rhythmic contractions. The heart has four compartments - two atria and two ventricles - that are enclosed by thick muscular walls. The upper part of the heart is divided by the right and left atria, and

the bottom part is divided into the right and left ventricles and separated by a wall called the interventricular septum. The circulatory system works closely with other systems in the body, especially with the pulmonary system. Blood flow through the aorta artery from the left ventricle to other tissues and then returns from organs by the superior and inferior cava veins in the right atria in a circuit called systemic circulation. Afterwards, blood pass to right ventricle and leaves through the pulmonary artery to the lungs where it is oxygenated and returns to the left atria through the pulmonary vein. This second circuit is called pulmonary circulation [190].

The heart is a complex organ, composed of different cell types (cardiomyocytes, vascular cells, and fibroblasts) that contribute to its structural, biochemical, mechanical, and electrical properties. Among them, cardiomyocytes are well differentiated cells without regeneration capacity and are responsible for the contractile function of the myocardium. Cardiomyocytes are linked together by the intercalated discs that ensure the transmission of action potentials. Cardiomyocytes have low amount of energy reserves in the form of lipids or glycogen that it compensated with the high mitochondrial content, therefore they obtain energy from the OXPHOS system rather than from glycolysis [191].

The most drastic changes in the heart function occurring with aging are: left ventricular hypertrophy, alterations in the diastolic function, aortic valve calcification, arrhythmia and heart failure [192, 193]. These alterations are, at least in part, due to a decrease in the number of cardiomyocytes, as well as to hypertrophy and fibrosis due to increased collagen content and alterations in the extracellular matrix[193, 194]. As postmitotic cells, cardiomyocytes are highly susceptible to age-related mitochondrial damage and ROS production, which results in an overall enhancement in the rate of cardiomyocyte death with age. Thus, the progressive decline of cardiomyocyte mitochondrial function is considered the major mechanism underlying heart senescence [195].

CVDs, mainly atherosclerosis, hypertension, myocardial infarction, and stroke, are the main cause of mortality in the world and its prevalence increases with age. [196]. Atherosclerosis is the main cause of CVDs and it is promoted by the accumulation of cholesterol and fatty acids in tissues leading to the production of inflammatory

cytokines and ROS [197]. Arrhythmia might be due to the accumulation of mtDNA mutations and the subsequent ETC deficiency [198]. Heart valves calcification (especially aortic and mitral valves) leading to cardiac pressure/volume overload is also associated with advanced age [199]. These changes may be independent of conventional risk factors for CVDs: smoking, hypertension, obesity, cholesterol levels, diabetes, etc that further contributes to CVD prevalence and mortality. Since the heart is vital for life, it has been also widely studied as an aging model [200, 201].

Objetives

The working hypothesis of this PhD is that mitochondrial efficiency is optimized in CYB5R3 transgenic mice, which leads to an improvement in energy production and better preservation of mitochondria during aging. For this reason, our main objective has been to study several mitochondrial function markers in liver and heart tissues of CYB5R3-overexpressing mice submitted to nutritional interventions (CR and alterations in fat composition of the diet) and aging.

To this end, we have pursued the following 5 specific aims:

1. To study the physiological effects of CYB5R3 overexpression, dietary fats, CR and aging on body composition and biochemical profile.
2. To characterize the CYB5R3 overexpression in liver and heart in the three proposed interventions.
3. To determine the combined effect of CYB5R3 overexpression and CR on key markers of mitochondrial function.
4. To determine dietary conditions that maximize mitochondrial preservation in mice overexpressing CYB5R3.
5. To determine the effects of aging on the mitochondria parameters studied in the previously indicated aims in CYB5R3 overexpressing mice.

Material & Methods

Animal models

1. Mice husbandry

For this study, male mice of the C57BL/6J strain and overexpressing the cytochrome *b5* reductase 3 enzyme (CYB5R3) were used. These mice were generated as previously reported by Martin-Montalvo et al [138], by transgenesis of the CYB5R3 rat gene.

Transgenic mice were kindly provided by Dr. Rafael de Cabo (Translational Gerontology Branch, National Institute on Aging, Baltimore, Maryland, USA) to establish a colony in the Servicio de Animales de Experimentación (SAEX) of the University of Córdoba. To this purpose, transgenic males in a C57BL/6J background were transferred from the Animal Facilities in Baltimore to the laboratory of Charles River (Écully, France) for rederivation of embryos. Transgenic males were crossed with females of the same background and the embryos were then transferred to pseudo-pregnant females, that were then transferred to the University of Córdoba. Founding couples were established with the CYB5R3-Tg males that were obtained from this progeny and wild-type females in the same genetic background obtained from Charles River (Barcelona, Spain) to establish a colony that was maintained under barrier conditions at the Service of Experimentation Animals (SAEX) of the University of Córdoba. The resulting litters were subsequently identified by PCR genotyping using genomic DNA (see below).

The mice were housed in sterile filter-capped cages in a fully controlled environment with 12 hours light/dark cycle at 22 °C with free access to water and were fed a standard chow until they reached the age of three months. The animals were group-housed, except mice from caloric-restricted (CR) groups that were housed individually to have an exhaustive control of the intake.

All animal procedures were approved by the Bioethics and Biosafety Committee of the University of Cordoba (Spain), according to the current EU regulations, and were authorized by the Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía (Spain; authorization code: 20/04/2016/053).

Genotyping

The genotyping was performed by isolating genomic DNA obtained from tail tissue. The tissue was digested in lysis buffer containing 1M Tris pH 8.5, 0.5M EDTA pH 8, 10% SDS, 1M NaCl, and proteinase K (Qiagen, Hilden, Germany) diluted at 1:200 in DEPC-water. The sample was incubated at 55 ℃ in a dry block heater with shaking every 30 min, in order to degrade the tissue and break the cells to release the genomic DNA. After the digestion, the tissue lysate was centrifuged at 13,000 g for 30 min. The supernatant was transferred to a new tube and an equal volume of isopropanol was added for DNA precipitation. The solution was mixed by inversion of the tube. A second centrifugation was run at 13,000 g for 30 minutes. After centrifugation, the supernatant was discarded and the pellet washed with 70% ethanol. The sample was dried at room temperature and the pellet was subsequently resuspended in 100 µL of DEPC-water. Once the DNA extraction was completed, the samples were stored at -20 ℃ for genotyping on the following day using selected primers for the *CYB5R3* transgene and for *IL2* that was used as positive control of the reaction. The PCR was performed using the specific primers listed in Table 1.

Table 1. Primers sequences for genotyping

Gene	Forward	Reverse
CYB5R3	5’ CACCAAAATCAACGGGACTT 3’	5’ AGACCGGGGAGAGTACCACT 3’
IL2	5’CTAGGCCACAGAATTGAAAG ATCT 3’	5’ GTAGGTGGAAATTCTAGCATCATCC 3’

2. Cohorts and diets

Three cohorts of mice were established. All cohorts consisted of CYB5R3-Tg mice and wild-type mice of the same genetic background that were obtained from the same litters and used as controls.

A first cohort, called the aging intervention group, consisted of two experimental groups as a function of age. These mice were fed *ad libitum* (AL) for 4 or 21 months respectively (7 or 24 months of age at the end of the intervention) with standard AIN93M diet (containing soybean oil as main source of fat).

A second cohort, called the dietary fat intervention group consisted of four experimental groups that differed in the source of fat used for diets formulation. These mice were fed *ad libitum* for 4 months (7 months of age at the end of the intervention) with an AIM93M-based diet containing either soybean (S), lard (L), olive oil (O) or fish oil (F) as the main source of fat.

A third cohort called the CR intervention group consisted of two experimental groups that differed in calory intake. Both of them were fed for 4 months (7 months of age at the end of the intervention) with an AIM93M-based diet containing soybean oil. The two dietary groups were *ad libitum* (AL) and caloric restriction (CR) with a 40% reduction of the *ad libitum* intake.

The 7-months old mice fed *ad libitum* with the AIM93M diet containing soybean oil were the same in all the cohorts, and were used as controls. All diets were prepared monthly by the Animal Experimentation Service of the University of Granada (Spain) and stored at −20 °C to minimize fatty acid oxidation.

The exact composition of the experimental diets, as well as their fatty acid content, are described in Table 2 and Table 3. Lard, olive oil, and fish oil diets were supplemented with adequate amounts of soybean oil to meet linoleic acid requirements [202]. Mice were weighed once a week until their sacrifice.

Table 2. Ingredients of the four diets used in our animal interventions

Ingredients	Soybean oil (g/Kg)	Lard (g/Kg)	Oilve oil (g/Kg)	Fish oil (g/Kg)
Casein	140.00	140.00	140.00	140.00
Corn starch	465.692	465.692	465.692	465.692
Maltodextrin	155.00	155.00	155.00	155.00
Sucrose	100.00	100.00	100.00	100.00
Cellulose	50.00	50.00	50.00	50.00
Soybean oil	40.00	9.00	11.00	12.00
Lard	0	31.00	0	0
Olive oil	0	0	29.00	0
Fish Oil	0	0	0	28.00
t-Butylhydroquinone	0.008	0.008	0.008	0.016
Mineral mix #94049	35.00	35.00	35.00	35.00
Vitamin mix #94047	10.00	10.00	10.00	10.00
L-Cysteine	1.80	1.80	1.80	1.80
Choline bitartrate	2.50	2.50	2.50	2.50

Table 3. Specific fatty acid content of each diet

Fatty acid	Soybean oil (%)	Lard (%)	Olive oil (%)	Fish oil (%)
Lauric acid C12:0	0	0.09	0	0.1
Myristc acid C14:0	0.06	1.46	0.01	6.67
Palmitic acid C16:0	9.47	25.84	10.83	19.16
Palmitoleic acid C16:1	0.08	2.67	0.91	7.35
Margaric acid C17:0	0.11	0.3	0.05	0.94
Margaroleic acid C17:1	0.05	0.27	0.1	0.29
Stearic acid C18:0	4.64	13.67	3.15	4.07
t- Oleic acid C18:1	0.03	0.24	0.02	0.16
Oleic acid C18:1	21.75	46.45	79.27	20.47
t- Linoleic acid C18:2	0.04	0.03	0	0.01
Linoleic acid C18:2	55.22	7.17	4.24	2.46
Linolenic acid C18:3	7.57	0.43	0.65	1.3
t- Linolenic acid C18:3	-	-	-	0.23
Arachidic acid C20:0	0.35	0.2	0.38	0.61
Gadoleic acid C20:1	0.17	1.07	0.24	0.74
Behenic acid C22:0	0.33	0.02	0.11	0.25
Lignoceric acid C24:0	0.12	0.07	0.05	0.15
EPA C20:5	-	-	-	11.15
DHA C22:6	-	-	-	23.89
SFAs	15.8	41.45	14.58	31.95
MUFAs	22.08	50.7	80.54	29.00
PUFAs	62.83	7.63	4.89	39.04

3. Collection of tissue samples

At the end of each intervention, mice were fasted for 24 hours and anesthetized with isoflurane. Blood was then withdrawn by cardiac puncture to allow the measurement of several biochemical parameters, and mice were finally euthanized by cervical dislocation. The liver and heart were quickly dissected and weighed. A thin slice of the tissues was processed immediately for electron microscopy, another small portion was frozen in RNA later (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The remaining tissue was frozen in liquid nitrogen with a cryopreserving buffer (25

mM Tris–HCl pH 7.6, 0.21 M mannitol, 0.07 M sucrose, 20% DMSO), and then stored at −80 ºC until used for biochemical determinations.

4. Preparation of tissue extracts

A thin slice weighing approximately 30 mg of liver and heart were cut off and homogenized in radioimmunoprecipitation assay (RIPA) buffer at 4ºC, which contains 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/mL each of chymostatin, leupeptin, antipain, and pepstatin A (CLAP) and phosphatase inhibitor cocktails 2 and 3 (Sigma- Aldrich) diluted at 1:100. The tissues were homogenized using Qiagen TissueLyser II MM400 (Qiagen) at 2.5 Hz during 1.5 min. The homogenates were centrifuged at 10,000g for 15 min at 4 °C to separate supernatants containing the protein extracts, which were transferred to new tubes. Afterward, the samples were sonicated for 30 seconds and stored frozen at −80 °C until subsequent use.

5. Determination of protein contents

The total amount of protein in the extracts was determined by using the Stoscheck modification [203] of the dye-binding method of Bradford [204]. Briefly, 5 μl of each sample were taken and mixed with 50 μl of 1 N NaOH. Then 1 ml of Bradford's reagent was added and mixed by vortex. Then, samples were incubated in darkness for 15 min at room temperature and the absorbance of each sample was measured at a wavelength of 595 nm in a DU-640 spectrophotometer (Beckman Coulter). For each sample, the analysis was carried out in triplicate. The standard was made using known and increasing concentrations of γ-globulin ranging between 0 and 20 μg/μl.

Western Blot Immunodetection

1. Protein sample preparation

In order to prepare samples for electrophoresis, 50 μg of protein extracts were taken and mixed with 1X SDS-DTT loading buffer containing 60 mM Tris–HCl pH 6.8, 10% sucrose, 2 mM EDTA, 1.5% SDS, 20 mM dithiothreitol, and 0.01% bromophenol blue. In the routine procedure, samples were boiled at 100 ºC for 5 min

before electrophoresis. However, for membrane proteins. Heating of the samples was limited to 45 ºC for 15 min to avoid aggregations. The only exception were those samples prepared to measure mitochondrial complexes, in which heating was further limited to 37 ºC for 5 min, in order to preserve the complex IV signal.

2. Gel electrophoresis, transfer and loading control

Denatured protein samples were loaded in Criterion TGX 4-20% polyacrylamide gradient Precast Gels (Bio-Rad, Hercules, California, USA). For the identification of proteins and to facilitate the cropping of membranes, two lanes of each gel were used to load the molecular weight markers (Dual Color Precision Plus Protein Standards, Bio-Rad).

Proteins were separated by electrophoresis in a Criterion system B (Bio-Rad) for 45 min at a constant voltage of 200 V and Imax of 150 mA in a running buffer solution containing 25 mM Tris pH 8.3, 192 mM glycine, 0.1% SDS.

Once the electrophoresis was completed, proteins embedded in polyacrylamide gels were transferred to nitrocellulose membranes included in the Trans-Blot Turbo kit (Bio-Rad). The proteins were transferred at a voltage of 25 V, for 7 minutes, in the presence of 1X Trans-Blot Turbo Transfer Buffer (Bio- Rad).

After completion of the transfer, the membranes were stained for 5 min with 0,1% Ponceau S red dye diluted in 1% acetic acid, with gentle agitation and at room temperature. The excess staining was removed by washing the membranes several times with 1% acetic acid solution. Protein patterns obtained were digitized using a ChemiDoc Image System (Bio-Rad) and used as a loading control. Afterward, membranes were destained from Ponceau S and, membranes were then blocked with TTBSL buffer containing 50 mM Tris-HCl pH 7.6, 0.85% NaCl, 0.05% Tween 20 and 5% skimmed milk powder in two incubations steps of 30 min each. After this step, the membranes were used immediately for immunostaining.

3. Immunoblotting, imaging, and quantification

Once membranes were blocked, they were incubated with the corresponding primary antibody diluted in TTBSL buffer to the optimum concentration in each case. The incubation was performed overnight at 4 ºC with shaking. After incubation, the

membranes were washed three times at room temperature for 5 min each in TTBS containing 50 mM Tris-HCl pH 7.6, 0.85% NaCl and 0.05% Tween 20 to remove remnants of the antibody. After the washes, the membranes were incubated during 1h with the corresponding species-specific secondary antibody at room temperature diluted in TTBSL. In all cases, we used secondary antibodies conjugated with horseradish peroxidase for the immunodetection of the proteins by enhanced chemiluminescence. Once again, the membranes were washed three times as described above to remove the remains of the antibody.

Finally, the membranes were incubated using the Clarity Western ECL Blotting Substrates kit (Bio-Rad) for 5 min. Chemiluminescent signal was recorded using a ChemiDoc Imaging System (Bio-Rad) to obtain digital images and were quantified with Image Lab Software (Bio-Rad). The data obtained from quantification of stained bands (in arbitrary units) were normalized to the values of the corresponding lane stained with Ponceau S to correct for differences in protein loading between samples.

Primary antibodies used in this study, as well as their corresponding secondary antibodies, working concentrations, and their references are listed in the Table 4.

Table 4. Details about sources and concentrations of the antibodies used in the study

Antibody	Dilution	Company (catalog #)
Primary antibodies		
anti-VDAC1	1:1,000	Santa Cruz Biotechnology (sc-98708)
anti-TFAM	1:1,000	Santa Cruz Biotechnology (sc-166965)
anti-MFN1	1:1,000	Santa Cruz Biotechnology (sc-50330)
anti-MFN2	1:1,000	Santa Cruz Biotechnology (sc-50331)
anti-NRF1	1:1,000	Santa Cruz Biotechnology (sc-33771)
anti-FIS1	1:500	Santa Cruz Biotechnology (sc-98900)
anti-Total OxPhos Complex kit*	1:1,000	Life Technologies (458099)
anti-MFF	1:1,000	Cell Signaling (#86668)
anti-CYB5R3	1:10000	Proteintech (10894-1-AP)
Secondary antibodies		
anti-rabbit	1:2,500	Sigma Aldrich (A-9169)
anti-mouse	1:2,500	Sigma Aldrich (A-9044)

* OXPHOS primary antibody cocktail: Complex I subunit NDUFB8 (NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8). Complex II subunit SDHB (Succinate dehydrogenase (ubiquinone) iron-sulfur subunit). Complex III subunit UQCRC2 (Cytochrome b-c1 complex subunit). Complex IV subunit MTCO1 (mitochondrially encoded cytochrome *c* oxidase I). Complex V subunit ATP5A (Complex V alpha subunit)

Ultrastructural analysis of mitochondria

1. Sample preparation for electron microscopy

Tissue processing for electron microscopy and mitochondrial analysis for ultrastructure and stereology were performed according to Khraiwesh *et al* [205]. Briefly, small sections from the liver and the left ventricle were cut into 3 or 4 pieces of approximately 1 mm3. These pieces were washed for two minutes in 0.1 M sodium cacodylate buffer pH 7, and fixed in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in the same buffer, for a period of at least 12 hours at 4 °C. Then, a standard embedding protocol for electron microscopy was followed. Post-fixation was carried out for 1 hour at 4 °C in the dark in a 1% solution of osmium tetroxide in the same buffer. After post-fixation, the samples were dehydrated in a series of ascending concentrations of ethanol, and then transferred to the carrier agent propylene oxide to facilitate embedding in epoxy resins. The samples were finally embedded in Embed-812 resin (Electron Microscopy Sciences, Hatfield, PA, USA). Thin sections (~60 nm width) were obtained and mounted on nickel grids. After staining with uranyl acetate and lead citrate, the sections were viewed and photographed using a Jeol Jem 1400 electron microscope at the Servicio Centralizado de Apoyo a la Investigación (SCAI; University of Córdoba).

2. Planimetric and stereological calculations

Six mice were used per experimental group. Random images of hepatocytes were obtained at 4000X magnification in order to calculate the mitochondrial mass. We also obtained 15,000X magnification of perinuclear portions of the cells in order to determine the planimetric parameters of the mitochondria. Pictures at 6,000X and 20,000X magnification were obtained randomly from different zones of the cardiomyocytes. The mitochondrial mass was calculated on cardiomyocyte images taken at 6000X and the mitochondrial volume was calculated on the images taken at 20000X magnification. In these images mitochondrial figures were analysed to obtain several planimetric and stereological parameters such as area, circularity, maximum and minimum diameter, number of figures per area (Na), volume density (Vv) and numerical density (Nv). For the stereological measures, we used a simple square lattice test system as described by Weibel [206]. This method employs the

superposition of a virtual grid over the micrographs where the user performs a point-counting method. After that, volume density (Vv) was calculated from the number of points that concur with the mitochondria, and this parameter was referred to the total number of points of the grid contained in the area of interest (the whole cell). Planimetric data were obtained using ImageJ software (NIH, USA).

Quantitative polymerase chain reaction (qPCR)

1. Genomic DNA extraction

Tissue genomic DNA (gDNA) was isolated using the kit NucleoSpin Tissue (Macherey-Nagel) according to the manufacturer's instructions. Briefly, 15 mg of the tissue were pre-lysed in a lysis buffer containing 1/8 diluted proteinase K. The samples were incubated overnight at 56 ºC in a shaking incubator and once the lysis was completed, 1 µl of RNase I (Thermo Scientific) was added to remove RNA. Samples were incubated for 30 min in a shaking incubator. After RNA digestion, lysis buffer was added at a 1:1 volume ratio, samples were vortexed vigorously and then incubated at 70 ° C for 10 min in a shaking incubator. To precipitate the DNA, an equal volume of 100% ethanol was added and the solution was mixed by vortexing. The solution obtained from each sample was placed on a NucleoSpin Tissue Column (provided in the kit) and centrifuged for 2 min at 11,000 g. The gDNA bound to the column was washed twice to remove impurities, by centrifuging again for 2 min at 11,000 g in each step. An additional centrifugation was carried out to remove residual ethanol and to dry the silica membrane. gDNA was subsequently eluted in a new tube in 100 µl of nuclease-free water. The gDNA concentration was measured using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) and a final concentration of 10 ng/µl was used for qPCR.

2. Analysis of relative mtDNA/nuDNA ratio

Quantification of the relative mitochondrial to nuclear DNA ratio in the tissues was performed as described in Quiros *et al* [207].

A set of genes were measured to evaluate the relative copy number of mtDNA and nuDNA (Table 5). qPCR reactions were performed in triplicate for each experimental sample and were placed in 384-well plates at a total volume of 5 µl per well. This

total volume consisted of 2 µl gDNA, 0.5 µl specific forward and reverse primer mix (10 µM working concentration) and 2.5 µl qPCR Syber Green Mix (Roche)

Primer-specific amplification cycles were run as follow: one preamplification cycle at 95 ºC for 5 min, followed by forty-five cycles of amplification at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 20 s. The qPCR reactions were performed using the Light-Cycler 480 system (Roche Applied Science).

The mtDNA/nDNA ratio was calculated using the ΔΔCt method. First, the ΔCt is calculated as follows: ΔCt = Ct (mtDNA gene) − Ct (nDNA gene). Then we calculate the ΔΔCt for each sample as follows: ΔΔCt = ΔCt (Sample of interest) − ΔCt (control sample). In this step, the calculation was performed in two ways: to analyze the effects of the diet or age, young *ad libitum* mice (7 months), subjected to AIM93M diet with soybean oil, were used as a control sample. To analyze the effect of the genotype within each experimental group, group-specific control mice were used. Finally, the relative mtDNA levels of each sample was calculate as $2^{-\Delta\Delta Ct}$.

Table 5. Primers sequences for mt/nuDNA ratio

Gene	Forward	Reverse
UCP2	5' CTACAGATGTGGTAAAGGTCCGC 3'	5' GCAATGGTCTTGTAGGCTTCG 3'
b2M	5' ATG GGA AGC CGA ACA TAC TG 3'	5' CAG TCT CAG TGG GGG TGA AT 3'
HK2	5' GCCAGCCTCTCCTGATTTTAGTGT 3'	5' GGGAACACAAAAGACCTCTTCTGG 3'
16S rRNA	5' CCGCAAGGGAAAGATGAAAGAC 3'	5' TCGTTTGGTTTCGGGGTTTC 3'
ND1	5' CTAGCAGAAACAAACCGGGC3'	5' CCGGCTGCGTATTCTACGTT3'
d-loop 1	5'AATCTACCATCCTCCGTGAAACC 3'	5' TCAGTTTAGCTACCCCCAAGTTTAA 3'

ATP determinations

For measurement of ATP levels, 15-20 mg of tissue sample were homogenized in RIPA buffer as described above. The ATP concentration was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega), in triplicate for each experimental sample. The protocol consisted in adding 20 µg of protein samples per well in a 96-well plate with white walls and clear bottom. The volume in each well was brought to 100 µl with PBS and then mixed with 100 µl of CellTiter-Glo reagent.

The mixture was incubated in the dark during 10 min at room temperature. Luminescence signal was recorded with a Victor X4 multilabel plate reader (Perkin-Elmer). The standard curve was made using known and increasing concentrations of ATP, from 0 to 1 μM.

Images

All illustrative figures were created with BioRender.com.

Statistics

Statistical analyses were carried out using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). All the data shown here are mean ± standard error of the mean (SEM). Data normality was checked by the Kolmogorov-Smirnov normality test. The means were compared using the two-way ANOVA followed by a post hoc Tukey test for multiple comparisons. We assessed overall differences due to "diet" (independently of genotype), "genotype" (independently of diet), and the interaction "diet × genotype", as well as individual differences between experimental groups. Data regarding size, area, and circularity of mitochondria were analyzed using the D'Agostino – Pearson normality test. In case the data did not pass the normality test, the Mann – Whitney nonparametric analysis was used. Significant differences were expressed as follows: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Chapter I. Characterization of CYB5R3 transgenic mice subjected to different nutritional interventions

Results

Body and organ weight

The body weight of mice from the different experimental groups was assessed once a week throughout the entire intervention (see Material and Method section for a detailed description of the different interventions). In a first experimental cohort, we wanted to know whether different types of predominant dietary fat and the overexpression of CYB5R3 were enough to modify the weight of the young mice. After four months of intervention, no differences in weight were observed due to the source of fat in the diet alone. Tg mice exhibited a similar body weight as WT animals except in lard WT mice and fish oil Tg. In this case, lard-fed WT animals weighed more than those fed with soybean oil (control group). Also, fish-oil fed Tg mice showed higher weight than mice of the control group (Fig. 9A).

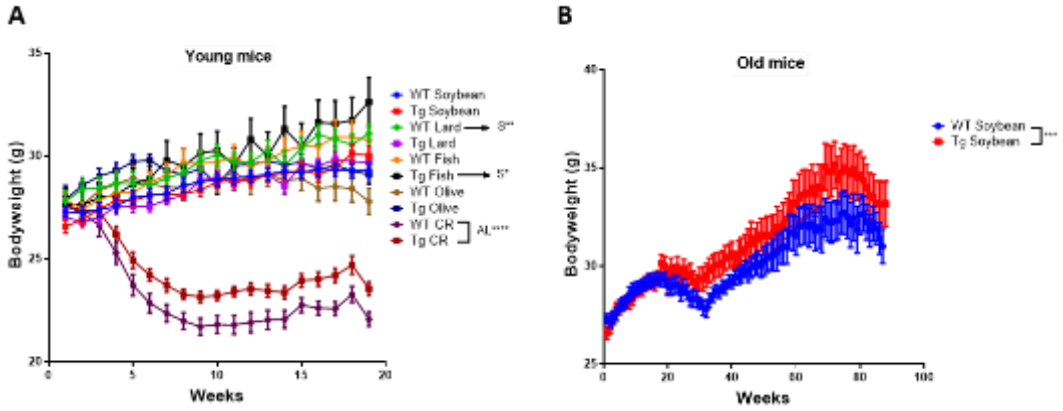


Figure 9. Bodyweight under different intervention and aging. (A) Representation of bodyweight profile in young animals fed with AIM93M-based diets with different fat sources in *ad libitum* and CR intervention. (B) Representation of bodyweight profile during 21 months of *ad libitum* intervention with standard AIM93M diet (containing soybean oil). Data represent mean ± SD. "AL" refers to significant differences with respect to *ad libitum* group and "S" refers to significant differences with respect to soybean oil-enriched diet. When detected, general effects of diets and genotype x intervention interaction are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

We also determined the body weight in WT and Tg mice that had been fed for 4 months either *ad libitum* or under 40% CR. No differences in body weight between genotypes were observed when mice were fed *ad libitum* and, as expected, CR produced a significant decrease of body weight in both genotypes. However, weight loss due to CR intervention was significantly more pronounced in WT than in Tg mice (Fig. 9A).

We finally analysed whether age had any effect on the weight of the animals. Mice were fed *ad libitum* with AIN93M diet for 21 months. During the young stage, WT and Tg mice maintained a very similar weight. However, at the age of five months the weights of the mice begun to diverge in such a way that Tg mice were heavier than the controls, as reported by Matin-Montalvo *et al.* [138]. This difference in weight between the groups became increasingly higher as the animals became older (Fig. 9B).

In order to determine possible differences in the weight of the organs between the different nutritional interventions in both genotypes and the effect of age, once mice were euthanized, the organs were weighed. Statistically significant differences were found in liver, heart, kidneys, and brain weights (Fig. 10). Also, the organ weights in relation to body weight were calculated, and the results are shown in Fig. 11.

Liver weight was reduced in soybean oil-fed Tg mice compared to mice of the same genotype fed with the diets based in lard or fish oil. However, no such difference was observed in the case of WT mice (Fig. 10A). This reduction in liver weight was still maintained when normalized to body weight (Fig. 11A). No differences in liver weight or in liver weight normalized to body weight were found when comparing mice of the two genotypes when fed *ad libitum*.

Liver weight was also reduced in WT mice fed under CR conditions in comparison with mice of the same genotype fed *ad libitum* (Fig. 10A) although no difference between the two dietary conditions was observed when liver weight was normalized to body weight (Fig. 11A). Of note, in contrast to what was found for WT mice, CR did not result in a significant decrease of liver weight in Tg mice (Fig. 10A). Moreover, liver weight normalized to body weight was significantly increased in Tg mice fed under CR in comparison with mice of the same genotype fed *ad libitum* (Fig. 11A).

Liver weight increased in both genotypes in aged mice (Fig. 10A). However, when normalized to body weight, liver weight was significantly increased only in aged Tg mice, and no differences were found in WT aged mice compared to young animals (Fig. 11A).

Heart weight decreased in Tg mice fed a fish oil-based diet compared to mice of the same genotype fed lard or olive oil-based diets (Fig. 10B). As in liver, no differences in heart weight were found when comparing mice of the two genotypes fed *ad libitum.* Nevertheless, when normalized to body weight heart underwent a reduction in both WT and Tg mice that had been fed a fish oil rich diet in comparison with mice fed the olive oil-based diet. Heart weight normalized to body weight was also higher in WT mice fed the olive oil-based diet in comparison with mice of the same genotype fed the lard-based diet (Fig. 11B).

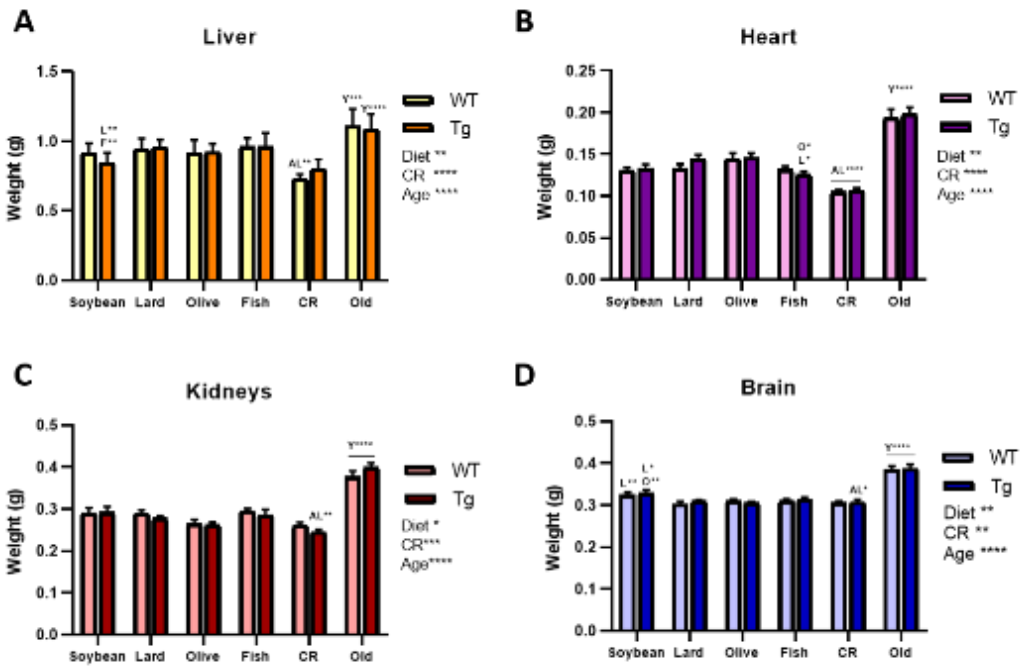


Figure 10. Tissue/organ weight under different intervention and aging. (A) Representation of liver (A), heart (B), kidney (C) and brain (D) weights in young /adult and aged Tg and WT mice fed *ad libitum* or under CR. Data represent mean values ± SEM. In all graphs, "S", "L" "O" and "F" refers to significant differences with respect to soybean oil, lard, olive oil and fish oil-enriched diet respectively. "AL" refers to significant differences with respect *ad libitum*, "Y" denotes significant differences with respect to young mice. When detected, general effects of diets and interaction between genotype, diet, CR or age are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Heart weight was decreased in mice of both genotypes under CR conditions in comparison with mice fed *ad libitum* (Fig. 10B) However, a remarkable increase was found in CR mice when data were normalized to body weights (Fig. 11B).

In contrast to CR, an increase in heart weight was observed in aged mice from both genotypes (Fig. 10B). However, this increase was lost when the weight of the organ

is normalized with the body weight, remaining at the same levels as in young mice (Fig. 11B). Same was shown in kidneys of aged mice (Fig. 10C y Fig. 11C).

Although this work was focused on liver and heart, we also determined the weight of other organs to assess possible changes due to diet or genotype. No significant differences were found due to dietary fat or genotype in kidney weight, either as absolute values or when normalized to body weight (Fig. 10C). However, in caloric-restricted Tg mice, the kidney weights decreased in comparison with mice of the same genotype fed *ad libitum* (Fig. 10C). Nevertheless, when normalized with body weight, kidneys were heavier than in *ad libitum* controls in both genotypes (Fig. 11C).

Brain weight in mice fed the soybean oil-based diet was increased when compared to lard in both genotypes, and when compared to olive oil in the case of Tg mice (Fig. 10D). However, once normalized by the body weight, a greater preservation of the organ weight was observed in Tg mice fed diets with different fat sources, whereas in the case of WT animals it was found that brain weight normalized to body weigh was decreased in those mice fed diets based in fish oil or lard in comparison with mice fed diets based in soybean oil or olive oil (Fig. 11D).

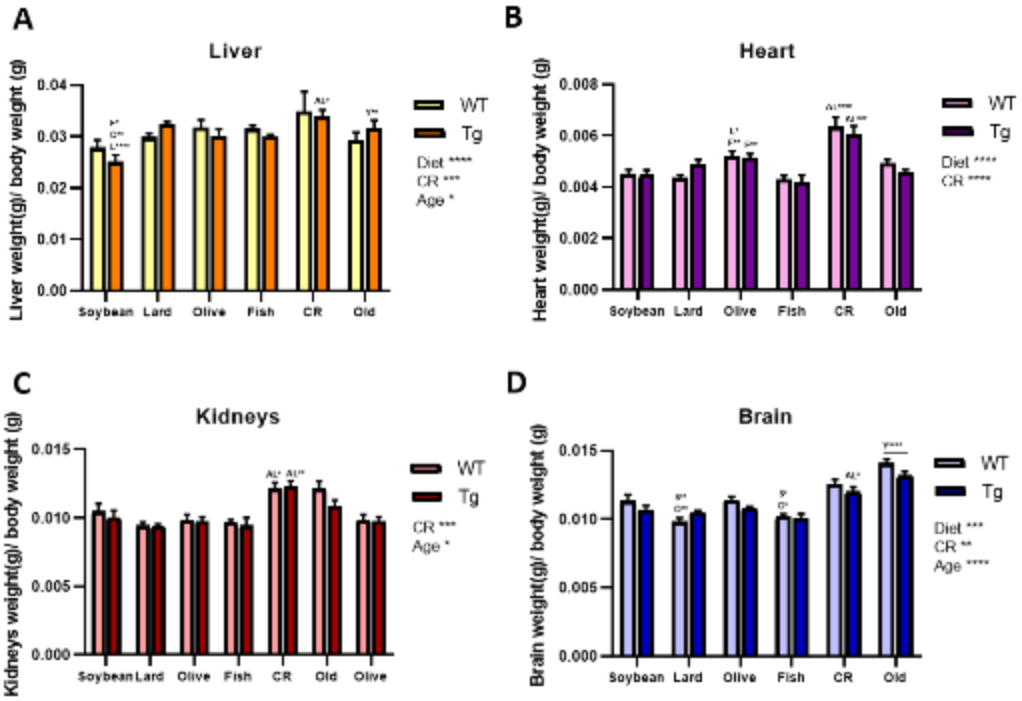


Figure 11. Relative tissue to body-weight under different intervention and aging. Representation of the weight of liver (A), heart (B), kidney (C) and brain (D) relative to bodyweight in mice fed *ad libitum* or under CR. Data represent mean ± SEM. In all graphs, "S", "L" "O" and "F" refers to significant differences with respect

to soybean oil, lard, olive oil and fish oil-enriched diet, respectively. "AL" denotes significant differences with respect to *ad libitum*, and "Y" refers to significant differences with respect to young mice. When detected, general effects of diets and interaction between genotype, diet, CR or age are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Brain weight was reduced in Tg mice fed under CR conditions in comparison with mice of the same genotype fed *ad libitum* (Fig. 10D) although an increase in CR *vs.* *ad libitum* was observed when brain weight was normalized to body weight (Fig. 11D).

Finally, both brain weight and brain weight normalized to body weight were increased with aging in mice of the two genotypes (Fig. 10D & Fig. 11D).

Biochemical parameters

Biochemical analyses were carried out at the end of the nutritional intervention with blood samples obtained from mice that had been fasted from the day previous to sacrifice. Concentrations of glucose, triglycerides, total cholesterol, creatinine, urea, and total protein were determined by the personnel of the SAEX, and the results indicate that some of these parameters were affected by the diet. Thus, glucose was significantly higher in WT animals fed the lard-based diet compared to mice of the same genotype fed the fish oil-based diet. Interestingly, glucose values in mice fed the lard-based diet were normalized by CYB5R3 overexpression. Finally, in CR Tg mice we also found a slight decrease in glucose compared to WT animals (Fig. 12A). Concerning total cholesterol, small elevations were found with consumption of the lard-based diet, both in WT and Tg mice (Fig. 12B), but no changes in triglycerides were found for these groups (Fig. 12C). In CR WT animals, triglycerides were lower compared to *ad libitum* mice, but this difference disappeared with the transgenic. Furthermore, triglycerides were significantly lower in aged mice of both genotypes in comparison with their young counterparts (Fig. 12C). Creatinine concentration was slightly decreased in Tg animals fed the olive oil-based diet in comparison with mice of the same genotype fed the soybean oil or lard-based diets, but increased in Tg mice fed under CR. This metabolite was significantly lower in aged mice (Fig. 12D). The urea concentrations and total proteins were strongly higher in aged mice, which might be due to renal dysfunction (Fig. 12E & 12F.). Finally, urea concentrations were

statistically lower in WT animals subjected to CR (Fig. 12E) while total protein was higher in Tg mice fed under CR (Fig. 12F).

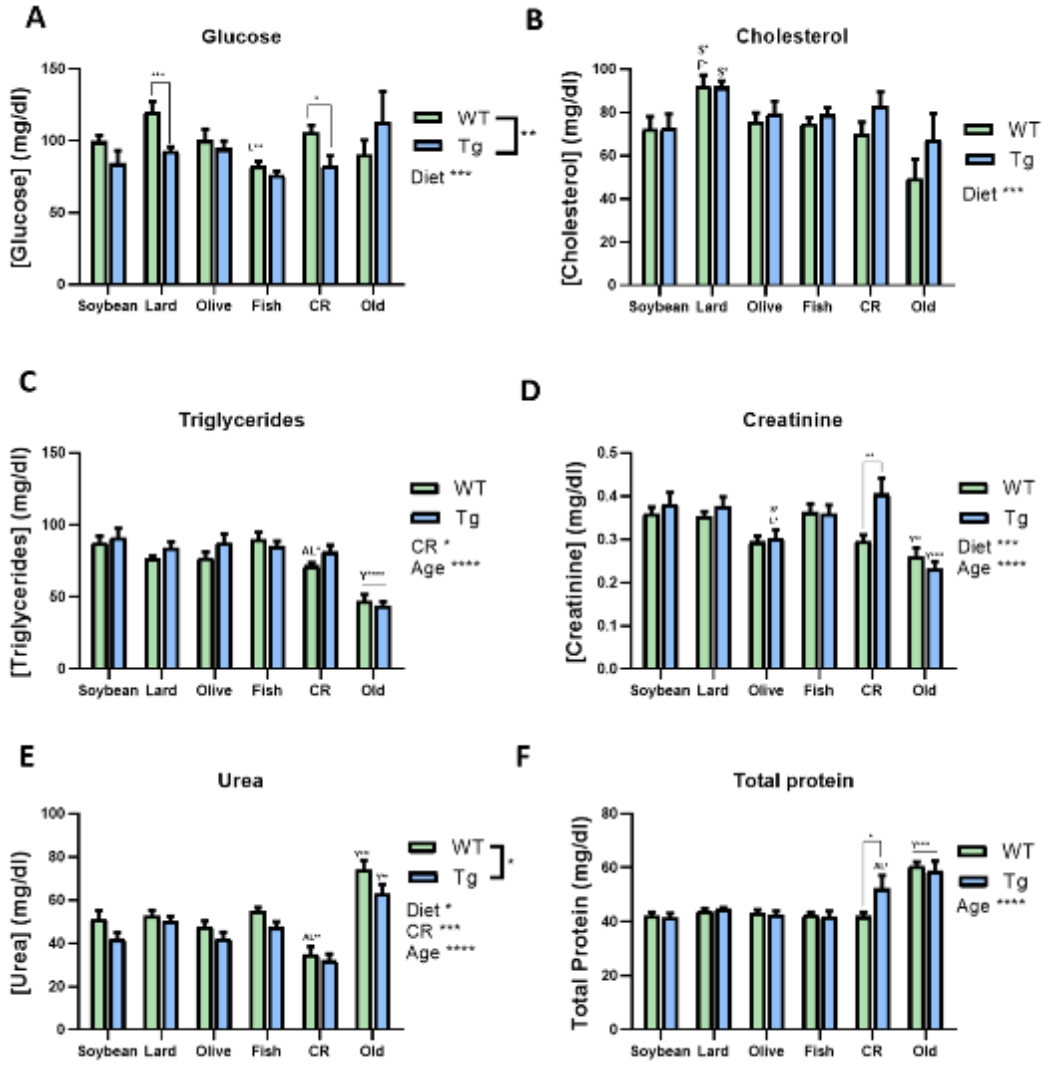


Figure 12. Biochemical analysis. Representation of glucose (A), cholesterol (B), triglycerides (C), creatinine (D), urea (E) and total protein (F) levels measured in blood samples from mice of the different experimental groups. Depicted data are means ± SEM. In all graphs, "S", "L" "O" and "F" refers to significant differences with respect to soybean oil, lard, olive oil and fish- enriched diet, respectively. Asterisks without a letter refer to statistically significant differences between genotypes. "AL" denotes significant differences with respect to *ad libitum* group. "Y" refers to significant differences with respect to young mice. When detected, general effects of diets and interaction between genotype, diet, CR or age are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

CYB5R3 polypeptide in liver and heart from WT and Tg mice fed diets with different dietary fats

Before studying the effects of CYB5R3 overexpression on the parameters evaluated in the different nutritional interventions, we first determined the expression levels of CYB5R3 polypeptide in total homogenates of tissues obtained of mice fed with the diets based on the AIM93M formulation with different fat sources. We observed that dietary fat affected steady-state levels of CYB5R3 polypeptide in a tissue-dependent manner. Thus, in the liver PUFAs decreased the abundance of this enzyme, as it was observed with the soybean oil-based diet (n-6 PUFAs) and, particularly, in the fish oil-based diet (n-3 PUFAs) where we found that the lowest levels of CYB5R3 regardless of genotype. No differences were detected when comparing WT and Tg mice within each dietary group (Fig. 13A).

A completely different pattern of CYB5R3 abundance was observed in the heart. In this organ, dietary fat did not affect CYB5R3 levels. Contrary to what was found in liver, a clear overexpression of CYB5R3 was found in Tg mice in all dietary conditions (Fig. 13B).

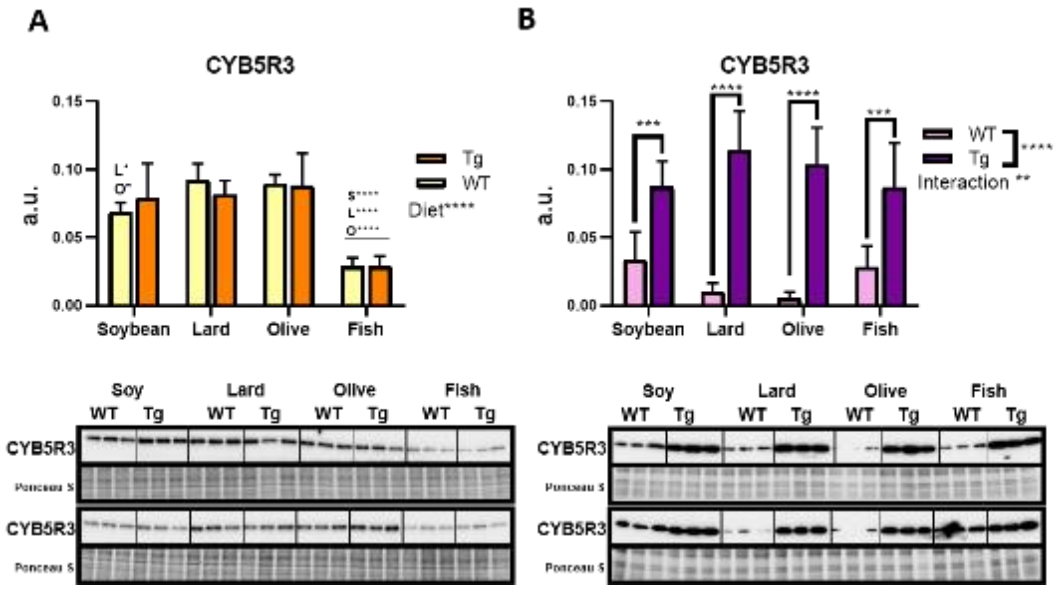


Figure 13. Levels of CYB5R3 polypeptide measured by Western Blots in tissues from mice fed diets with different fat source. The panel depicts the blots and immunoblots quantification showing the levels of CYB5R3 in liver (A) and heart (B) homogenates of mice fed with *ad libitum* with different fat sources. The corresponding Ponceau S staining is shown below each blot. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S. Data represent mean ± SEM. In all graphs, asterisks without a letter refer to statistically

significant differences between genotypes, "AL" refers to significant differences for *ad libitum*. "S", "L" and "O" refers to significant differences for soybean oil, lard, olive oil-enriched diet, respectively. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

CYB5R3 polypeptide in liver and heart from WT and Tg mice fed *ad libitum* or under caloric restriction

Next, we studied the levels of CYB5R3 polypeptide in liver and heart tissue extracts obtained from WT and Tg mice that had been fed *ad libitum* or under CR. As shown in Fig. 14, the effects of CR and overexpression of the CYB5R3 gene on the abundance of CYB5R3 polypeptide were also strongly tissue-dependent. Overexpression of the CYB5R3 gene led to a modest albeit significant increase of the CYB5R3 polypeptide in liver in Tg mice fed *ad libitum*, but this increase was blunted by CR so no differences between genotypes were noted in the latter dietary group. Interestingly, levels of hepatic CYB5R3 polypeptide were significantly decreased by CR in mice of both genotypes, and this effect was much more intense in Tg mice (Fig. 14A). In heart, overexpression of the CYB5R3 gene led to a dramatic increase in the levels of the CYB5R3 polypeptide regardless caloric intake. In contrast with the results obtained in liver, the levels of CYB5R3 polypeptide in heart were not affected by CR regardless of the genotype (Fig. 14B).

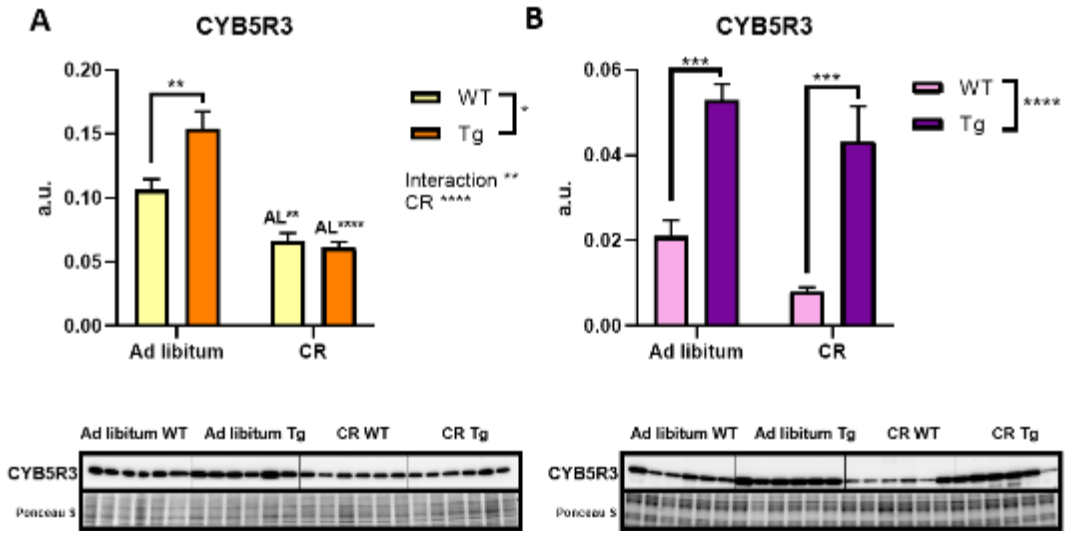


Figure 14. Levels of CYB5R3 polypeptide measured by Western Blots in tissues from mice fed *ad libitum* or under CR. The panel depicts the blots and immunoblots quantification showing the levels of CYB5R3 in liver (A) and heart (B) homogenates of mice fed either *ad libitum* or under CR. The corresponding Ponceau S staining

is shown below each blot. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S. Data represent mean ± SEM. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refers to significant differences in comparison with the *ad libitum* group. When detected, general effects of CR and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p< 0.001$) and **** ($p < 0.0001$).

Effect of aging on CYB5R3 polypeptide in liver and heart from WT and Tg mice

In order to know the effect of aging in our model, we evaluated the expression of CYB5R3 in tissue extracts obtained from the liver and heart from 24 months-old WT and Tg mice in comparison with their young counterparts. CYB5R3 overexpression produced an increment in protein levels in both tissues (Fig. 15A & 15B). Interestingly, unlike the results observed in liver, heart CYB5R3 levels increased with age in mice of both genotypes (Fig. 15B).

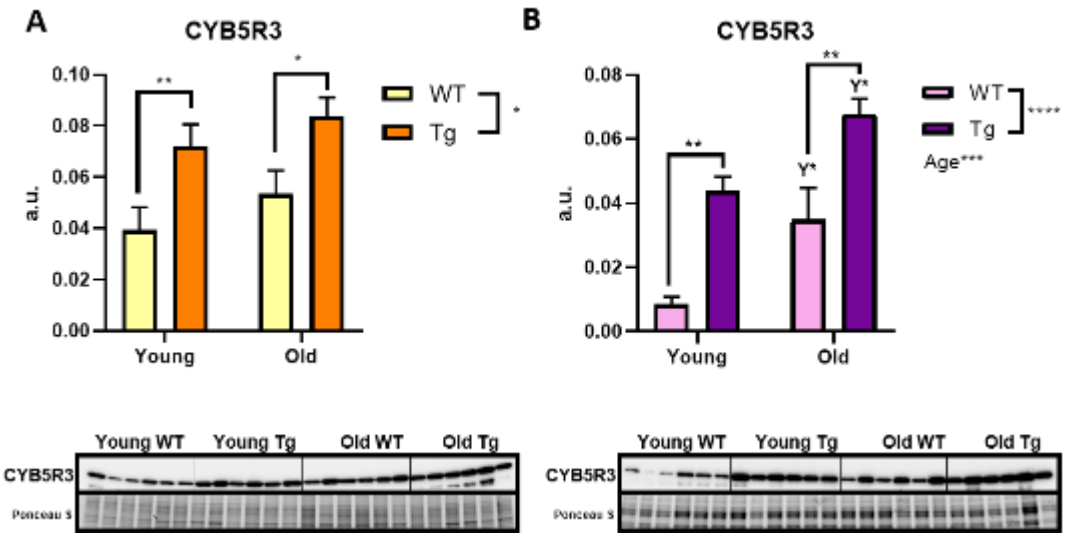


Figure 15. Levels of CYB5R3 polypeptide measured by Western Blots in tissues from young/adult or old mice. The panel depicts the blots and immunoblots quantification showing the levels of CYB5R3 in liver (A) and heart (B) homogenates in young/adult and old mice. The corresponding Ponceau S staining is shown below each blot. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. Data represent mean ± SEM. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "Y" refers to significant differences for young mice. When detected, general effects of aging or between genotype are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p< 0.001$) and **** ($p < 0.0001$).

Chapter I. Characterization of CYB5R3 transgenic mice subjected to different nutritional interventions

Discussion

Characterization of CYB5R3 transgenic mice subjected to different nutritional interventions

CYB5R3 is a flavoprotein that participates in a plasma membrane redox system accepting electrons from cytosolic NADH to transfer them to coenzyme Q, thus regenerating its antioxidant [138]. Outer mitochondrial membrane-associated CYB5R3 plays an important role in the regeneration of cytosolic ascorbate with the participation of a high-potential and outer mitochondrial membrane-specific isoform of cytochrome b_5 (CYB5B) [149]. CYB5R3 associated to the endoplasmic reticulum participates in the elongation and desaturation of fatty acids, biosynthesis and metabolism of cholesterol and steroids, inhibition of chronic pro-inflammatory pathways and mono-oxygenations through the cytochrome P450 system, with the participation of a low-potential isoform of cytochrome b_5 (CYB5A) [150, 151]. These functions link CYB5R3 activity with protection against metabolic diseases [140].It has been reported that plasma membrane-bound CYB5R3 is highly expressed under long-term caloric restriction conditions in liver tissue [208], and culture of SH-SY5Y neuroblast cells with serum obtained from animals subjected to caloric restriction recapitulated this response [208, 209]. Moreover, its overexpression in transgenic mice extends longevity, improves insulin sensitivity and glucose homeostasis, and decreases oxidative damage and inflammation, suggesting that CYB5R3 overexpression strategies may constitute a viable intervention to confer protection against metabolic pathologies and improve healthspan [138].

Since the maintenance of an adequate mitochondrial function can aid in the prevention of metabolic diseases, and is a hallmark of successful aging [2, 3], we aimed to gain novel insights into the mechanisms by which the overexpression of CYB5R3 can result in the optimization of mitochondrial functions and therefore, improve metabolism and longevity in transgenic mice.

Inadequate diets are key risk factors for metabolic diseases. We thus investigated the role dietary fat, CR and age had on the health of CYB5R3-overexpressing mice in comparison with WT littermates. Previous studies have shown that young/adult CYB5R3 Tg mice exhibit a higher body fat and lower lean percentage that their WT counterparts, without differences in weight, both when fed a standard diet and after consumption of a high fat diet. This effect has been related to metabolic

reprogramming in Tg mice toward a preferential use of carbohydrates [138]. In our cohort, CYB5R3- Tg mice had similar body weights to WT animals after 4 months of *ad libitum* feeding with diets containing different dietary fats. However, we found that a lard-rich diet increased the weight of WT mice compared to a soybean oil-enriched diet. This result is consistent with a recent study showing that a short-term intervention with a diet containing lard was enough to induce weight gain in mice [210]. Interestingly, Tg mice fed with a lard diet showed similar weights than those found in control group (soybean oil), suggesting a protective effect of CYB5R3 transgenesis against the harmful effects associated with saturated fats. This idea is in accordance with the results reported showing that CYB5R3 Tg mice displayed increased levels of long-chain PUFA [138] which can be due to the role of CYB5R3 in elongation and desaturation of fatty acids [150].

On the other hand, we also observed an increase in the body weight of Tg mice fed with fish oil-based diet. This observation is apparently at odds with the well-known beneficial effects of diets enriched in EPA and DHA against obesity-associated metabolic disorders due to the increase in β-oxidation and insulin sensitivity [211]. However, due to the metabolism of CYB5R3 Tg mice, which exhibit a greater preference to obtain energy from carbohydrates (and less by β-oxidation), the possibility exists that Tg mice accumulate free fatty acids as occurs in animals fed a high fat diet (HFD). Thus, the accumulation of free fatty acids can block the mitochondrial electron transfer chain affecting the production of energy from fatty acid oxidation, which might be due to a decrease in mitochondrial CoQ [212, 213]. In agreement with this idea, previous research has shown that increasing n-3 PUFA in tissues can be beneficial to mice fed *ad libitum* [214], while a diet containing fish oil as the predominant fat source resulted in shorter lifespan in mice fed under caloric restriction compared to other fat sources [215]. The negative effects of fish oil-enriched diet in combination with interventions such as CR or CYB5R3 overexpression could explain the effect on body weight in caloric-restricted Tg mice. Furthermore, a recent lipidomic approach has demonstrated that CR significantly altered the hepatic lipidome in male C57BL/6 mice and caused a change in the relative abundance of specific triglycerides and phosphatidylethanolamines and reduced hepatic 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphatidylcholine content, a specific product of phospholipid peroxidation. Less susceptibility of membranes to

peroxidation was explained on the basis of a redistribution in the type of unsaturation: CR increased MUFA in liver, whereas the levels of PUFA were decreased without any observed changes in SFA. These specific changes in FA composition may be the result of a metabolic reprogramming leading to lower levels of oxidative damage which could contribute to the increased lifespan of CR mice [120]. It is very likely that these changes may be hindered by consumption of a fish oil-enriched diet.

CR is also accompanied by changes in the distribution and amount of fat in the body. Although CR produced a significant decrease of body weight in both genotypes, CYB5R3 overexpression led to a greater preservation of fat. According with previous reports, the maintenance of body weight in Tg animals on CR may be due to a higher accumulation of fat in these animals in comparison with WT mice [138]. This effect could account also for the beneficial effect of CYB5R3 overexpression on lifespan. It is worth mentioning that a recent study showed a positive correlation between mouse longevity and the preservation of body fat with aging [216], which is in agreement with our observations.

Despite the fact that in young mice there were no significant changes in body weight regardless the genotype, as reported in a previous study [138], our longitudinal study showed that Tg mice gained more weight as they aged. It has been suggested that saturated fatty acids are necessary to trigger β-oxidation [217]. Thus, the increase of unsaturated fatty acids achieved in CYB5R3 Tg mice due to a higher desaturase activity might inhibited β-oxidation [138], which could explain our observed changes in body weight with aging.

Previous studies have shown that organs weight decrease with CR regardless the fat source in the diet [215, 218]. In our study, we found some differences in mice fed *ad libitum*, such as a larger heart in animals fed MUFAs and a higher weight of the organs in aged mice. However, the most striking changes were found in the group of mice subjected to CR. Although this intervention indeed produced a significant decrease of body weight in both genotypes, CYB5R3 overexpression led to a greater preservation of body weight which was reflected in all the tissue weights. Thus, CYB5R3 emerges for the first time as a key determinant in the control of organ and body weight in mice under CR.

To characterize the metabolic changes that take place in CYB5R3 Tg mice in the different experimental conditions, a biochemical analysis in blood plasma was carried out and several changes were noted. We found the highest cholesterol levels in mice fed *ad libitum* with the lard-enriched diet. However, we observed less glucose levels in Tg mice that had been fed this diet, which is in accordance to previous studies showing that CYB5R3 overexpression in Tg mice resulted in an improvement of glucose homeostasis. Lower glucose levels observed in Tg mice could be related to an enhancement of insulin sensitivity and mTOR pathway [138].

Aging and aberrant fatty acid metabolism are both associated with the risk of metabolic diseases [219]. Interestingly, plasma triglycerides levels decreased in older mice *versus* young mice regardless of the genotype. This change had been already observed in previous studies where fatty acid metabolism was affected by aging. In this sense, although plasma triglycerides decreased with age, an increase in free fatty acids followed by decreased β-oxidation and plasma acylcarnitines levels was also observed [220]. According to these data, decreased triglycerides in plasma could indicate deposition of these components in the tissues.

On the other hand, aging is characterized by a gradual decline of cellular function that irreversibly affects all tissues of the organism causing structural changes, loss of function and inevitably leads to its consequent failure. As occurs in other organs, the kidney is also affected by aging [221, 222]. In fact, this organ can be considered as a marker of this process, being the alterations in glomerular filtration and in markers as plasma creatinine and urea hallmarks of renal aging [223].Although urea itself is not a good marker of glomerular function, there is the suspicion that decreased creatinine values are related with renal failure, which is linked with glomerulonephritis [223].. Other changes are also associated with aging renal function, such as the permeability of the capillary wall, abnormal tubular reabsorption and secretory capacity damage in podocytes, as well as changes in the production of urine [224-226]. Although we found decreased kidney function in aged mice, these changes seem to be less pronounced in CYB5R3 overexpressing mice, which suggest a better protection of Tg animals against kidney failure, as proposed in the studies carried out by Dr. Calvo-Rubio (Calvo-Rubio, M. PhD Thesis).

In this study, we characterized how overexpression of the CYB5R3 gene affected the levels of the CYB5R3 polypeptide in different tissues and as a function of the interventions. We found that CYB5R3 polypeptide levels are highly influenced by dietary fat in mitotic organs such as liver, probably related with the desaturase activity of this enzyme. While in liver there were no differences between genotypes for a given diet, those animals fed a lard or olive oil-enriched diets showed higher amounts of CYB5R3 polypeptide, whereas the lowest amount was observed in mice fed the fish oil-enriched diet. Similar results were observed in another mitotic tissue as kidney (Calvo-Rubio, M. PhD Thesis). Whereas dietary fat appears as one of the predominant factors that influences the CYB5R3 content in mitotic organs, we found a totally opposite pattern in post-mitotic organs. CYB5R3 polypeptide in heart homogenates from mice fed *ad libitum* was dramatically increased in Tg animals when compared with WT mice regardless of the diet, and similar results were observed in skeletal muscle (López-Bellón S. PhD Thesis, in preparation). The results obtained with our control group, constituted by mice fed a soybean oil-based diet (as a standard AIN93M formulation), also confirmed our previous observations of a modest, although statistically significant, increase of CYB5R3 polypeptide in liver, and its dramatic augmentation in heart muscle from in Tg in comparison with WT animals.

While CR did not affect CYB5R3 levels neither in heart nor in skeletal muscle [136], this intervention induced a decrease in liver, particularly in Tg mice. The decrease of hepatic CYB5R3 in CR- young mice is in accordance with previous research carried out in Fischer-344 rats [208] and in C57BL/6J mice [227]. These results allow us to suggest that CR has a dual effect on CYB5R3 levels in hepatocytes plasma membrane. Of note, while a decrease in a CYB5R3-dependent activity was observed in young animals fed a CR diet, a remarkable increase was observed in old animals fed the CR diet lifelong, and we also observed increased CYB5R3 content in old animals fed *ad libitum* both in mitotic and post-mitotic organs. Furthermore, hepatic expression and polypeptide levels of CYB5R were also decreased in C57BL/6J mice after 18h fasting, which was confirmed in HepG2 and Hepa1.6 cell lines cultured under glucose deprivation [228]. In brain, CYB5R3 levels were unchanged by CR in young, but markedly increased in old Ficher-344 rats fed a CR diet. Also, a substantial increase of CYB5R3 levels was observed in SH-SY5Y neuroblast cells cultured with

serum obtained from rats that had been fed under CR, in comparison with cells cultured with serum obtained from rats fed *ad libitum* [209].

Of note, whereas the expression of ectopic CYB5R3 in Tg mice is controlled by a viral promoter (CMV), expression of the endogenous CYB5R3 gene is controlled by SP1 transcription factor [143], which can be further upregulated through FOXO3a and NFE2L2 under conditions of nutritional and oxidative stress [144]. Since the levels of hepatic CYB5R3 polypeptide were similarly affected by CR in both WT and Tg mice, it is very likely that this regulation is post-transcriptional.

Taken together, these observations point out the existence of tissue and age specific mechanisms regulating CYB5R3 levels by CR or diet, being post-mitotic organs the most appropriate models to study the direct effects of CYB5R3 overexpression on mitochondrial physiology.

Chapter II. The crosstalk between CYB5R3 overexpression and caloric restriction in transgenic mice

Results

Mitochondrial dynamics and biogenesis markers in liver

In order to study the impact that CR and/or overexpression of CYB5R3 gene impose on mitochondrial dynamics, we analysed the levels of key marker proteins related with mitochondrial fusion and fission in liver tissue.

Mitofusin 1 and 2 (MFN 1 and 2, respectively) are transmembrane GTPases essential for mitochondrial fusion and morphology [86]. CYB5R3 overexpression did not affect significantly the levels of hepatic MFN1, either in *ad libitum* or in CR-fed mice (Fig. 16A). However, MFN2 was significantly increased in WT mice fed a CR diet in comparison with mice of the same genotype fed *ad libitum* although the increase of MFN2 with CR was attenuated in Tg animals (Fig. 16B). FIS1 participates in mitochondrial fission [86], and significantly increased levels of this protein were found in Tg mice fed *ad libitum* in comparison with WT mice fed the same diet, and also when compared with CR Tg mice (Fig. 16C). Neither CR nor CYB5R3 overexpression altered the content on VDAC1 levels in liver (Fig.16D). The western blots used for quantifications are depicted in Fig. 16E & 16F.

We also assessed the levels of TFAM and NRF1, two key transcription factors regulating mitochondrial biogenesis [66]. TFAM polypeptide exhibited a dramatic increase with caloric restriction both in WT and Tg mice, although, as found for other markers, overexpression of CYB5R3 attenuated this effect in such a way that a decrease of TFAM was observed in Tg mice fed under CR in comparison with WT mice fed the same diet (Fig. 17A). NRF1 levels also tended to increase with CR in WT mice in comparison with their *ad libitum* controls, although in this case the differences did not reach statistical significance ($p = 0.1$). Interestingly, a significant decrease of this mitochondrial biogenesis marker was again detected in Tg mice fed under CR in comparison with WT mice fed the same diet (Fig. 17B). These data fit with those obtained when measuring mitochondria/nuclear DNA (mt/nu DNA) ratio (see Fig 17D & Fig. S.1). The western blots used for quantifications are depicted in Fig. 17C.

Taken together, these results indicate that liver mitochondria of mice subjected to CR undergo changes in their dynamics that directly produce an alteration in the mt/nu DNA ratio, and that CYB5R3 plays a role at modulating these changes, particularly under CR.

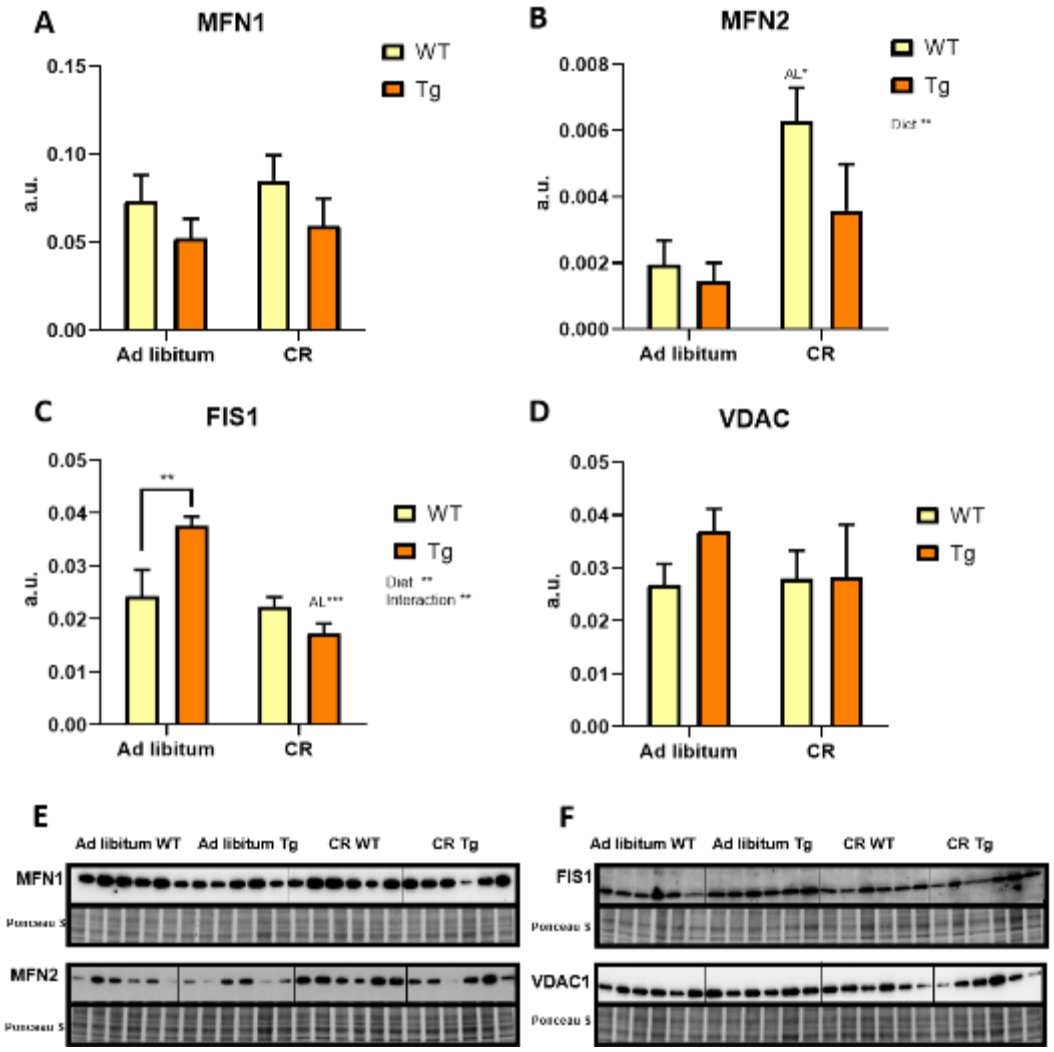


Figure 16. Mitochondrial dynamics and mass markers in liver. Immunoblots show the levels of MFN1, MFN2, FIS1 and VDAC1 in liver homogenates from WT and Tg mice fed *ad libitum* or CR diets. The corresponding Ponceau S staining is shown underneath each blot. (A-D) Quantification of the blots. (E-F) Immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized to the density of the corresponding lane stained with Ponceau S. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refers to significant differences with respect to the corresponding *ad libitum* group. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

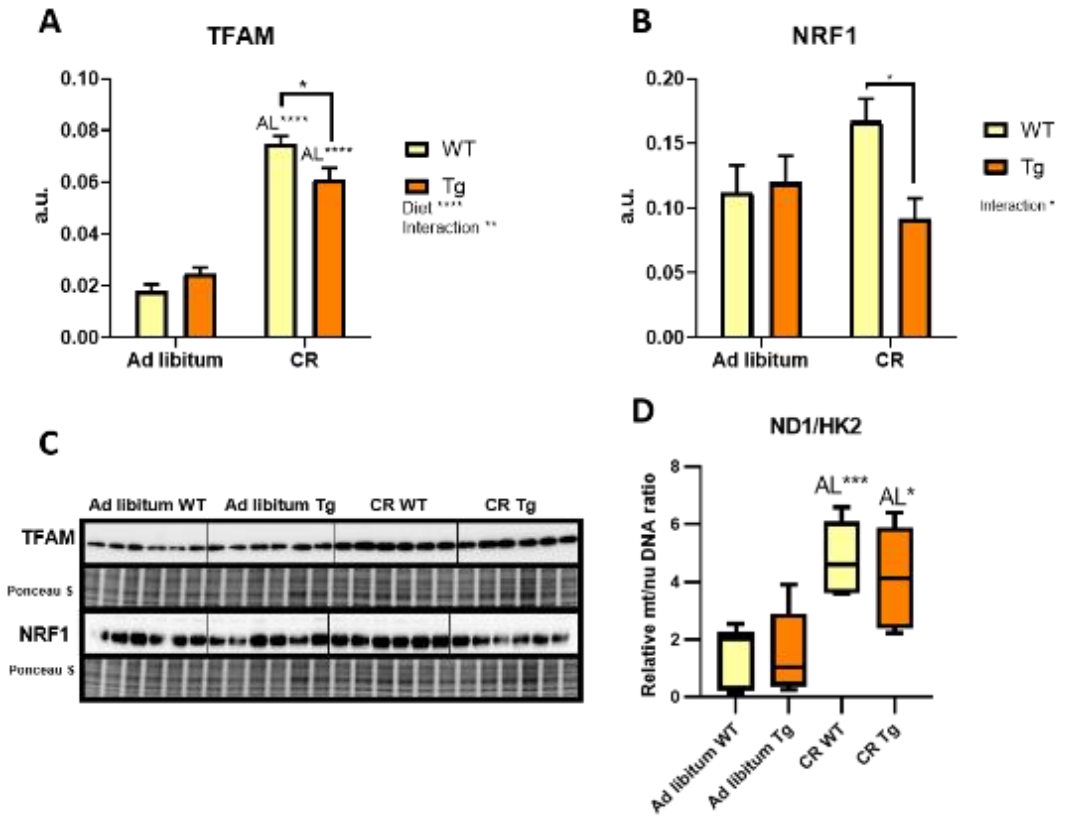


Figure 17. Mitochondrial biogenesis markers in liver. Immunoblots show the levels of TFAM and Nrf1, as well as the quantification of mt/nu DNA ratio in liver homogenates from WT and Tg mice fed with AIN93M diet either *ad libitum* or under CR. The corresponding Ponceau S staining is shown underneath each blot. (A-B) Quantification of the blots. (C) Images of the immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized to that of the corresponding lane stained with Ponceau S. (D) Mitochondrial/nuclear DNA ratio. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refers to significant differences with respect to the corresponding *ad libitum* control. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Mitochondrial ultrastructure in hepatocytes

The changes observed in mitochondrial dynamics and biogenesis markers prompted us to study possible ultrastructural changes in mitochondria using planimetric and stereological techniques. Figure 18 shows representative pictures of hepatocytes as examples of the material used for this purpose. We first calculated the mitochondrial volume density (Vv) and the number of mitochondria per cell unit area (Na) as a relative stereological parameter related to mitochondrial abundance. Mitochondrial Na did not change across experimental groups (Fig. 19A). There were also no changes in Vv (Fig. 19B), indicating that overall mitochondrial mass remained unchanged in *ad libitum* and CR mice of both genotypes, confirming the data obtained for VDAC1.

Although no changes were found in mitochondrial mass in the different experimental groups, significant changes were indeed detected when ultrastructural quantitative parameters were analysed in individual organelles, specifically mitochondrial area and circularity (see Fig. 19D). Thus, mice undergoing CR showed significantly decreased mitochondrial circularity compared to their corresponding fed *ad libitum*. On the other hand, in mice under CR this parameter was notoriously higher in those animals overexpressing CYB5R3 *versus* their WT controls. Interestingly, mitochondrial sectional area in hepatocytes from Tg mice was significantly higher in both diets compared with their WT counterparts (Fig. 19C).

Some of these changes were already evident by direct observation of the electron microscopy pictures (see Figs. 18B, 18D, 18F and 18H). Our results indicate that CYB5R3 overexpression induces changes in the planimetric parameters of mitochondria (area and circularity) without altering the overall mitochondrial mass.

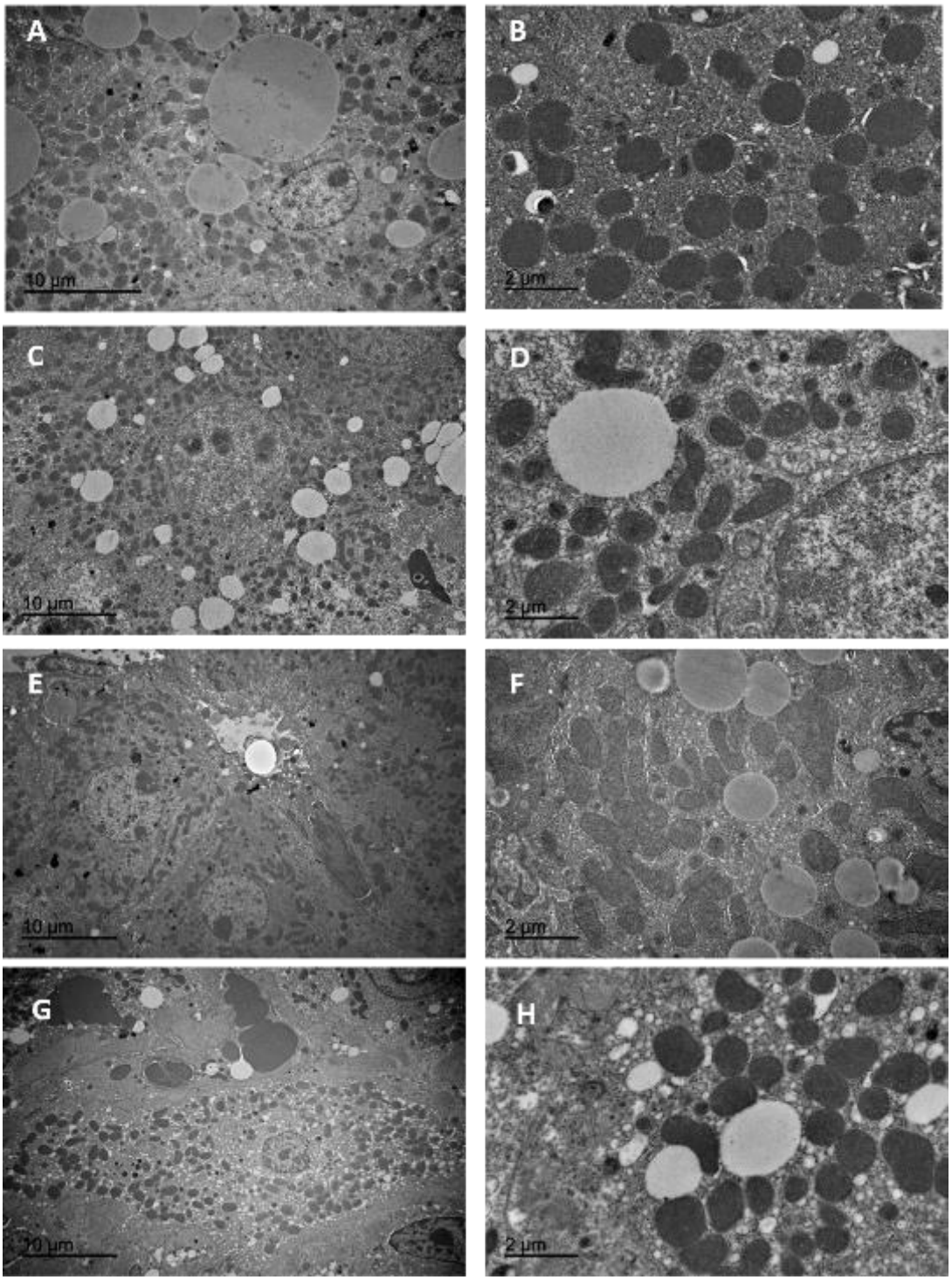


Figure 18. Mitochondrial ultrastructure in hepatocytes. Representative electron microscopy images from livers of mice fed *ad libitum* or CR diets. In left panels we show portions of liver tissue at low magnification, while in right panels portions of hepatocyte cytoplasm at higher magnification from each group are displayed. (A and B) *Ad libitum* WT. (C and D) *Ad libitum* Tg. (E and F) CR WT. (G and H) CR Tg.

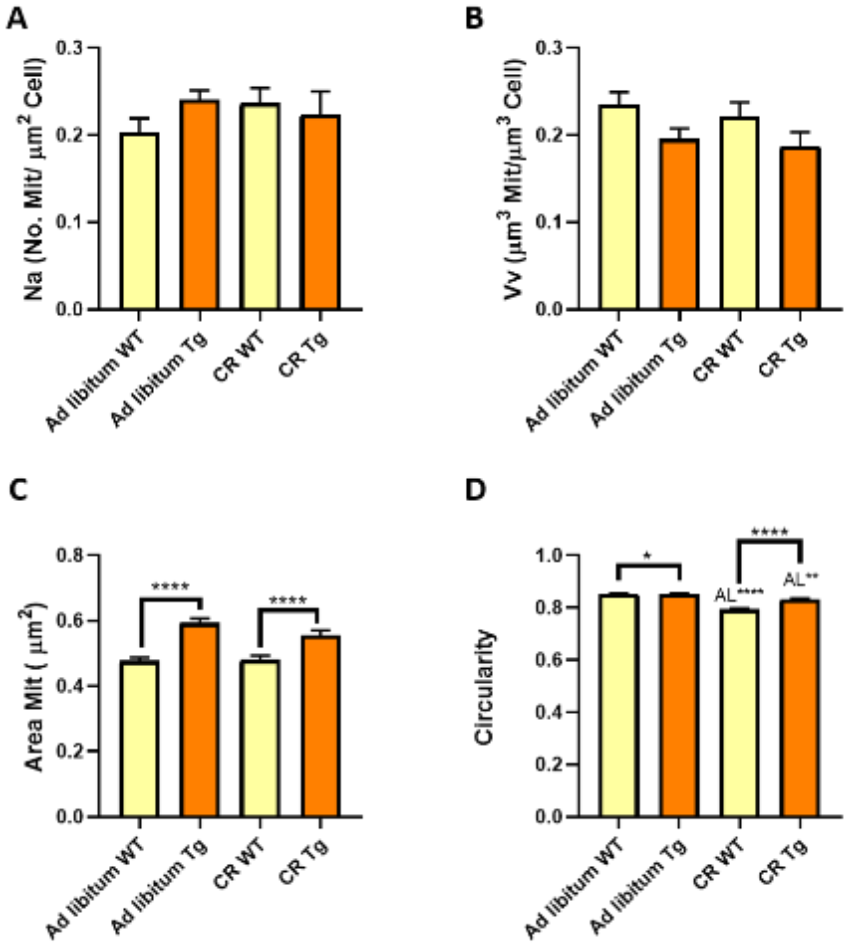


Figure 19. Mitochondrial planimetric and stereological analysis in hepatocytes. Mitochondrial numerical profile density (Na) and volume density (Vv) are displayed in panels A and B. Panels C and D, show mitochondrial area and circularity, respectively. Data represent mean ± SEM. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refers to significant differences in comparison with the *ad libitum* controls. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), and **** ($p < 0.0001$).

Mitochondrial electron transport chain complexes in liver

In addition to the analyses performed to investigate mitochondrial dynamics, biogenesis and ultrastructure, we also assessed the abundance of marker subunits of the ETC complexes as representative proteins of the inner mitochondria membrane (IMM).

We found no changes attributable to diet or genotype in hepatic levels of complexes I (Fig. 20A) and II (Fig. 20B). In the case of complex V, although our statistical analysis did not reveal significant differences among the experimental groups, we

evidenced a general effect of CR increasing its abundance irrespective of genotype (Fig. 20E). However, CR produced substantial increases of hepatic complex III in both genotypes (Fig. 20C) and complex IV in Tg animals (Fig. 20D). The western blots used for quantifications are depicted in Fig. 20F.

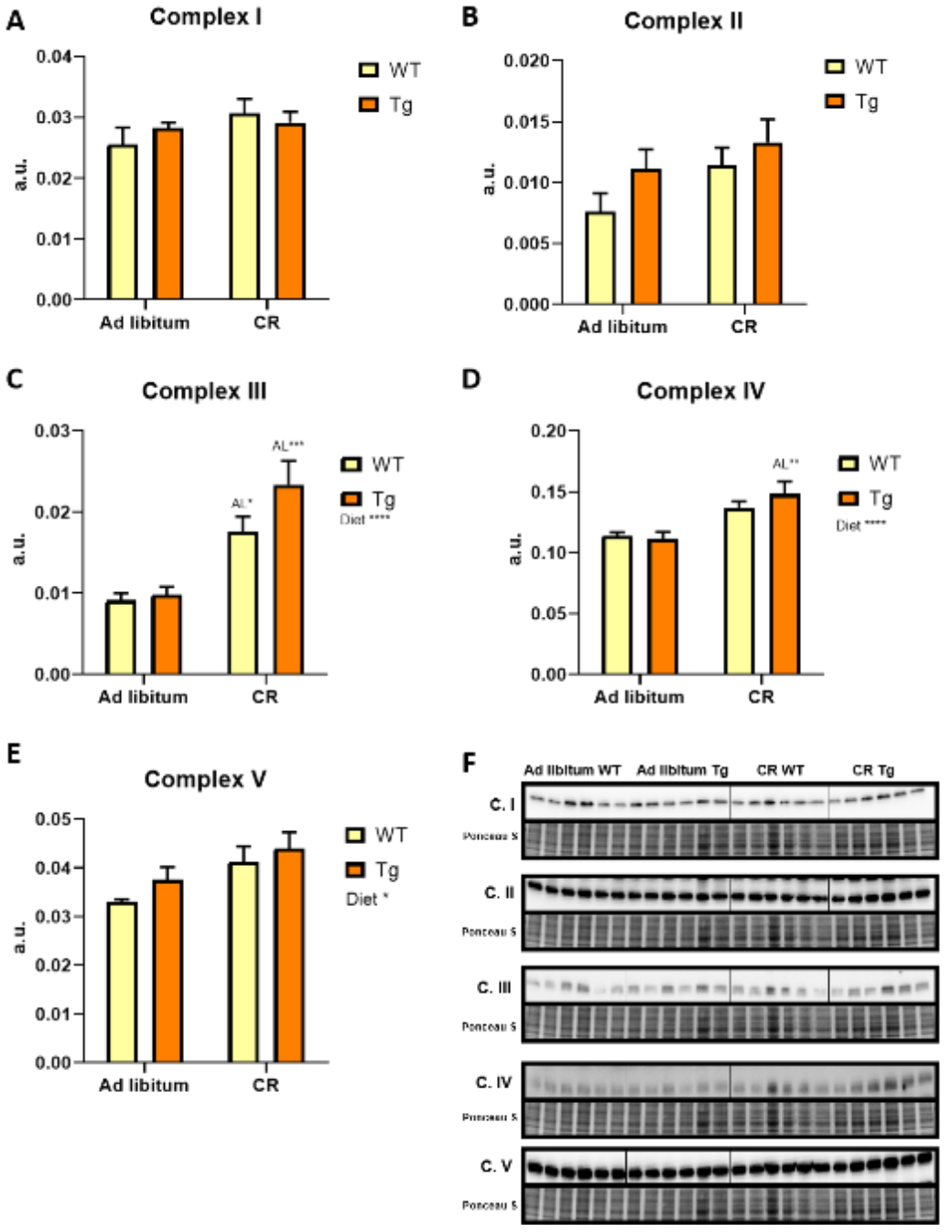


Figure 20. Expression levels of mitochondrial electron transport chain complexes in liver homogenates. Immunoblots show the levels of mitochondrial complexes I to V markers in liver homogenates of WT and CYB5R3 Tg mice fed with *ad libitum* or CR diet. The corresponding Ponceau S staining is shown underneath each blot. (A-E) Quantification of the blots. (F) Pictures of the immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized to that of the corresponding lane stained with Ponceau S. Data represent

mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refers to significant differences with respect to *ad libitum* controls. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Mitochondrial dynamics and biogenesis markers in heart

As highly dynamics organelles, mitochondria undergo different patterns of fission or fusion cycles depending on the tissue and on how it adapts to alterations in the diet. Thus, a parallel study was carried out in heart as a representative post-mitotic organ.

Changes elicited by CR and/or CYB5R3 overexpression in heart were different to those found in liver. In heart, CR induced a significant increase in MFN1 levels in WT but not in Tg mice. CYB5R3 overexpression by itself did not alter the basal levels of MFN1 in any diet (Fig. 21A). MFN2 significantly decreased in Tg mice fed *ad libitum* in comparison with WT mice fed the same diet. However, under CR, CYB5R3 overexpression rescued the normal values (Fig. 21B). The fission marker FIS1 showed a dramatic increase by CR in both genotypes, but to a greater extent in Tg animals in such a way that levels of FIS1 were significantly higher in CR Tg than in CR WT mice (Fig. 21C). With respect to the mitochondrial abundance marker VDAC1, the most prominent finding was a decrease with CR in the Tg group compared to WT mice (Fig. 21D).

Consistent with our previous findings in liver, we also found an increase of mitochondrial transcription factors under CR in heart. Although the increase of TFAM with CR was only evidenced in WT animals (Fig. 22A), NRF1 was substantially increased by CR in both genotypes (Fig. 22B). The western blots used for quantifications are below the graphs. However, changes in the mt/nu DNA ratio did not follow this pattern. In fact, a significant reduction of this ratio was found in WT mice under CR in comparison with the *ad libitum* controls, while no changes were found in Tg animals in this diet compared to *ad libitum* fed mice (Fig. 22C& Fig. S.2). No changes in ATP contents due to CYB5R3 overexpression or CR were noted.

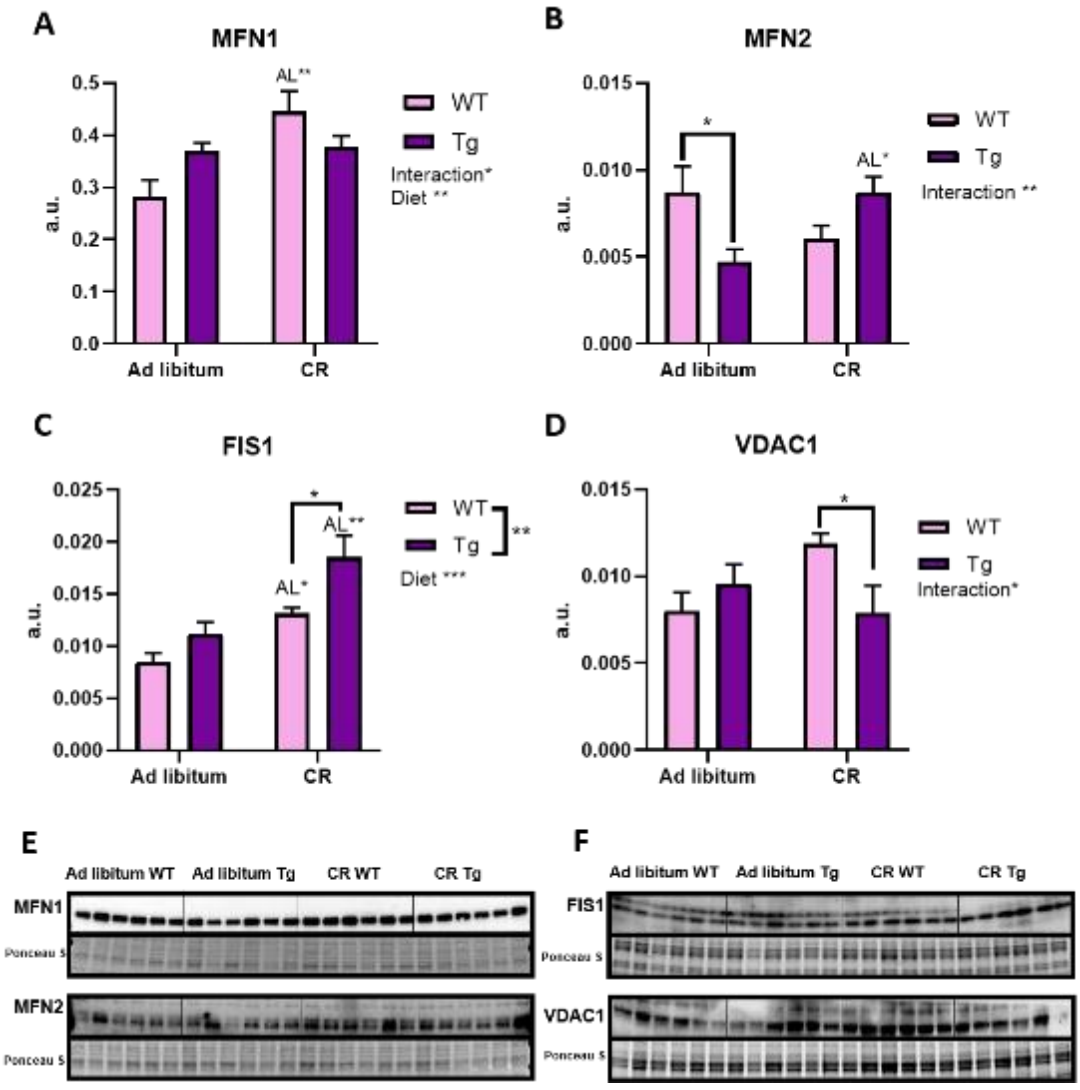


Figure 21. Mitochondrial dynamics and mass markers in heart. Immunoblots show the levels of MFN1, MFN2, FIS1 and VDAC1 in heart homogenates obtained from WT and Tg mice fed with *ad libitum* or CR diet. The corresponding Ponceau S staining is shown underneath each blot. (A-D) Quantification of the blots. (E-F) Images of the immunoblots and Ponceau S. In all cases, the intensity of the bands has been normalized by the corresponding Ponceau S staining. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refers to significant differences with respect to the corresponding *ad libitum* control. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

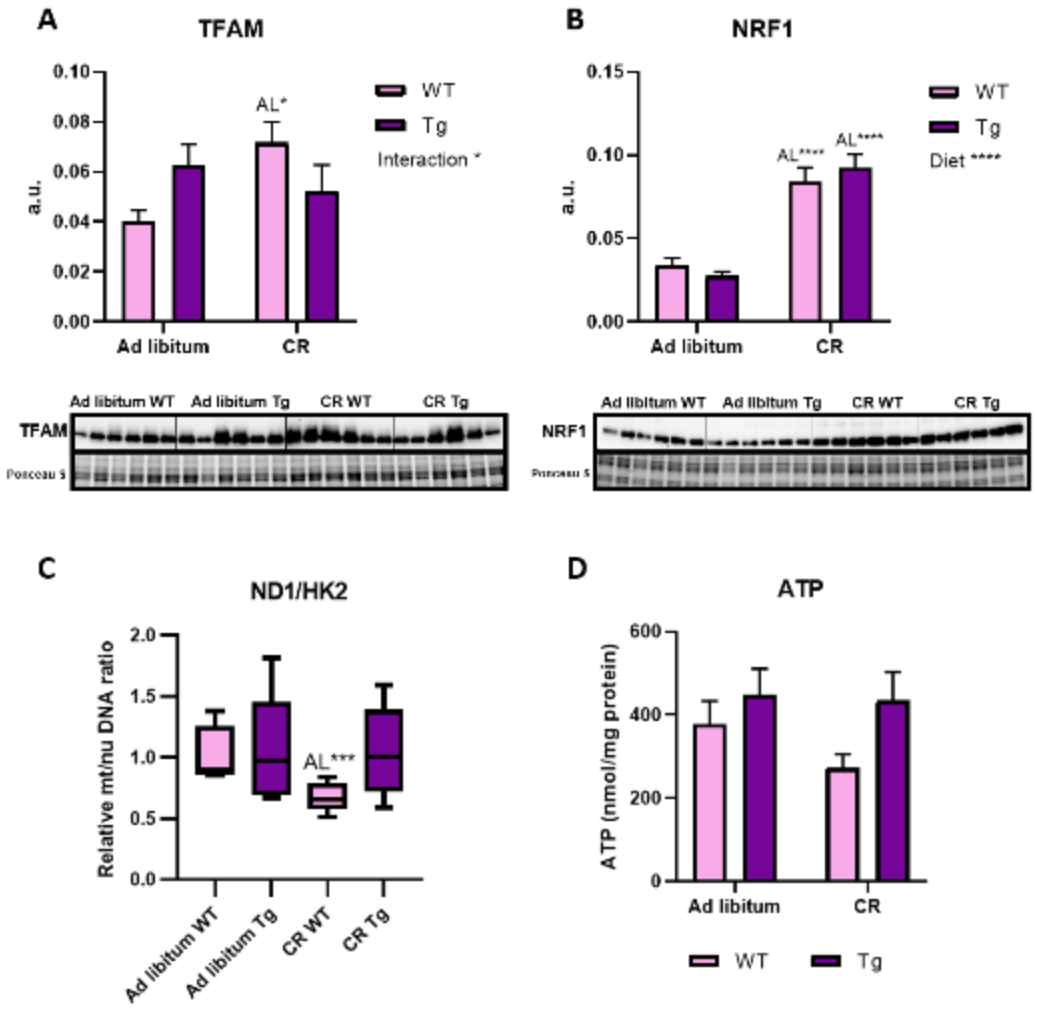


Figure 22. Mitochondrial biogenesis markers in heart. (A-B) Quantification of the immunoblots show the levels of TFAM and NRF1. The corresponding Ponceau S staining is shown underneath each blot. (C) The quantification of mt/nu DNA ratio in heart homogenates obtained from WT and Tg mice fed with *ad libitum* or CR diet. (D) Cardiac ATP contents from WT and Tg mice fed with *ad libitum* or CR diet were determined with a luminescent kit (Promega). In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. (D) Mitochondrial/nuclear DNA ratio. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refers to significant differences with respect to the corresponding *ad libitum* controls. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Mitochondrial ultrastructure in cardiomyocytes

Mitochondria play a fundamental role in the aging process, and the morphology of mitochondria in cardiomyocytes may undergo alterations over time as described for hepatocytes. We thus carried out a quantitative study on cardiomyocyte mitochondria in the different experimental groups.

As in skeletal muscle red fibers, cardiomyocytes contain two distinct mitochondria subpopulations that differ functionally. Subsarcolemmal mitochondria (SSM) are located just under the sarcolemma and contain large cristae of lamellar shape. Intermyofibrillar (IMF) mitochondria are located between the myofibrils and are smaller and are usually found in compact groups compared with SSM (Fig. 23). Both types of mitochondria may differ not only in morphology but also in response to dietary alterations. To explore this possibility, we have determined various planimetric parameters of both mitochondria subpopulations.

Our planimetric study confirmed the existence of notable changes in circularity and size of IMF and SSM from the different experimental groups, these changes being similar in both mitochondrial subpopulations. In both cases, Tg mice showed larger mitochondria than their corresponding WT controls, occurring this phenomenon either in animals with *ad libitum* or CR diets (Fig. 24A & 24B). However, the increase in mitochondrial area was especially marked in the *ad libitum* group. Mitochondrial volume showed a pattern nearly identical to that described for the previous parameter and again, the largest mitochondria were found in Tg animals in both subpopulations regardless the ingested diet. As in the previous case, the increase in mitochondrial volume was especially marked in animals of *ad libitum* group, an effect that occurred in both IMF and SSM (Fig. S. 3A & 3B.). Mitochondria were more numerous in the CR animals (Fig. 24C), but the total mitochondrial mass did not change between the two interventions (Fig. 24D) On the other hand, circularity also showed changes depending on the genotype and the diet. IMF and SSM showed a more spherical shape in samples from mice fed *ad libitum* than in those subjected to CR. Moreover, an increase in circularity was also observed in IMF from Tg mice summited to CR as compared to WT littermates (Fig. 24E & 24F).

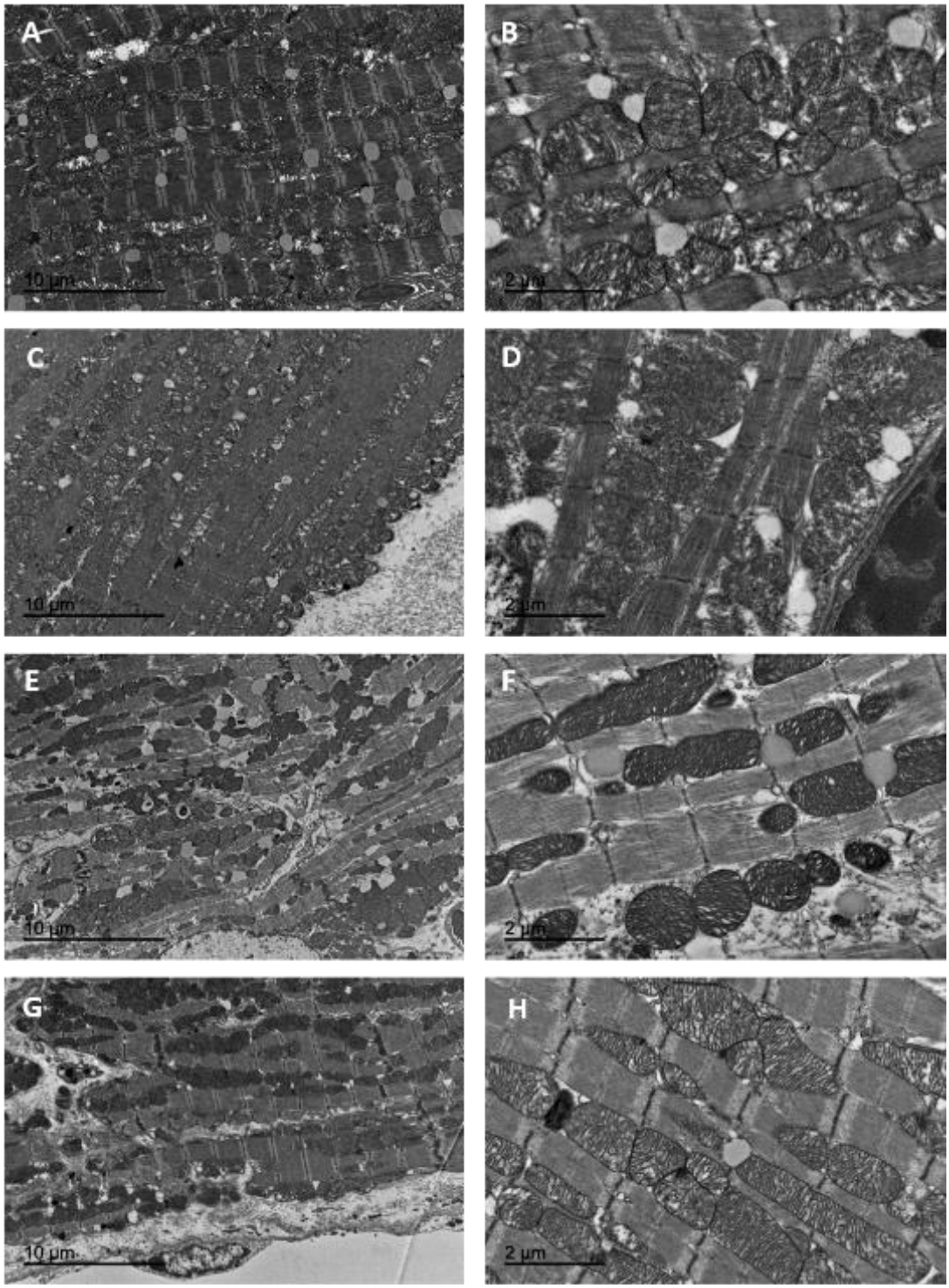


Figure 23. Mitochondrial ultrastructure in cardiomyocytes. Representative electron microscopy images of cardiomyocytes from WT and Tg mice fed either *ad libitum* or under CR. Left panels show portions of cardiomyocytes at low magnification, while right panels display areas of cardiomyocyte cytoplasm at higher magnification for better observation of mitochondrial ultrastructure. (A and B) *Ad libitum* WT; (C and D) *Ad libitum* Tg; (E and F) CR WT, and (G and H) CR Tg.

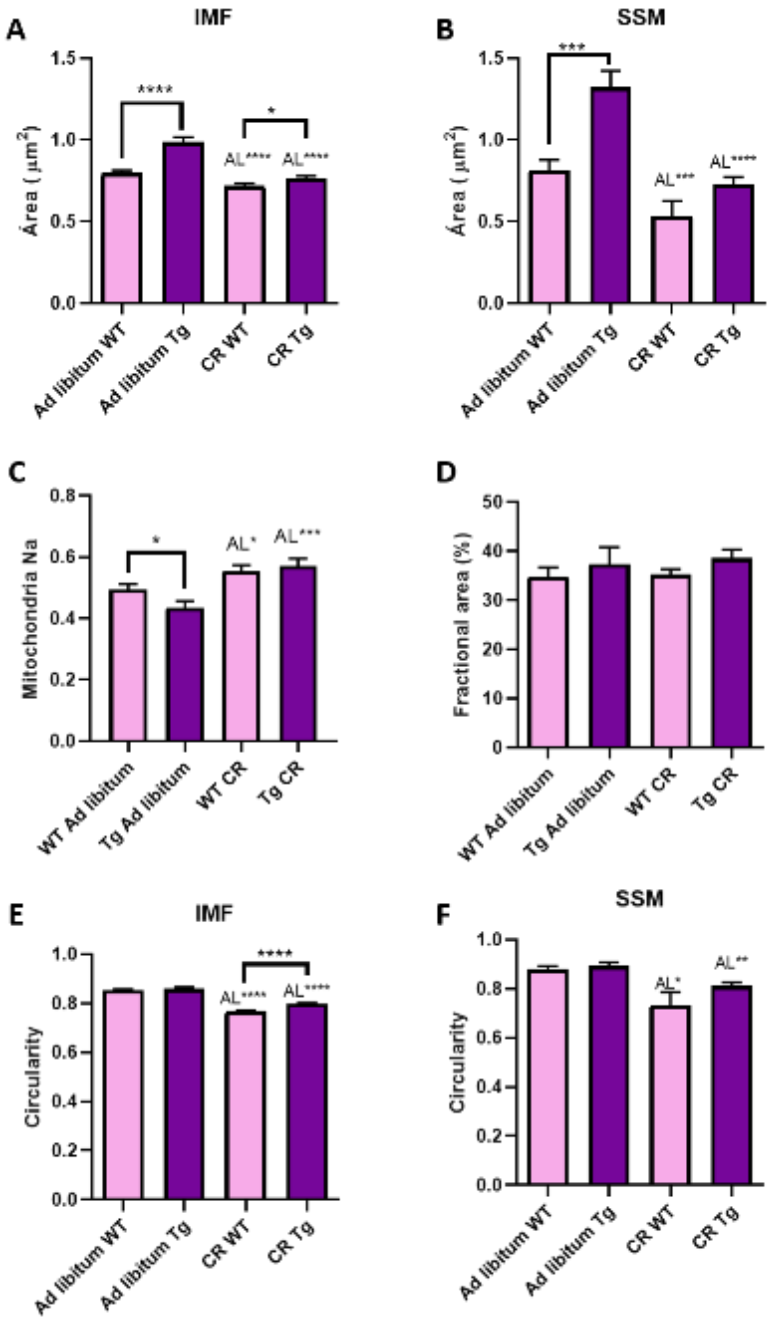


Figure 24. Mitochondrial planimetric and stereological analysis in cardiomyocytes. A and B, mitochondrial area. The results for intermyofibrillar mitochondria (IMF) are represented in left panels, while right panels display the results obtained for subsarcolemmal mitochondria (SSM). C and D, mitochondrial numerical profile density (Na) and fractional aera (in %). E and F, mitochondrial circularity results for intermyofibrillar mitochondria (IMF) are represented in left panels, while right panels display the results obtained for subsarcolemmal mitochondria (SSM). Data represent mean ± SEM. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refers to significant differences with respect to *ad libitum* controls. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Mitochondrial ETC complexes in heart

We also examined possible changes in the expression levels of ETC complexes in heart from mice of both genotypes fed *ad libitum* or under CR. No changes were found in most complexes (Fig. 25) except for complex II which was increased significantly in Tg compared with WT mice in *ad libitum* diet (Fig. 25B), and in complex IV with a higher expression in WT mice under CR compared to mice of the same genotype fed *ad libitum* (Fig. 25D). The western blots used for quantifications are depicted in Fig. 25F.

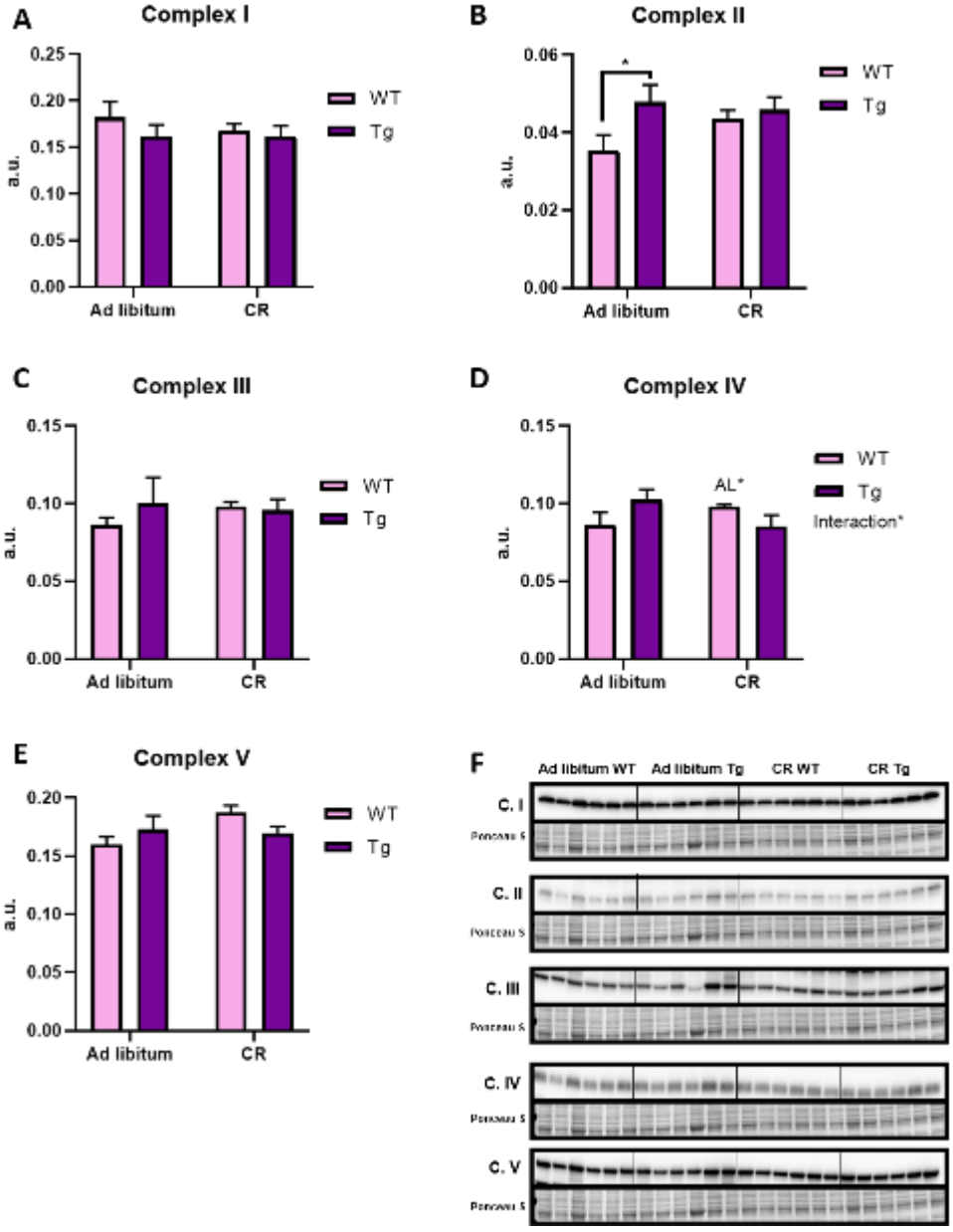


Figure 25. Expression levels of mitochondrial electron transport chain complexes in heart. Immunoblots show the levels of mitochondrial complexes I to V in heart homogenates of mice fed either *ad libitum* or under

CR. The corresponding Ponceau S staining is shown underneath each blot. (A-D) Quantification of the blots. (E-F) Images of the immunoblots and Ponceau S staining. In all cases, intensity of the bands has been normalized by the corresponding Ponceau S staining. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refer to significant differences with respect to the *ad libitum* control. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$).

Chapter II. The crosstalk between CYB5R3 overexpression and caloric restriction in transgenic mice

Discussion

The crosstalk between CYB5R3 overexpression and caloric restriction in transgenic mice

Several experimental interventions have been reported to delay aging and attenuate a wide variety of diseases associated with this process. Among them, caloric restriction without malnutrition is the most robust nongenetic intervention that promotes a healthy aging phenotype and extends lifespan [106]. Closely related to the regulation of cellular energy state are sirtuins, a family of deacetylase enzymes that require NAD^+ for their enzymatic activity and have been involved in a multitude of cellular processes [134]. Importantly, it has been reported that sirtuins expression levels and activity increase under CR and their upregulation mediates some of the salutary effects of this dietary intervention [229]. Homeostasis of NAD^+ is important for the maintenance of many metabolic functions and for protection against aging and aging related diseases. Decreased levels of NAD^+ are associated with some hallmarks of aging while, conversely, the increase in cellular levels of NAD^+ and the activation of sirtuins have been shown to extend longevity in several species such as yeast, flies, worms, and mice [127-130]. Several strategies have been designed to increase intracellular NAD^+, which include the limitation of NAD^+ consumption by genetically or chemically inhibiting enzymes that use (and degrade) NAD^+, such as PARPs or CD38, the activation of NAD^+ production, and the preservation of NAD^+ by providing precursors as dietary supplements [230]. However, the methods aimed to achieve genetic or pharmacological inhibition of NAD^+ consuming enzymes do not seem to be feasible approaches *in vivo* since these enzymes play essential roles in the physiology of cells, as DNA repair [231].

The overexpression of enzymes capable of generating NAD^+ is another strategy that is currently in progress. We have carried out a genetic intervention based on the overexpression of CYB5R3 [138]. CYB5R3 is a flavoprotein that catalyses the reduction of cytochrome b_5 and additional alternative acceptors as coenzyme Q using NADH as electron donor. CYB5R3 plays an important role in the regulation of metabolic pathways associated with healthspan and aging through mechanisms likely related to the decrease of oxidative damage and the alteration of fatty acids composition, consistent with the Free Radical Theory of Aging and the Theory of Membranes in aging [141, 142].

CYB5R3 expression is enhanced in response to nutritional and oxidative stress [144]. CYB5R3-deficient cells exhibit a lower NAD^+/NADH ratio, together with decreased mitochondrial respiration, ATP production and electron transport, all these phenomena being associated with greater sensitivity to oxidative stress and the onset of cell senescence. In summary, CYB5R3 appears to play an important role in the regulation of metabolic pathways associated with life expectancy and aging.

Moreover, it has been considered that some of the beneficial effects CR exerts on health could be related, at least in part, to CYB5R3 induction [141, 227, 232]. However, whereas some of the salutary effects of CR are indeed mimicked in transgenic mice overexpressing CYB5R3 [138], the mechanisms involved in metabolic adaptation of Tg mice might be independent of those described for CR since key aspects of energetic metabolism are different regulated by CR and by CYB5R3 overexpression. To this sense, CYB5R3 mice fed a standard diet *ad libitum* were fatter than wild-type controls and preferentially used carbohydrate to meet their energy needs, which departs from other studies using CR or metformin, where increased longevity required the use of fat as a source of fuel [233, 234].

Metabolic flexibility has been defined as the capacity of cells to adapt their metabolic pathways to different situations of energy availability or fuel oxidation. This phenomenon is negatively correlated with aging and positively with the maintenance of a healthy state of cells and tissues. Liver, skeletal muscle and heart are examples of important contributors to systemic metabolism, being necessary a correct coordination in the utilization of available energy among them. In this sense, mitochondria play a fundamental role in preserving metabolic flexibility since mitochondrial dysfunction and the perturbation of nutrient-sensing pathways lead cells to shift towards a glycolytic phenotype, which is a hallmark of aging [235]. Mitochondria are highly dynamic organelles continuously undergoing processes of fusion, fission and recycling by mitophagy/biogenesis. The hepatocyte is a cell type with abundant mitochondria and the liver is one of the main contributors to whole animal energy expenditure. Studies using this cellular model are thus especially relevant in the context of metabolism and aging. In this context, the liver contributes to the systemic metabolism of carbohydrates, lipids and proteins, as well as to detoxification processes that are relevant for aging [236]. Adaptation to diets, including those of caloric restriction or those involving different nutritional

variations, is mainly regulated by the liver, which in turn reflects the positive effects of these dietary variations [237].

MFN2, TFAM and the relative mt/nu DNA ratio were dramatically increased and NRF1 tended to increase by CR in liver of WT mice, which is partially in accordance with a previous research documenting the upregulation of hepatic mitochondrial biogenesis parameters in 3 and 12 months-old mice fed under CR [73]. However, this study also evidenced an increase of MFN1 that we have not confirmed here at the level of protein. On the other hand, Khraiwesh *et al*. [205] reported increased levels of hepatic FIS1 in 9 months old mice fed under CR for 6 months, which is in contrast with the results we have obtained in our model. However, it is worth noting the differences between the experimental diets used for these two interventions. While the AIM93G diet – which contains 7% fat - was used in the study by Khraiwesh *et al*. [205], the diet used in our study (AIN93M) contains 4% fat. This difference in the amount of fat provided in the diet, together with a different duration of the intervention period (6 *versus* 4 months) might be key determinants in the rate of mitochondrial hepatic adaptations to CR. This fact was also evident in the studies carried out by Calvo-Rubio (Calvo-Rubio, M. PhD Thesis) where mice of the same age were subjected to 30% caloric restriction with two different diets (NIA-1-87 and WNPRC), resulting in different outcomes in mitochondrial parameters. One of the main differences between these two diets was the percentage of fat: 5% for NIA-1-87 diet and 10.6% for WNPRC diet.

CYB5R3 overexpression abated (for MFN2 and FIS1) or attenuated (for TFAM) the effect of CR on hepatic markers of mitochondrial dynamics and biogenesis. CYB5R3 overexpression by itself led to an increase of hepatic FIS1 in mice fed *ad libitum*, but this effect was suppressed by CR. Moreover, CYB5R3 overexpression also led to decreased levels of hepatic NRF1, indicating a global effect on mitochondrial metabolism, dynamics and biogenesis in mice fed under CR. MFN2 is a key protein that facilitates contacts between the endoplasmic reticulum and the mitochondria in the regulation of several processes including mitochondrial dynamics, calcium homeostasis and, of particular relevance in this case, in the processing of fat deposits, which is accelerated [238, 239]. The absence of CR effect on hepatic levels of MFN2 in Tg mice, and the decrease of TFAM and NRF1 in Tg mice fed under CR in

comparison with WT mice fed the same diet might be related with the enhancement of carbohydrate metabolism in mice overexpressing CYB5R3 [138].

VDAC1 is an abundant protein of the outer mitochondrial membrane whose levels serve as a biochemical assessment of mitochondrial mass [240]. Previous works have established that CR results in increased mitochondrial number and mass, and that may reduce ROS production without decreasing cellular respiration [241, 242]. However, our study revealed no significant differences among experimental groups for hepatic VDAC1. In addition, a stereological study on TEM micrographs indicated that neither CR nor CYB5R3 overexpression induced changes in mitochondrial mass, which is in accordance with the data obtained for VDAC1 levels by western blot. The observations reported here are, however, in contrast with previous studies carried out by our group based on a quantitative approach using electron microscopy, which documented that a 6-month CR intervention based on the AIN93G diet was sufficient to increase mitochondrial abundance in mouse liver [205, 236]. Furthermore, other authors have suggested that CR and resveratrol (a CR mimetic) induce an increase in mitochondrial biogenesis and in bioenergetic efficiency [241, 243], although if CR truly increases mitochondrial biogenesis, it has been also questioned [244, 245]. The differences in mitochondrial abundance between these studies could be due to various factors as the age of the animals and the type of lipids included in the diet. Moreover, controversial results regarding changes of mitochondrial mass with CR and aging may also arise from the heterogeneity in the methods used to assess mitochondrial content [246, 247].

On the other hand, the studies by Khraiwesh *et al*. [205] showed that CR did not produce changes in the mean size of mitochondria, which agrees with our results. However, it is noteworthy that CYB5R3 overexpression induced an increase in the average mitochondrial area in the two dietary conditions, which might be related with an improvement in mitochondrial function if, as previous studies have proposed, an increase in size is accompanied by increased number of mitochondrial cristae [236]. Changes in mitochondrial shape have been also related to alterations in the organelle dynamics [248]. Ultrastructural changes, as increased circularity, have been suggested to be related with a loss of mitochondria homeostasis, being this phenomenon frequently observed in aged animals and in several pathologies [249, 250]. Additionally, other studies have shown that more elongated mitochondria were

preserved from mitophagy [251]. In our model, CR induced a decrease in circularity in WT animals, and this effect was attenuated in Tg mice, which is consistent with the idea that the effect of CR is partially blunted by the overexpression of CYB5R3.

An increase of fatty acid β-oxidation in response to CR could influence the activity and abundance of mitochondrial complex subunits [252]. We observed no changes in mitochondrial mass (as indicated by VDAC1 levels and TEM analysis), but the levels of complex III were increased by CR both in WT and Tg mice, with a much larger effect seen in the case of mice overexpressing CYB5R3. Additionally, a general effect of CR increasing the abundance of mitochondrial complexes independently of genotype was observed for complex IV (with significant differences between the two groups of Tg mice) and complex V. Together, these data suggest that mitochondrial function, rather than its structure or abundance, is primarily affected by CYB5R3 overexpression in liver. An increase in the abundance of mitochondrial complexes downstream ubiquinone (i.e., complexes III and IV) may favour the reoxidation of ubiquinol and redox sites the upstream complexes (I and II), which could decrease ROS generation during mitochondrial electron transport, particularly in mice overexpressing CYB5R3.

The heart shows a highly aerobic metabolism with a great energy demand, in order to perform muscle contraction. This is why alterations in mitochondrial bioenergetics can lead to heart dysfunction and failure. Numerous studies have demonstrated that CR is capable to avoid the development of cardiovascular disease [253, 254]. To analyse how CR and CYB5R3 overexpression affect post-mitotic tissues, we studied how both interventions affected several mitochondrial parameters in the heart.

In heart homogenates, we observed increased levels of MFN1 under CR in WT animals but not in Tg mice. Meanwhile, MFN2 decreased in Tg mice fed *ad libitum* but was increased under CR. Nisoli *et al.* reported an upregulation of gene expression related to mitochondrial dynamics in heart and skeletal muscle under caloric restriction [73]. Interestingly, in *ad libitum* conditions, Tg mice displayed similar values of MFN2 expression than those obtained in caloric restricted WT animals, which suggest that the effect of CYB5R3 overexpression may be similar to that obtained with CR. Nonetheless, when applied together, both interventions seem to

interfere. A dramatic increase was observed in FIS1 levels in mice of both genotypes fed under CR, and this increase was more evident in Tg animals.

CR is related to multiple benefits on cardiovascular diseases through attenuation of oxidative stress, mitochondrial dysfunction and inflammation. FIS1, a marker directly involved in mitochondrial fission, could play a key role in protection against heart attacks in various rodent models [255]. In organs with high energetic demand and, therefore, with a high number of mitochondria, as the heart, the control of mitochondrial dynamics is especially relevant. However, most of the results published on mitochondrial fusion and fission in the heart come from studies in cultured cells and not cardiac tissue itself [255]. Taken together, our observations could indicate that CYB5R3 overexpression targets mitochondrial dynamics promoting fission over fusion events. Since the same has been observed in skeletal muscle (López-Bellón S. PhD Thesis, in preparation), this could be a general feature of CYB5R3 overexpression in muscular tissue.

Our results showed an increase in VDAC1 levels under CR which was reverted by CYB5R3 overexpression. Once again, overexpression of CYB5R3 appears to counteract the effect of CR on another biochemical parameter. These data contrast however with those obtained from electron microscopy studies, showing that mitochondrial mass was similar in all the experimental groups. However, due to the existence of two different populations of mitochondria in cardiomyocytes [256], the possibility exists that aging and/or CR, or even CYB5R3 overexpression, differentially affect these populations. Although electron microscopy can discriminate both populations allowing separate quantitative studies, protein quantification was performed on heart total homogenates, and the results are therefore referred to both mitochondrial subpopulations together. An increase of VDAC1 levels under CR and its prevention by CYB5R3 overexpression has been also found to occur in skeletal muscle by our group [136]. Since mice overexpressing CYB5R3 showed a preferential use of carbohydrates [138], the decrease of this mitochondrial marker in post-mitotic tissues could be related with the inhibition of β-oxidation.

As in liver (see above), levels of TFAM were affected by CR but not by CYB5R3 overexpression, and increased NRF1 was observed for both genotypes in cardiac tissue. Some studies have shown that *Tfam* and *Nrf1* mRNAs were increased with CR

in rodents and human muscle [73, 242], which is in accordance to our data. Additional results, partially in agreement with ours, were also obtained by Gesing *et al*. in the heart of mice subjected to CR for six months, where a slight increase in TFAM expression was detected without changes in NRF1 [257]. However, another study carried out in rats submitted to CR for three months showed a decrease in the levels of TFAM and PGC1α polypeptides in both males and females. Discrepancies among the different studies may be caused by differences in animals models used, and also by differences in duration of the intervention. On the other hand, the study carried out by Colom *et al.* reported that the heart of animals subjected to CR exhibited a decrease in the content of mtDNA [258]. However, this does not necessarily indicate a compromised mitochondrial functionality, since it is possible that the heart of mice subjected to CR shown a higher respiratory efficiency. This study has also provided evidence of sexual dimorphism in mice regarding mitochondrial biogenesis. Although females showed a greater number of differentiated mitochondria than males, ATP production was not affected by CR regardless the animal gender. These data agree with ours and partially explain the lower levels of mtDNA found in WT mice fed under CR, without altering mitochondrial function or ATP content. Regarding our results, we noted a discrepancy between TFAM (which increased with CR in WT mice) and mtDNA (which decreased with CR in WT mice) data. However, a recent study has shown that TFAM is not directly related to cardiac mtDNA in adult mice, as it would be only essential for the formation of the heart during development. This study also reported that the short-term ablation of TFAM in the adult, and therefore a decrease in protein levels, did not directly affect the content of mtDNA in heart. In fact, the mtDNA content was preserved, as well as mitochondrial and cardiac function. This is not unreasonable since, as an organ fully dedicated to mitochondrial respiration, the heart should be expected to remain resilient to acute disruptions [259]. This fact highlights the differences between a developing *versus* an adult tissue, and how the heart faces different environmental alterations, as CR, differently to other organs as the liver.

As stated above, it has been suggested that the two types of heart mitochondria (IMF, SSM) may display different metabolic adaptations to oxidative damage and, therefore, a different susceptibility to aging or changes in food intake. In fact, the separation of the two mitochondrial subpopulations using fractionation techniques

has shown that aging affects mainly the IMF mitochondria subpopulations in rats, in which increased ROS and decreased levels of antioxidants were associated with a loss of complex IV activity, while SSM population remained practically unaltered [260]. These authors also pointed out that, excepting for their location, both types of mitochondria are practically indistinguishable. In *ad libitum* conditions, CYB5R3 overexpression induced a significant increase in the area and volume parameters of both mitochondrial populations when compared with their respective WT counterparts. On the other hand, CR induced a decrease in mitochondrial area and volume in all experimental groups, especially in Tg mice. Despite these alterations in the size of individual organelles, our analysis of total mitochondrial mass evidenced by fractional area parameter did not reveal notable changes. Thus, a decrease in the mean size in some of the mitochondrial subpopulations is accompanied by an increase in the number of mitochondria (Na) to maintain a uniform and constant mitochondrial mass regardless as occurs in CR animals. These data are in accordance with those obtained in rats under CR, which also exhibited a lower mitochondrial content without deficiency of mitochondrial functionality [258].

A parallel study carried out on skeletal muscle (red gastrocnemius) from mice of the same cohort as we have used here has shown that CYB5R3 overexpression also altered mitochondrial ultrastructural parameters. In this case, Tg mice exhibited a decrease of mitochondrial size and abundance in white (glycolytic) but not in red (respiratory) fibers (López-Bellon S., PhD Thesis in preparation), indicating that the effect of CYB5R3 overexpression on the ultrastructure of mitochondria in muscular tissues is dependent on the fiber type.

As observed in the liver, in heart from CR mice there is a morphological change towards decreased circularity, producing more elongated mitochondria. Interestingly, we also found a loss of circularity under CR in skeletal muscle tissue (López-Bellón S. PhD Thesis, in preparation). In general, there is no clear consensus when correlating changes in mitochondrial morphology and functionality. It has been proposed that changes in mitochondrial shape toward more elongated profiles seem to optimize ATP production and these mitochondria become protected from autophagy in times of starvation [261]. Mitochondrial shape is also altered with aging (leading in this case to a less elongated and more rounded shape) [262], and the area of the mitochondrial inner membrane per mitochondria is reduced in aged

myocardium without affecting cristae configuration [263, 264]. According to these ideas, changes in mitochondrial morphology with CR may be indicative of a greater preservation of these organelles by autophagy, which would represent an advantage compared to animals fed *ad libitum*. Once again, we observed how CR and CYB5R3 overexpression produced opposite effects when combined.

It is well known that animals under to CR show lower levels of free radical production in heart and other tissues. This has been associated with less generation of H_2O_2 and, therefore, with less oxidative damage in these organs. This adaptation prevents against numerous aging-related diseases associated with oxidative damage like cardiovascular disorders, which could be also related to the extension of healthspan in CR animals. The mitochondrial production of free radicals is directly correlated with the activities of complexes I and III [265, 266].

The activity and expression levels of mitochondrial complexes seem to depend on the tissue, the age of the animals and the experimental intervention. Regarding the protein levels of mitochondrial electron transport chain complexes, our group has obtained partially diverging results regarding the effects of CR in skeletal muscle of mice. A first study involving six-month-old animals fed either *ad libitum* or under CR conditions did not reveal appreciable changes in the levels of any of the complexes [246]. However, another recent study reported a generalized increase in the levels of mitochondrial complexes in this tissue [136]. Again, it is important to consider he the different designs of both studies, particularly with respect to the composition of diets in terms of the percentage of fat. Moreover, both in cardiac and skeletal muscle of aged mice, long periods of CR did not affect ATP production neither in control animals nor in those subjected to CR, which suggests a marked stability of these complexes during aging [267]. On the other hand, previous studies described that complex I and I+III activities were increased in CYB5R3 Tg mice in liver tissue without changes in protein expression. In our case, we also did not detect any change in protein levels of mitochondrial complexes, except for complex IV in WT mice fed under CR, as we have also observed in skeletal muscle [136]. CR has been shown to enhance efficiency of complex IV in skeletal muscle [268], a phenomenon also reported to occur in heart [258]. These results are likely related to a higher affinity and an increase in the binding sites of O_2 to complex IV. Under these conditions, both electrons flow and redox state of the complexes are improved resulting in decreased

generation of mitochondrial ROS [269]. Interestingly, complex I activity appears to be a valid surrogate of mitochondrial mass in the muscle of mice, outperforming other suggested markers [270]. Although we did not measure its enzymatic activity, the stability of complex I levels across experimental conditions seems to confirm the overall absence of change in mitochondrial mass as measured by TEM.

In summary, our data support the idea that many beneficial effects of CR are determined by a better preservation of the mitochondrial physiology and ultrastructural features which results, not only in lower production of ROS, but also in the protection of this organelle from mitophagy. These outcomes are partially mimicked by CYB5R3 overexpression in mice fed under *ad libitum* conditions as described in detail here. Nevertheless, as we stated in our initial hypothesis, the accrual of beneficial effects produced by CR and CYB5R3 overexpression appear to operate by different metabolic pathways when studied separately and, when applied both interventions simultaneously, their effects become partially blunted.

Chapter III. Effect of the dietary fat source on mitochondrial structure and function in CYB5R3 -overexpressing mice

Results

Mitochondrial dynamics and biogenesis markers in liver

The impairment of mitochondrial function is associated with changes in the mitochondrial network, and it has been suggested that dietary fats affect mitochondrial function and dynamics [271]. Therefore, we analysed the interplay between dietary fat and CYB5R3 overexpression with regards to key aspects of mitochondrial biology.

Overall, we detected a great influence of the diet on fusion proteins. Higher levels were found when using a MUFA- and n-3 PUFA-enriched fat sources (olive oil and fish oil, respectively), whereas lower levels were found with fat sources rich in SFA (lard) and n-6 PUFA (soybean oil). Expression levels of the fusion proteins MFN1 and MFN2 were significantly increased in Tg mice on an olive oil-containing diet compared with the other fats, and despite a lack of changes in their WT counterparts fed the same diets (Fig. 26A & 26B). Regarding the markers related with mitochondrial fission, a small decrease in FIS1 levels was found in WT animals fed the soybean oil-based diet, whereas an overall increase in all experimental groups was found with Tg mice (Fig. 26C). Regarding MFF, a dramatic increase was observed with the lard diet, an effect that was attenuated with CYB5R3 overexpression. Of note, very low levels of MFF were found in mice on the soybean oil diet regardless of the genotype (Fig. 26D). VDAC1 expression was analysed not only to get an insight on mitochondrial mass, but also due to its participation in PINK/Parkin-dependent mitophagy, which is functionally linked to mitochondrial fission [100, 101]. Across the WT groups fed diets with different fat source, we only found a decreased expression in those animals that had been fed an olive oil-containing diet (Fig. 26E). Regarding the effect of CYB5R3 overexpression, as displayed in Fig. 26E, VDAC1 was significantly increased in Tg mice fed a lard-based diet. The western blots used for quantifications are depicted in Fig. 26F.

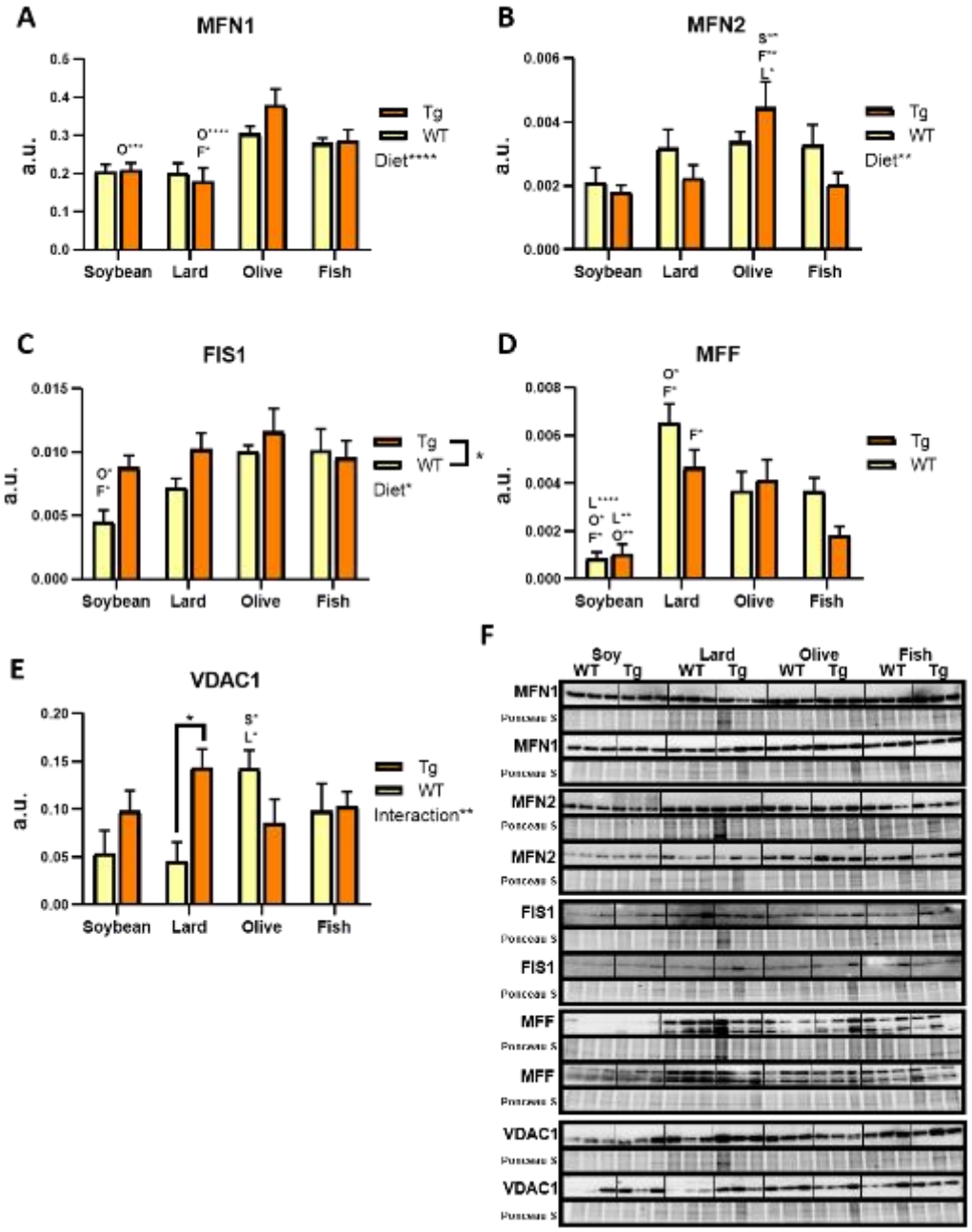


Figure 26. Mitochondrial dynamics and mass markers in liver from WT and Tg mice fed diets containing different fat sources. Immunoblots show the levels of MFN1, MFN2, FIS1, MFF and VDAC1 in liver homogenates from WT and Tg mice fed *ad libitum* with experimental diets containing soybean oil, lard, olive oil or fish oil as the predominant source of fat. The corresponding Ponceau S staining is shown below each blot. (A-E) Quantification of the blots. (F) Immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. Data represent means ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "S", "L", "O" and "F" refers to significant differences with respect to soybean oil, lard, olive oil and fish oil-enriched diet, respectively. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

The mitochondrial transcription factor TFAM was highly expressed in WT mice that had been the lard-based diet, whereas its protein levels were the lowest in the case of those mice fed the soybean oil-based diet, a pattern that closely resembled that of MFF (Fig. 27A). On the contrary, NRF1 displayed higher expression in WT mice fed the soybean oil-based diet (Fig. 27B). However, none of these parameters correlated with the relative levels of mtDNA, since a significant increase in the mt/nuDNA ratio was found in the olive oil- and fish oil-based diet, especially in the case of Tg mice (Fig.27C-E & Fig. S.4-6.).

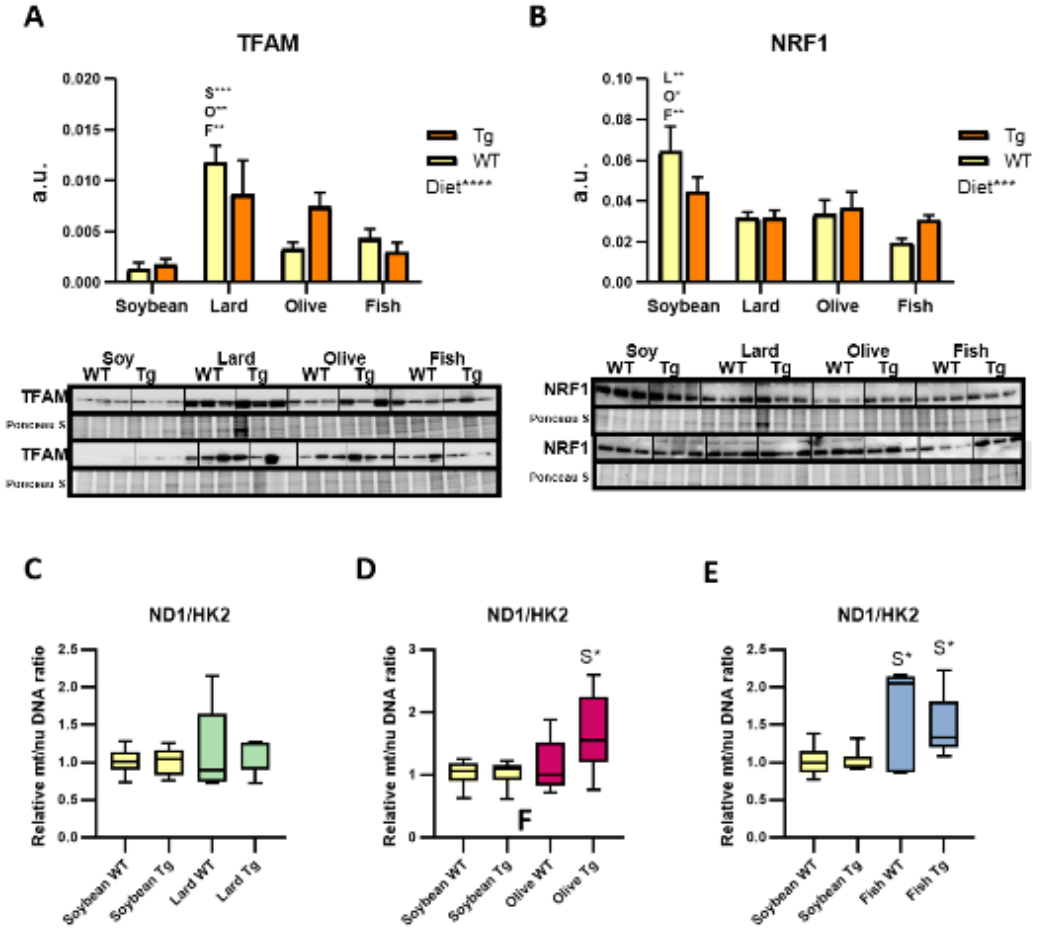


Figure 27. Mitochondrial biogenesis markers in liver from WT and Tg mice fed diets that differed in the predominant source of fat. Immunoblots show the levels of TFAM and NRF1, and quantification of mt/nu DNA ratio in liver homogenates is also shown. The corresponding Ponceau S staining is shown below each blot. (A-B) Quantification of the blots and images of the immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. (C-E) Mitochondrial/nuclear DNA ratio. Data represent means ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes. "S", "L", "O" and "F" refers to significant differences with respect to soybean oil, lard, olive oil and fish oil-containing diet, respectively. When detected, general effects of diets are represented

on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

In summary, a trend towards increased mitochondrial fusion and decreased fission appears to occur in liver from mice fed olive oil, soybean oil or fish oil-based diets, while lard, when used as the predominant dietary fat, increases the levels of proteins related with mitochondrial fission.

Mitochondrial electron transport chain complexes in liver

Expression levels of selected marker proteins for the five ETC were also studied. Only soybean oil affected the expression levels of complex I and complex II markers. Specifically, complex I levels were the highest in liver from WT mice that had been fed a soybean oil-enriched diet, compared to all other groups (Fig. 28A). With regard to complex II, mice from both genotypes experienced a decrease in protein levels when fed the same soybean oil-based diet in comparison with the other three diets (Fig. 28B). Complex IV was only affected by olive oil, which caused a decrease in the case of WT mice compared to the rest of dietary groups of the same genotype (Fig. 28D). Finally, neither complex III (Fig. 28C) nor complex IV (Fig. 28E) were affected by either diet or genotype. The western blots used for quantifications are depicted in Fig. 28F.

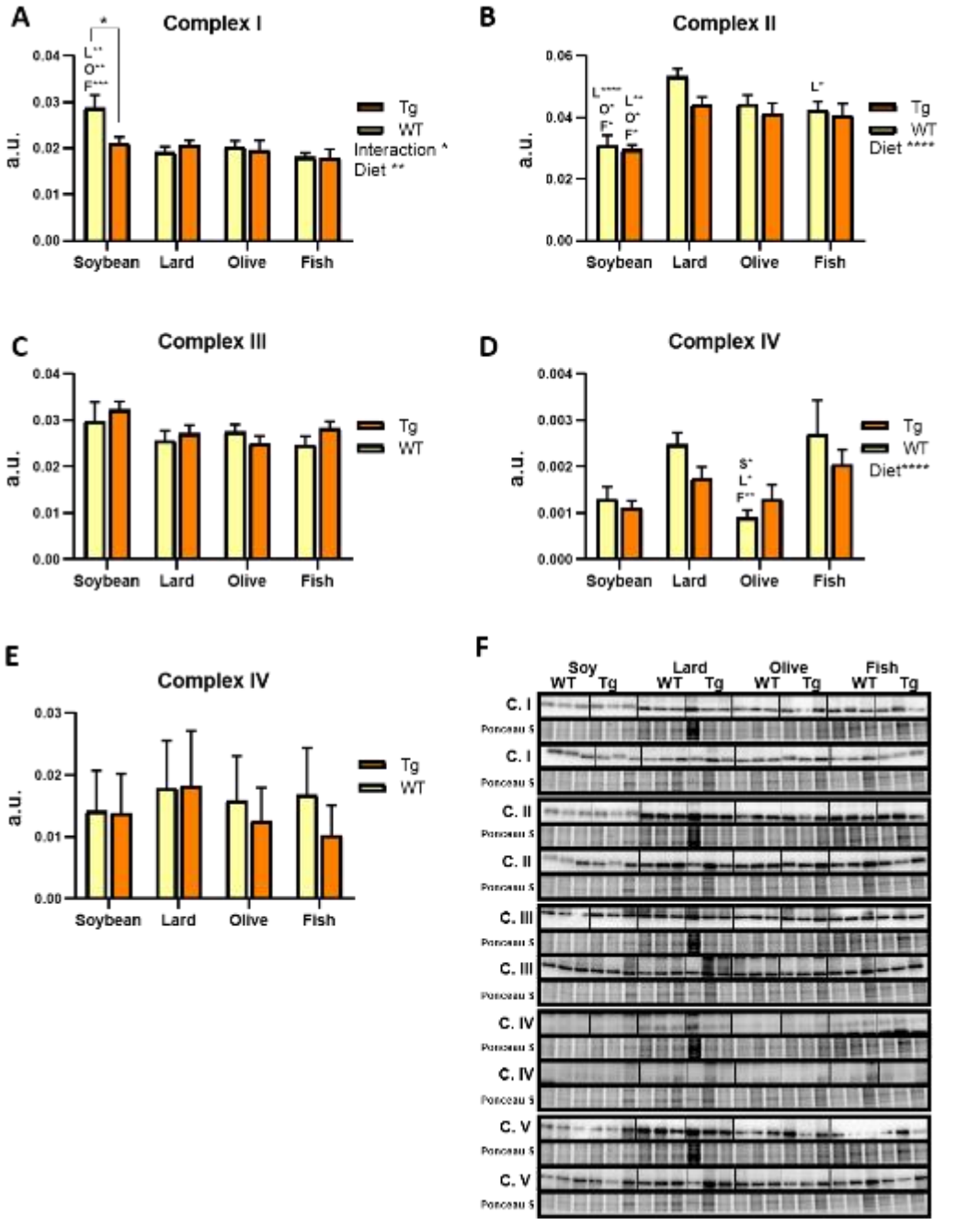


Figure 28. Expression levels of mitochondrial electron transport chain complexes in liver homogenates from WT and Tg mice fed diets with different fat sources. Immunoblots show the levels of mitochondrial complexes I to V in liver homogenates from WT and Tg mice fed *ad libitum* with diets containing soybean oil, lard, olive oil or fish oil as the predominant sources of fat. The corresponding Ponceau S staining is shown underneath each blot. (A-E) Quantification of the blots. (F) Immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "S", "L", "O" and "F" refers to significant differences with respect to soybean oil, lard, olive oil and fish oil-enriched diet, respectively. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Mitochondrial dynamics and biogenesis markers in heart

As reported for other organs, dietary patterns may lead to structural and physiological changes that in some cases could induce mitochondria dysfunction within the cardiac muscle. In addition, CYB5R3 expression has been shown to influence lipid composition of membranes [138, 144]. Therefore, we wanted to elucidate the interplay between CYB5R3 overexpression and dietary fat in the modulation of key mitochondrial features that may influence the rate of aging. To achieve this goal, we analysed mitochondrial dynamics and biogenesis in the heart from WT and Tg mice that had been fed diets containing different dietary fat sources.

A decrease in MFN1 was found in WT animals under an olive oil-enriched diet, while no differences were found with other dietary fats or as a result of the genotype (Fig. 29A). Decreased MFN2 content was found in Tg animals under a fish oil-based diet. In contrast, we detected an increase in this parameter in Tg mice fed an olive oil-enriched diet. (Fig. 29B). In relation to mitochondrial fission, we found a general increase in FIS1 expression under the lard-based diet (Fig. 29C). However, the pattern of the other protein related with mitochondrial fission studied here was different, as increased levels of MFF were found in Tg mice fed with diets containing soybean oil or olive oil in comparison with mice of the same genotype that had been bed a fish oil-based diet, for which the lowest levels of the fission factor were found (Fig. 29D). No differences were found in VDAC1 except for a slight increase in WT animals fed a fish oil-based diet in comparison with those fed a soybean oil-based diet (Fig. 29E). The western blots used for quantifications are depicted in Fig. 29F.

The expression levels of the transcription factors TFAM and NFR1 were also investigated. We found increased TFAM levels in Tg animals fed with the lard-based diet, both when compared with mice of the same genotype belonging to other dietary groups, and when compared with their WT counterparts fed the same diet (Fig. 30A). We also evidenced an increase in NRF1 levels in WT animals fed with a fish oil-enriched diet (Fig. 30B). However, despite the observed alterations in TFAM and NRF1, two key factors in the regulation of mitochondrial biogenesis, no changes were found in the relative mt/nuDNA ratio, except for a slight decline in WT mice fed the fish oil-based diet (Fig. 30C-E & Fig. S.7-9).

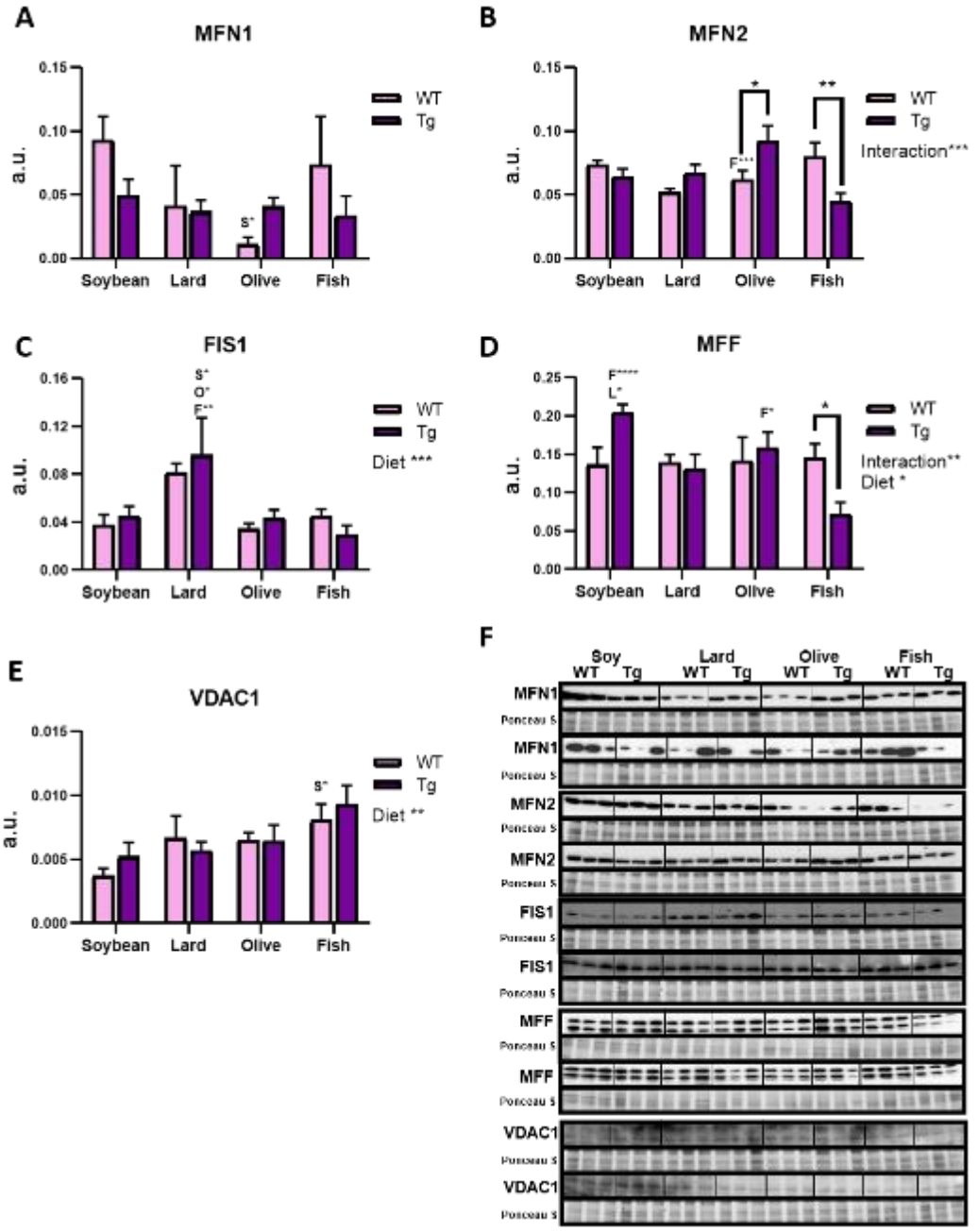


Figure 29. Mitochondrial dynamics and mass markers in heart from WT and Tg mice fed diets containing different fat sources. Immunoblots show the levels of MFN1, MFN2, FIS1, MFF and VDAC1 in heart homogenates obtained from WT and Tg mice fed *ad libitum* with diets containing soybean oil, lard, olive oil or fish oil as the predominant sources of fat. The corresponding Ponceau S staining is shown underneath each blot. (A-E) Quantification of the blots. (F) Immunoblots and Ponceau S. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "S", "L", "O" and "F" refers to significant differences with respect to soybean oil, lard, olive oil or fish oil-enriched diet, respectively. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

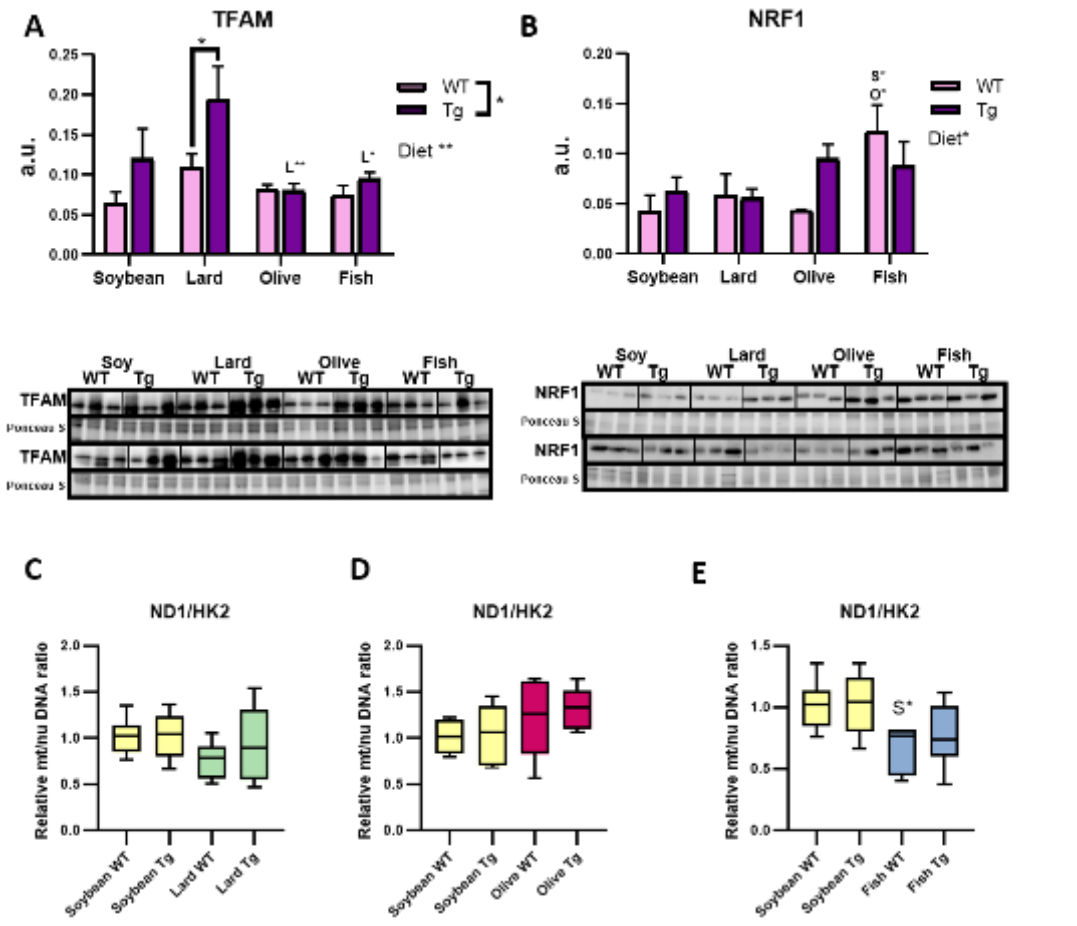


Figure 30. Mitochondrial biogenesis markers in heart from WT and Tg mice fed diets containing different fat sources. This panel depicts immunoblots showing levels of TFAM and NRF1, and the quantification of mt/nu DNA ratio in heart homogenates from WT and Tg mice fed *ad libitum* with diets containing soybean oil, lard, olive oil or fish oil as fat source. The corresponding Ponceau S staining is shown underneath each blot. (A-B) Quantification of the blots and images of the immunoblots with Ponceau S. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. (C-E) Mitochondrial/nuclear DNA ratio. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "S", "L", "O" and "F" refers to significant differences with respect to soybean oil, lard, olive oil or fish oil-enriched diet, respectively. When detected, general effects of diets are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$) and ** ($p < 0.01$).

Mitochondrial ETC complexes in heart

Finally, we studied in heart the expression levels of key ETC protein markers. We found higher levels of complex I with PUFAs-enriched diets (soybean and fish oils), especially in WT mice (Fig. 31A). Complexes II, III, and IV were also increased in WT animals fed with a fish oil diet (Fig. 31B-D), whereas no differences were found with any diet for complex V (Fig. 31E). No changes due to CYB5R3 overexpression

were noted. The western blots used for quantifications are depicted in Fig. 31F.

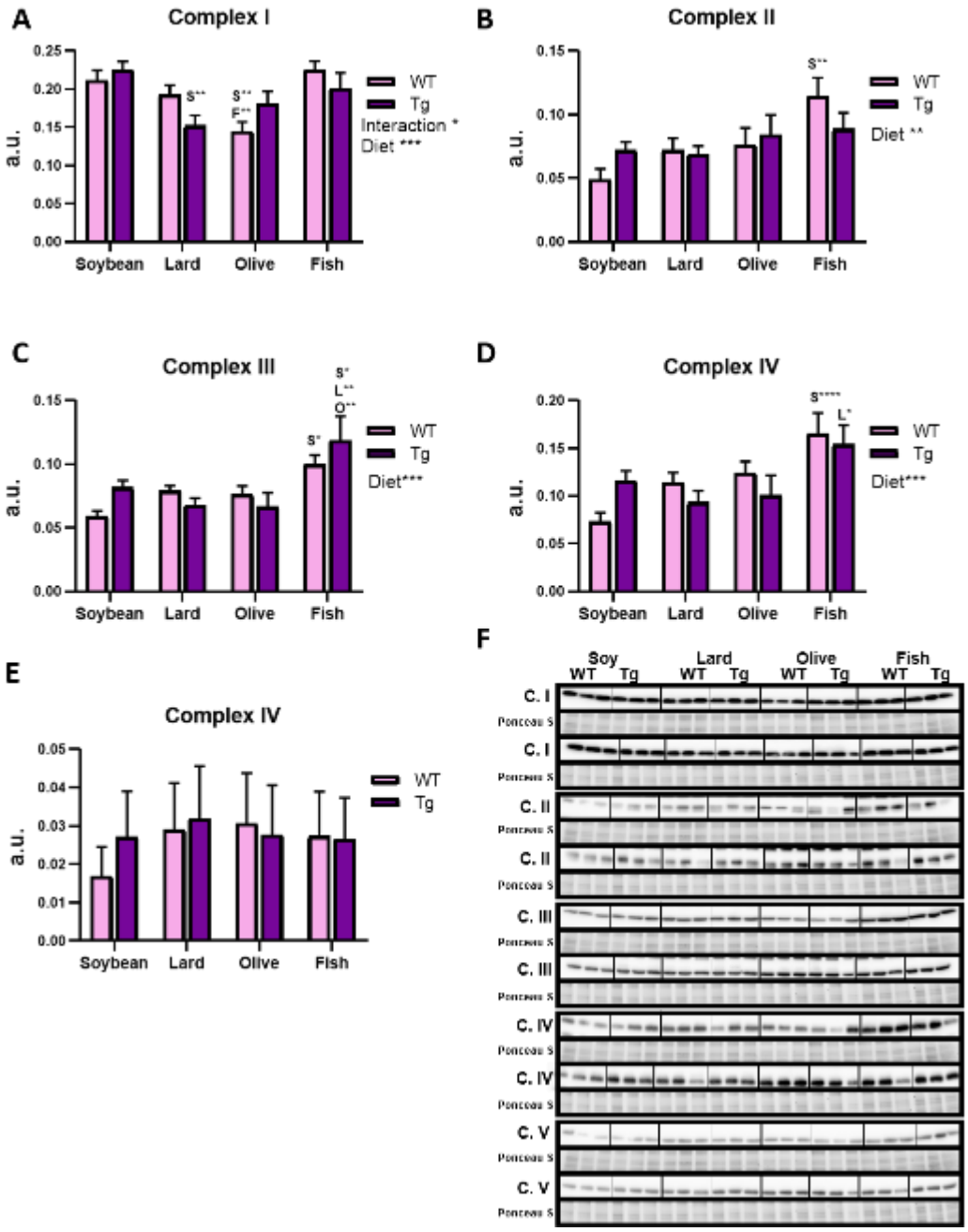


Figure 31. Expression levels of mitochondrial electron transport chain complexes in heart homogenates from WT and Tg mice fed diets containing different fat sources. This panel depict immunoblots showing the levels of mitochondrial complexes I to V respectively in heart homogenates from WT and Tg mice fed *ad libitum* with diets containing soybean oil, lard, olive oil or fish oil as fat sources. The corresponding Ponceau S staining is shown underneath each blot. (A-E) Quantification of the blots. (F) Immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "S", "L", "O" and "F" refers to significant differences with respect to soybean oil, lard, olive oil and fish oil-enriched diet, respectively. When detected, general effects of diets and interaction between genotype and

diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

In summary, in heart the expression levels of proteins related to mitochondrial biogenesis, fission and fusion as well as VDAC1 and mitochondrial complexes from animals fed with different dietary fats, seem to follow a similar pattern to that described for liver: a trend to increase fusion proteins in the olive oil- (especially with Tg mice) and with the fish oil-based diet. For some parameters, overexpression of CYB5R3 appears to collide with the fish oil-based diet, blunting some of the effects found to be produced by this diet in WT mice. Concerning the lard- and soybean oil-based diets, mitochondrial balance seems to tend towards fission, being this effect more accentuated with CYB5R3 overexpression.

Chapter III. Effect of the dietary fat source on mitochondrial structure and function in CYB5R3 -overexpressing mice

Discussion

CYB5R3-overexpressing mice submitted to different dietary fats

It is well accepted that environmental factors such as diet and exercise exert profound effects that determine the state of health in the organisms [272-275]. In this sense, the effects of dietary fatty acids on chronic diseases are particularly relevant [275, 276]. Fatty acids are not only necessary as a fuel source and as structural components of the cell. Barr and Burr already demonstrated in the first half of the past century the importance of these components for the correct growth and development of the organism, which led them to establish the term “essential fatty acids” in reference to those fatty acids that are needed by the organism and can only be obtained by food intake [155].

Animal fats (predominantly SFAs) have traditionally been classified as deleterious, while fats obtained from plant sources (enriched in MUFAs and PUFAs) have been better accepted among nutritionists due to the favourable correlation observed with the prevention of certain diseases. The concentrations of LDL, HDL, cholesterol, and triglycerides in plasma are related to the type and amounts of fatty acids ingested in the diet, which, in turn, influence insulin sensitivity and glucose metabolism [277, 278]. In this sense, while some studies have shown that a higher intake of SFA results in elevated blood cholesterol levels, which is the major risk factor for cardiovascular diseases, others showed the opposite effect in diets rich in PUFAs [279, 280].

Metabolic and cardiovascular diseases are on the rise, particularly in Western societies where these pathologies have become a social, health and economic problem [281]. For a long time, the general nutritional advice has recommended the public to prevent weight gain, cancer, and other diseases reduce dietary fat and substituting it with carbohydrates (including highly refined grains and sugar), assuming that these were mostly innocuous. Nevertheless, the opposite effect has been found [282, 283]. Nowadays, high rates of obesity and diabetes resulting from the spreading influence of the Western dietary style, consisting of overnutrition, consumption of high-sugar, high intake of saturated fats, as well as a sedentary lifestyle, have been extended all over the world [281]. Currently, an alternative approach has grown in popularity among nutritionists and other scientists, consisting of diets that tend to be lower in carbohydrates and higher in fat, the ketogenic diets being an extreme example.

However, the association of a high intake of fat, especially from dairy or red meat with an increase in the risk of heart disease and cancer remains valid [284]. Other studies have reported that a reduction in body fat extends maximum lifespan [285-287], suggesting that weight loss is primarily achieved by reducing energy intake, as occurs in CR interventions. In accordance with this idea, excessive energy consumption is the cause of fat accumulation regardless of whether the energy is obtained from fat or carbohydrates [288].

Several nutritional approaches have led to the conclusion that the determining factors in health and longevity, are not only the balance between fat and carbohydrates, but, importantly, which particular nutrients from each category are included in the diet. On the other hand, it has been shown that dietary fat can modulate longevity probably through its influence on the composition and function of the biological membranes. The Membrane Theory of Aging proposes that lifespan is related to the amount of unsaturation in membrane phospholipids. Across different species, animals with greater longevity show lower degree of fatty acid unsaturation in tissues, and this has been explained on the basis that PUFAs, particularly those of the n-3 series, are much more sensitive to oxidation [43-46].

Caloric restriction promotes the redistribution of FA in mitochondrial membranes depending on the type of unsaturation, causing increased MUFA, decreased PUFAs and no changes in SFA levels in liver. Decreased PUFAs in mitochondrial membrane phospholipids results in lower susceptibility to peroxidation, potentially contributing to the increased life and health span of CR mice [106, 120, 215]. On the other hand, a study carried out in mice subjected to CR with different dietary fats, showed an extension of lifespan in mice fed with a lard-based diet (SFA) compared to those fed with PUFAs (either n-3 or n-6), a result which is consistent with the Membrane Theory of Aging [215], but questioning the efficacy of PUFAs-enriched diets in CR regimens. The effects of dietary fish oil on health are very similar to those produced by CR, including a decrease in inflammation and ROS production. Therefore, n-3 PUFAs within a CR context do not appear to provide additional benefits. On the contrary, apparently PUFAs would only provide their negative side effects arising from their increased sensitivity to oxidation or their role in gene transcription regulation [215]. However, n-3 PUFAs do appear to be beneficial to mice fed *ad*

libitum, at least in short-term interventions, as observed when these fatty acids were tested to aid in the treatment of chronic inflammatory diseases such as CVDs, rheumatoid arthritis and diabetes [289, 290]. In addition, decreased ROS production by hepatic ETC complex was observed in *fat-1* mice that overexpress a desaturase from *C. elegans* which allows them to synthesize n-3 from n-6 fatty acids [291].

The metabolic improvements detected in mice over-expressing CYB5R3 may be due also to the increased elongation and desaturation of fatty acids provided by their genotype [138]. Thus, the possibility that its beneficial effects, such as the optimization of mitochondrial function elicited by CYB5R3 over-expression, could be either maximized or abated by modulating the fatty acid composition of membranes through a dietary intervention, prompted us to determine the possible effects of feeding Tg mice with diets based on different fat sources under *ad libitum* conditions.

Dietary fats have been related to a variety of pathological conditions, especially those concerning the liver, since FAs can modulate hepatic lipogenesis [292]. Excessive SFA consumption promotes lipid accumulation in liver, increase visceral fat and inflammation, whereas MUFAs and PUFAs may have a protective role reducing hepatic lipid content [293]. We thus focused our work on the liver of WT and Tg mice fed diets formulated with different fat sources and found a strong relationship between diet and mitochondrial biogenesis and dynamics markers.

While diets rich in MUFA lead to increased mitochondrial fusion markers, especially in CYB5R3 Tg mice, a diet rich in SFA exerted the opposite effect. According to the literature, a reduction in mitochondrial fusion may be involved in the development of obesity and insulin resistance [294]. Fat accumulation triggers insulin resistance that increases hepatic FFA synthesis [295]. To restore the equilibrium, the increased fat input is compensated by forcing the entry of FFA into the mitochondria and increasing β-oxidation. However, this balance is achieved by expanding the liver deposits of FFA and triglycerides, thus promoting hepatic steatosis [296]. In addition, it has been described in liver and skeletal muscle that lard diet increased β-oxidation as a measure to process the excessive amounts of fatty acids acquired in the diet, leading to increased ROS production [297]. We observed that a lard-enriched diet resulted in reduced fusion and increased mitochondria fragmentation. This is

consistent with the idea of the existence of damaged mitochondria that must be eliminated. On the other hand, a high SFA diet can compromise the structure of the mitochondrial membrane by altering its viscosity and fluidity [298]. In our experimental groups, we found a positive effect of a lard-based diet in Tg mice, since increased VDAC1 and decreased MFF could indicate a change in mitochondrial function towards a healthier phenotype.

Some studies on HFD have reported decreased expression levels of genes related to mitochondrial biogenesis (PGC1α, NRF1, and TFAM) [299]. In our model we observed an increase in TFAM with the lard-based diet, which suggests that the deleterious effects on the organism found in these previous studies may be more related with an excess of fat than with its saturated nature. This idea is supported by the fact that no changes in the mt/nuDNA ratio were observed. Moreover, together with a shift in mitochondrial dynamics in favour to fission, this could indicate that damaged mitochondria are being efficiently eliminated by mitophagy whereas mitochondrial biogenesis is enhanced to maintain the normal population of healthy mitochondria. However, we should also consider the possibility that an impairment of mitochondrial function might be produced by a long-term intervention with a lard-based diet, despite not being on an HFD.

Due to their anti-inflammatory effects helping to counteract ROS action and lipotoxicity, MUFAs and PUFAs have been described to mitigate some of the harmful effects associated to SFA [300]. In contrast with the data obtained in the lard-enriched diet, we observed a significantly decrease in the mitochondrial fission marker MFF, compatible with an improvement of mitochondrial function in the olive oil-, soybean oil- and fish oil-based diets. A shift towards mitochondrial fusion has been associated with enhanced mitochondria function and ATP production [301]. In addition, increased mt/nuDNA was found in those mice fed diets containing MUFA or n-3 PUFA, revealing the different effects that olive and fish diets exerts on mitochondria.

In general, the relative abundance of SFA and MUFA in mitochondrial membranes appears to remain essentially constant over a wide dietary range. The fact that neither n-6 nor n-3 PUFAs can be entirely synthesized de novo in mammals necessarily implies that the composition of membrane phospholipids is strongly influenced by the ratio and abundance of n-6 and n-3 in the diet, particularly by n-3 PUFA.

Ultimately, the fatty acid composition of the membrane affects its function; in the case of PUFAs through an increase in fluidity [302, 303].

It should be noted that although the n-3 and n-6 PUFAs can exert very similar effects on membranes due to their biophysical similarities, the biological processes in which they take part are often largely divergent. While linoleic acid (LA) is one of the most biologically relevant n-6 PUFAs, α-linolenic acid (ALA) is a basic, 18-carbon n-3 fatty acid. LA is metabolized to arachidonic acid (AA), while ALA is transformed into EPA and DHA [304, 305]. There is competition between n-6 and n-3 PUFAs since the desaturation enzymes Fatty Acid Desaturase 1 (FADS1) and 2 (FADS2) show higher affinity for ALA than for LA [306, 307]. However, high concentrations of LA can interfere with the desaturation and elongation of ALA, as occurs in Western diets, in a similar way to how *trans* fatty acids interfere with the desaturation and elongation of both LA and ALA [308]. An unbalanced n-6/n-3 ratio in favour of n-6 PUFAs is highly proinflammatory and can contribute to the prevalence of obesity, and diabetes [308].

PUFAs act on mitochondria at different levels. In rats fed supplemented diets and in *fat-1* mice, they increase mitochondrial fusion and reduce H_2O_2 production [291, 309]. In addition, dietary n-3 and n–6 PUFAs are strong inhibitors of hepatic FA synthesis, and the ETC enzyme activities depend on specific membrane phospholipids that can be influenced by dietary fat. Among them, cardiolipin composition depend on the availability of linoleic acid, and an imbalance in cardiolipin composition can alter the activity of ETC and result in several pathologies [310]. However, an excess in levels of n-6 PUFAs can induce detrimental effects due to a profound loss of cardiac mitochondrial cardiolipin resulting in abnormal ETC activity that affect the production of ATP [311]. Therefore, inefficiency in ETC activity due to high n-6 PUFA content could explain the increase observed in complex I protein levels and the decrease of complex II as a compensatory mechanism in mice fed soybean oil. Furthermore, it has been reported that AA selectively inhibits complex I and III activity causing a substantial increase in ROS production [312].

Several studies described a decrease in ATP production and respiration rate with diets rich in PUFA, while others reported no changes in respiration [313, 314]. In our experimental groups, in general, we did not observe changes in ETC protein levels.

Based on the current literature, we propose that dietary fats target respiratory enzyme activity, supercomplexes formation, or both, rather than modulate protein levels. In young animals fed under *ad libitum* conditions, as well as in PUFAs- and MUFAs-based diets, we could expect decreased OXPHOS in a similar way to that described with CR and improved assembly of the supercomplexes. These phenomena might be some of the underlying mechanisms behind the healthy effects of this diet. However, n-3 PUFAs-based diet could bring beneficial effects only in the short-term. In the long-term, we might expect enhanced oxidative injury in n-3 PUFA since highly unsaturated membranes are prone to oxidation. In addition, the loss and failure of antioxidant capacities as a consequence of aging could result in a greater production of ROS. This possibility remains for further investigation.

On the other hand, it is important to consider that dietary fats modulate mitochondrial membrane lipid profiles in a tissue-specific manner [315]. Thus, another objective of our study was to evaluate the role of dietary fat in relation to possible changes in different tissues, using liver and heart as examples of mitotic as post-mitotic organs, respectively.

Heart, a postmitotic tissue, exhibits a highly aerobic metabolism due to the presence of abundant large mitochondria and the highly expressed fusion proteins. This balanced state towards mitochondrial fusion allows the mitochondrial function to be optimized and to compensate mtDNA mutations in heteroplasmic cell under conditions that favour the appearance of damage [316, 317]. Mitochondrial fusion also controls the exchange of Ca^{2+} between this organelle and the endoplasmic reticulum [318, 319], which reveals the importance of mitochondrial dynamics for adequate myocardial function. However, unlike liver, adult postmitotic tissues such as heart and skeletal muscle are characterized by low rates of mitochondrial fusion and fission [320] except during starvation or pathological conditions, when changes in mitochondrial dynamics have been reported. Mitochondrial fusion is beneficial for the heart since it prevents mitophagy and strengthens the ability of the mitochondria to produce energy [321]. On the other hand, mitochondrial fission has been associated with heart failure both in human and rat models [322], and unbalanced fusion and fission have been also reported in cardiac hypertrophy [323] or during challenging nutritional conditions, as consumption of HFD [324].

It is well-known that fish oil consumption lowers plasma triglycerides [325], reduces blood pressure [326] and have anti-inflammatory effect and provides general protection from cardiovascular pathologies and heart failure [327]. On the other hand, CVDs have been associated with mitochondrial dysfunction and dietary fat through changes in membrane profiles [315, 328].

As expected, in our model of mice fed with different dietary fat sources, we did no observe pronounced changes regarding mitochondrial dynamics and biogenesis in heart. An increase in proteins involved in mitochondrial fusion was observed in PUFA-rich diets, especially with fish diet, which agrees with the previous reports documenting that supplementation with n-3 PUFA is beneficial for the maintenance of heart function and has a protective effect in this tissue [329]. However, we found that CYB5R3 overexpression in Tg mice apparently eliminated this effect in a context of fish oil-enriched diet consumption, but enhanced fusion in a context of an olive oil-based diet. As mentioned in Chapter 1, the fatty acid elongation and desaturation activity of CYB5R3 might result in negative effects when the diet is already rich in n-3 PUFA, possibly due to an excess of PUFAs in membranes. On the other hand, we observed once again that consumption of the lard-based diet increased mitochondrial fission, as it occurs in the liver.

Regarding mitochondrial respiratory complexes, no major changes have been found except for the increment in levels of some ETC markers in heart from mice fed the fish oil-based diet, which could be related with the reported benefits of these fatty acids against cardiovascular diseases (see above).

It has been reported that a lack of essential fatty acids is associated with decreased activity or turnover of ETC enzymes and, therefore, with decreased mitochondrial respiration in heart, being these effects probably due to the reduction of cardiolipin content [330-332]. An intervention with n-3 PUFA-enriched diet restored the cardiolipin levels, meanwhile an n-6 PUFA-enriched diet produced a decrease in this phospholipid [333]. Enhanced cardiolipin content has been associated with increased respiration in heart [334, 335].

Therefore, the incorporation of n-3 PUFAs in the cardiac membrane driven by diets enriched in this component has beneficial effects in both young and adult rats as

reported in several studies. Some of these effects are the improvement in Ca_2^+ homeostasis, the recovery of contractile work and the reduction of arrhythmias even after long-term diet rich in n-6 PUFA [336-339].

Although we did not measure different phospholipid classes, our results in heart from mice fed the fish oil-based diet could indicate an increase of cardiolipin content that interacts with respiratory protein complexes enhancing mitochondrial function for optimal ATP production. The increments in complex II, III and IV could be related to an n-3 PUFAs stimulation of electron flux channelled through complex I, thus limiting ROS production and preventing this complex from remaining in a reduced state.[340, 341] Complex I can generate ROS through reverse electron transfer (RET) and it is associated with pathology condition as occurs in ischemia [342, 343]. In RET the electrons from CoQ are transferred back to complex I, reducing NAD^+ to NADH and generating a significant amount of ROS which requires a high membrane potential. This ROS generation during RET can be prevented by n-3 PUFAs due to their uncoupling action [343, 344].

However, two considerations must be taken into account. First, it is important to consider that our results refer to young mice, so it is possible that some of the effects of the diets on mitochondrial function that could arise later in the life are not yet visible. Second, measurements of mitochondrial ETC proteins expression do not necessarily reflect changes in mitochondrial ETC activity or supercomplexes assembly and, therefore, do not provide information on alterations in the activity of specific ETC enzymes, or on protein damage by lipotoxicity or ROS. For this reason, future research will be essential to assess the activity of individual ETC enzyme complexes, antioxidant capacity of the cell, and the alteration in the formation of supercomplexes to truly determine the influence of dietary fats and CYB5R3 overexpression on mitochondrial biology.

In summary, the dietary source of fat seems to have a different impact in mitotic and postmitotic tissues regarding metabolism and mitochondrial function. CYB5R3 protein levels in mice overexpressing the *CYB5R3* gene is strongly regulated by dietary fats in liver but not in heart, which highlights the existence of tissue-dependent mechanisms regulating the abundance of the CYB5R3 polypeptide. CYB5R3 can produce different effects on mitochondrial function depending on the

dietary fat source used. Although overexpression of CYB5R3 does not appear to confer a marked phenotype by itself, in the case of mice fed the lard-based diet it prevents mitochondrial fragmentation, which could be indicative of a healthier mitochondria state compared to their WT counterparts. In the case of mice fed the olive oil-enriched diet, CYB5R3 appears to promote a healthy phenotype by increasing mitochondrial fusion proteins. However, some interference between CYB5R3 overexpression and the consumption of a fish oil-enriched diet appears to exist, and some of the beneficial actions of each isolated intervention may be hampered, as evidenced by a decrease in mitochondrial dynamics and greater weight gain.

Chapter IV. CYB5R3 overexpression in aged mice

Results

Mitochondrial dynamics and biogenesis markers in liver

As described in Chapter 1 (see Fig. 15A & 15B), the abundance of CYB5R3 protein were increased with age in heart, a postmitotic tissue model, whereas its levels were found unchanged in liver, a mitotic tissue model. The existence of tissue- and age-specific mechanisms regulating CYB5R3 protein content led us to study the effects of CYB5R3 overexpression on several markers related with mitochondrial metabolism during aging.

Due to its role on the sorting of intermediate metabolite (glucose, fatty acids, amino acids, etc.) among different tissues and organs in the body, the liver emerges as a crucial organ in the regulation of the systemic metabolism. Liver mitochondrial function declines with age, and this has been associated with disorders such as diabetes, cirrhosis, and cancer [345]. To get novel insights on the impact of CYB5R3 on mitochondrial dynamics with aging, we first analysed the levels of key marker proteins related to mitochondrial abundance, fission, fusion and biogenesis in liver of aged mice and the results were compared with those obtained in young animals.

No changes were found for MFN1 regardless of genotype or age (see Fig. 32A), while MFN2 increased in old WT mice, and this effect was blunted in mice overexpressing CYB5R3 (Fig. 32B). On the other hand, a significant increase of FIS1 was observed in old Tg mice compared with young mice of the same genotype (Fig. 32C), although no change with age was detected in the case of WT mice. Interestingly, MFF levels were dramatically increased in old WT mice, and this effect was abated by CYB5R3 overexpression (Fig.32D). Hepatic levels of VDAC1 were significantly decreased by aging in both genotypes (Fig.32E). The western blots used for quantifications are depicted in Fig. 32F.

Then, we focused on TFAM and NRF-1, two key transcription factors which regulate mitochondrial biogenesis. We observed increased TFAM levels with aging in mice of both genotypes (Fig. 33A), but no changes with either age or genotype were detected for NRF1 (Fig. 33B). Strikingly, the increase in TFAM was not correlated with a parallel increase of the mt/nuDNA ratio (Fig. 33D & Fig. S.10). The western blots used for quantifications are depicted in Fig. 33C.

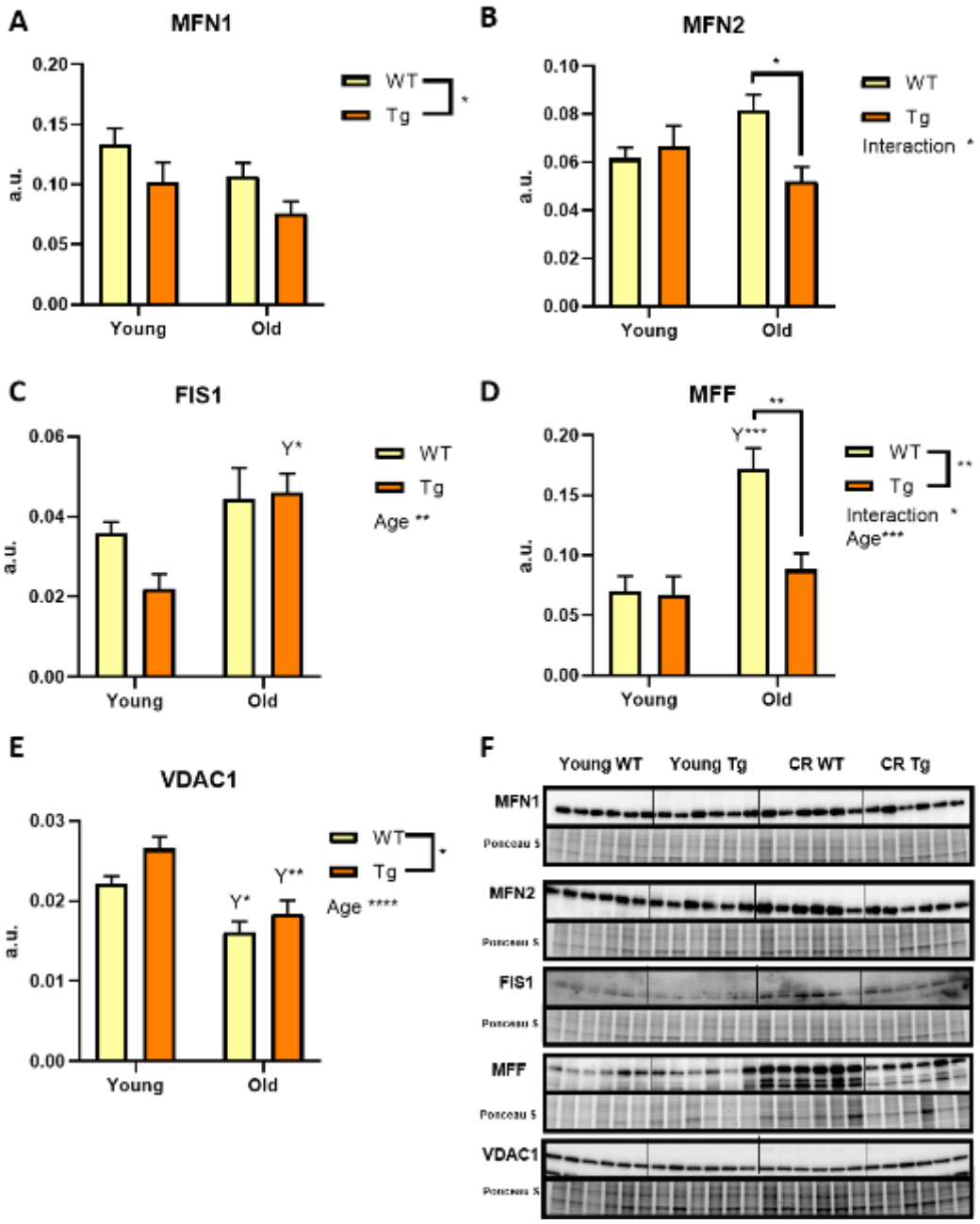


Figure 32. Markers of mitochondrial dynamics and mass in liver. Immunoblots show the levels of MFN1, MFN2, FIS1, MFF and VDAC1 in liver homogenates from WT and Tg mice of 7 or 24 months of age. The corresponding Ponceau S staining is shown below each blot as a control of protein loading. (A-E) Quantification of the blots. (F) Immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "Y" refers to significant differences in comparison with young mice. When detected, general effects of age and interaction between genotype and age are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

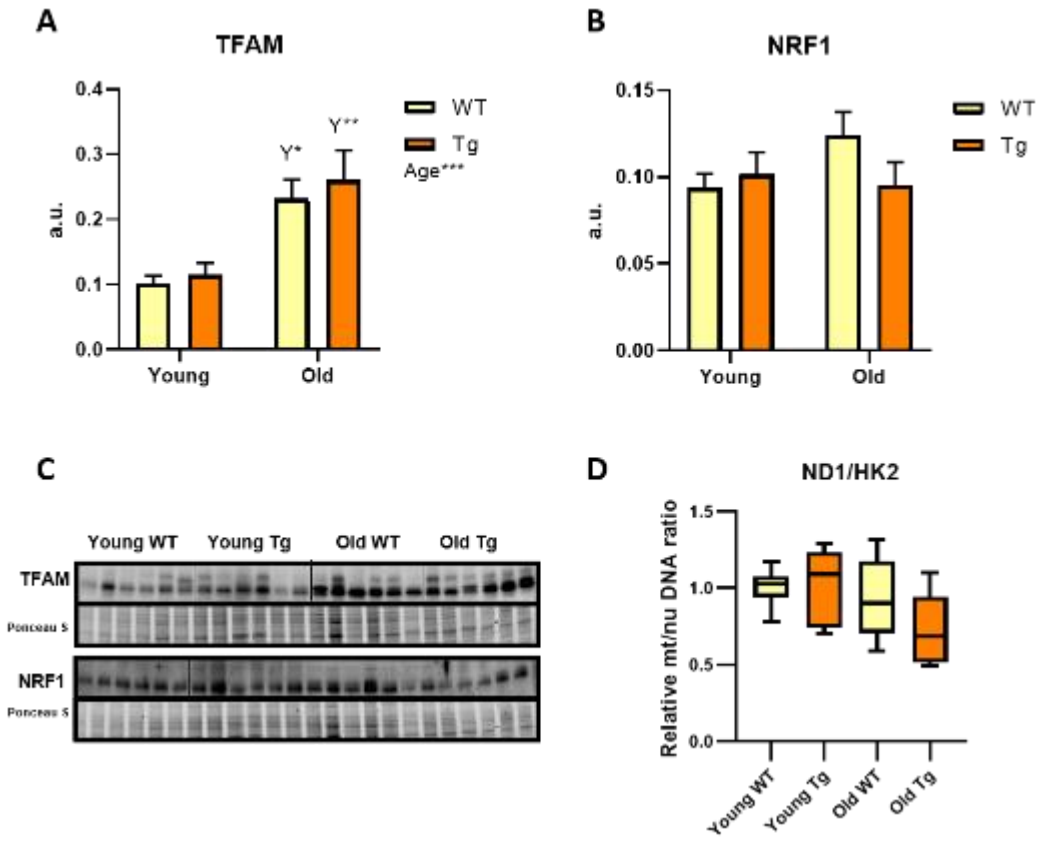


Figure 33. Mitochondrial biogenesis markers in liver. Immunoblots show the levels of TFAM and NRF1, and the quantification of mt/nu DNA ratio in liver homogenates from WT and Tg mice at 7 or 24 months of age. The corresponding Ponceau S staining is shown below each blot. (A-B) Quantification of the blots. (C) Images of the immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. (D) Mitochondrial/nuclear DNA ratio. Data represent mean ± SEM of 6 replicates. "Y" refers to significant differences with respect to young mice. When detected, general effects of age are depicted on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Hepatic levels of mitochondrial electron transport chain complexes

We next studied the possible impact that age and/or CYB5R3 overexpression may impose on the content of ETC complexes as measured by abundance of marker subunits. Aging induced significant increases in the abundance of complexes I, II, III and IV in WT mice, with no appreciable effect on complex V (Fig. 34A to 34E). Concerning the effect of CYB5R3 overexpression, we evidenced the existence of noticeably higher levels with age for most of the selected ETC markers, even when compared to the old mice, as observed for complexes I, II, IV and V (Fig. 34A, 34B, 34D & 34E). In the case of complex III, although no such increase was observed due to CYB5R3 overexpression in aged mice, livers from young Tg mice presented a 2-

fold higher protein level compared to their WT littermates (Fig. 34C). The western blots used for quantifications are depicted in Fig. 34F.

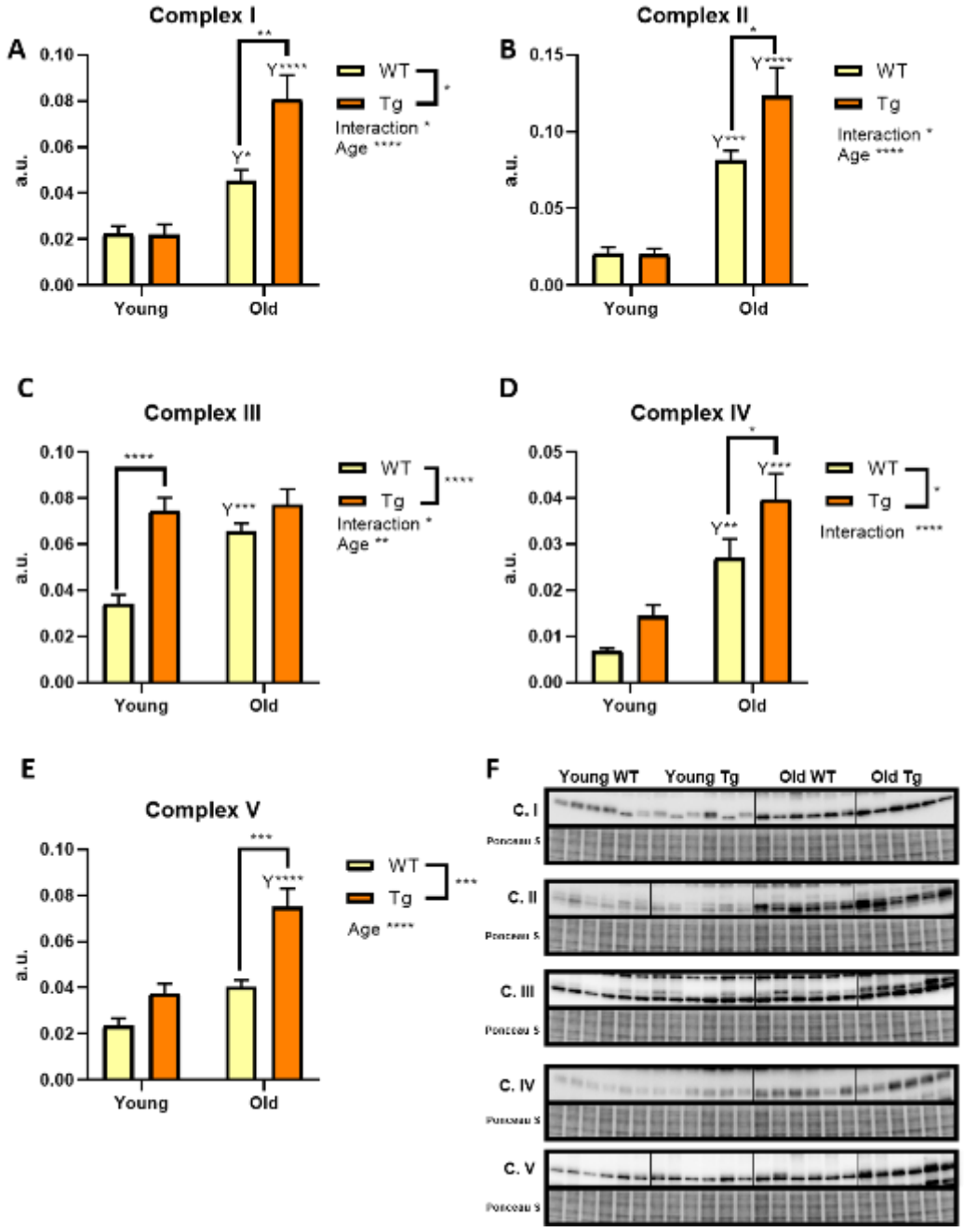


Figure 34. Expression levels of mitochondrial electron transport chain complexes in liver homogenates. Immunoblots show the levels of mitochondrial complexes I to V respectively in liver homogenates from 7- or 24-months old mice. The corresponding Ponceau S staining is shown below each blot. (A-E) Quantification of the blots. (F) Pictures of the immunoblots and Ponceau S staining. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "Y" refers to significant differences with respect to young mice. When detected, general effects of age and interaction between genotype and age are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Mitochondrial dynamics and biogenesis markers in heart

Several heart pathologies are characterized by the presence of cells that are unable to produce the necessary amount of energy for the contraction and relaxation of the cardiac muscle, an effect which has been linked to mitochondrial dysfunction [346]. Mitochondrial fusion and fission are necessary to regulate cardiac homeostasis and adaptation to stress and aging [347]. To understand how aging affects mitochondria in a context of CYB5R3 overexpression, we studied possible changes in these parameters in Tg aged mice, using heart as a model of post-mitotic tissue.

In heart, aging produced no changes in MFN1 (Fig. 35A), but MFN2 was significantly increased in old WT compared to young mice, a phenomenon that was reversed by CYB5R3 overexpression (Fig. 35B). The fission marker FIS1 was increased in old WT mice (Fig. 35C) without any change being observed with aging in old Tg mice. No changes were found for MFF (Fig. 35D) or VDAC1 (Fig. 35E) in any condition. any condition. The western blots used for quantifications are depicted in Fig. 35F.

Regarding the markers of mitochondrial biogenesis, we also evidence a significant increase in TFAM content in old Tg mice compared to young mice (Fig. 35A). However, neither NRF1 (Fig. 36B) nor the mt/nu DNA ratio (Fig. 36D & Fig. S.11) exhibited any change with age or genotype. The western blots used for quantifications are depicted in Fig. 36C.

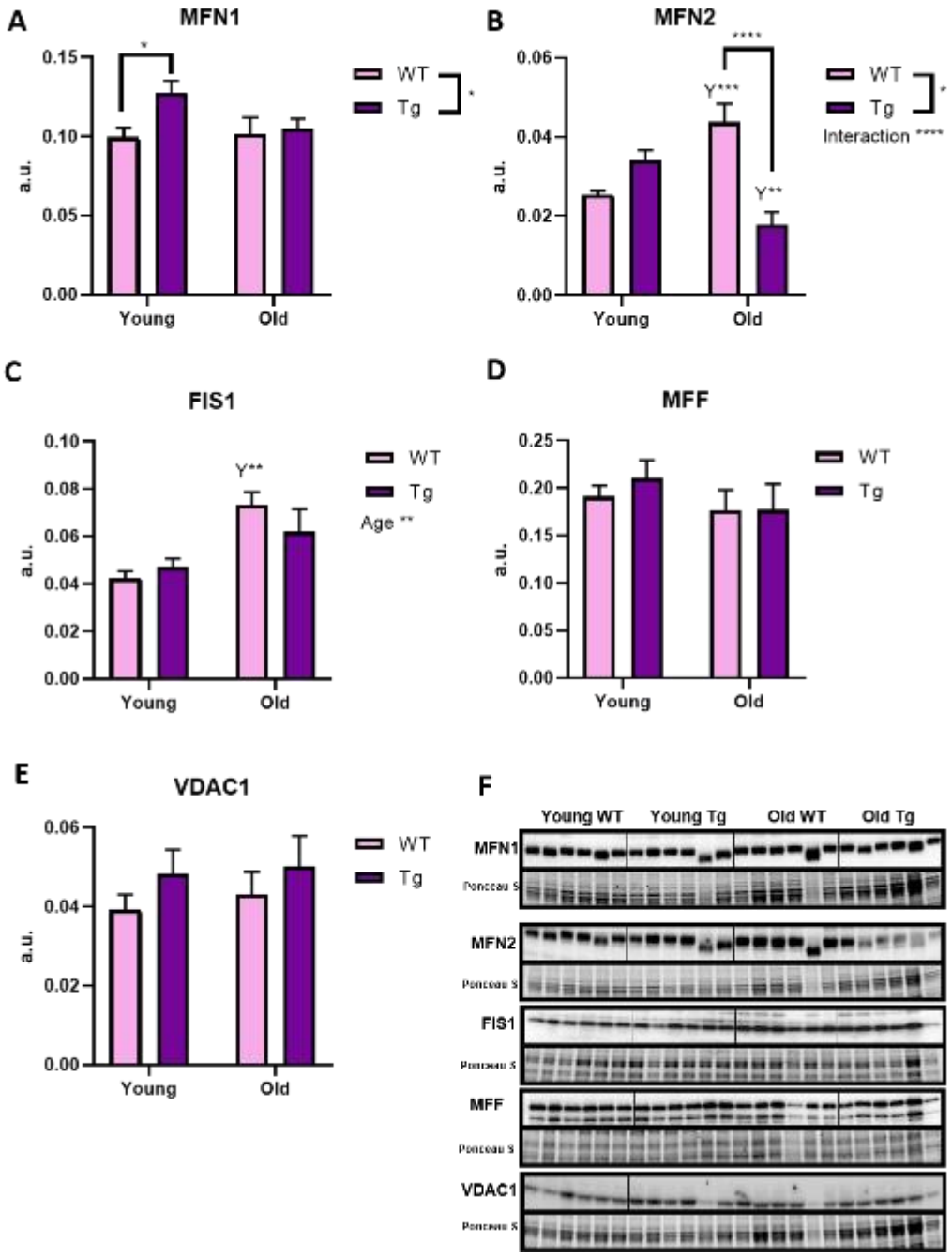


Figure 35. Markers of mitochondrial dynamics and mass in heart. Immunoblots show the levels of MFN1, MFN2, FIS1, MFF and VDAC1 in heart homogenates obtained from 7- or 24-months old mice. The corresponding Ponceau S staining is shown below each blot. (A-E) Quantification of the blots. (F) Images of the immunoblots and Ponceau S staining. In all cases, the intensity of the bands has been normalized by the corresponding Ponceau S staining. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "Y" refers to significant differences with respect to young mice. When detected, general effects of age and interaction between genotype and age are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

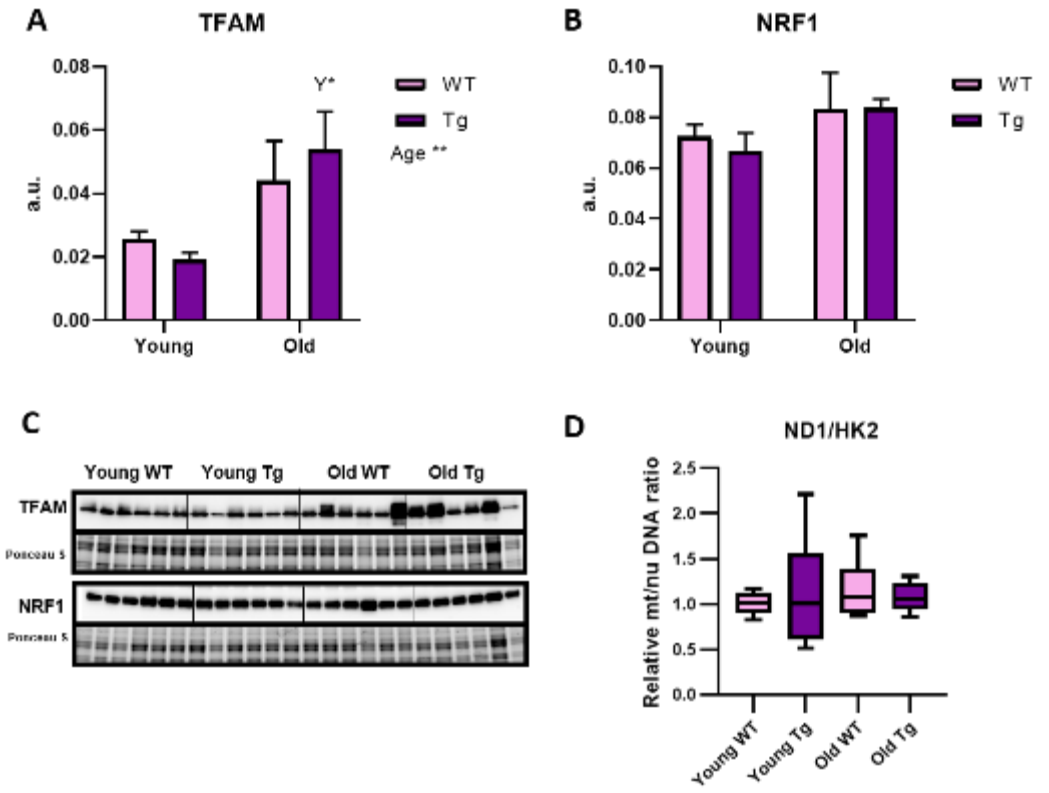


Figure 36. Mitochondrial biogenesis markers in heart. Immunoblots showing levels of TFAM, NRF1 and quantification of mt/nu DNA ratio in heart homogenates from WT and Tg of 7 and 24 old months. The corresponding Ponceau S staining is shown below each blot. (A-B) Quantification of the blots. (C) Pictures of the immunoblots and Ponceau S. In all cases, the intensity of the bands was normalized with the corresponding Ponceau S staining. (D) Mitochondrial/nuclear DNA ratio. Data represent mean ± SEM of 6 replicates. "Y" refers to significant differences for young mice. When detected, general effects of age are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$) and ** ($p < 0.01$).

Mitochondrial ETC complexes in heart

As in liver, we found a significant increase in complexes expression with age. Thus, in WT animals, the abundance of protein markers of complexes II (Fig. 37B), III (Fig. 37C), IV (Fig. 37D) and V (Fig. 37E) was higher in old compared with young mice, although no changes in complex I were found (Fig. 37A). Again, the increase in the abundance of some ETC markers with aging was more pronounced in CYB5R3-overexpressing animals, as observed for complex I (Fig. 37A), III (Fig. 37C) and V (Fig. 37E). On the contrary, complex IV was decreased in old TG mice in comparison with WT animals of the same age (Fig. 37D). The western blots used for quantifications are depicted in Fig. 37F.

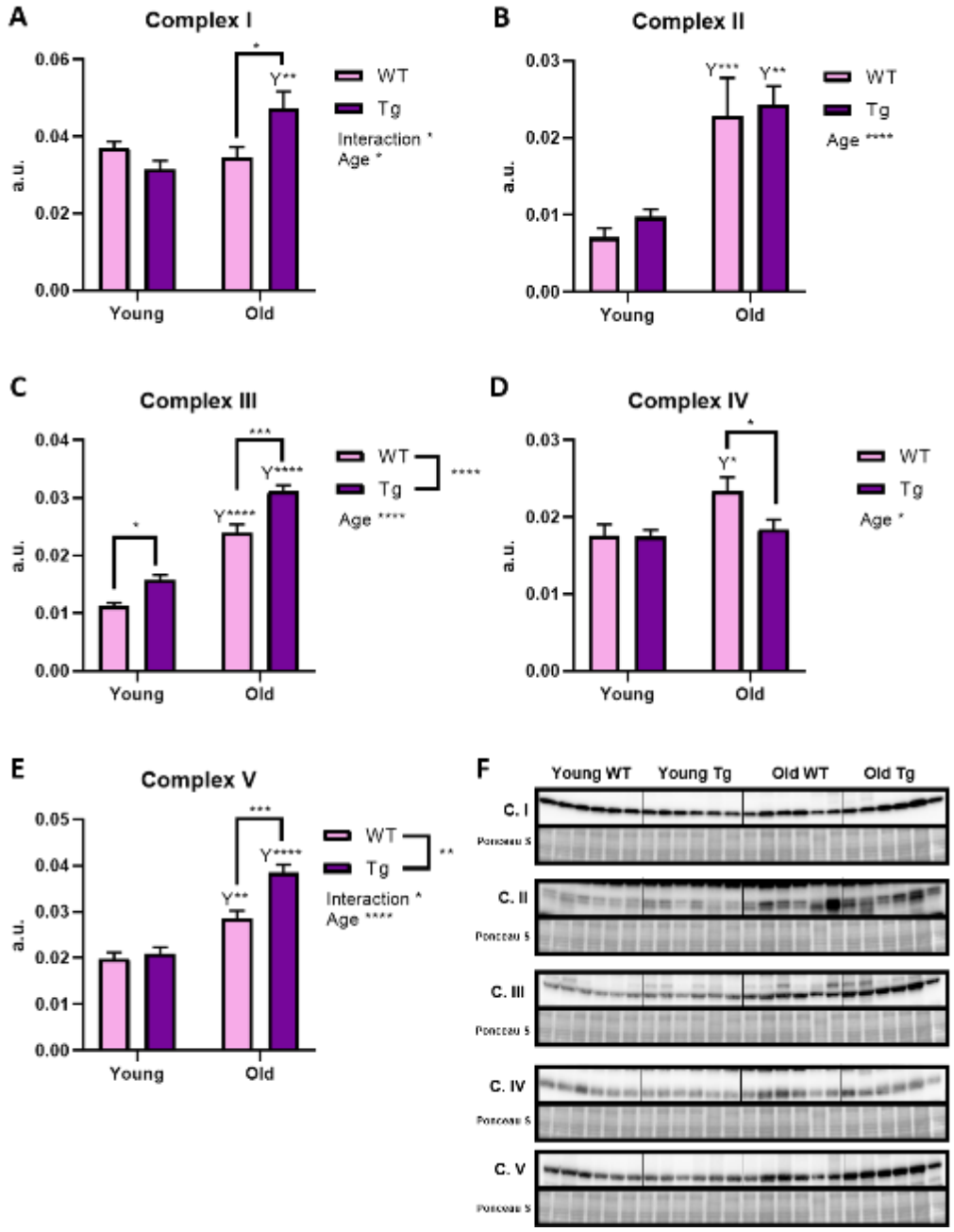


Figure 37. Expression levels of mitochondrial electron transport chain complexes in heart. Immunoblots show the levels of protein markers for mitochondrial complexes I to V in heart homogenates of 7- or 24-months old mice. The corresponding Ponceau S staining is shown below each blot. (A-E) Quantification of the immunoblots. (F) Images of the immunoblots and Ponceau S staining. In all cases, intensity of the bands has been normalized by the corresponding Ponceau S staining. Data represent mean ± SEM of 6 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "Y" refer to significant differences with respect to young mice. When detected, general effects of age and interaction between genotype and age are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Chapter IV. CYB5R3 overexpression in aged mice

Discussion

CYB5R3 overexpression in aged mice

Aging is a natural process characterized by accumulation of damage in cellular structures that leads to tissue and organ dysfunction and, ultimately, to organismal death. The loss of cell function leads to a different response to the disease, presenting a greater susceptibility to infections and a higher incidence of chronic and degenerative pathologies. Therefore, aging is the most significant risk factor for chronic diseases including cancer, cardiovascular disease (CVDs) and neurodegeneration [3]. In this process, mitochondria play a key role since these organelles are responsible of ATP production, calcium uptake and the regulation of apoptosis, among other functions [348].

Mitochondria are highly dynamic organelles that undergo continuous changes in size, shape, number, and distribution. Mitochondrial dynamics is determined by the balance between the frequency of fusion and fission events [84, 349]. Dysfunctional mitochondrial dynamics during aging results in accumulation of damaged mitochondria, leading to alteration in energy production and increased ROS production [116, 350], being these effects involved in the development of different age-related diseases such as cardiovascular or metabolic disorders. To maintain homeostasis under these conditions, mitochondria activate a highly regulated machinery involving mitochondrial fusion, fission and biogenesis, which is fundamental to palliate the negative effects of aging.

CYB5R3 expression under conditions of oxidative stress is controlled by the coordinate action of FOXO3A and NFE2L2 [144]. CYB5R3 is naturally overexpressed in some tissues during aging, especially in post-mitotic tissues, and genetic ablation of NFE2L2 in mouse embryonic fibroblasts results in lower content of CYB5R3 protein, an effect that has been associated with lower proliferation rates and a strong senescent phenotype [351]. Recently, it has been proposed that CYB5R3 participates in the regulation of mitochondrial function by controlling the rate of ATP production and providing cells with antioxidants to neutralize ROS generated by oxidative phosphorylation [352].

CYB5R3 plays an important role in the regulation of metabolic pathways associated with healthspan and aging through mechanisms apparently related with protection against oxidative stress, production of cellular NAD^+ and alterations in fatty acids

composition [141, 142]. Previously, studies reported that the mechanisms by which CYB5R3 improves lifespan and healthspan were independent from those described for CR [136, 138]. Thus, to elucidate whether CYB5R3 overexpression targets key markers related with mitochondrial adaptation during aging, we compared young adult (7 months old) *versus* old (24 months old) mice. As shown in the present work, the detrimental effects of aging regarding mitochondrial function were partially counteracted by CYB5R3 overexpression, which is in accordance with previous studies [138]. We also observed weight gain in aged mice and fewer TGs in blood, which might be related with the re-organization of body fat promoting its accumulation in tissues [353, 354], a process associated with the risk of suffering age-related diseases [355] (see Results Chapter 1). Additionally, mitochondrial dysfunction induced by changes in lipid metabolism which promote the accumulation of triglycerides in the tissues and decrease fatty acid oxidation [220] has been related with decreased autophagy [356]. In consequence, interventions targeting lipid metabolism may be valuable strategies to improve the metabolic state associated with age. Among then, exercise is one of the most used to stimulate lipolysis, improve insulin metabolism and enhance mitochondria biogenesis [357, 358]. Among nutritional interventions, CR produce a decrease in PUFA content in mitochondrial membrane phospholipids and therefore decreases the production of ROS [359]. Other strategies are the overexpression of lipid synthesis/degradation related enzymes, as CYB5R3 that increase levels of polyunsaturated fatty acids in hepatic membranes but a reduction in lipid peroxidation [138].

Mitochondrial fusion (which is mainly controlled by MFN1, MFN2 and OPA1), results in a more branched and interconnected mitochondrial network, which has been reported to improve communication with the endoplasmic reticulum [360]. However, how aging affects this process is a matter of controversy. While some studies showed that aging did not alter the expression levels of these proteins [361], others reported upregulation [246, 362] or even downregulation [363]. Our data in liver yielded mixed results since aging induced a trend towards increased levels of MFN2 (a change that was not observed in Tg mice) without changes in MFN1, suggesting different adaptation mechanisms to aging in WT compared with Tg animals.

It has also been reported that MFN2 exerts a protective effect against NASH [364], and also represses tumour growth and metastasis, and induce apoptosis in

hepatocellular carcinoma [365]. Mitochondrial fusion is involved in endoplasmic reticulum stress, and in fat accumulation in conditions of metabolic stress due to nutrient excess such as obesity [366]. MFN2 is enriched in mitochondria associated membranes (MAMs) and modulates the contact between ER with OMM which is involved in lipid synthesis and Ca^{2+} homeostasis [238, 367]. Taken together, these results support the idea of a highly complex and differential mechanism of adaptation of the tissues and/or organs to different conditions, positioning mitochondrial fusion as a crucial process to achieve an improved cell function and to provide mtDNA complementation by mixing damaged and normal mtDNA [317].

Mitochondrial fission is another important phenomenon which is closely related to mitophagy, since it is necessary to fragment the mitochondria prior to its lysosomal degradation [368]. In fact, it has been proposed that excessively elongated mitochondria cannot be efficiently removed by autophagy [261]. The assembly of the fission machinery is assisted by the endoplasmic reticulum which forms contact sites with mitochondria creating a microdomain where DRP1, MFF, and pro-apoptotic proteins get assembled [92]. The fission process together with fusion disruption results in smaller mitochondria, a fact that has been observed in apoptosis [369], during cell cycle progression [370], or prior to the generation of ROS during stress [371]. In our model we observed increased MFF in aged WT mice, an effect possibly aimed to eliminate damaged mitochondria but at the same time, our results could indicate that mitophagy was not effective in these mice since we found a decrease in VDAC1, which is a crucial mitochondrial substrate required for PINK/PARKIN-mediated mitophagy [372]. The attenuation of fission (MFF) together with fusion (MFN2) in CYB5R3 Tg animals during aging, might suggest a healthier state of mitochondria in Tg mice in which the molecular mechanisms to control mitochondrial damage has not been activated yet.

Mitochondrial function was also evaluated by studying the proteins involved in the ETC. As previously mentioned, one of most widely accepted theories of aging suggests that it is the result of progressive ROS accumulation, which causes harm to macromolecules and mitochondrial complexes, making oxidative phosphorylation inefficient and decreasing ATP production. If mitochondrial damage is not repaired, the cellular malfunction can become irreversible due to the complete failure of the ETC. In this sense, our analysis of protein levels of mitochondrial complexes subunits

in liver indicated an overall increase with age in both genotypes, although the change was more pronounced in Tg mice. Several studies have shown an age-related decrease in mitochondrial complexes activity, being complexes I and IV the most affected [373]. This decrease may be due to several factors: to the modification of proteins by ROS-driven impairment, to the inhibition of enzymatic activities by products generated during aging, or even to decreased expression of proteins. *Miquel et al.* reported a decline in ETC complexes with age in mouse liver mitochondria [374]. Meanwhile, other studies showed a decrease in protein levels in some tissues such as skeletal muscle [375], or only a decrease in complex I while the rest of the complexes remained unchanged or even increased [246].

The variability of these results may be partially explained based on physiological differences in aged mitochondria depending on the organ or tissue analysed, as it has been shown by Kwong and Sohal in a study focused on complexes activity [376] and by Srivastava through a transcriptomic analysis [377]. Furthermore, aging is a process that affects animals individually and therefore the data obtained may diverge due to the stochastic effects experienced by each one [378]. According to this idea, no single uniform pattern in protein expression or activities is expected to occur in all different tissues or murine strains. Hence, it is possible that the increase in the protein levels of the complexes observed in our mice due to aging was related to the need to relieve the dysfunction in their enzymatic activity.

The mitochondrial activity dysfunction can also set in motion other compensatory mechanisms to satisfy the energy demand of the cell and counteract the cellular damage. One of these mechanisms is enhanced mitochondrial biogenesis. This process is mediated by PGC-1α and requires the activation of TFAM and nuclear respiratory factors (NRF1, NRF2). In our model, we found an increase in TFAM in liver during aging regardless of the genotype. This is consistent with other studies showing increased TFAM levels in cerebellum, liver, and kidney during aging [379] and in human skeletal muscle along with increased abundance of mtDNA [380, 381]. Although TFAM is closely related to mitochondrial transcription and replication, being also a downstream target of NRF1, it has also been shown to participate in other mechanisms such as mtDNA copy number control, nucleoid mtDNA constitution, and mtDNA repair due to oxidative damage [81, 382-384]. Therefore, the possibility exists that the observed increase in TFAM in liver could be related to its other roles

such as the repair of mitochondrial damage. With regard to other markers, we found no changes in NRF1 or in the mt/nuDNA ratio

Some studies have shown that age-related changes are more evident in post-mitotic tissues. To address this matter, we studied the mitochondrial network in heart, a model of post-mitotic organ.

Morphological and physiological changes in the heart are associated with cardiomyopathies and heart failure in fly and mouse models [385, 386]. The heart mainly depends on the energy generated in the mitochondria, mainly from the beta-oxidation of fatty acids and, although the mitochondria of adult cardiomyocytes are hypo-dynamic, recent results showed that fusion and fission proteins were highly abundant in this tissue [387]. MFN2 have different roles depending on the degree of cellular differentiation, being necessary for the proper functionality in the neonatal myocyte. Deletion of MFN2 delays mitochondrial permeability transition pore opening in adult cardiac myocytes which partly contributes to the fact that these cardiomyocytes are protected from death in hypoxia and H_2O_2 exposure assays. However, the opposite outcome was observed in cultured neonatal cardiac myocytes where MFN2 deficiency could promote early apoptotic events due to a mitochondrial membrane depolarization and changes in mitochondrial morphology in response to ROS stress (Papanicolaou KN, 2011). In addition, other studies have shown that, in the adult heart, MFN1-deficient cardiomyocytes were resistant to oxidative stress-induced cell death [388]. Also, MFN2-deficient adult cardiomyocytes were protected against simulated ischemic reperfusion injury [388].

The balance in favour of fusion causes a "hyper-fused" state of the mitochondria, as described by Tondera *et al.* and Mitra *et al.*, and its persistence can cause a pathological effect [370, 389]. Furthermore, overexpression of MFN1 or MFN2 has also been reported to induce mitochondrial aggregation and collapse at the perinuclear region [390, 391]. We found a dramatic increase in MFN2 in old WT, whereas a decrease was observed in mice overexpressing CYB5R3, an effect that could have a positive outcome in these animals.

However, is important to take into account that MFN2 is not only involved in mediating mitochondrial fusion. MFN2 is also located in the endoplasmic reticulum, where it promotes the tethering of endoplasmic reticulum and mitochondria,

controlling the exchange of Ca_2^+ between both organelles [318, 319]. Thus, proper communication between mitochondria and ER is vital since a continuous increase in the concentration of mitochondrial calcium can lead to the release of cytochrome *c* and the onset of programmed cell death [392]. Mice overexpressing CYB5R3 may therefore have different requirements of MFN2, compared with WT, in order to maintain mitochondrial fitness during aging.

Regarding mitochondrial biogenesis, some studies have reported a loss of mtDNA content and decreased TFAM with aging [81]. However, TFAM decline appears to be tissue-specific and is also highly variable across individuals [393]. On the other hand, a possible role of TFAM in mtDNA repair has also been suggested [384, 394]. In our model, the slight increase in TFAM with aging could be attributed to this role in mtDNA repair. Although we did not observe changes in the mt/nuDNA ratio, TFAM was significantly increased in Tg mice, which again suggests the maintenance of improved mtDNA repair systems with aging in animals overexpressing CYB5R3.

Several studies conducted both in murine models and in humans have examined the enzymatic function of mitochondrial complexes during aging. While in aged human heart there are no changes associated with the function of the complexes [395, 396], a decrease in the enzymatic activity of mitochondrial complexes, especially complex I, III and IV, as well as a reduction in ATP production and increased oxidative stress in mitochondria is one of the conditions contributing to the appearance of cardiomyopathies during aging in rodents is [397]. In our model, we evidenced that most of the complexes were increased in aging heart, and its levels were even higher in Tg mice, which depicts a pattern similar to that observed in liver, excepting for complex IV, which was decreased in CYB5R3-overexpressing mice. There is evidence that at the IMM, complexes I, III, and IV, but not II, together with the electron carriers, coenzyme Q and cytochrome *c*, can be arranged in different combinations to produce supramolecular structures called supercomplexes, which provide kinetic advantages to the electron transfer [398]. Previous studies carried out in murine hearts showed a loss of supercomplexes with age, while ETC complex subunits protein levels remained stable [399]. However, other studies have shown that the loss of ETC activities is more relevant than the variation in the amount of protein. Thus, alterations in complex IV function, which is the last component to be incorporated into this structure [398, 400], could destabilize the supercomplexes as

demonstrated by COX17 gene ablation in HeLa cells [401]. According to this, decreased complex IV activity has been reported in aged rats [260]. It is tempting to speculate that the increase found in complex IV levels in old WT mice shown in this work could be part of a compensatory mechanism aiming to re-establish the assembly of supercomplexes. According to this hypothesis, the decrease in complex IV levels found in Tg mice could imply the stabilisation of these supercomplexes. Additionally, overexpression of the lncRNA LINC00116, which encodes the peptide Mitoregulin (Mtln), in the heart preserves the interaction of complex I with other complexes and its assembly into supercomplexes [402, 403]. Interestingly, it has been shown that the action of Mtln to preserve mitochondrial function relies on its interaction with CYB5R3 [402]. Nevertheless, the demonstration of our hypothesis and the elucidation of the role played by CYB5R3 in aged mitochondria will deserve further investigation.

Mitochondria constitute a complex, interconnected and highly dynamic network, maintained by permanent, opposed and balanced, events of mitochondrial fusion and fission that are modulated in response to changes in energy and stress status. Under physiological conditions, mitofusins are highly expressed in tissues with high energy demand and rich in mitochondria, such as the heart and skeletal muscle and tend to maintain a fused mitochondrial network that compensates the appearance of mtDNA mutations in heteroplasmic cells (through a mixture of content that dilutes mitochondrial dysfunction), contributes to the dissipation of energy in cells, and improves the tethering of the ER to the mitochondria. Other tissues, tend to have a more fragmented network which serves to eliminate damaged mitochondria by mitophagy in order to maintain their bioenergetic capacity [404]. Both mechanisms coexist and are regulated by the energy demand of the cell.

When the bioenergetic state of mitochondria changes, mitochondrial dynamics events adapt to new conditions, fusion events increase metabolic efficiency, while mitochondrial fission favours uncoupled respiration, which can also occur during high levels of cellular stress, as observed when apoptosis is activated. An abnormal mitochondrial dynamic has been observed in association with aging and aged-related diseases. Mitochondrial fragmentation has been associated with the development of insulin resistance in obese mice, and has been observed in several disease states as cardiomyopathies, ischemic myocardia and chronic heart failure. Meanwhile, the

presence of giant mitochondria is observed in dilated cardiomyopathy and cardiomyocyte necrosis in mice. Both hyper- and hypo- fusion or fission states can be deleterious to the cell, promoting abnormal mitochondrial phenotypes that lead mitochondrial dysfunction, which is a hallmark of aging (see Fig. 6).

Conclusions

1. CYB5R3 overexpression led to greater preservation of body weight, an effect that was also reflected in all the tissues weight. However, the outcome of overexpression on weight can be modulated through dietary fats. Moreover, CYB5R3 emerges for the first time as a key determinant in the control of organ and body weight in mice under caloric restriction.

2. Our observations support the existence of tissue- and age-specific mechanisms regulating CYB5R3 levels and highlight the importance of the particular tissue in studying the influence of dietary interventions and aging on mice overexpressing CYB5R3. In this sense, post-mitotic organs appear as the most appropriate models to study the effects of CYB5R3 overexpression.

3. CYB5R3 overexpression and caloric restriction do not induce synergistic changes, but the beneficial effects on mitochondrial function described in *ad libitum* conditions are blunted when both interventions are combined. These results support the idea that CYB5R3 overexpression and caloric restriction may often act through different pathways.

4. In *ad libitum* fed animals, dietary fat has a greater effect and prevalence than CYB5R3 overexpression on the mitochondrial markers analysed. With lard as dietary fat, CYB5R3 overexpression seems to exert a positive effect on mitochondria function. However, fish oil as the source of fat in the diet appears to result in negative interactions with CYB5R3.

5. The increase in electron transport chain enzymes levels of observed in mitotic (liver) and post—mitotic (heart) tissues with aging, particularly in CYB5R3-transgenic mice, is consistent with a possible enhancement mitochondrial function in both tissues.

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Appendix I.

Supplementary figures

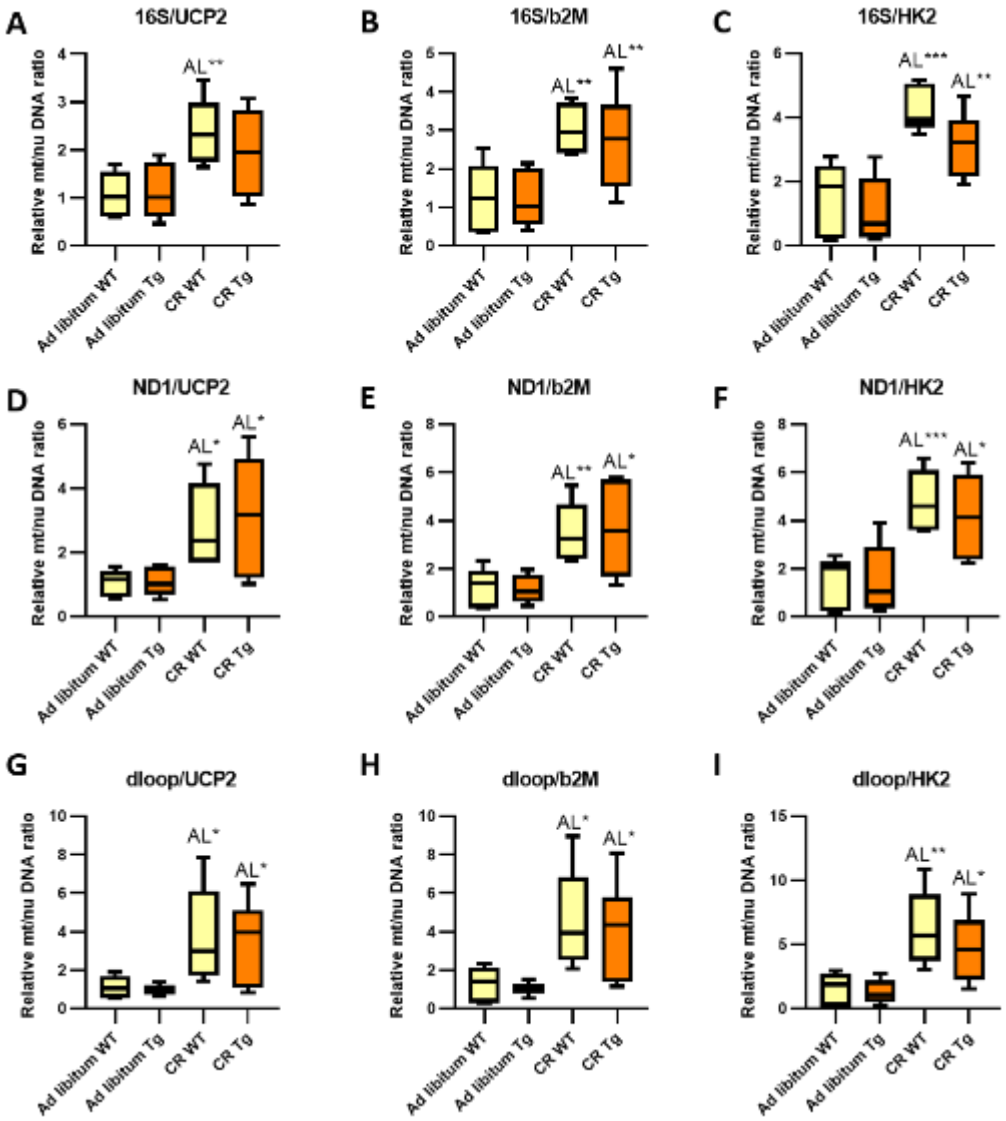

Fig. S. 1 mtDNA/nuDNA ratio in *ad libitum* and CR mice in liver. Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried out and normalised to UCP2, b2M and HK2. Data represent mean ± SEM of 6 replicates. In all box-and-whisker plots "AL" refers to significant differences with the corresponding *ad libitum* group. Statistical signification is represented as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

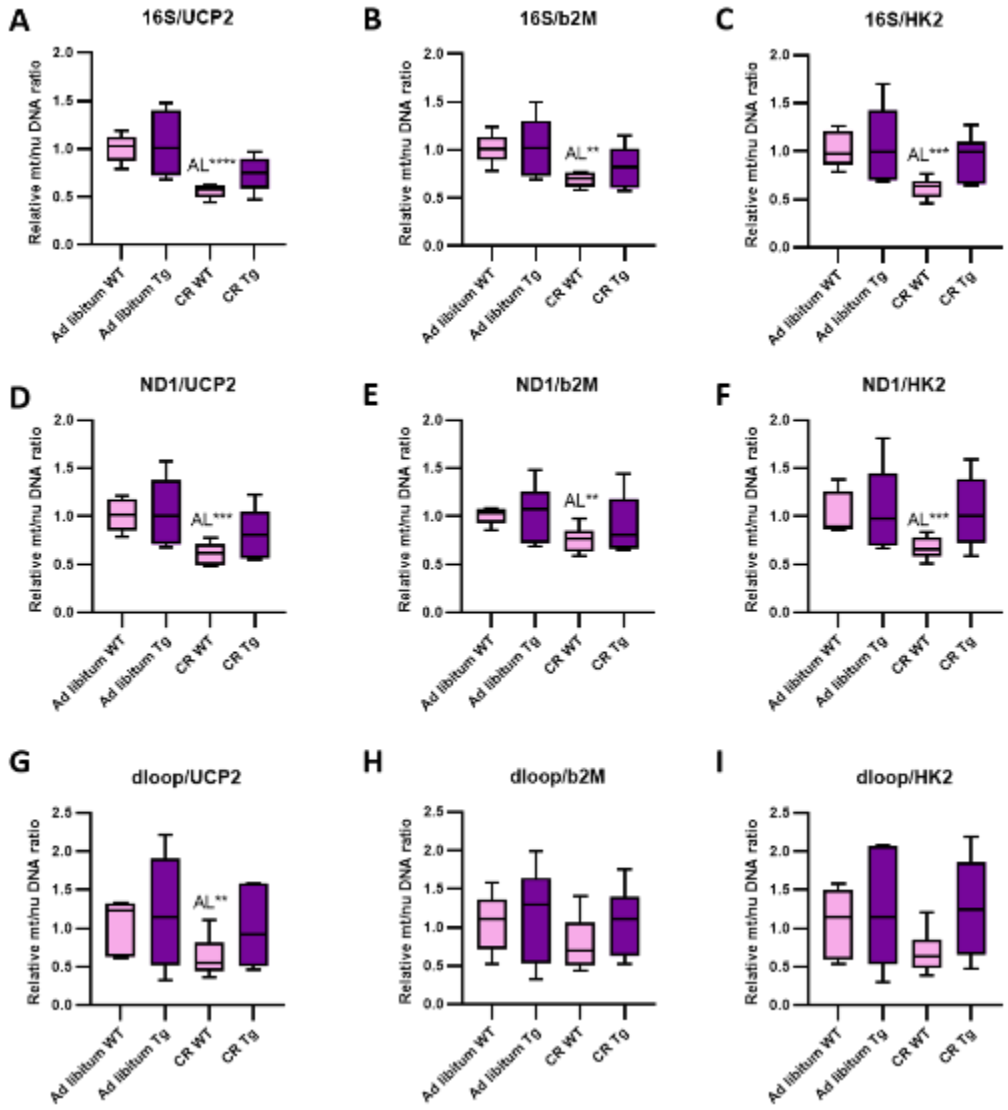

Fig. S. 2. mtDNA/nuDNA ratio in heart from mice fed *ad libitum* or under CR. Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried out and normalised to UCP2, b2M and HK2. Data represent mean ± SEM of 6 replicates. In all box-and-whisker plot "AL" refers to significant differences with respect to the corresponding *ad libitum* control. Statistical signification is represented as ** ($p < 0.01$) and *** ($p < 0.001$) and **** ($p < 0.0001$).

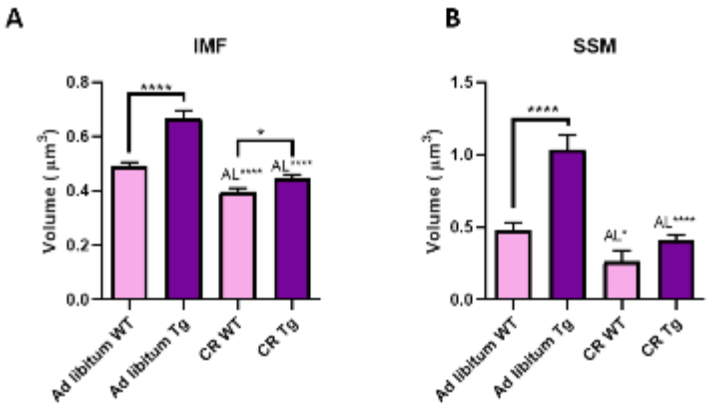

Fig. S. 3. TEM parameters. The results for intermyofibrillar mitochondria (IMF) are represented in left panels, while right panels display the results obtained for subsarcolemmal mitochondria (SSM). A and B, mitochondrial volume. Data represent mean ± SEM. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, "AL" refers to significant differences with respect to *ad libitum* controls. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Statistical signification is represented as * ($p < 0.05$) and **** ($p < 0.0001$).

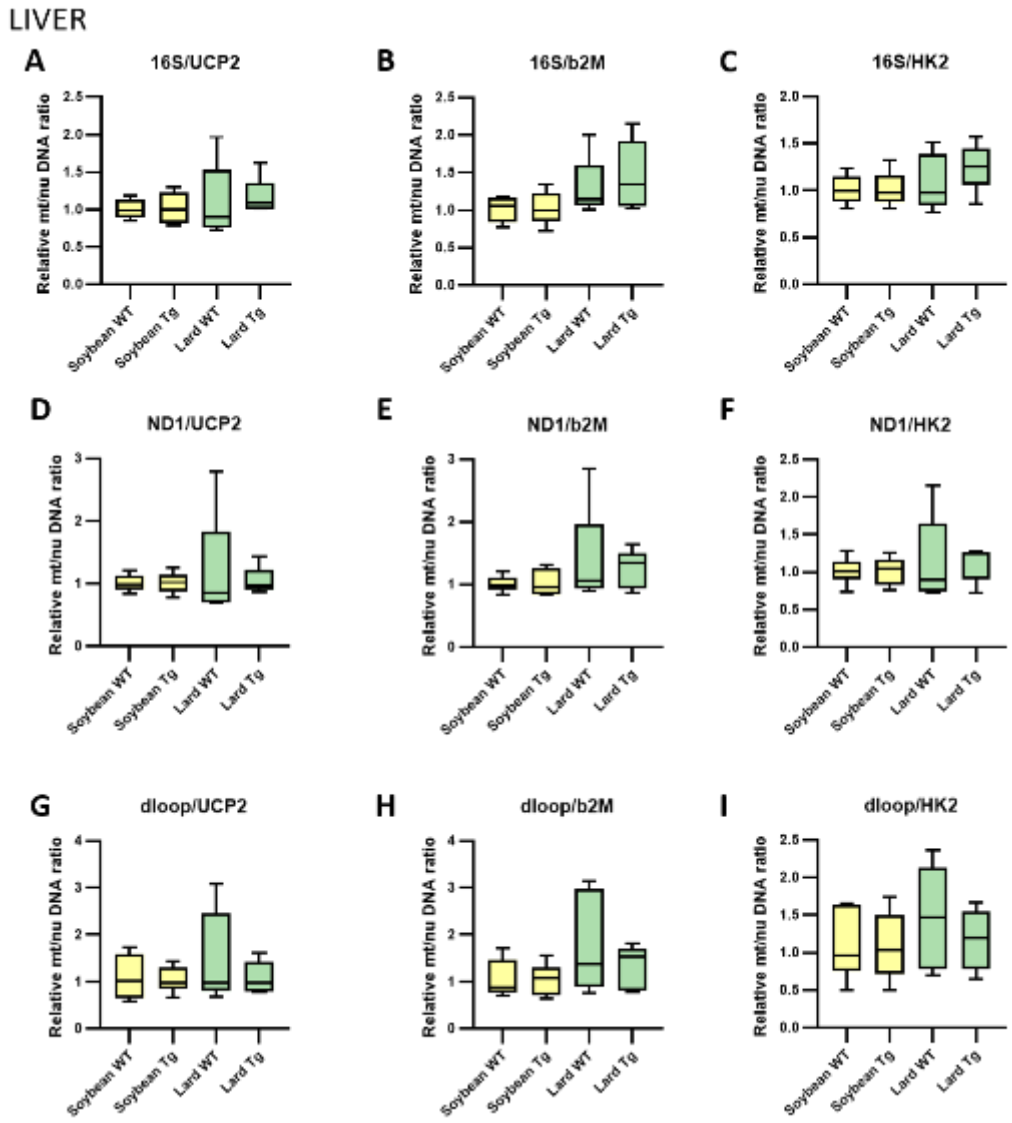

Fig. S. 4. **Hepatic mtDNA/nuDNA ratio in mice fed *ad libitum* with soybean oil- or lard-containing diets.** Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried out and normalised against UCP2, b2M and HK2. Data represent mean ± SEM of 6 replicates.

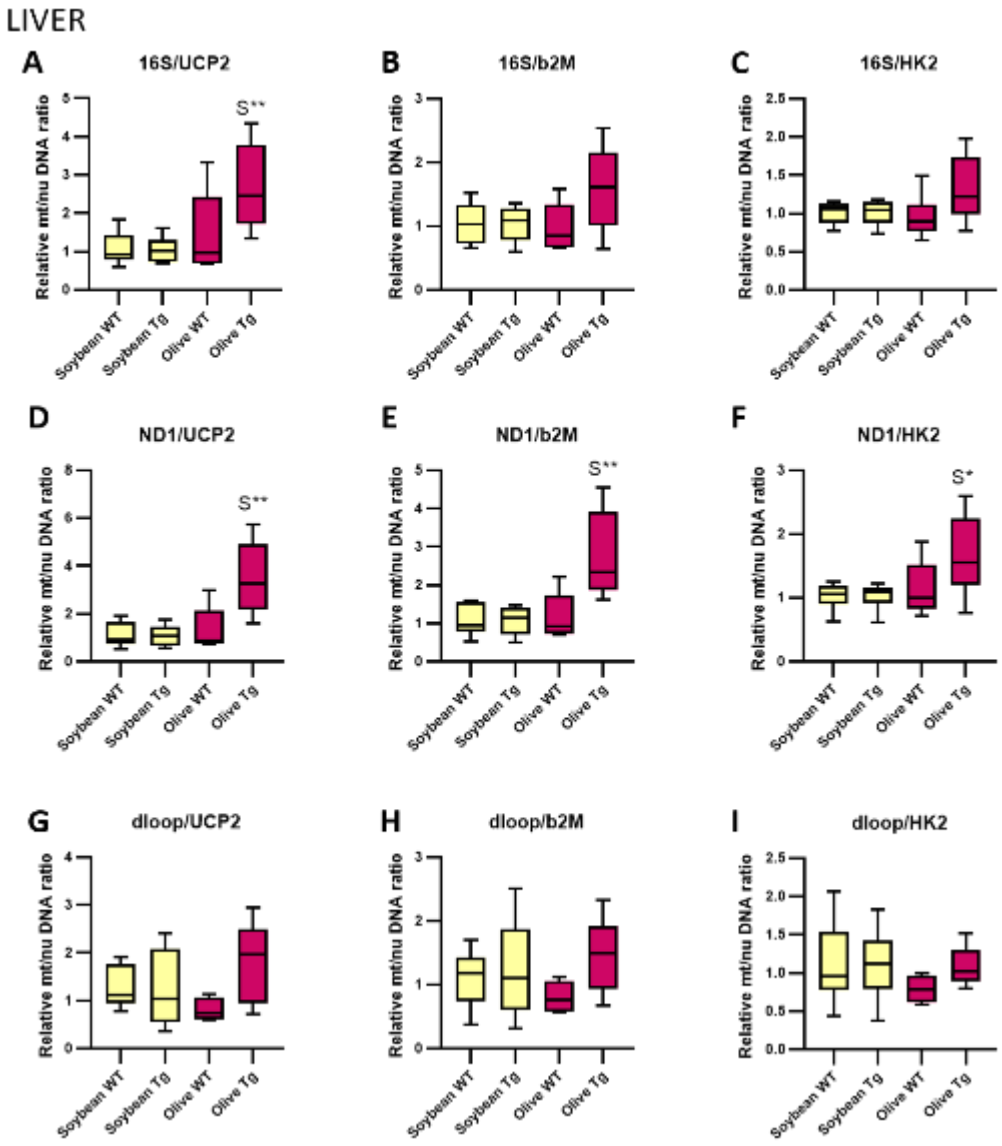

Fig. S. 5. Hepatic mtDNA/nuDNA ratio in mice fed *ad libitum* with soybean oil- or olive oil-enriched diets. Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried out and normalised against UCP2, b2M and HK2. Data represent mean ± SEM of 6 replicates. In all box-and-whisker plot, differences between genotypes, "S" refers to significant differences with respect to soybean oil diet (control group). Statistical signification is represented as * ($p < 0.05$) and ** ($p < 0.01$).

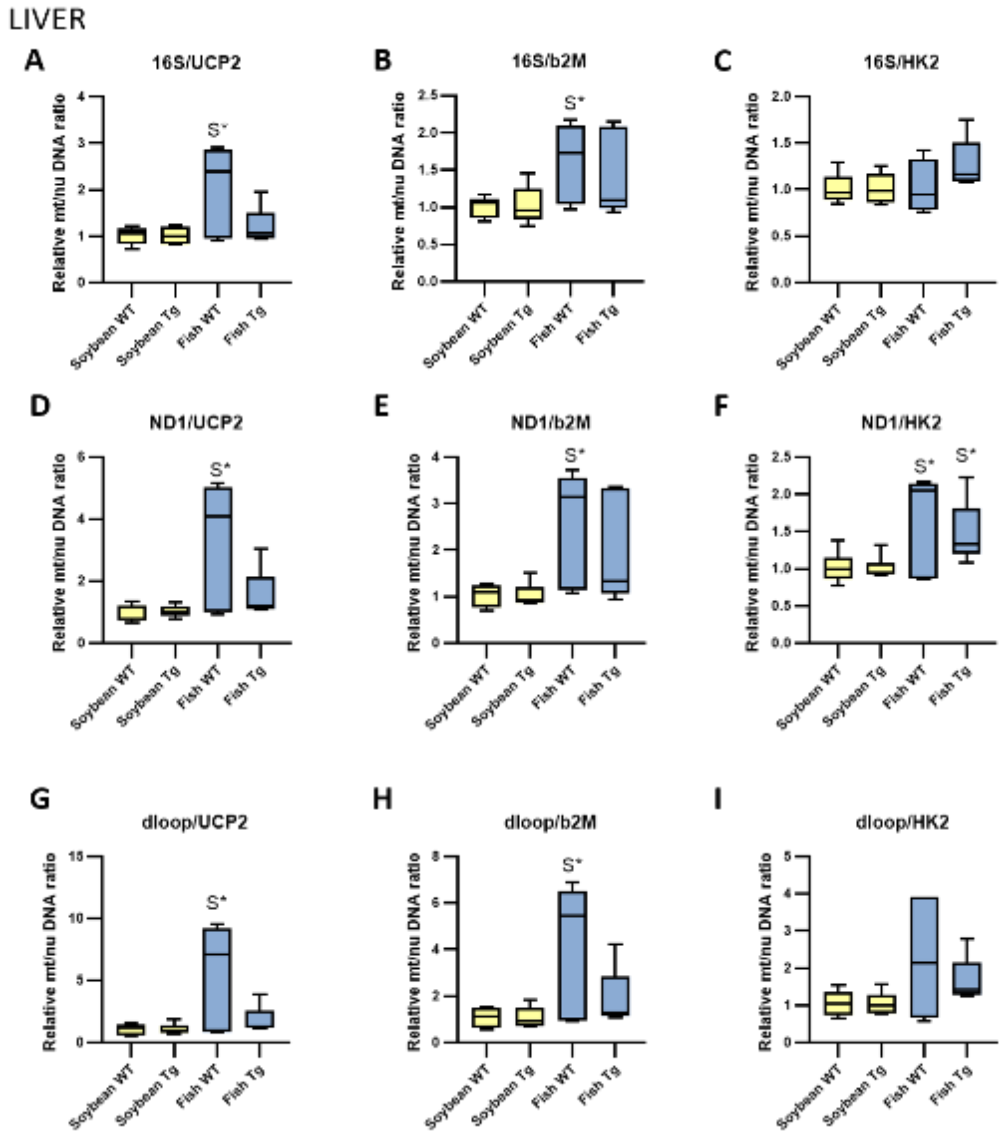

Fig. S. 6. Hepatic mtDNA/nuDNA ratio in mice fed *ad libitum* with soybean oil- or fish oil-enriched diets. Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried and normalised against UCP2, b2M and HK2. Data represent mean ± SEM of 6 replicates. In all box-and-whisker plot, differences between genotypes, "S" refers to significant differences with respect to soybean oil diet (control group). Statistical signification is represented as * ($p < 0.05$).

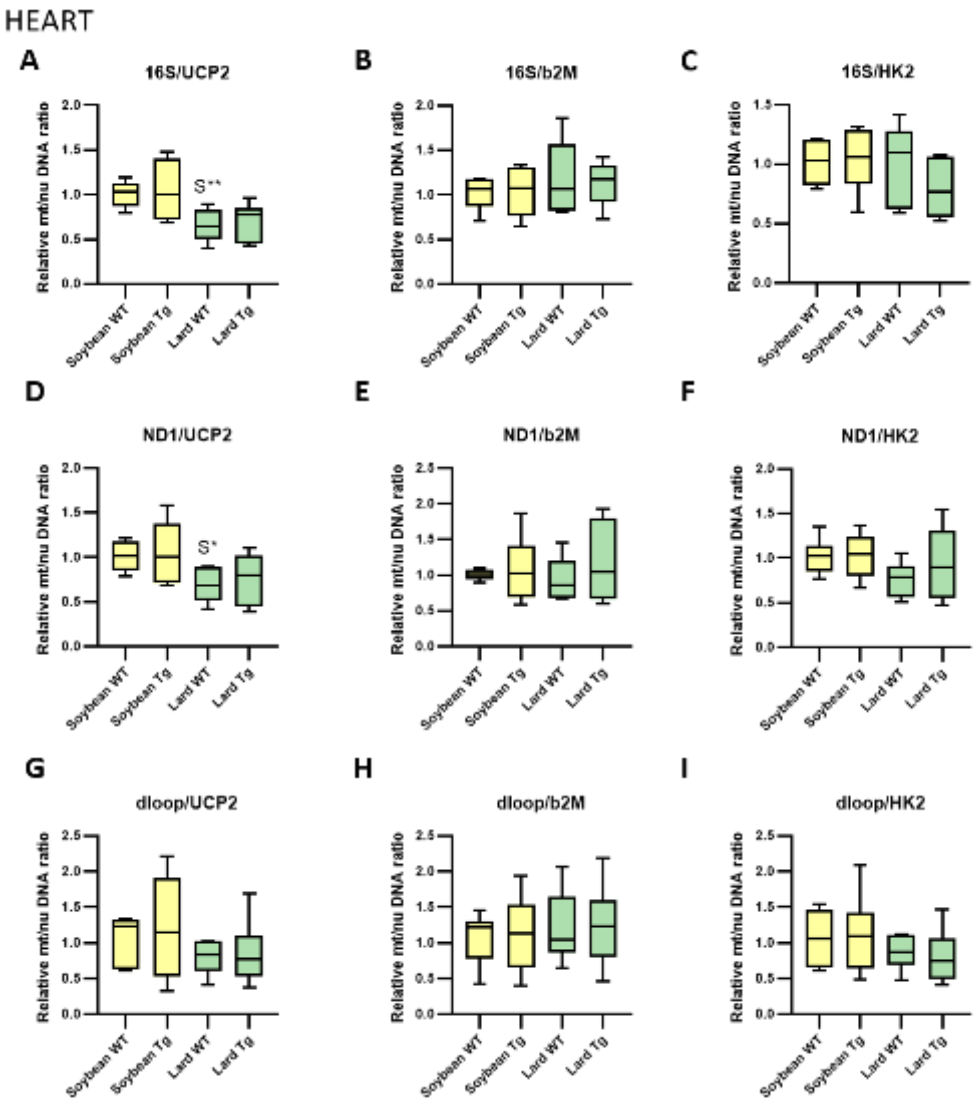

Fig. S. 7. Cardiac mtDNA/nuDNA ratio in mice fed *ad libitum* with soybean oil- or lard-enriched diets. Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried out and normalised against UCP2, b2M and HK2. Data represent mean ± SEM of 6 replicates. In all box-and-whisker plot, differences between genotypes, "S" refers to significant differences with respect to soybean oil diet (control group). Statistical signification is represented as * ($p < 0.05$) and ** ($p < 0.01$).

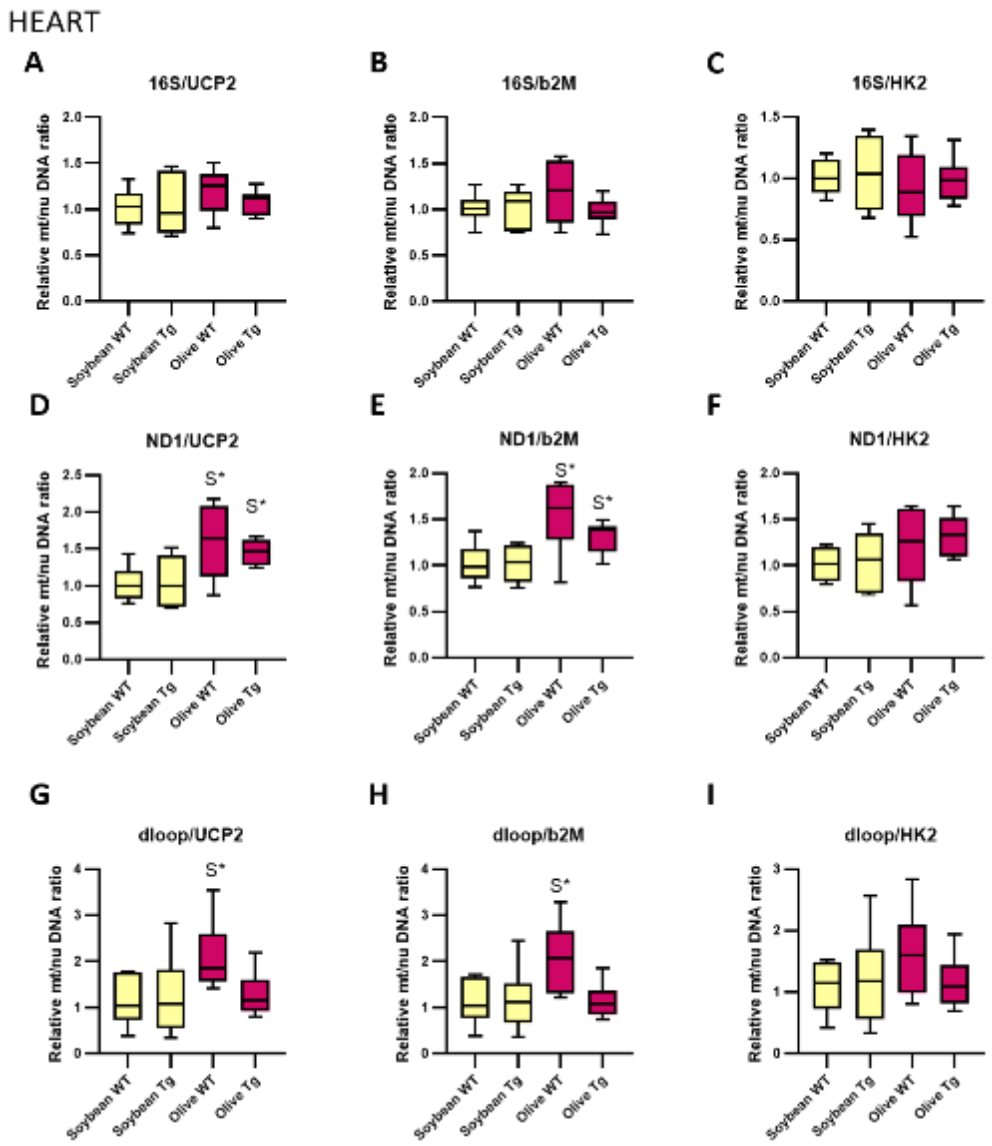

Fig. S. 8. Cardiac mtDNA/nuDNA ratio in mice fed *ad libitum* with soybean oil- or olive oil-enriched diet. Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried out and normalised against UCP2, b2M and HK2. Data represent mean ± SEM of 6 replicates. In all box-and-whisker plot, differences between genotypes, "S" refers to significant differences with respect to soybean oil diet (control group). Statistical signification is represented as * ($p < 0.05$).

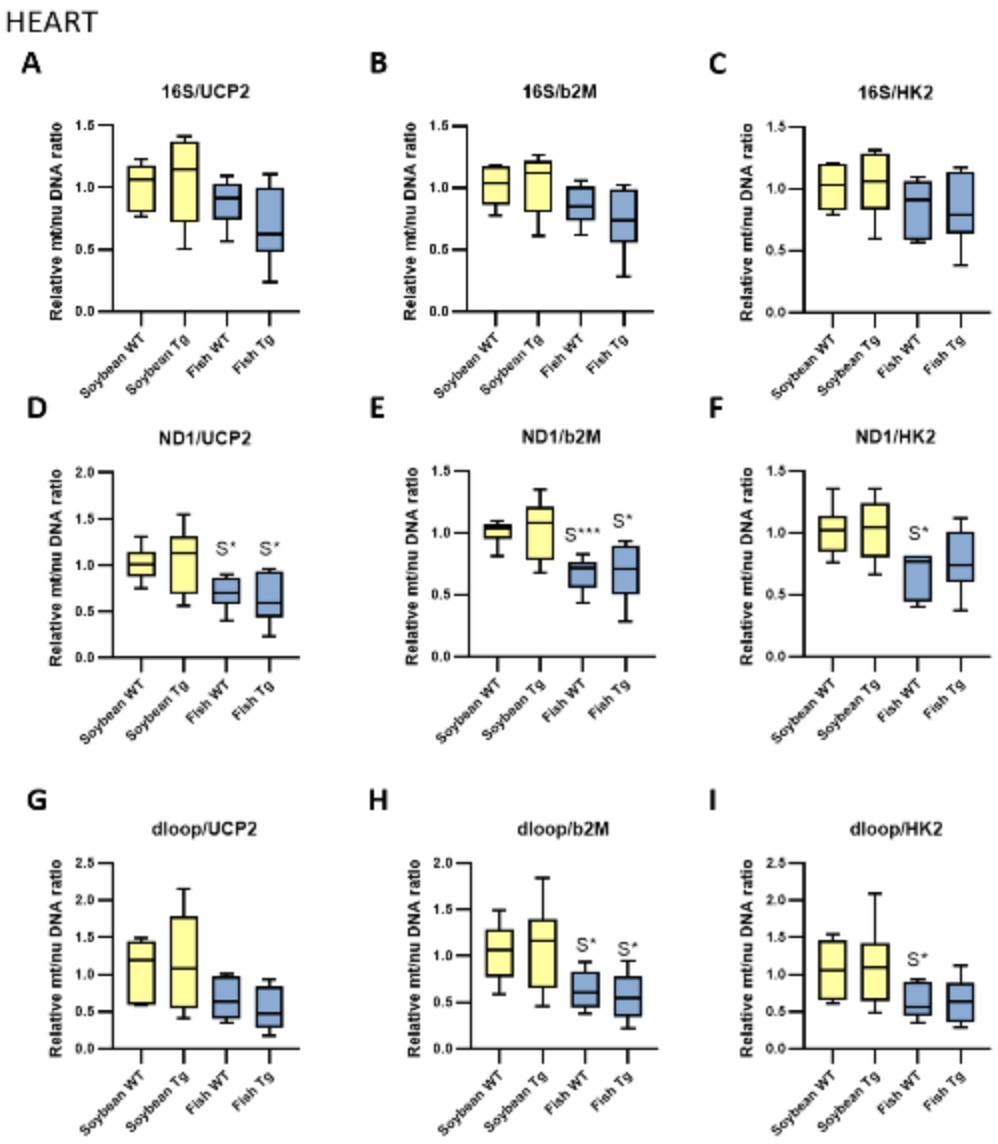

Fig. S. 9. Cardiac mtDNA/nuDNA ratio in mice fed ad libitum with soybean oil- or fish oil-enriched diets. Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried out and normalised against UCP2, b2M and HK2. Data represent mean ± SEM of 6 replicates. In all box-and-whisker plot, differences between genotypes, "S" refers to significant differences with respect to soybean oil diet (control group). Statistical signification is represented as * ($p < 0.05$) and *** ($p < 0.001$).

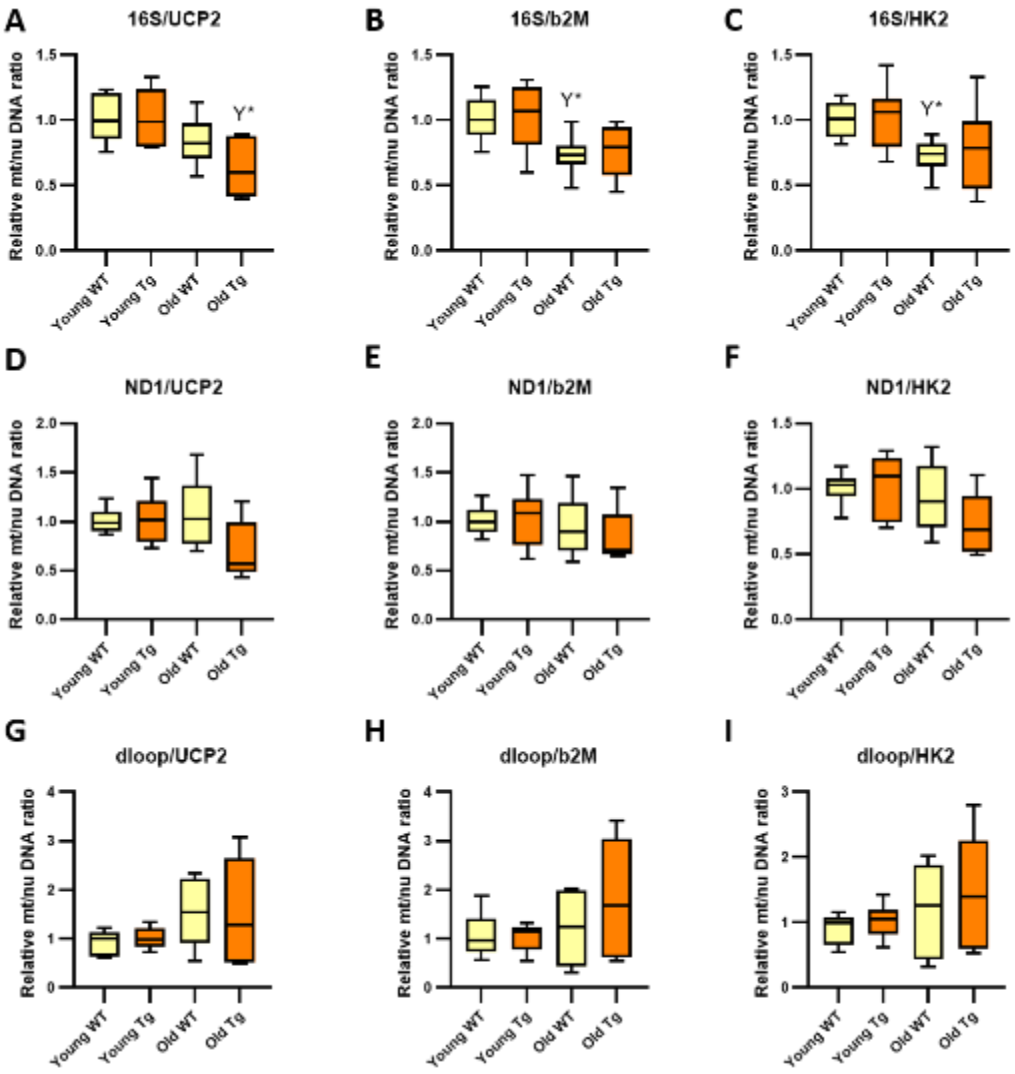

Fig. S. 10. mtDNA/nuDNA ratio in liver from young and old mice. Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried out and normalised against UCP2, b2M and HK2. Data represent means ± SEM of 6 replicates. "Y" refers to significant differences with respect to young mice. Statistical signification is represented as * ($p < 0.05$).

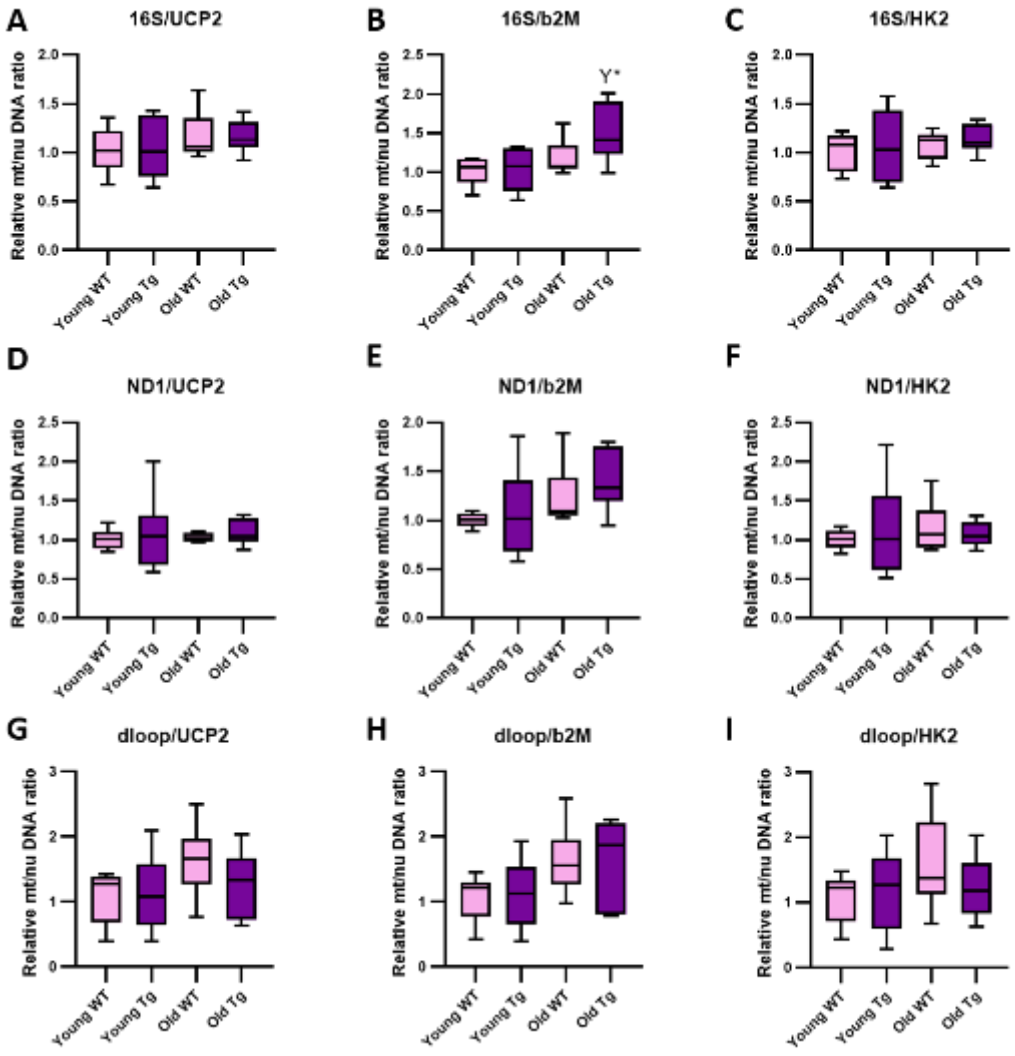

Fig. S. 11. mtDNA/nuDNA ratio in heart from young and old mice. Relative quantification was performed using qPCR. Amplification of 16S, ND1 and d-loop genes, belonging to stable part of mtDNA, was carried out and normalised against UCP2, b2M and HK2. Data represent mean ± SEM of 6 replicates. "Y" refers to significant differences with respect to young mice. Statistical signification is represented as * ($p < 0.05$).

Appendix II. Articles derived from the present work

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ORIGINAL ARTICLE

Mitochondrial adaptations in liver and skeletal muscle to pro-longevity nutritional and genetic interventions: the crosstalk between calorie restriction and CYB5R3 overexpression in transgenic mice

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Abstract Calorie restriction without malnutrition (CR) is considered as the most effective nongenetic nor pharmacological intervention that promotes healthy aging phenotypes and can extend lifespan in most model organisms. Lifelong CR leads to an increase of cytochrome b_5 reductase-3 (CYB5R3) expression and activity. Overexpression of CYB5R3 confers some of the salutary effects of CR, although the mechanisms involved might be independent because key aspects of energy metabolism and lipid profiles of tissues go in opposite ways. It is thus important to study if some of the metabolic adaptations induced by CR are affected by CYB5R3 overexpression. CYB5R3 overexpression greatly preserved body and liver weight in mice under CR conditions. In liver, CR did not modify mitochondrial abundance, but lead to increased expression of mitofusin Mfn2 and TFAM, a transcription factor involved in mitochondrial biogenesis. These changes were prevented by CYB5R3 overexpression but resulted in a decreased expression of a different mitochondrial biogenesis-related transcription factor, Nrf1. In skeletal muscle, CR strongly increased mitochondrial mass, mitofusin Mfn1, and Nrf1. However, CYB5R3 mice on CR did not show increase in muscle mitochondrial mass, regardless of a clear increase in expression of TFAM and mitochondrial complexes in this tissue. Our results support that CYB5R3 overexpression significantly modifies the metabolic adaptations of mice to CR.

Keywords Calorie restriction · Cytochrome b_5 reductase · Liver · Mitochondria · Skeletal muscle

Sandra Rodríguez-López and Sara López-Bellón contributed equally to this work.

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Introduction

Aging can be defined as the time-dependent progressive functional decline with decreased fertility and increased susceptibility of the organism to endogenous and external threats. Aging is the greater risk factor for all chronic diseases including metabolic, degenerative and neoplastic disorders (Harman 1956). The free radical theory of aging, despite some draw backs and criticisms (Liochev 2015), is still one of the most accepted theories enunciated to explain the causes of aging (Barja 2013; Barja 2014). This theory is based on the overproduction of several oxidant species—mainly of mitochondrial origin—with advancing age as the main causative factor of aging, which may be also concomitant with a decrease of antioxidant protective mechanisms (Harman 1956). Indeed, mitochondrial dysfunction has been increasingly identified as a causative factor in many aging-related diseases (Sure et al. 2018). Since the preservation of mitochondrial function is a viable strategy to counteract some of the deleterious manifestations of aging (Csiszar et al. 2019), understanding the alterations of mitochondrial metabolism with aging is of utmost importance (Sakamuri et al. 2018).

Several interventions have been reported to delay aging and to provide protection against aging-related diseases. Among them, calorie restriction without malnutrition (CR) has revealed as the most effective nongenetic intervention that preserves health and extends lifespan in many model organisms (Sohal and Weindruch 1996). There is a strong interest in elucidating how CR produces its antiaging effects, not only to get basic knowledge about the molecular mechanisms involved, but also because this research could give us novel clues on the identification of targets susceptible of intervention. As a general agreement, it has been established that optimization of mitochondrial function plays a key mechanistic role in the prolongevity action of CR (Lopez-Lluch et al. 2006). In accordance, our recent comparative study aimed to elucidate the role of sex, strain and energy intake on hallmarks of aging in mice has shown that the maintenance of mitochondrial function is indeed one of the major predictors of longevity extension in mice fed a CR diet age. Interestingly, the two other main predictors of longevity extension highlighted in our previous study were the maintenance of NAD^+ levels and the preservation of an optimal amount of fat mass with age (Mitchell et al. 2016).

NADH-cytochrome b_5 reductases (EC 1.6.2.2) constitute a family of flavoproteins encoded by 4 different genes in mammals, designed as CYB5R1–4, which catalyse one electron transfer from NADH (which is thus oxidized to NAD^+) not only to cytochrome b_5 but also to a variety of alternative acceptors including coenzyme Q and other redox cyclers (de Cabo et al. 2009). Recently, a great interest has been paid to CYB5R3 in the fields of metabolism and aging research (de Cabo et al. 2009; Diaz-Ruiz et al. 2018; Martin-Montalvo et al. 2016). Two CYB5R3 isoforms can be generated by alternative splicing. A soluble cytosolic isoform, known as methaemoglobin reductase, is expressed exclusively in the erythroid lineage, whereas a membrane-bound isoform expressed in many cell types is attached to the cytosolic side of the mitochondrial outer membrane, the endoplasmic reticulum and the plasma membrane and participates in many physiological processes including elongation and desaturation of fatty acids (Oshino et al. 1971), cholesterol biosynthesis (Reddy et al. 1977) and drug metabolism (Jansson and Schenkman 1973; Sacco and Trepanier 2010). A transplasma membrane redox system which relies on membrane-bound CYB5R3 and coenzyme Q protects cells against oxidants (Navas et al. 2007) and is upregulated by CR, thus potentiating resilience of cells against oxidative damage (De Cabo et al. 2004; Lopez-Lluch et al. 2005; Navas et al. 2007).

To gain new insights into the physiological role this enzyme plays in metabolism and aging, we generated transgenic *Drosophila melanogaster* flies overexpressing CYB5R and transgenic mice overexpressing CYB5R3 (Tg mice) and, interestingly, both experimental models were found to live longer than their wild-type (WT) counterparts (Martin-Montalvo et al. 2016). Tg mice exhibited increased insulin sensitivity and improved regulation of glucose homeostasis both when fed standard and high-fat diets. Conversely, mice with a ß-cell-specific deletion of CYB5R3 had impaired insulin secretion, resulting in glucose intolerance and diet-induced hyperglycaemia. Moreover, respiratory response to glucose was blunted in CYB5R3-deficient cells, which also displayed impaired NAD^+ homeostasis and extensive mitochondrial abnormalities (Fan et al. 2020). On the other hand, Tg mice overexpressing CYB5R3 showed less inflammation and decreased oxidative damage, and were protected against induced cancer, which resembled the healthy effect of CR. However, Tg mice were fatter but not heavier than WT

controls when fed a standard diet ad libitum and preferentially used carbohydrate to meet their energy needs, which differs from other anti-aging interventions as CR or metformin supplementation, where increased longevity required the use of fat as a source of fuel (Guarente 2008; Martin-Montalvo et al. 2013). Thus, CYB5R3 overexpression could contribute to extend lifespan in mice by mechanisms that may be distinct from those described for CR. Further studies aimed at gain knowledge on mitochondrial modifications in tissues from mice overexpressing CYB5R3 are lacked. Furthermore, the existence of a putative crosstalk between CR and CYB5R3 overexpression has not been explored.

The aim of this research was to study the impact of CR on key markers related to mitochondrial function in Tg mice in comparison with their WT littermates. We extended our studies to both liver and skeletal muscle because these tissues are major contributors to whole animal energy expenditure, and they represent mitotic (liver) (Spindler and Dhahbi 2007) and postmitotic (skeletal muscle) tissue models (Ramsey et al. 2000). Our results support that metabolic adaptations of mice fed under CR can be altered by CYB5R3 overexpression.

Materials and methods

Animals and diets

Tg mice were generated as previously reported (Martin-Montalvo et al. 2016). Briefly, the rat CYB5R3 gene was cloned into the pRC/CMV-rDTD plasmid (Belcourt et al. 1998). The transgene insert was cleaved from the DNA cloning vector by digestion with SwaI and NruI restriction enzymes and the purified transgene (under the control of the human cytomegalovirus immediate-early promoter and the SV40 poly- adenylation sequences) was then microinjected into fertilized C57BL/6J eggs at the University of Michigan Transgenic Animal Model Core Facility (http://www.med.umich.edu/tamc/). Surviving eggs were transferred to pseudopregnant B6D2F1 female mice. Stable incorporation of the construct into the genome was validated as described in our previous report (Martin-Montalvo et al. 2016). Tg males were crossed with WT females of the C57 BL/6J background obtained from Charles River (Barcelona, Spain) to establish a colony that was maintained under barrier conditions at the Service of Experimentation Animals (SAEX) of the University of Córdoba. Tg and WT mice were distinguished by PCR genotyping with DNA obtained from tail tissue using the primers CACCAAAATCAACGGGACTT (forward) and AGACCGGGGAGAGTACCACT (reverse) to reveal the presence of the transgene. As internal control, we used amplification of the IL2 gene with the primers CTAGGCCACAGAATTGAAAG ATCT (forward) and GTAGGTGGAA ATTC TAGCATCATCC (reverse).

Experimental groups were established with males of the two genotypes (WT and Tg in C57BL/6J background). The animals were maintained from weaning on 12-h light/dark cycle at 22 °C with a free access to water and a standard chow until they reached an age of 3 months. Then, they were switched to a purified AIN93M diet and separated into the two dietary groups: ad libitum (AL) and calorie restriction (CR) with a 40% reduction of the ad libitum intake. Mice were fed with experimental diets for 4 months and then sacrificed by cervical dislocation. Muscle from hind limb and liver were rapidly excised and frozen by immersion in liquid nitrogen in a buffered medium containing 10% DMSO as cryoprotectant and then stored at − 80 °C. Procedures with experimentation animals were approved by the bioethics committee of the University of Córdoba and authorized by the *Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía* (authorization code: 20/04/2016/053).

Preparation of tissue extracts

Muscle and liver tissues were trimmed and homogenized in radioimmunoprecipitation assay (RIPA) buffer, which contains 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/mL each of chymostatin, leupeptin, antipain, and pepstatin A (CLAP) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich) diluted at 1:100. Tissues were homogenized using a mechanical tissue disrupter (Ultra-Turrax T25, IKA, Staufen, Germany) for 30 s. Homogenates were centrifuged at 10,000*g* for 15 min at 4 °C to separate supernatants containing the protein extracts, which were transferred to new tubes and stored frozen at − 80 °C until use. Total amount of protein in the extracts was estimated by using the Stoscheck modification

(Stoscheck 1990) of the dye-binding method of Bradford (Bradford 1976).

Electrophoresis and Western blot immunodetection

Polyacrylamide gel electrophoresis was performed as described by Lopez-Dominguez et al. (2013). Membranes were incubated with primary antibodies (obtained from Santa Cruz Biotech unless otherwise stated) raised against the following mitochondrial components: CYB5R3 (Proteintech, 10894-1-AP), VDAC1/2/3 (sc-98708), TOTAL OxPhos Complex Kit (Life technologies, 458099), NRF1 (sc-33771), TFAM (sc-2358), Mfn1 (sc-50330), Mfn2 (sc-50331) and Fis1 (sc-98900). Antibody against CYB5R3 was used at 1:10,000 dilution, antibodies against Mfn2 and Fis 1 were used at 1:500 dilution, antibodies against TFAM and VDAC were used at 1:1000 dilution, antibody against NRF1 was used at 1:2000 dilution and TOTAL OxPhos Complex antibody Kit was used at 1:4000 dilution. Appropriate species-specific secondary antibodies coupled to horseradish peroxidase were used to reveal binding sites by enhanced chemiluminescence (ECL-Plus, GE Healthcare Life Sciences). The signal was recorded using a ChemiDoc Imaging System (Bio-Rad) and staining intensity of positive bands was quantified with Image LabTM Software (Bio-Rad). To correct for possible differences in protein load between samples, data obtained from the quantification of the immunostained bands (in arbitrary units) were normalized to density data of the corresponding lanes stained with Ponceau S.

Statistics

Data values were analysed using the GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). All the data shown in this paper are means ± SEM. Normality of data was verified using the Kolmogorov-Smirnov normality test. The means were compared using the two-way ANOVA. We assessed overall differences due to "diet" (independently of genotype), "genotype" (independently of diet) and the interaction "diet × genotype", as well as individual differences between experimental groups. Significant differences were expressed as follows: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Results

Body and liver weight

We first determined body and liver weight in WTand Tg mice that had been fed an AIN93M diet for 4 months, either ad libitum or under conditions of 40% restriction. No differences in body weight between genotypes were observed when mice were fed ad libitum and, as expected, CR produced a significant decrease of body weight in both genotypes ($p < 0.0001$). However, weight loss due to CR intervention was significantly more pronounced in WT than in Tg mice. As a result, Tg mice weighed significantly more than WT littermates when fed under CR conditions ($p < 0.0001$) (Fig. 1a).

Liver weight was also reduced in WT mice fed under CR conditions in comparison with mice of the same genotype fed ad libitum (Fig. 1b) although no difference between the two dietary conditions was observed when liver weight was normalized to body weight (Fig. 1c). No differences in liver weight or in liver weight normalized to body weight were found when comparing mice of the two genotypes when fed ad libitum. Of note, in contrast to what was found for WT mice, CR did not result in a significant decrease of liver weight in Tg mice (Fig. 1b). Moreover, liver weight normalized to body weight was significantly increased in Tg mice fed under CR in comparison with mice of the same genotype fed ad libitum (Fig. 1c).

CYB5R3 polypeptide in liver and skeletal muscle from WT and Tg mice

Next, we studied the levels of CYB5R3 polypeptide in tissue extracts obtained from liver and hind limb skeletal muscle from WT and Tg mice fed the two experimental diets. As shown in Fig. 1a, b, the effect of CR and CYB5R3 overexpression on this parameter was strongly tissue-dependent. Overexpression of the CYB5R3 gene led to a modest albeit significant increase of the CYB5R3 polypeptide in liver from Tg mice fed ad libitum, but this increase was blunted by CR in such a way that no differences between genotypes were noted in mice submitted to this dietary intervention. Interestingly, levels of hepatic CYB5R3 polypeptide were significantly decreased by CR in mice of both genotypes, with this effect being much more striking in Tg mice (Fig. 2a). In skeletal muscle, overexpression of the CYB5R3 gene led to a dramatic increase in the levels

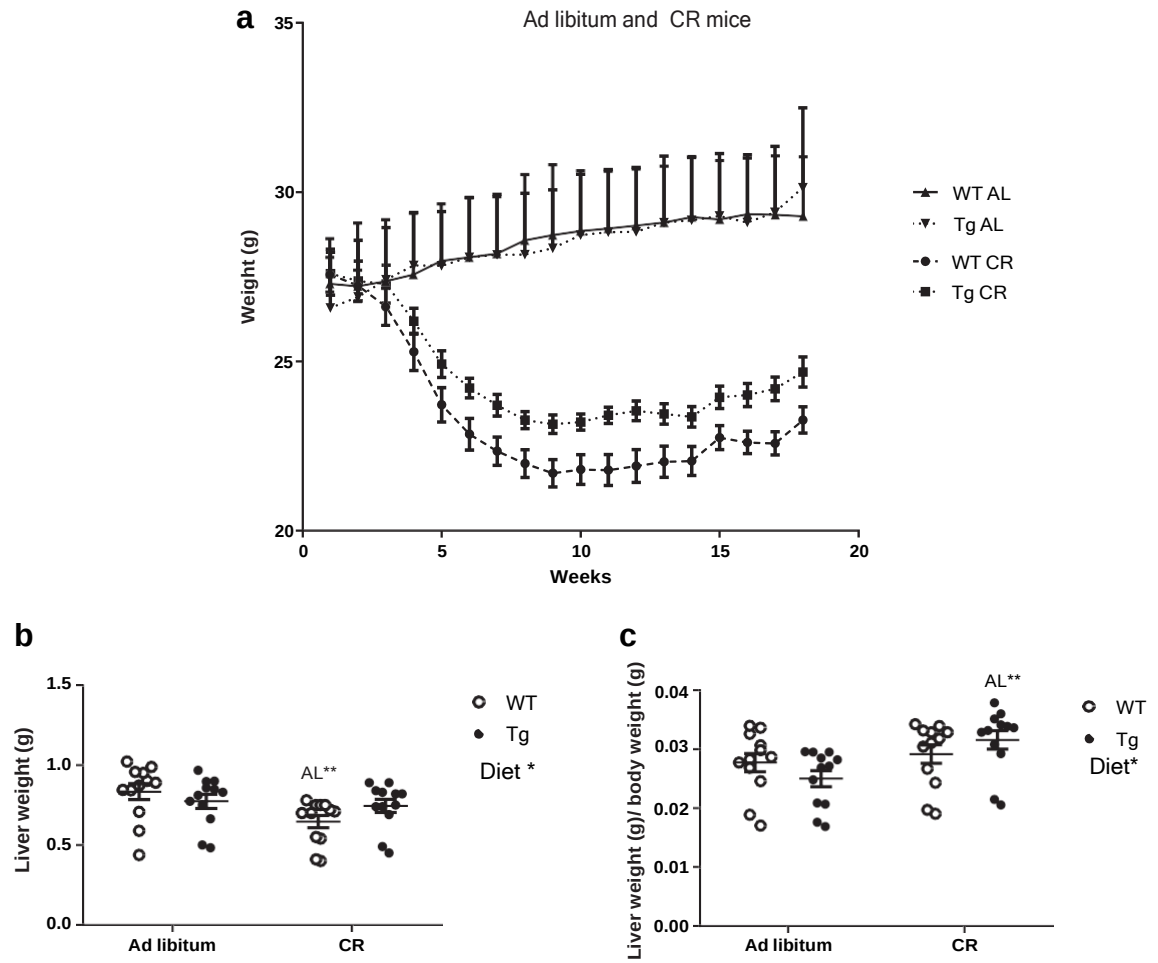

Fig. 1 Changes in body (a) and liver (b) weight in WT and Tg mice overexpressing CYB5R3, fed ad libitum or under CR. The body weight (a) was monitored weekly during 19 weeks of intervention. b displays liver weights after 19 weeks of intervention and c shows the relative liver-to-body weight at the end of the intervention. Data are represented as mean ± SEM of at least 12 mice

of the CYB5R3 polypeptide regardless the intake of calories. In contrast with the results obtained in liver, the levels of CYB5R3 polypeptide in skeletal muscle was not affected by CR regardless of genotype (Fig. 2b).

Mitochondrial dynamics and biogenesis markers

We next investigated the impact that CR and/or overexpression of CYB5R3 gene imposes on mitochondrial dynamics by studying the levels of key marker proteins related to mitochondrial fusion and fission phenomena. As depicted in Fig. 3a, no significant differences were detected for the levels of hepatic Mfn1 in both ad libitum and CR-fed mice, but Mfn2 was significantly increased in WT mice fed under CR in comparison with WT mice fed ad libitum. Interestingly, the extent of this increase by CR was attenuated in Tg mice in such a way that no significant differences were observed when comparing Tg mice fed ad libitum or under CR (Fig. 3b). The fission marker Fis1 was not altered by CR in WT mice, but it was significantly increased in Tg mice fed ad libitum in comparison with WT mice fed the same diet. As observed for CYB5R3 polypeptide, this effect was also vanished in mice fed under CR conditions. Consequently, Fis1 levels were significantly lower in Tg mice fed under CR in comparison with animals of the same genotype fed ad libitum (Fig. 3c). We also measured the levels of TFAM and NRF1, two key transcription factors regulating mitochondrial biogenesis. TFAM polypeptide exhibited a dramatic increase by CR both in WT and in Tg mice

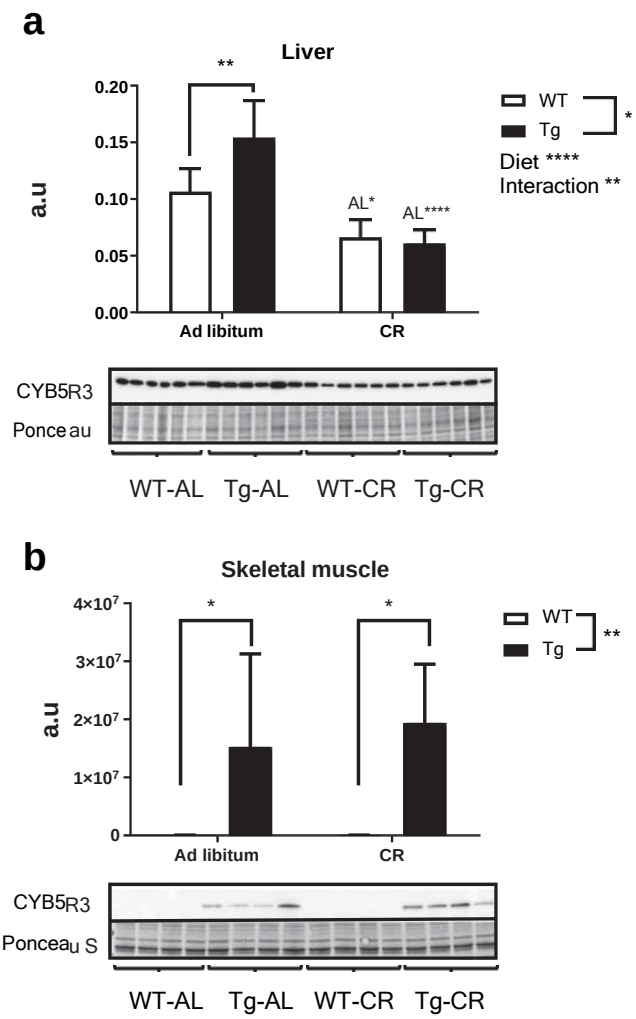

Fig. 2 Expression levels of CYB5R3 measured by Western blots in protein extracts obtained from liver (a) and hind limb skeletal muscle (b) of wild-type and Tg mice. Depicted data are mean ± SEM of 4 replicates. In all graphs, asterisks without a letter refer to statistically significant differences between genotypes, whereas "AL" followed by asterisks indicates statistically significant differences between ad libitum and CR for a given genotype. Significant interactions between diet and genotype are also indicated when appropriated. In Figs. 2, 3, 4, 5, 6, and 7, representative Western blots and Ponceau S-stained lanes as loading control are included below each graph. a.u. arbitrary units

although, as found for another markers, a decrease was also observed in Tg mice fed under CR in comparison with WT mice fed the same diet (Fig. 3d). NRF1 levels also tended to increase with CR in WT mice in comparison with their ad libitum controls, although in this case the differences did not reach statistical significance (p = 0.1). Interestingly, a significant decrease of this mitochondrial biogenesis marker was again observed in Tg mice fed under CR in comparison with WT mice fed the same diet (Fig. 3e). Figure 3f depicts Western blots used for quantification of protein levels in liver with their corresponding Ponceau S-stained lanes used for normalization of protein loading.

Changes elicited by CR and/or CYB5R3 overexpression in skeletal muscle were different to those observed in liver. In skeletal muscle, CR produced a significant increase in the levels of Mfn1 in mice of both genotypes without any change attributable to CYB5R3 overexpression (Fig. 4a), while the levels of Mfn2 (Fig. 4b) and Fis1 (Fig. 4c) were unaffected by either CR or CYB5R3 overexpression. We found an increase of TFAM levels by CYB5R3 overexpression regardless of the diet (Fig. 4d), while NRF1 was substantially increased by CR in WT mice but no significant change was observed in Tg mice (Fig. 4e). Figure 4f depicts Western blots used for quantification of protein levels in skeletal muscle with their corresponding Ponceau S-stained lanes used for protein loading normalization.

Mitochondrial mass and electron transport chain complexes

To further study the impact CR and/or CYB5R3 overexpression exert on mitochondria, we measured the

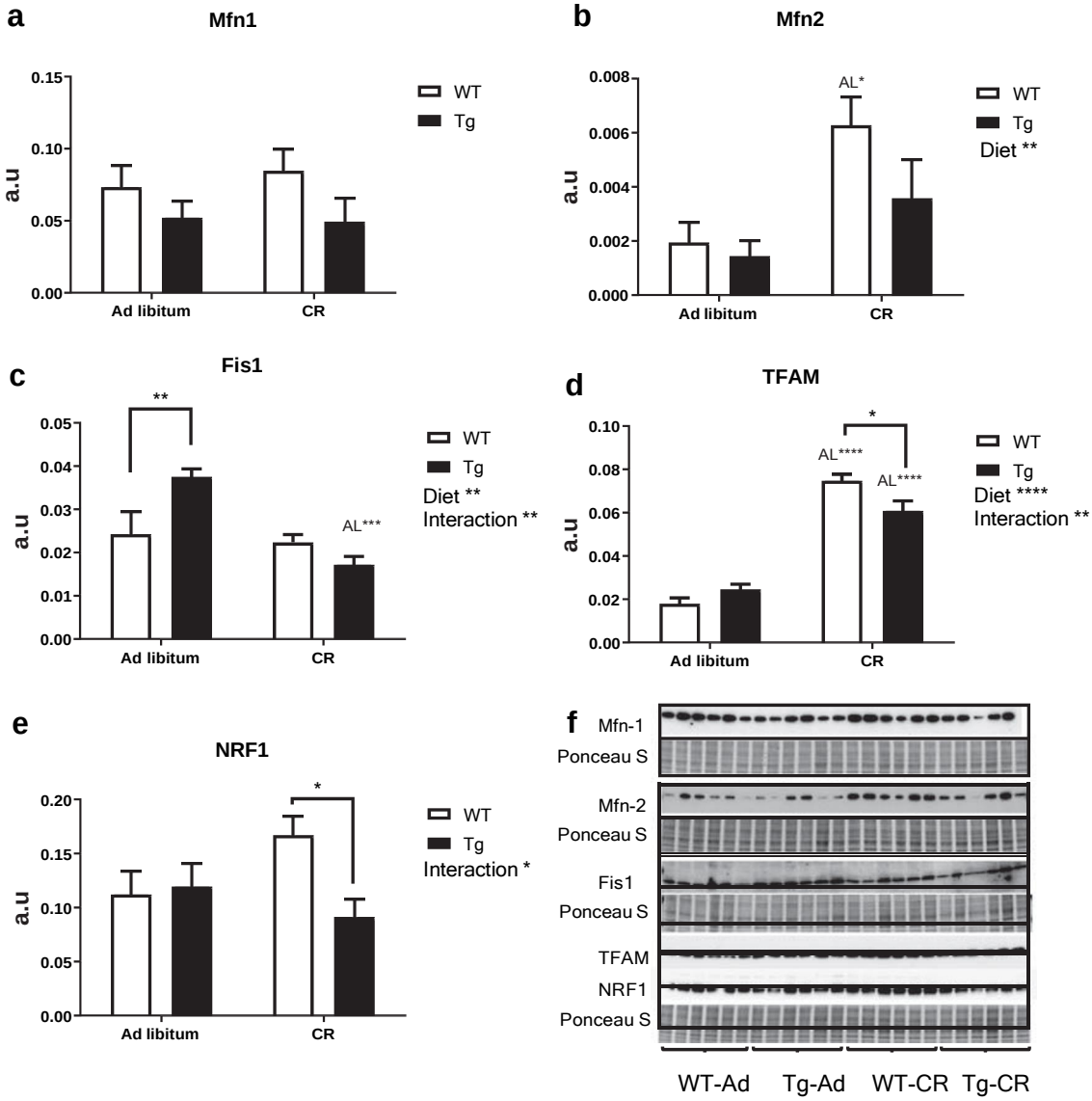

Fig. 3 Expression levels of proteins related to mitochondrial fusion: Mfn-1 (a) and Mfn-2 (b); fission: Fis1 (c); and biogenesis: TFAM (d) and NRF1 (e) in liver of wild-type and Tg mice fed ad libitum or under CR. When detected, general effects of diets and interaction between genotype and diet are represented on the corresponding panels. Data are represented as mean ± SEM of 6 replicates. Western blots used for quantification of protein levels with their corresponding Ponceau S-stained lanes used for normalization of protein loading (f)

levels of VDAC (porin), an outer membrane protein which is considered as a biochemical marker of mitochondrial abundance (Grünewald et al. 2014), and several marker subunits of the electron transport chain (ETC) complexes in the inner membrane. Neither CR nor CYB5R3 overexpression altered VDAC levels in liver (Fig. 5a), but CR strongly increased VDAC in skeletal muscle from WT mice. It is noteworthy that the effect of CR was again abated in mice overexpressing CYB5R3, so that VDAC levels in Tg mice fed under CR were not only much lower than those of WT mice fed the same diet, but even lower than those of Tg mice fed ad libitum (Fig. 5b).

Regarding the abundance of marker subunits of the electron transport chain complexes, we found no changes attributable to diet or genotype in hepatic levels of complexes I and II (Fig. 6a, b), but CR produced substantial increases of hepatic complex III (with statistical

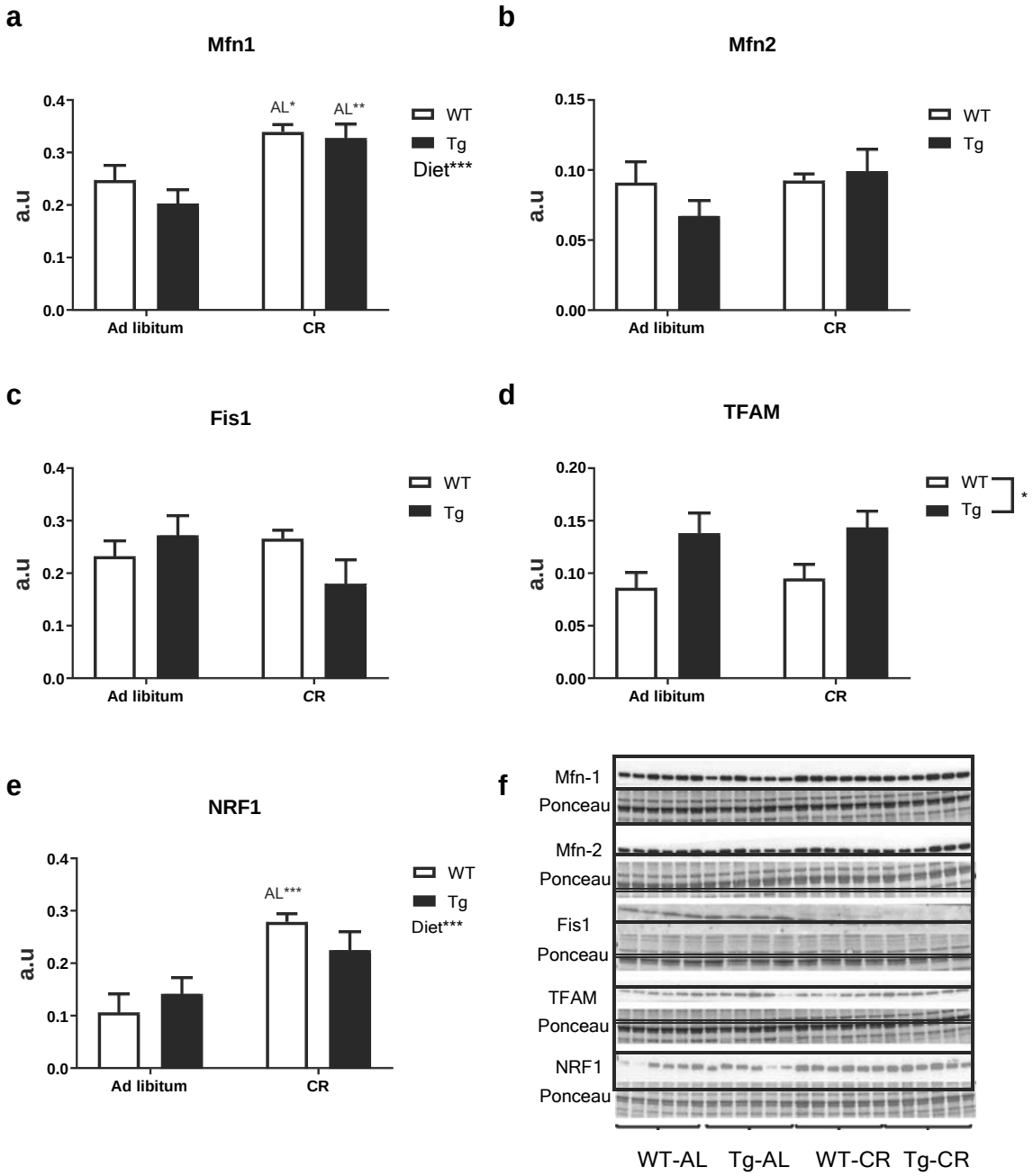

Fig. 4 Expression levels of protein related to mitochondrial fusion Mfn-1 (a) and Mfn-2 (b), fission Fis1 (c) and biogenesis TFAM (d) and NRF1 (e) in skeletal muscle of wild-type and transgenic animals fed ad libitum or under CR. General effects of diet and interaction between genotype and diet are represented, when detected, on the corresponding panel. Data are shown as mean ± SEM of 6 replicates except in c (Fis1) with 4 replicates. Western blots used for quantification of protein levels with their corresponding Ponceau S-stained lanes used for normalization of protein loading (f)

significance in both genotypes, see Fig. 6c), complex IV (with statistical significance in Tg mice, see Fig. 6d) and complex V (where statistical significance for the effect of diet was obtained when mice of both genotypes were combined, see Fig. 6e). Figure 6f depicts the Western blots used for quantification of protein levels, with their

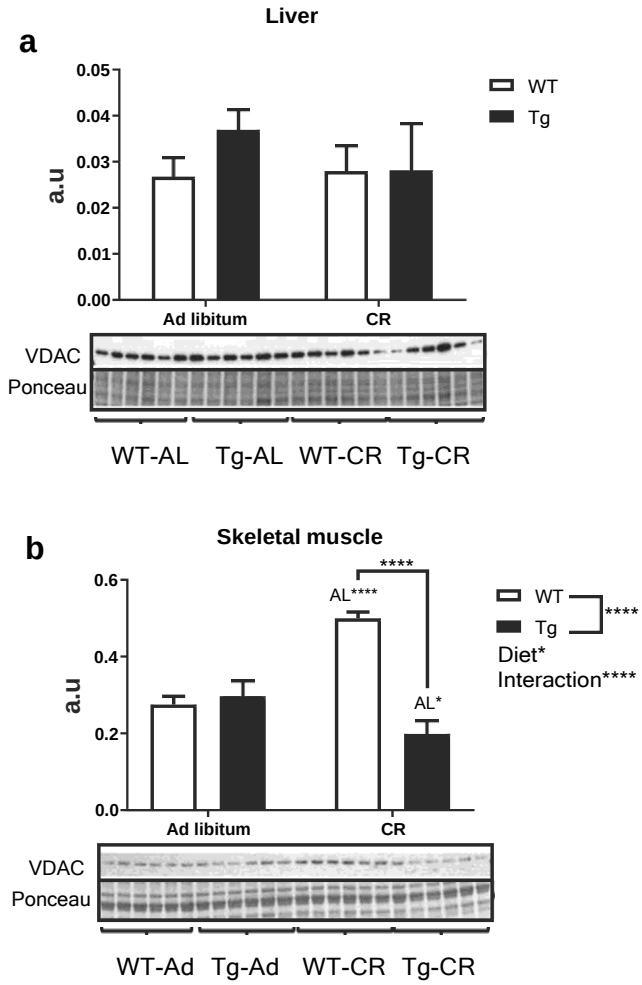

Fig. 5 Expression levels of VDAC as mitochondrial mass marker in protein extracts obtained from liver (a) and hind limb skeletal muscle (b) of WT and Tg mice. Data are depicted as mean ± SEM of 6 replicates

corresponding Ponceau S-stained lanes used for protein loading normalization.

In the case of skeletal muscle, we evidenced a global and genotype-independent effect of CR to increase the abundance of marker subunits of complexes I (where statistical significance was obtained when mice of both genotypes were combined, see Fig. 7a) and IV (with statistical significance in mice of both genotypes, see Fig. 7d). On the other hand, complexes II and III shared another pattern of changes as a function of diet and/or genotype, i.e. the abundance of these complexes was increased in WT mice fed a CR diet and in Tg mice fed ad libitum, in comparison with their WT controls fed ad libitum. No differences were however found when comparing the two dietary groups of Tg mice, and CR did not affect significantly the levels of complexes II and III in these mice (see Fig. 7b, c). Finally, the pattern of complex V changes with diet and/or genotype was similar to that exhibited by VDAC (see above), i.e. CR produced a significant increase in WT mice, but this increase was abated by CYB5R3 overexpression. Complex V abundance was thus significantly lower in Tg mice fed under CR in comparison with WT mice fed the same diet, and no change as a function of genotype was observed in mice fed ad libitum (Fig. 7e). Figure 7f depicts Western blots used for quantification of protein levels, and their corresponding Ponceau S-stained lanes used for protein loading normalization.

The alterations in the levels of several mitochondrial complexes with diet and/or genotype we document here could be accounted by changes in mitochondrial abundance and/or by modifications in the intrinsic composition of mitochondrial membranes. To distinguish between these two possibilities, we

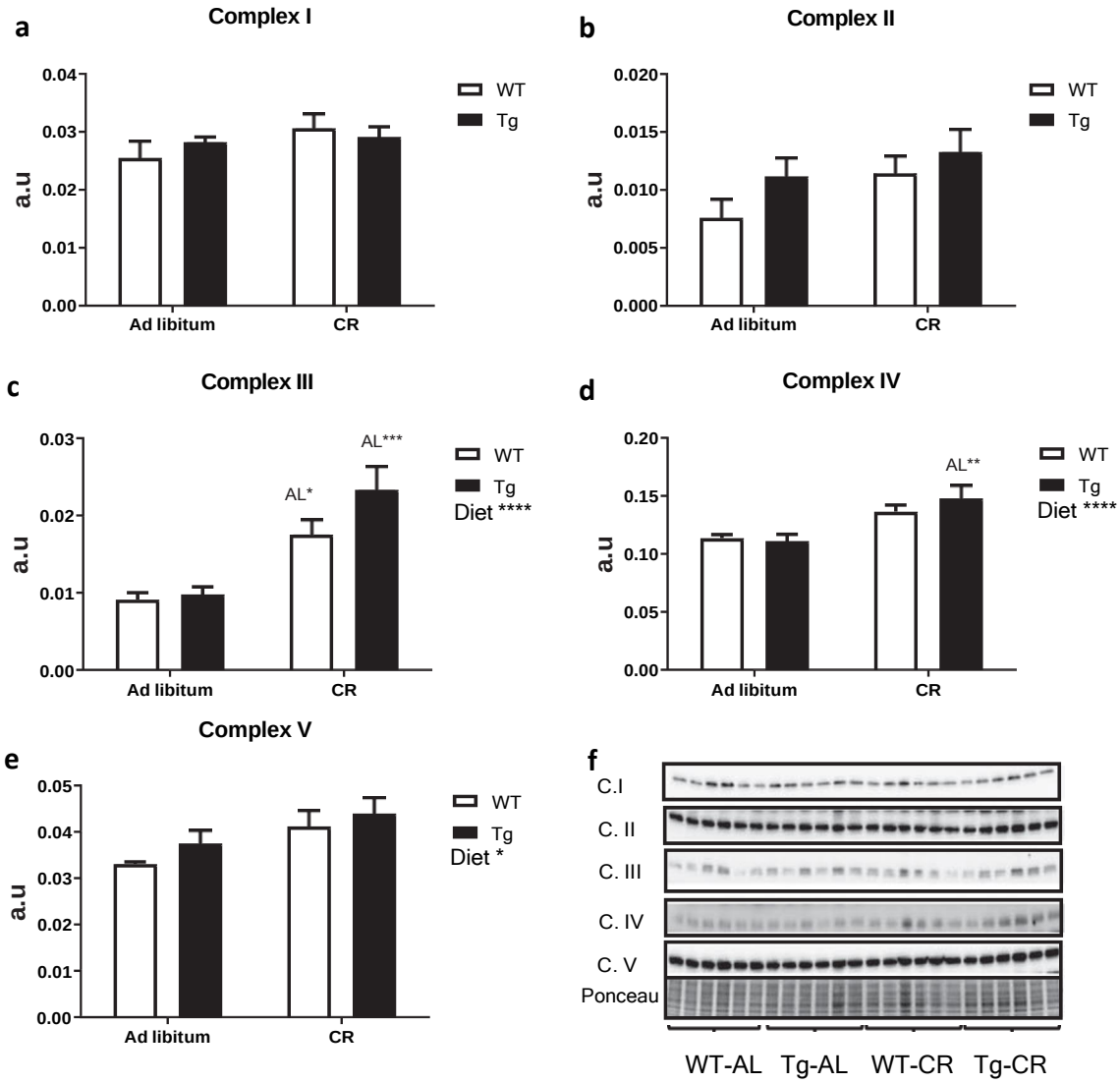

Fig. 6 Expression levels of mitochondrial electron transport chain complexes in liver from WT and Tg mice fed ad libitum or under CR. a to e represent the levels of mitochondrial complexes I to V, respectively. Statistically significant differences between groups are indicated where detected. Data are mean ± SEM of 6 replicates. Western blots used for quantification of protein levels with their corresponding Ponceau S-stained lanes used for normalization of protein loading (f)

calculated the levels of mitochondrial complexes relative to VDAC, as previously published (Grünewald et al. 2014), and the results are depicted in Figs. 8 (for liver) and 9 (for skeletal muscle). Interestingly, a consistent pattern of changes was highlighted for both tissues when the abundance of each mitochondrial complex was referred to the corresponding value of VDAC. In liver, CYB5R3 overexpression did not alter the abundance of ETC complexes relative to VDAC in mice fed ad libitum, but a general trend towards the increase of all complexes was found in Tg mice fed under CR (see Fig. 8a–e), with statistically significant differences being obtained for complex IV:VDAC in comparison with Tg mice fed ad libitum (Fig. 8d).

In skeletal muscle, it was highlighted again that abundance of most complexes relative to VDAC was not altered by CYB5R3 overexpression in mice fed ad libitum (see Fig. 9a, c–e), resembling the results obtained in liver, with the sole exception of complex II which was increased significantly in Tg compared with WT mice (Fig. 9b). Of note, the abundance of all complexes relative to VDAC was consistently

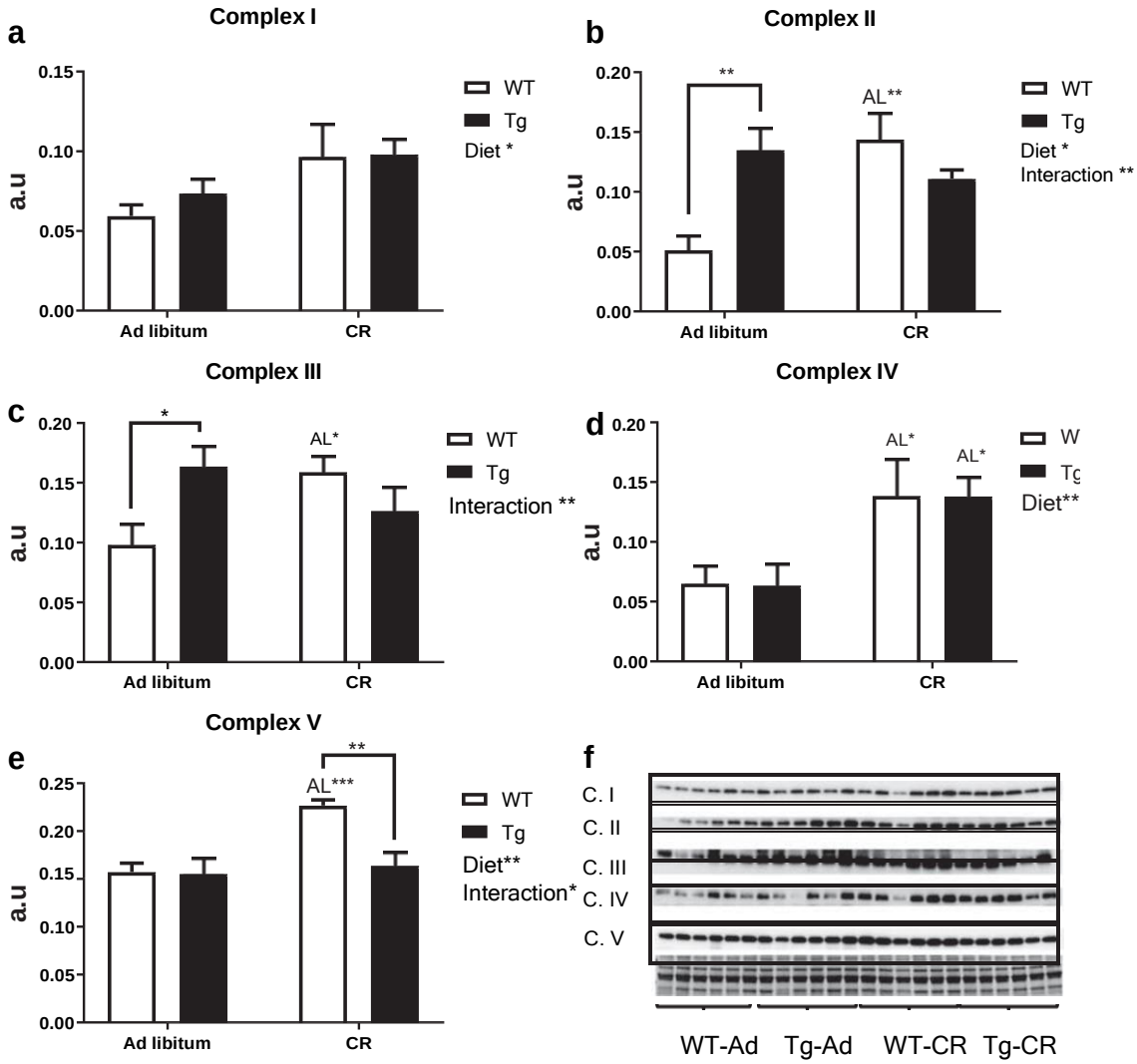

Fig. 7 Expression levels of mitochondrial electron transport chain complexes quantified in skeletal muscle of WT and Tg mice fed either ad libitum or under CR. a to e represent complexes I to V, respectively. Statistical differences between groups as well as general effect of diet and interactions genotype-diet are indicated on the corresponding panel. Data were represented as mean ± SEM of 6 replicates. Western blots used for quantification of protein levels with their corresponding Ponceau S-stained lanes used for normalization of protein loading (f)

increased in Tg mice fed under CR in comparison with their WT littermates fed the same diet (see Fig. 9a–e). Moreover, statistically significant increases with respect to Tg mice fed ad libitum were also observed for complexes I, IV, and V (see Fig. 9a, d, e).

Discussion

The NADH dehydrogenase CYB5R3 has direct implications in cell protection against oxidative stress and in the regulation of NAD^+ levels, and thus, a fundamental role for CYB5R3 in metabolism and aging has been proposed (de Cabo et al. 2009; Siendones et al. 2014). Moreover, it has been considered that some of the beneficial effects CR exerts on health could be related, at least in part, to CYB5R3 induction (De Cabo et al. 2004; Lopez-Lluch et al. 2005; Navas et al. 2007). However, whereas some of the salutary effects of CR are indeed mimicked in transgenic mice overexpressing CYB5R3 (Tg mice) (Martin-Montalvo et al. 2016), the mechanisms involved in metabolic adaptation of Tg mice

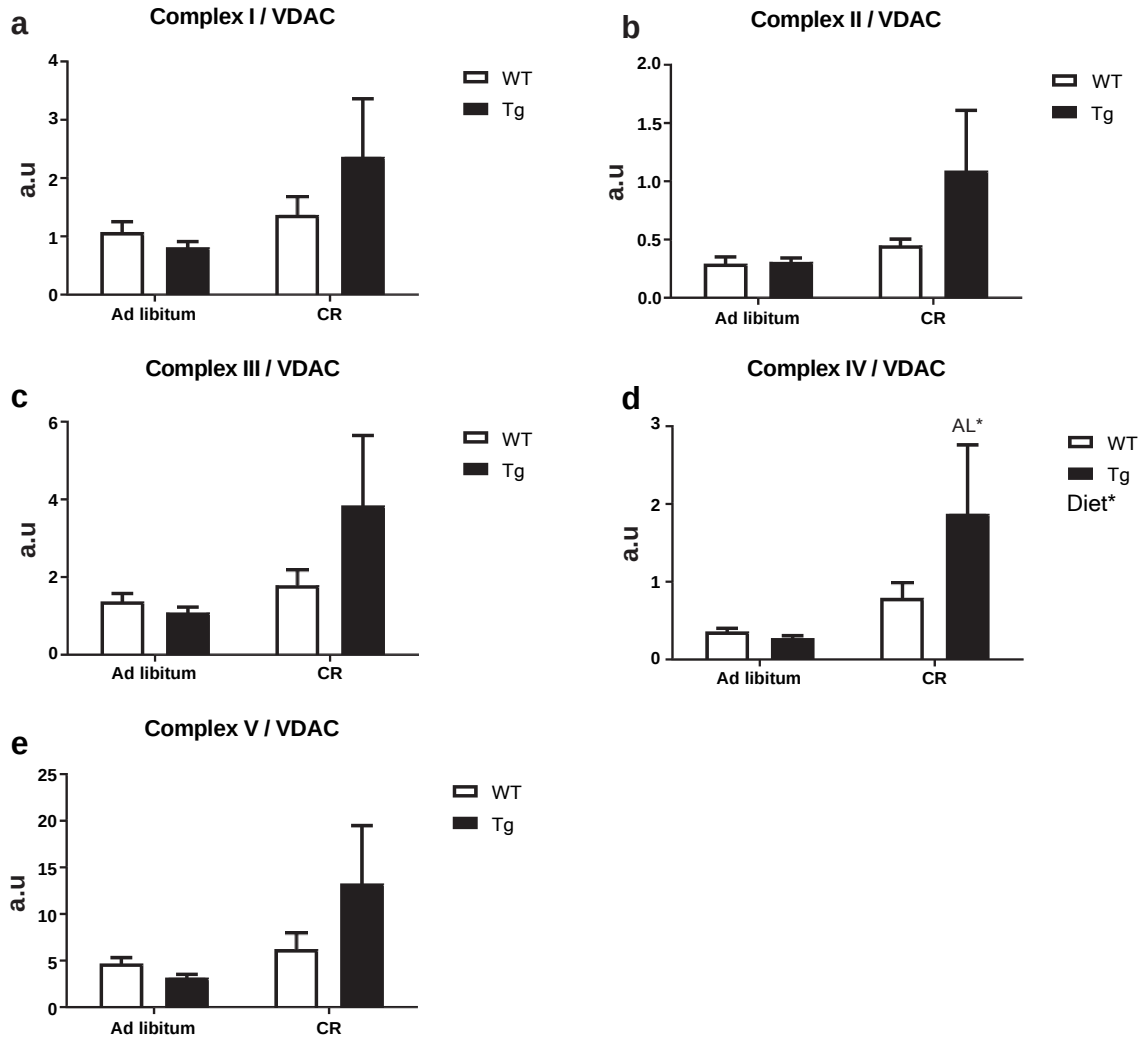

Fig. 8 Levels of mitochondrial complexes relative to VDAC in liver. a to e as in Fig. 5. Statistical differences were found only for complex IV/VDAC (d)

might be independent of those described for CR. In this sense, Tg mice fed a standard diet ad libitum were fatter but not heavier than the corresponding WT controls, and preferentially used carbohydrate to meet their energy needs. These two traits clearly depart from a typical CR phenotype, which is characterized by the decrease of body weight, adiposity and the use of fat as a fuel source (Guarente 2008; Martin-Montalvo et al. 2013). Since saturated fatty acids are required to allow mitochondrial ß-oxidation to proceed (Kunau et al. 1995), metabolic adaptations elicited by CYB5R3 overexpression might be related to the action of this enzyme in enhancing elongation and desaturation of fatty acids, which may lead to the inhibition of fatty acid ß-oxidation and, thus, to the preferential use of carbohydrate metabolism (Martin-Montalvo et al. 2016). Lipid composition of membranes is also modified by CR, but the changes again differ from those produced by CYB5R3 overexpression. In this sense, CR decreases the levels of long-chain PUFA and increases monounsaturated fatty acids without any change in saturated fatty acids, as result of a metabolic reprogramming leading to lower levels of oxidative damage which could contribute to the extension of lifespan (Jove et al. 2014; Laganiere and Yu 1989; Laganiere and Yu 1993).

Because CR and CYB5R3 overexpression modify key aspects of energy metabolism and the lipid profile of tissues in opposite ways, it is important to study if

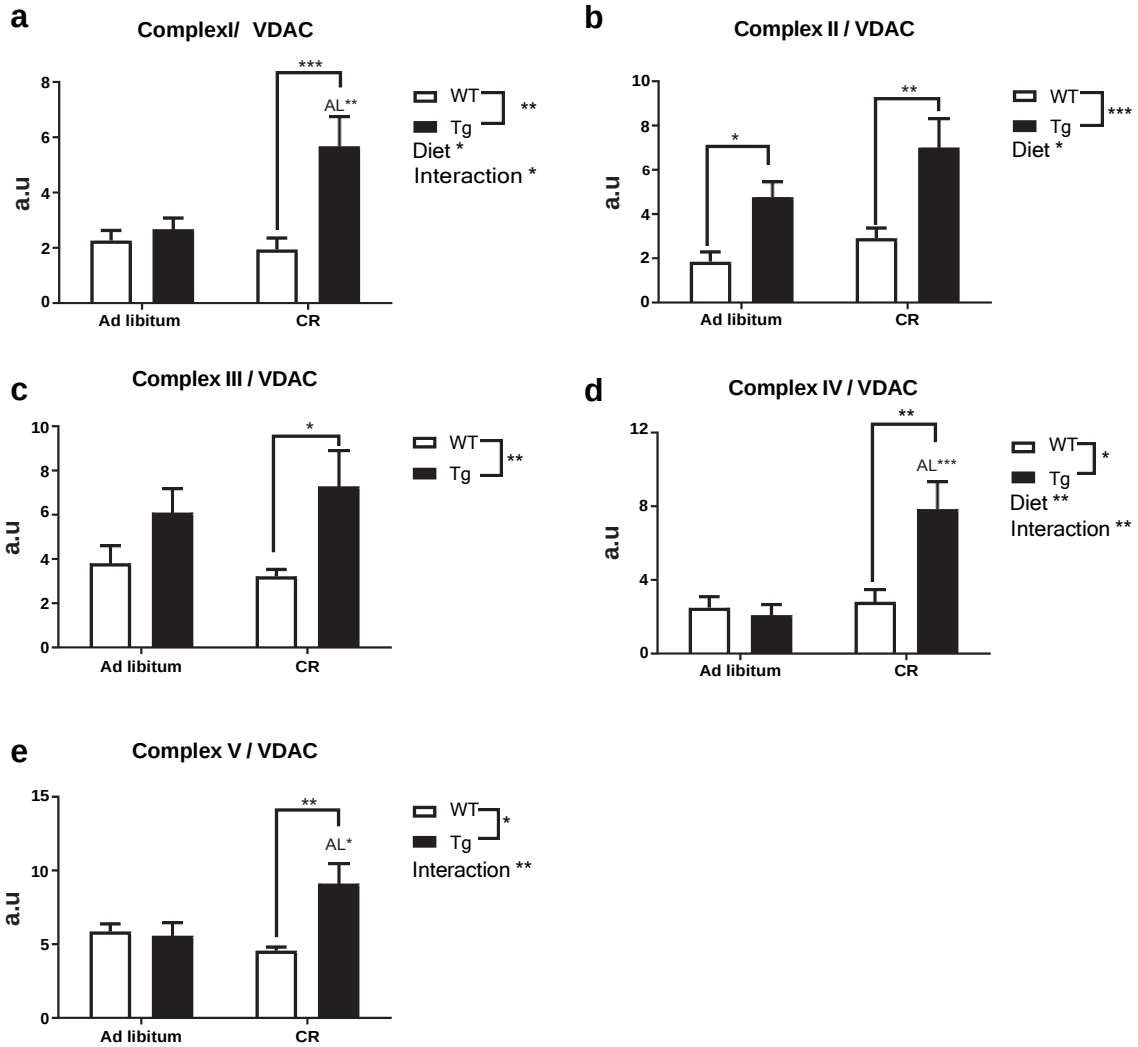

Fig. 9 Levels of mitochondrial complexes relative to VDAC in hind limb skeletal muscle. a to e, as in Fig. 6. Significant differences between groups or diets and genotype-diet interaction were found where noted

some of the metabolic adaptations induced by CYB5R3 overexpression are affected by CR. In accordance with this idea, our previous research has shown that increasing n-3 PUFA in tissues can be beneficial to mice fed ad libitum (Hagopian et al. 2010), while shortening lifespan in mice fed a CR diet (Lopez-Dominguez et al. 2015).

After being fed a standard diet for 4 months ad libitum, CYB5R3 mice showed no significant differences in body weight with respect to their WT controls, which is in agreement with our previous research in these mice fed closely related standard or high-fat diets (Martin-Montalvo et al. 2016). However, although CR produced a significant decrease of body weight in both genotypes, CYB5R3 overexpression led to a greater preservation of body weight—and to a greater extent of liver weight. Thus, CYB5R3 emerges for the first time as a key determinant in the control of organ and body weight in mice under CR.

Quantification of CYB5R3 polypeptide levels in liver and skeletal muscle homogenates from mice fed ad libitum also confirmed our previous observations, with a modest although statistically significant increase of CYB5R3 polypeptide in liver and a dramatic augmentation in skeletal muscle from Tg in comparison with WT mice (Martin-Montalvo et al. 2016). Skeletal muscle is thus a suitable model to study the direct effects of CYB5R3 overexpression in the cellular physiology.

While CR did not affect CYB5R3 levels in skeletal muscle, this intervention produced a decrease in liver, particularly in Tg mice. The decrease of hepatic CYB5R3 in young mice submitted to CR is in accordance with our previous research carried out in Fischer-344 rats (De Cabo et al. 2004) and C57BL/6J mice (Lopez-Lluch et al. 2005), which allowed us to document that CR has a dual effect on CYB5R3 levels in liver plasma membrane. Of note, while a decrease in a CYB5R3-dependent activity was observed in young animals fed a CR diet, a remarkable increase was observed in old animals fed the CR diet lifelong. Furthermore, hepatic expression and polypeptide levels of CYB5R were also decreased in C57BL/6J mice after 18-h fasting, which was confirmed in HepG2 and Hepa1.6 cell lines cultured under glucose deprivation (Jakobs et al. 2014). In brain, CYB5R3 levels were unchanged by CR in young, but markedly increased in old Ficher-344 rats fed a CR diet. A substantial increase of CYB5R3 levels was also observed in SH-SY5Y neuroblast cells cultured with serum obtained from rats that had been fed under CR, in comparison with cells cultured with serum obtained from rats fed ad libitum (Hyun et al. 2006). Taken together, these observations demonstrate the existence of age and tissue-specific mechanisms regulating CYB5R3 levels by CR.

Of note, whereas the expression of ectopic CYB5R3 in Tg mice is controlled by a viral promoter (CMV), expression of the endogenous CYB5R3 gene is controlled by SP1 transcription factor (Toyoda et al. 1995), which can be further upregulated through FOXO3a and Nrf2 under conditions of nutritional and oxidative stress (Siendones et al. 2014). Since the levels of CYB5R3 polypeptide were similarly affected by CR in both WT and Tg mice, it is very likely that this regulation is post-transcriptional.

Previous research has firmly established that CR results in increased mitochondrial number and mass, and it may reduce ROS production without decreasing cellular respiration (Civitarese et al. 2007; Lopez-Lluch et al. 2006). However, how CR targets mitochondrial metabolism in mice overexpressing CYB5R3 is completely unknown. To elucidate the putative crosstalk between CR and CYB5R3 overexpression in the regulation of mitochondrial mass and function, we used here a battery of antisera raised against key mitochondrial proteins, including VDAC, different subunits of mitochondrial complexes, the mitofusins Mfn1 and Mfn2, the fission factor Fis1 and two transcription factors involved in mitochondrial biogenesis: Nrf1 and TFAM.

VDAC is an abundant protein of the outer mitochondrial membrane whose levels serve as a biochemical estimate of mitochondrial mass (Grünewald et al. 2014). No significant differences among experimental groups were found for hepatic VDAC, but a substantial increase was observed in skeletal muscle from WT mice on CR in comparison with their ad libitum controls, supporting a higher mitochondrial abundance in this tissue under CR conditions. In accordance with previous investigations (Villalba et al. 2015), our results support that mitochondrial metabolism is rapidly adapted to CR in skeletal muscle compared with other tissues as liver, although another pathways which also affects the rate of aging, as mTOR signalling and the ubiquitin-proteasome pathway, were not affected by CR in skeletal muscle from young rats but a substantial inhibition was achieved in middle-age animals (Chen et al. 2019). The observations reported here are however in contrast with our previous studies based on a quantitative approach using electron microscopy, which allowed us to document that a 6-month CR intervention based on AIN93G diet was sufficient to increase mitochondrial abundance in mouse liver (Khraiwesh et al. 2014; Khraiwesh et al. 2013) whereas these changes were not detected in skeletal muscle (Gutierrez-Casado et al. 2019). Since the amount of fat constitutes the main difference between AIN93G (7% fat) and AIN93M (4% fat) diets, the possibility exists that this factor plays a key role in determining the rate of mitochondrial adaptations of different tissues to CR. However, controversial results regarding changes of mitochondrial mass with CR and aging may also arise from the heterogeneity in the methods used to assess mitochondrial content (Cartee et al. 2016), as discussed in our previous publication (Gutierrez-Casado et al. 2019).

Interestingly, CYB5R3 overexpression abated the increase of VDAC levels in skeletal muscle from mice fed under CR, an effect that might be related to the inhibition of mitochondrial β-oxidation and the preferential use of carbohydrate metabolism in Tg mice (Martin-Montalvo et al. 2016). Mfn2 (but not Mfn1) and TFAM were dramatically increased and NRF1 tended to increase by CR in liver of WT mice, which is partially in accordance with a previous research that documented the upregulation of hepatic Mfn2 and TFAM mRNA in mice fed under CR for 3 or 12 months starting at 2 months of age (Nisoli et al. 2005). However,

this research also evidenced an increase of Mfn1 mRNA that we have not confirmed here at the level of polypeptide. In accordance with our results, NRF1 mRNA levels increase in HeLa cells cultured with CR serum and this effect is inhibited by insulin (Lopez-Lluch et al. 2006), and TFAM and NRF1 protein levels decrease with aging in rat liver, and this decrease can be prevented by CR (Picca et al. 2013). According to our results, CR not only prevents the aging-related decrease of hepatic TFAM in old animals, but it even increases the level of this factor in young mice. The lack of Fis1 changes in WT mice fed under CR is in contrast with the increase reported for this fission factor in mice fed an AIN-93G diet for 6 months under CR conditions (Khraiwesh et al. 2013). This highlights again the importance of dietary fat content (AIN-93G vs. AIN-93M) and/or duration of intervention (6 months vs. 4 months) in determining the outcome of CR on mitochondrial dynamics.

Of note, CYB5R3 overexpression abated (for Mfn-2 and Fis1) or at least attenuated (for TFAM) the effect of CR in lever. CYB5R3 overexpression led to an increase of hepatic Fis1 in mice fed ad libitum, but this effect was abated by CR. Moreover, CYB5R3 overexpression also led to decreased hepatic NRF1, indicating a global effect on mitochondrial metabolism, dynamics and biogenesis in mice fed under CR. Mfn2 is a key protein of facilitating of contacts between endoplasmic reticulum and mitochondria which might result in an improvement in the processing of fat deposits (de Brito and Scorrano 2008; Rieusset 2018). The absence of CR effect of hepatic levels of Mfn2 in Tg mice and the decrease of TFAM and NRF1 in Tg mice fed under CR in comparison with WT mice fed the same diet might be related to the enhancement of carbohydrate metabolism in mice overexpressing CYB5R3 (Martin-Montalvo et al. 2016).

Changes elicited by CR and/or CYB5R3 overexpression in skeletal muscle were different to those observed in liver, indicating the existence of tissue specificity for mitochondrial adaptations to the interventions. In skeletal muscle, we evidenced an increase in the level of Mfn1 protein by CR in both genotypes, although Mfn2 was unaffected. It has been demonstrated elsewhere that CR increases the protein levels of Mfn2 in skeletal muscle of female swiss mice fed for 6 months with the same diet used here (AIN93M) starting at 1 month of age (Cerqueira et al. 2011). This discrepancy may be caused by differences in sex and strain (Mitchell et al. 2016). Previous results of our own group have also demonstrated an increase of Mfn2 levels in skeletal muscle of C57BL/6 mice fed the AIN93G diet under CR conditions for 6 months starting at 3 months of age (Gutierrez-Casado et al. 2019). This fact revels again the importance of the amount of fat in the diet. The increase of Mfn1 in skeletal muscle of mice of both genotypes fed under CR could indicate an increase in mitochondrial fusion needed to protect against autophagic degradation of these organelles (Tilokani et al. 2018). Additional studies will be necessary to elucidate putative changes in autophagy by CYB5R3 overexpression in mice fed under CR.

Unlike the results obtained in liver, levels of TFAM were not affected by CR but, interestingly, we evidenced a general effect of CYB5R3 overexpression increasing this factor independently of diet. While Civitarese et al. (2007) have demonstrated that TFAM mRNA is increased in muscle of healthy humans under CR, the intervention was not evaluated for TFAM at the levels of polypeptide. The dramatic increase of NRF1 polypeptide in skeletal muscle from WT mice fed under CR is in accordance with the similar increase observed for the mitochondrial abundance marker VDAC (see above). Of note, as also found for VDAC, the increase of NRF1 levels by CR was not observed in Tg mice, reinforcing the interference of both interventions.

While we found no major changes in the marker of hepatic mitochondrial abundance (VDAC) in liver tissue, an increase of fatty acid β-oxidation in response to CR could influence the activity and abundance of mitochondrial complex subunits (Chen et al. 2012a). Levels of complex III were increased by CR in both WT and Tg mice, which could facilitate electron flow through the mitochondrial chain. In skeletal muscle, CYB5R3 overexpression by itself increased the levels of complexes II and III in mice fed ad libitum, and CR apparently produced a generalized increase in the levels of mitochondrial complexes, although this effect seemed to be blunted when the dietary intervention was combined with CYB5R3 overexpression. Nevertheless, when the levels of mitochondrial complexes were normalized to those of VDAC to analyse those intrinsic changes taking place in the mitochondrial membranes independently of changes accounted by alterations in mitochondrial abundance, we were able to evidence a consistent effect of CYB5R3 overexpression to increase the levels of ETC complexes in both tissues, being the effect particularly striking in skeletal muscle. It has been shown that the increase in the levels of complex I by CR does not

produce an increase in the levels of ROS in skeletal muscle (Chen et al. 2012b). The increase of ETC complexes could indicate a higher production of ATP without higher ROS production in Tg mice, as we have already demonstrated in liver (Martin-Montalvo et al. 2016).

In summary, our data support that many alterations elicited by CR in mitochondrial abundance and dynamics can be hindered by CYB5R3 overexpression, but these effects are compensated by a greater abundance of mitochondrial ETC complexes in Tg mice submitted to this dietary intervention.

Authors' contributions JMVM and RdC conceived and designed the project; SRL was responsible of raising and maintaining the colony of mice; SRL and SLB performed the experimental determinations and conducted the data analysis; JAGR and MIB provided valuable advice; SRL, SLB and JMVM wrote the manuscript with approval from all authors.

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Compliance with ethical standards All animals were cared for in accordance with the University of Córdoba policy for animal welfare, which complies current European, Spanish and Andalusian regulations and is in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. This study was approved by the bioethics committee of the University of Córdoba and authorized by the Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía (authorization code: 20/04/2016/053).

Conflict of interest The authors declare that they have no conflicts of interest.

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Priority Research Paper

Cdkn1a transcript variant 2 is a marker of aging and cellular senescence

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ABSTRACT

Cellular senescence is a cell fate response characterized by a permanent cell cycle arrest driven primarily the by cell cycle inhibitor and tumor suppressor proteins $p16^{Ink4a}$ and $p21^{Cip1/Waf1}$. In mice, the $p21^{Cip1/Waf1}$ encoding locus, *Cdkn1a*, is known to generate two transcripts that produce identical proteins, but one of these transcript variants is poorly characterized. We show that the *Cdkn1a* transcript variant 2, but not the better-studied variant 1, is selectively elevated during natural aging across multiple mouse tissues. Importantly, mouse cells induced to senescence in culture by genotoxic stress (ionizing radiation or doxorubicin) upregulated both transcripts, but with different temporal dynamics: variant 1 responded nearly immediately to genotoxic stress, whereas variant 2 increased much more slowly as cells acquired senescent characteristics. Upon treating mice systemically with doxorubicin, which induces widespread cellular senescence *in vivo*, variant 2 increased to a larger extent than variant 1. Variant 2 levels were also more sensitive to the senolytic drug ABT-263 in naturally aged mice. Thus, variant 2 is a novel and more sensitive marker than variant 1 or total $p21^{Cip1/Waf1}$ protein for assessing the senescent cell burden and clearance in mice.

INTRODUCTION

The stringent cell growth arrest associated with cellular senescence is determined, among other mechanisms, by activities of cyclin-dependent kinase inhibitor proteins $p16^{Ink4a}$ and $p21^{Cip1/Waf1}$, encoded by the *Cdkn2a* and *Cdkn1a* loci, respectively [1]. The increased expression of these proteins is a major hallmark of senescence in most cells, and therefore have become markers of senescence both in culture and *in vivo*. Consistent with the fact that senescent cells increase with age in many mouse and human tissues, *Cdkn2a* ($p16^{Ink4a}$) mRNA levels also increase with age in these tissues [2]. Based on this association, transgenic mice have been generated

to detect [3] and selectively eliminate senescent cells *in vivo* [4, 5]. By contrast, despite having a key role in the senescence growth arrest, *Cdkn1a*/p21$^{Cip1/Waf1}$ upregulation during aging is often moderate or absent *in vivo*, and tissue-dependent [2, 6–8]. For example, p21$^{Cip1/Waf1}$ reporter mouse have shown increased reporter activity only in kidneys of 23.5 month-old mice [9]. Further, in most tissues, calorie restriction does not prevent the age-related increase in *Cdkn1a* expression, in contrast to *Cdkn2a* expression [7]. Consequently, the role of *Cdkn1a*/p21$^{Cip1/Waf1}$ as an *in vivo* marker of aging or cellular senescence remains uncertain.

Two transcript variants are currently annotated for the murine *Cdkn1a* gene (Figure 1A). The better-studied mRNA (*Cdkn1a* transcript variant 1, NM_007669.5, hereafter termed p21var1) contains three exons, the two latter of which encode the p21$^{Cip1/Waf1}$ protein. An alternative transcript (*Cdkn1a* transcript variant 2, NM_001111099.2, hereafter termed p21var2) differs from p21var1 in the first exon and therefore contains an almost entirely different 5' untranslated region (UTR), despite encoding the same protein [10]. Additionally, the p21var2 transcription start site (TSS) lies ~2.8 kb upstream of p21var1, and thus might be subject to regulation by elements not present in p21var1. Interestingly, the p21var2 is generally less abundant than p21var1, but the translation of the p21var2 transcript increases under nutrient stress; consequently, the relative contribution of each variant to the total pool of p21$^{Cip1/Waf1}$ protein likely varies depending on stress and possibly other conditions [11]. To date, possible changes in the expression of *Cdkn1a* transcript-specific variants during age or cellular senescence have not been explored.

To fill this gap in our knowledge, we explored the expression levels of each *Cdkn1a* transcript variant in several tissues from aged mice. We also analyze their expression levels in a cell culture model of mouse cells subjected to genotoxic stress-induced senescence to evaluate their relative utility as senescence markers both in culture and *in vivo*. Finally, we show that well-established regulators of *Cdkn1a* expression have variant-specific effects, which add a novel level of complexity to the biological roles of p21$^{Cip1/Waf1}$.

RESULTS

Cdkn1a transcript variant 2 is preferentially induced with age

To assess expression levels of the individual *Ckdn1a* mRNA transcript variants, we designed two primer sets in which the forward primers hybridize with the variant-specific first exons (Figure 1A). To determine whether differential regulation occurs during aging *in vivo*, we analyzed liver samples from male mice at 2, 11, 23, 25 and 30 months of age. Relative to 2 month-old mice, p21var2, but not p21var1, increased after 20 months of age (Figure 1B). We then obtained additional tissues from 2 and 24 month-old male and female animals (n = 12-15 per sex and age). p21var2 levels were higher than p21var1 levels in aged liver, white adipose tissue, kidney, heart and lung (Figure 1C–1G). Steady-state levels of p21var1 remained unaltered with age, and were even slightly reduced with age in liver. On average, p21var2 abundance increased 3-fold with age in liver, kidney and adipose tissue, and 2-fold in heart and lung. Mice at 4 months of age used as young control yielded equivalent results (data not shown). The transcript encoding p16^{Ink4a} also increased with age in all these tissues (Supplementary Figure 1A–1E).

When males and females were analyzed separately, p21var2 increased with age in all tissues and in both sexes (Supplementary Figure 2A–2J), with the only exception of heart in male mice, where the upwards trend did not reach statistical significance (Supplementary Figure 2D). Of note, we detected higher p21var1 levels in the kidney of aged females (Supplementary Figure 2H). Despite the propensity of p21var2 to increase with age, overall, in all organs tested, p21var1 was more abundant than p21var2, as reported [10]. In sum, p21var2 expression is consistently elevated with age, in contrast with an absence of age-related change in p21var1 levels.

Both *Cdnk1a* transcript variants are induced in cellular senescence

To determine whether either transcript variant is preferentially upregulated in senescent cells, we induced senescence in primary mouse dermal fibroblasts (MDFs) using 15 GY ionizing radiation (IR), which induces a senescence response in virtually all the irradiated cells. Seven days later, irradiated, but not sham-irradiated, MDFs showed hallmarks of senescence, including increased levels of the mRNA encoding p16^{Ink4a} and lower levels of the mRNA encoding lamin-B1, as expected [12] (Supplementary Figure 3A–3B). The levels of both p21var1 and p21var2 also increased. However, as reported [10], p21var2 levels were 6- to 8-fold lower compared to p21var1 (data not shown). These data show that both of the murine *Ckdn1a* transcript variants are valid readouts to evaluate cellular senescence in cultured MDFs.

We then tested the dynamics of *Cdkn1a* variant expression in MDFs after irradiation. Expression of p21var1 increased 3 hours after irradiation, then progressively declined to a level twice that of baseline

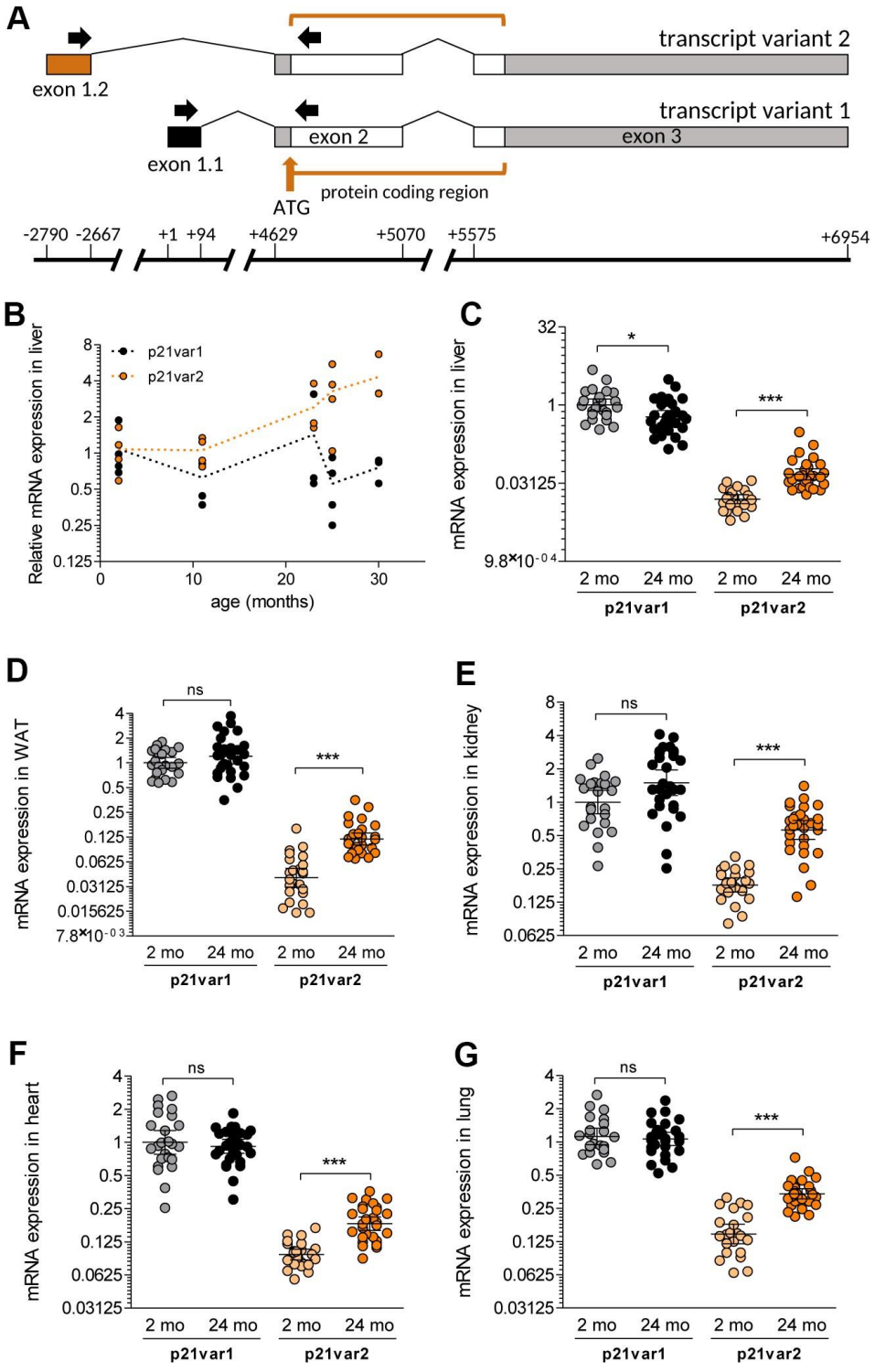

Figure 1. The *Cdkn1a* variant 2 transcript is preferentially induced during aging. (**A**) We designed primers (black arrows) to specifically detect *Cdkn1a* variant 1 and 2 transcripts, spanning the first and second exons in each case. The protein-coding region is indicated as well as the ATG start codon (brown arrow). Transcription starts at +1 for p21var1 and at -2790 for p21var2. The first and last bases of each exon are also indicated. (**B**) mRNA levels of p21var1 and p21var2 in the livers of male mice aged 2 to 30 months of age, normalized to levels in livers of 2 month-old animals. (**C**–**G**) Levels of each *Cdkn1a* transcript were assessed in 2 (young) and 24 (old) month-old mice. Animals were young males (n = 12), young females (n = 12), old males (n =14-15) and old females (n = 14-15). Results are shown for (**C**) liver, (**D**) adipose tissue, (**E**) kidney, (**F**) heart and (**G**) lung. In (**C**–**G**) data were normalized to p21var1 levels in young mice. Note Y axes are log-2 scales. 1-way ANOVA and Tukey post-tests were applied. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not-significant.

by 12 hours after irradiation. In contrast, p21var2 levels remained unaltered for the first 24 hours after irradiation (Figure 2A). Thereafter, both *Cdkn1a* variants steadily increased from day 3, without reaching a plateau by the end of the 12-day time course. Establishment of senescence was verified by increased $p16^{Ink4a}$ and decreased lamin B1 mRNA levels (Figure 2B). Treatment of MDFs with 250 nM doxorubicin (doxo), a chemotherapeutic agent known to cause cellular senescence in culture and *in vivo* [13], increased p21var1 levels within the 24 hours, followed by a smaller increase in p21var2 levels (Figure 2C). Similar to the pattern in irradiated cells, from day 5 onwards both variants were coordinately and increasingly upregulated, concomitant with senescence-associated changes in $p16^{Ink4a}$ and lamin B1 expression (Figure 2D). Together, our results show that p21var1 increases preferentially shortly after acute genotoxic stress, but both variants gradually rise as cells enter a senescent state.

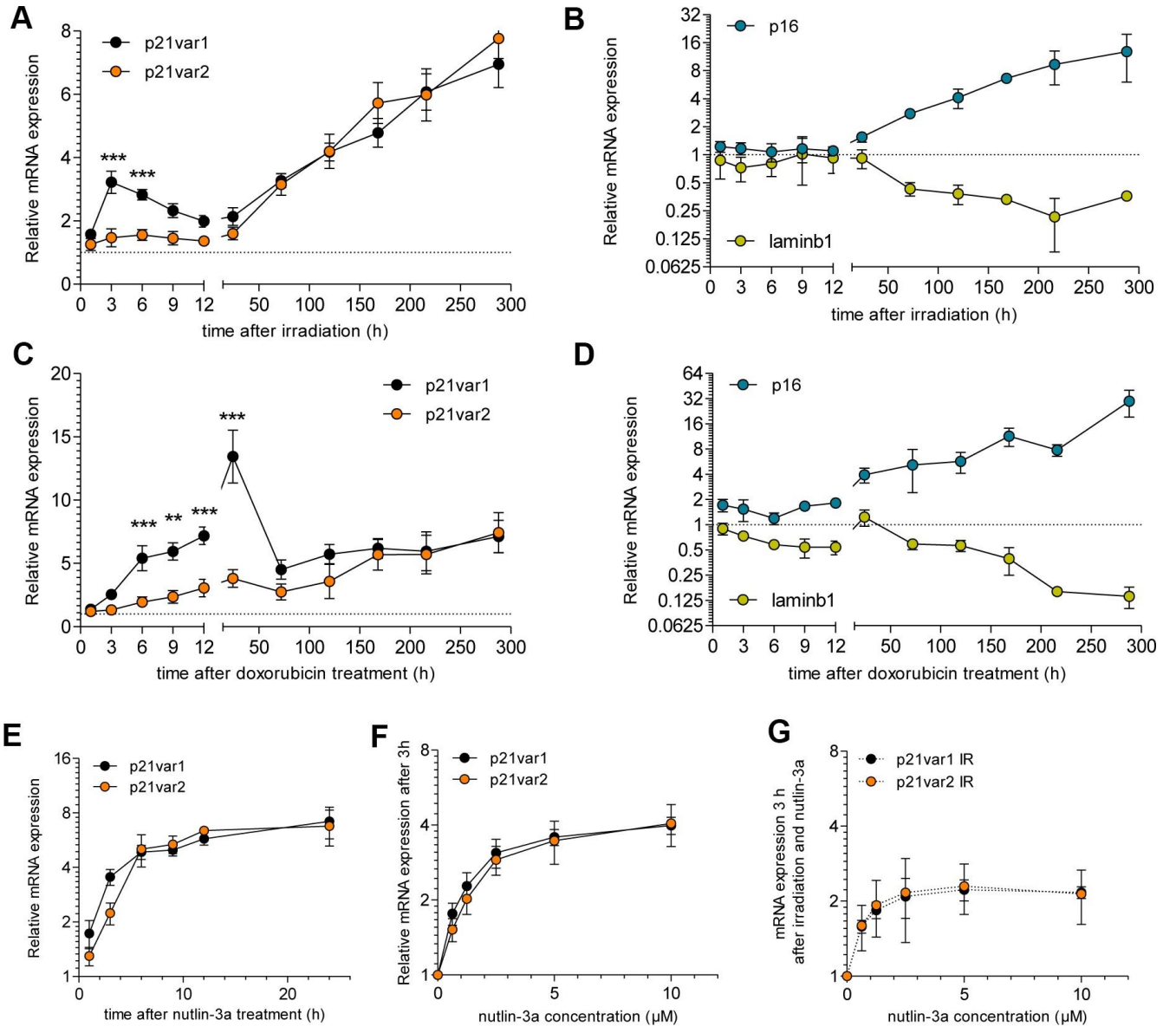

Figure 2. *Cdkn1a* variant 2 increases as cells acquire senescent phenotypes in culture. Time course of (**A**) p21var1 and p21var2 levels and (**B**) $p16^{Ink4a}$ and lamin B1 mRNA levels after 15 Gy irradiation. 2-way ANOVA test was applied. Time course of (**C**) p21var1 and p21var2 levels and (**D**) $p16^{Ink4a}$ and lamin B1 levels after a 24 h exposure to 250 nM doxorubicin. 2-way ANOVA test was applied. (**E**) p21var1 and p21var2 levels in MDFs after treatment with 10 μM nutlin-3a. (**F**) mRNA levels 3 hours after treatment with increasing doses of nutlin-3a. (**G**) mRNA levels 3 hours after irradiation (15 Gy) and treatment with increasing doses of nutlin-3a. Mean ± SEM is shown. Note Y axes are log-2 scales. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

p53 stabilization upregulates both *Cdkn1a* transcript variants

The basal expression of p21var1 and p21var2 is differentially regulated by p53, likely due to the proximity of the p21var2 transcription start site (TSS) to a p53-response elements (p53-Res) [10]. We performed a promoter analysis (TRANSFAC version 2018.3) for both *Cdkn1a* TSSs, spanning 2.5 kb upstream and 0.5 kb downstream of each TSS (Supplementary Information). Among a plethora of predicted transcription factor binding sites, p53-REs were detected within 500 bases upstream of both TSSs, even when we applied the most stringent algorithm. To understand the p53 responsiveness of the *Cdkn1a* transcript variants, we treated MDFs with 10 μM nutlin-3a, an MDM-2 inhibitor that stabilizes p53 [14]. The levels of both variants increased within 1 hour, reaching a plateau approximately 12 hours later (Figure 2E). Lower concentrations of nutlin-3a failed to reveal any sensitivity differences between the variants. Three hours after treatment, the dose-response curves were similar in shape for both *Cdkn1a* transcripts (Figure 2F). The same was true for senescent (irradiated) MDFs treated with 10 μM nutlin-3a (Figure 2G). These results suggest that p21var1 and p21var2 are equally sensitive to transcriptional upregulation upon p53 stabilization.

Circadian regulation of $p21^{Cip1/Waf1}$ does not involve transcript variant 2

To further explore the expression pattern of p21var2 *in vivo*, we asked whether it is subject to circadian regulation, as described for $p21^{Cip1/Waf1}$ [15]. We euthanized 2 month-old male mice at 3 hour intervals for 12 hours. In liver samples, p21var1 mRNA levels were highest at the end of the dark cycle (6:00 am Pacific time) and progressively decreased 8-fold to a minimum in the afternoon (Figure 3A). The p21var2 remained unaltered, at lower levels, throughout the same period. A similar pattern was observed in adipose and kidney tissue (Figure 3B, 3C). These results indicate that the circadian regulation of $p21^{Cip1/Waf1}$ is driven solely by expression of *Cdkn1a* transcript variant 1.

Expression of p21var2 *in vivo* is increased by doxorubicin and decreased by ABT-263

To determine how *Cdkn1a* transcript variants are expressed when cellular senescence is induced *in vivo*, we induced cellular senescence *in vivo* by intraperitoneal injection of a single dose (10 mg/kg) of doxorubicin. After 6 weeks, p21var1 increased 3-fold while p21var2 increased 25-fold (reaching levels comparable to those of p21var1 at baseline) in the livers of treated mice (Figure 3D and Supplementary Figure 4A, 4B). Similar elevations were observed in the adipose tissue and kidneys (Figure 3E, 3F and Supplementary Figure 4C–4F) of male and female mice, consistent with the widespread luminescence reported in doxorubicin-treated p21-reporter mice [9]. Our data suggest that p21var2 is a more sensitive readout for doxorubicin-induced senescence *in vivo* than p21var1.

ABT-263 (navitoclax) has been shown to clear senescent cells by inhibiting Bcl-2 and related anti-apoptotic proteins [16, 17]. In a mixed cohort of 20-22 month-old male and female mice, ABT-263 specifically reduced p21var2 levels in adipose tissue and kidney, whereas p21var1 levels remained unaltered (Figure 3H–3L). There were no significant changes in the levels of either variant in liver (Figure 3G). Together, these results reinforce the idea p21var2 is a better marker than p21var1 for assessing the presence of senescent cells *in vivo*.

DISCUSSION

Cdkn1a transcript variant 2 has received little attention since it was first described, likely because the encoded protein is identical to that encoded by variant 1, and both variants are regulated by p53. Our results are, to our knowledge, the first to study *Ckdn1a* transcript variants in the context of aging. We show that, although tissue-specific exceptions may arise, p21var2 but not p21var1 is a better candidate marker of aging and senescence in mice.

Our findings help explain previous reports regarding changes in $p21^{Cip1/Waf1}$ with age. Thus, given the architecture of the promoters for both transcript variants, a $p21^{Cip1/Waf1}$ reporter mouse that included 2.5 kb of the *Cdkn1a* upstream sequence [9] would not detect p21var2, unlike a reporter mouse containing 4.5 kb of *Cdkn1a* upstream sequences [18] or a knock-in $p21^{FLuc}$ reporter mouse [19]. In line with our data, the reporter mouse containing 2.5 kb of the *Cdkn1a* promoter did not detect an age-related increase in expression, except for the kidney, in which we also observed an increase in p21var1 levels in old females. Our findings clarify the interpretation of previous and future results on the age-related changes in expression of this locus.

Key to understanding the biological context of *Cdkn1a* transcriptional regulation is the different circadian expression pattern between both transcript variants. Thus, comparisons among previous studies may be confounded by an absence of data regarding the time of euthanasia, a limitation that could be, in principle, avoided by measuring p21var2. In the liver, the wide

circadian oscillation of p21var1 contrasts with the more subtle age-related changes in p21var2. This disconnect suggests that p21var1 would be, in accordance to its higher relative contribution to the *Cdkn1a* mRNA pool, the main source of $p21^{Cip1/Waf1}$ for homeostatic functions [9], whereas p21var2 could be more selectively responsive to stress signals and ultimately more reflective of cellular senescence *in vivo*.

Our data do not explain why an increase in p21var2, but not p21var1, which is also observed in cultured senescent cells, is detected in aged animals. One possibility is that some transcriptional modulators are evident *in vivo*, but not necessarily in cultured cells. In addition, there may be cell type-specific expression of *Cdkn1a* variants in senescent cells. Our data show that p53 stabilization cannot explain the expression dynamics of *Cdkn1a* variants upon genotoxic-stress. However, the differences between the variant promoters, of which our analysis provides only an initial hint, may well affect which variant is expressed under different circumstances.

A recent report describes how the dynamics of $p21^{Cip1/Waf1}$ levels in response to DNA damage determine whether cancer cells enter a permanent cell

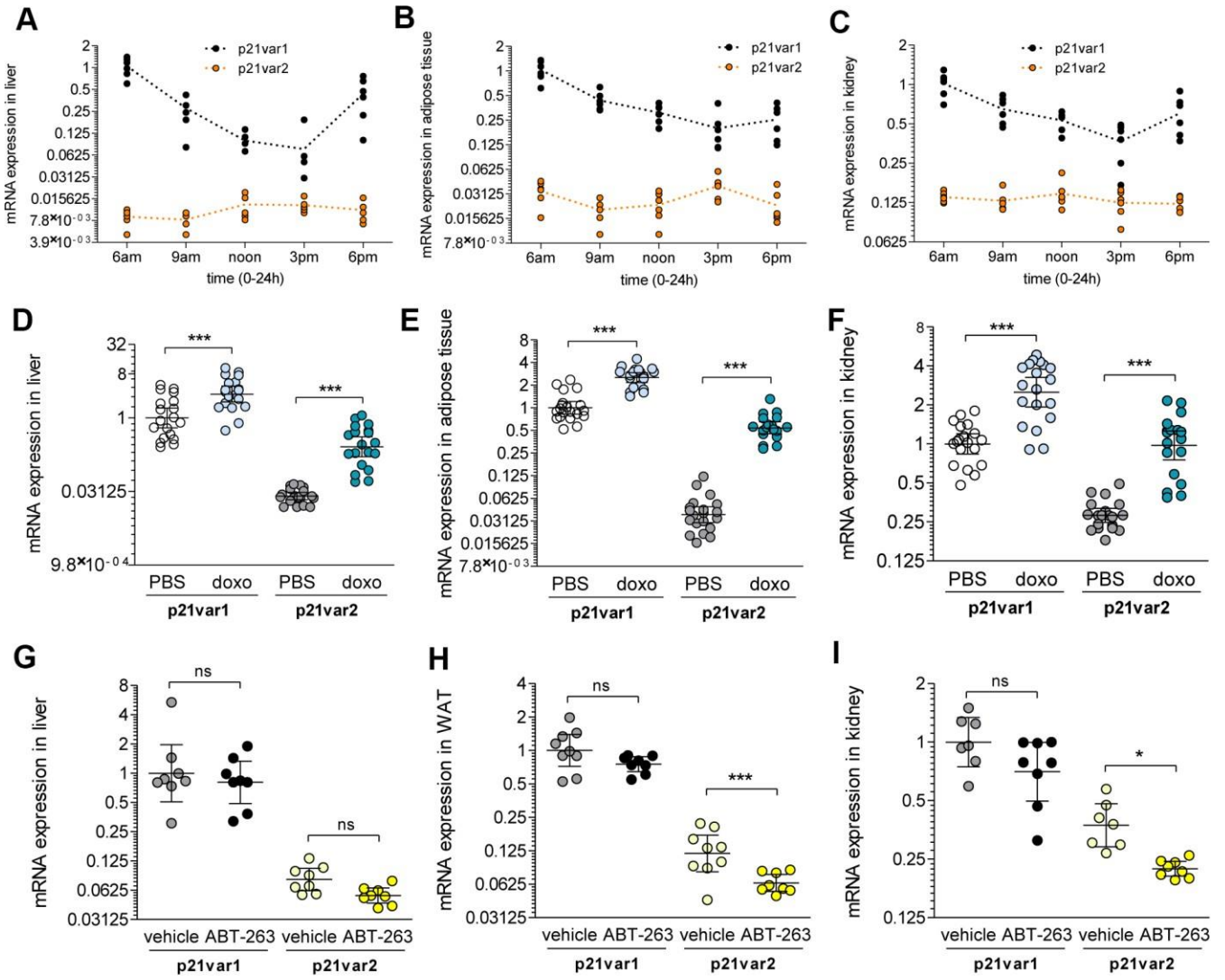

Figure 3. *Cdkn1a* variant 1 is circadian regulated, whereas variant 2 rises with senescence *in vivo*. (**A**) *Cdkn1a* variants expression throughout the light cycle (6 am to 6 pm) in the liver, (**B**) white adipose tissue and (**C**) kidney of 6 week-old male mice. (**D**–**F**) A cohort of 6 week-old mice were treated with doxorubicin or vehicle (n = 9-10 for either males or females, n = 9-10 for either vehicle or doxorubicin) and *Cdkn1a* variant levels were analyzed 6 weeks later. Results are shown for (**D**) liver, (**E**) adipose tissue and (**F**) kidney. (**G**–**I**) A cohort of 18 to 22 month-old mice were treated with ABT-263 or vehicle. Pooled results are shown for (**G**) liver, (**H**) adipose tissue and (**I**) kidney. 1-way ANOVA and Tukey post-tests were applied. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.

cycle arrest or return to proliferation [20]. It remains unexplored the possibility that the different transcript variants are preferentially associated with one or other cell fate. Additionally, translational regulation at the 5' UTR of the variants has been shown to affect cell fate [11, 21]. Human cells also express several *Cdkn1a* transcript variants [22]. Among the ten human transcript variants currently annotated, at least one (transcript variant 4) shares translational regulatory mechanisms with the murine p21var2 [21]. Interestingly, even though murine variant 2 and human variant 4 do not appear to share sequence homology, the translational regulation in both transcripts is driven by the integrated stress response and results in cell cycle arrest [21]. The potential relevance of this mechanism for cellular senescence in humans remains unknown, and the functions and interrelations of the different *Cdkn1a* transcript variants have not been studied in depth. Our findings may thus lead to a better understanding of the age-related functions of p21 and improve our ability to monitor the effectiveness of anti-aging therapies.

MATERIALS AND METHODS

Mouse models

Animal husbandry

C56BL/6 mice were purchased from The Jackson Laboratory and allowed to acclimate to the Buck Institute facilities. The animals were group housed under controlled conditions of temperature (22-24° C), humidity (40-60%), and a 12 h light-dark cycle. All animal procedures were approved by the Buck Institute Institutional Animal Care and Use Committee (IACUC).

Aging cohorts

C56BL/6 were aged at the Buck Institute vivarium and male and female littermates derived from the same colony were used as young controls.

Doxorubicin treatment

6-week old male and female C57BL/6 littermates were intraperitoneally (IP) injected with a single dose of doxorubicin (10 mg/kg) to induce widespread senescence [13]. After 6 weeks, the animals were euthanized by CO_2 inhalation followed by cervical dislocation, and tissues were harvested and flash frozen in liquid nitrogen.

ABT-263 treatment

ABT-263 or vehicle (5% DMSO, 95% corn oil) was delivered to 20-month old C57BL/6 littermates via IP injection at 50 mg/kg for 7 consecutive days per cycle for two cycles, with a 2-week interval between cycles. Animals were sacrificed one week after the last treatment cycle and tissues were collected and flash frozen in liquid nitrogen.

Cell culture

Mouse dermal fibroblasts

Primary mouse dermal fibroblasts (MDF) were obtained from postnatal day 2-3 C57BL/6 mice. Skin was excised from the mice and incubated overnight in 0.25% trypsin-EDTA. The dermis was mechanically disengaged and incubated with 10 mg/ml collagenase for 30 min at room temperature. The mixture was filtered and plated. MDF were cultured in 3% oxygen in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 100 U/ml streptomycin and penicillin.

Irradiation and doxorubicin treatment

MDFs were induced to senesce by X-irradiation (15 Gy), as described [23], or mock-irradiated (control). Alternatively, MDF were treated with 250 nM doxorubicin or vehicle (DMSO, control) for 24 h, then washed and cultured in fresh complete medium. Senescence markers were tested at different times (1, 3, 6, 9, 12 h and 1, 3, 5, 7, 9, 12 days) after treatment.

Nutlin-3a treatment

MDFs were incubated with 10 μM nutlin-3a or vehicle (DMSO, control) and RNA was collected 1, 3, 6, 9, 12 and 24 h later. MDFs were also treated with different nutlin-3a concentrations (0.6-10 μM), irradiated (15 Gy X-rays) or mock-irradiated and RNA collected 3 h later.

RNA extraction and RT-qPCR

RNA was extracted using Tri-reagent and isolated with the Direct-Zol RNA miniprep kit (Genesee Scientific). For adipose tissue, the RNeasy Lipid Tissue mini kit (Qiagen) was used. cDNA was synthesized using the High Capacity cDNA RT kit (Life Technologies). Transcripts were analyzed using a Roche LightCycler 480 II in 384-well plates and the UPL probe system. Bioline SensiFast Probe No-ROX was used as a master mix. Primer sequences and respective probes were as follows: Cdkn2a (p16), forward 5'-TCCTCGCAGTTCGAATCTG, reverse 5'-AACTCTTTCGGTCGTACCCC, with a custom designed probe (5'- /56-FAM/AGG TGA TGA /ZEN/TGA TGG GCA ACG TTC AC/3IABkFQ/ -3'); *Cdkn1a* (p21) variant 1, forward 5'-TCCACAG CGATATCCAGACA, reverse 5'-GGACATCACCA GGATTGGAC, with UPL probe 21; *Cdkn1a* (p21) variant 2, forward 5'-TTGCCAGCAGAATAAA AGGTG, reverse 5'-TTTGCTCCTGTGCGGAAC, with UPL probe 9; β-actin: forward 5'- CTAAG GCCAACCGTGAAAAG, reverse 5'- ACCAGAGG

CATACAGGGACA, with UPL probe 64; tubulin: forward 5'- CTGGAACCCACGGTCATC, reverse 5'-GTGGCCACGAGCATAGTTATT, UPL probe 88. Results were normalized to β-actin and tubulin. ΔΔCt values prior to logarithmic transformation were used for statistical analyses.

Promoter analysis

Transcription factor binding sites were analyzed using TRANSFAC version 2018.3 (geneXplain) and sequences between -2500 and +500 for each variant. For each variant, two analyses were performed, either minimizing false positives (minFP) or balancing false positives and false negatives (minSUM), using algorithms provided by the software.

Statistical analysis

Unless otherwise noted, results are shown as individual data points with geometric means and 95% CIs. Comparisons between multiple groups were analyzed using 1-way ANOVA with Tukey post-tests, or 2-way ANOVA with Bonferroni post-tests if a second variant (usually time) was present. No statistical outliers were removed.

AUTHOR CONTRIBUTIONS

J.A.L.-D., S.R.L. and U.A.C performed the experimental work. M.K. performed the *in vivo* ABT-263 experiment. J.A.L.-D., U.A.C, R.-M.L. P.-Y.D. and J.C. designed experiments and interpreted the results. J.M.V. and C.C. contributed to interpretation of the results. J.A.L.-D., P.-Y.D. and J.C. wrote the paper.

CONFLICTS OF INTEREST

J.C. is a cofounder of Unity Biotechnology, which is developing senolytic agents. R.-M.L. and J.C. are co-inventors on patent applications licensed to or filed by Unity Biotechnology.

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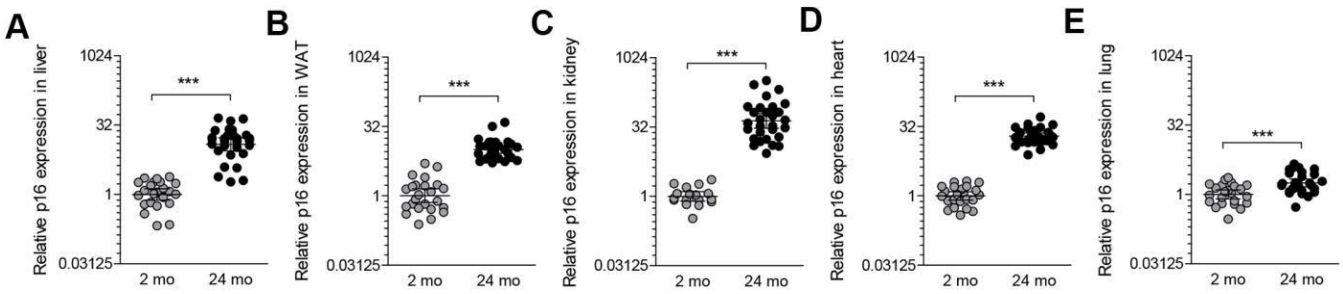
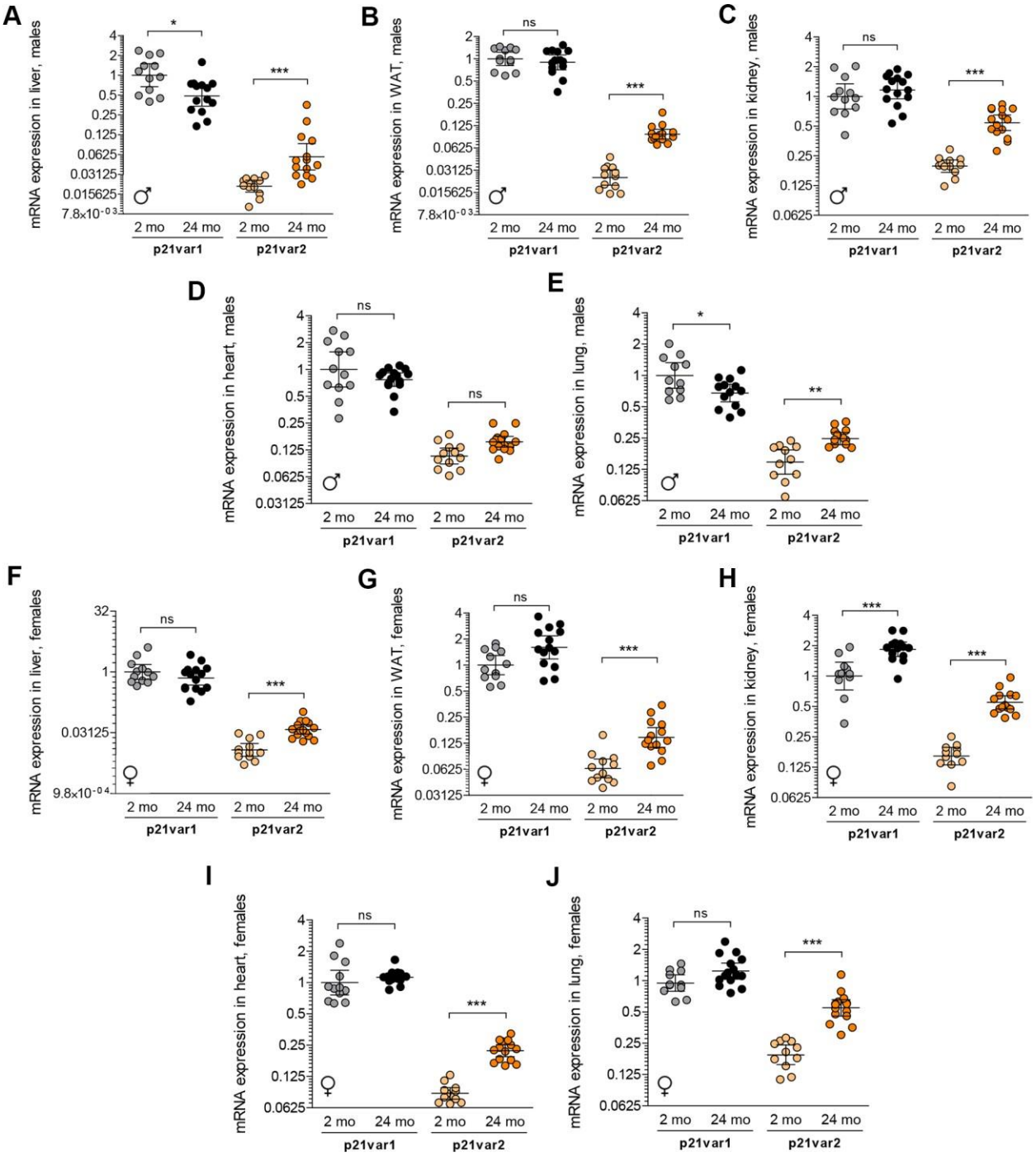
SUPPLEMENTARY MATERIALS

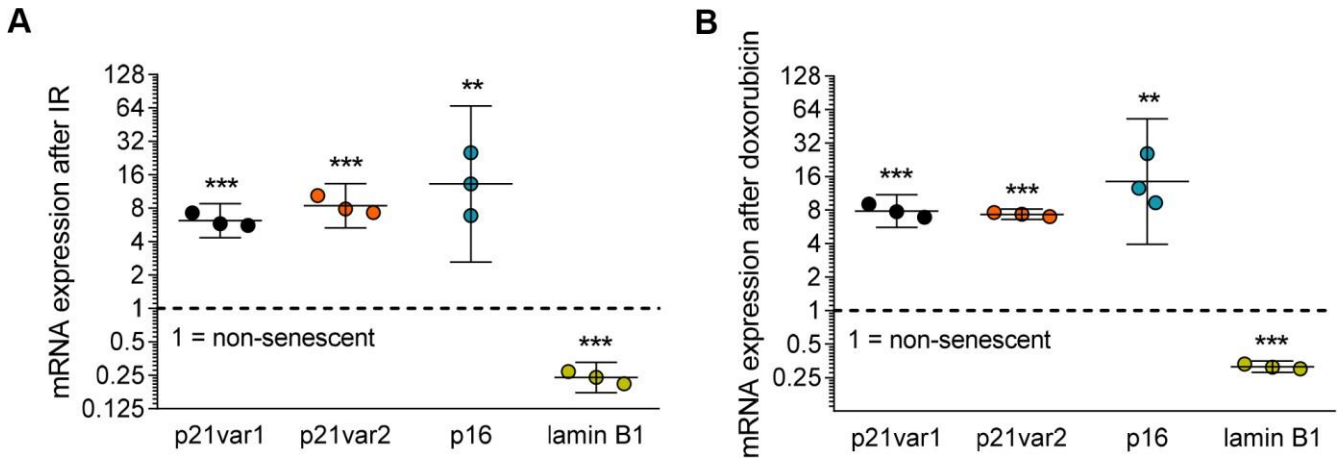
Supplementary Figures



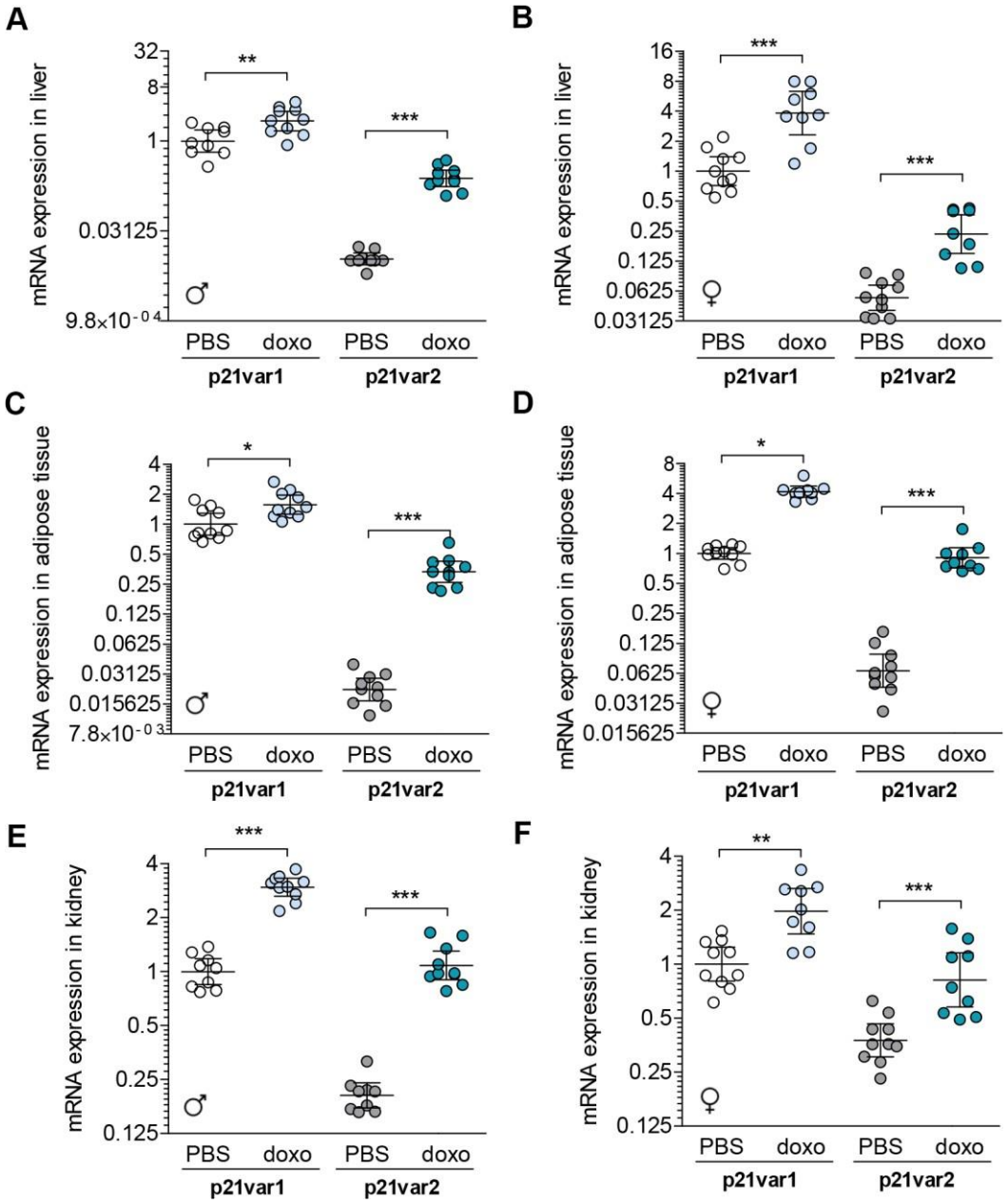
Supplementary Figure 1. p16^{Ink4a} mRNA levels increase with age in multiple tissues. mRNA levels were determined in (**A**) liver, (**B**) white adipose tissue, (**C**) kidney, (**D**) heart and (**E**) lung of 2 and 24 month old mice (n = 24-28). Males and females were pooled. Note Y axes are log-2 scales. t-tests were applied. *** $p < 0.001$.



Supplementary Figure 2. *Cdkn1a* transcript variants in young and aged male and female mice. Levels of *Ckdn1a* variants in the (**A**) liver, (**B**) white adipose tissue, (**C**) kidney, (**D**) heart and (**E**) lung of male 2 and 24 month old mice from Figure 1. Expression levels in the (**F**) liver, (**G**) white adipose tissue, (**H**) kidney, (**I**) heart and (**J**) lung of female mice at the same ages. Note Y axes are log-2 scales. 1-way ANOVA and Tukey post-tests were applied. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.



Supplementary Figure 3. Markers of senescence in cultured mouse dermal fibroblasts. (**A**) Levels of *Cdkn1a* transcript variants and p16Ink4a and lamin B1 mRNAs 7 days after irradiation (15 Gy). (**B**) Levels 7 days after a 24 h exposure to 250 nM doxorubicin. Note Y axes are log-2 scales. ** $p < 0.01$, *** $p < 0.001$ vs non-senescent control for each transcript (=1).



Supplementary Figure 4. *Cdkn1a* transcript variants in doxorubicin-treated male and female mice. Transcript levels were determined in (**A**, **B**) liver, (**C**, **D**) adipose tissue and (**E**, **F**) kidney of male or female 3.5 month old mice, 6 weeks after treatment with doxorubicin (n = 9-10 per sex or treatment). 1-way ANOVA and Tukey post-tests were applied. Note Y axes are log-2 scales. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = not significant.