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# Metabolic homeostasis and regulatory mechanisms of cellular senescence : roles of ITPR2-mediated calcium fluxes and mevalonate pathway

Dorian Ziegler

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Soutenue publiquement le 08/09/2020, par :

**Dorian ZIEGLER**

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# **Homéostasie métabolique et mécanismes de régulation de la sénescence cellulaire : Rôles des flux calciques médiés par ITPR2 et de la voie du mévalonate**

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- Griveau\*, A., Wiel\*, C., **ZIEGLER, D. V.**, Bergo, M.O., Bernard, D. The JAK1/2 inhibitor Ruxolitinib delays premature aging phenotypes. *Aging Cell* (2020) (**ANNEXE 2**)

### **SUBMITTED**

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## - TABLE OF ACRONYMS and ABBREVIATIONS -

### A

**ABCA1:** ATP Binding Cassette subfamily A member 1  
**ABCG1:** ATP Binding Cassette subfamily G member 1  
**ABPs:** AminoBiPhosphonates  
**ACAT:** Acetyl-CoA AcetylTransferase  
**AD:** Alzheimer Disease  
**AKT:** AKR mouse thymoma  
**AGE:** Advanced Glycation End products  
**ALE:** Advanced Lipid peroxidation End products  
**ALS:** Amyotrophic Lateral Sclerosis  
**AMD:** Age-related Macular Degeneration  
**AMP:** Adenosine MonoPhosphate  
**AMPK:** AMP-activated protein Kinase  
**ARF:** Alternative Reading Frame  
**ATF:** Activating Transcription Factor  
**ATM:** Ataxia Telangiectasia Mutated  
**ATR:** Ataxia Telangiectasia and Rad3 related

### B

**Bax:** BCL2 Associated X  
**Bcl2:** B-Cell CLL/Lymphoma  
**BrdU:** BromodeoxyUridine

### C

**CAMK:** CAIModulin Kinase  
**CCFs:** Cytoplasmic Chromatin Fragments  
**CCL:** CC motif chemokine Ligand  
**CCN1:** Cellular Communication Network 1  
**CDC:** Cell Division Cycle  
**CDK:** Cyclin-Dependent Kinase  
**cGAS:** Cyclic GMP-AMP Synthase  
**CHK:** CHeckpoint Kinase  
**CIP1:** CDK-Interacting Protein 1  
**CoQ:** Coenzyme Q  
**CpG:** C-phosphate-G  
**CR:** Caloric Restriction  
**CRE:** c-AMP Response Element  
**C/EBP $\beta$ :** CCAAT-Enhancer Binding Protein  $\beta$   
**CXCL:** Chemokine (C-X-C Motif) Ligand  
**CYP:** Cytochrome P450

### D

**DAG:** DiAcylGlycerol  
**DDR:** DNA Damage Response  
**DDS:** Dehydrodolichyl Diphosphate Synthase  
**Dedol-PP:** dehydrodolichyl PyroPhosphate  
**DHDDS:** DeHydrodolichyl Diphosphate Synthase subunit  
**DNA-SCARS:** DNA Segments with Chromatin Alterations Reinforcing Senescence  
**D/Q:** Dasatinib/Quercetin  
**DSD:** Double-Strand Breaks

### E

**ECM:** ExtraCellular Matrix  
**Edn:** Endothelin  
**EdU:** 5-Ethynyl-2'-deoxyUridine  
**EGF:** Epidermal Growth Factor  
**ER:** Endoplasmic Reticulum  
**ERK:** Extracellular-signal Regulated Kinase  
**ERP44:** Endoplasmic Reticulum Protein 44  
**ERR- $\alpha$ :** Estrogen-Related Receptor- $\alpha$   
**ETC:** Electron Transport Chain  
**EV:** Extracellular Vesicle

### F

**FPP:** Farnesyl-5-PyroPhosphate  
**FDPS:** Farnesyl DiPhosphate Synthase  
**FDFT1:** Farnesyl DiPhosphate Farnesyl Transferase  
**FOX:** Forkhead bOX protein  
**FUNDC1:** FUN14 Domain Containing 1

### G

**GF:** Growth Factor  
**GPP:** Geranyl-5-PyroPhosphate  
**GGPS:** GeranyGeranyl diPhosphate Synthase  
**GGDP:** GeranylGeranyl DiPhosphate  
**GIT:** G-protein-coupled receptor kinase-Interacting protein  
**GRP75:** Glucose Related Protein 75

### H

**HCC:** HepatoCellular Carcinoma  
**HGF:** Hepatocyte Growth Factor

**HGPS:** Hutchinson-Gilford Progeroid Syndrome

**hMEC:** human Mammary Epithelial Cell

**HMG-CoA:** 3-hydroxy-3-methylglutaryl-CoenzymeA

**HMGCS:** HMG-CoA Synthase

**HMGCR:** HMG-CoA Reductase

**HLH-LZ:** Helix Loop Helix Leucine Zipper

**HSP:** Heat Shock Protein

## **I**

**IDI:** Isopentenyl-Diphosphate Isomerase

**IGFBP:** Insulin-like Growth Factor-Binding Protein 1

**IFN:** InterFeroN

**IICR:** IP3-induced Calcium Release

**IL:** InterLeukin

**INK4:** INhibitor of cdK4

**IMM:** Inner Mitochondrial Membrane

**IPF:** Idiopathic Pulmonary Fibrosis

**IPP:** IsoPentenyl-DiPhosphate

**IP3:** Inositol TriPhosphate

**iPSCs:** induced Pluripotent Stem Cells

**IRE:** Inositol-Requiring Enzyme

**ITPR:** Inositol TriPhosphate Receptor

## **J**

**JMJD3:** JuMonJi Domain containing 3

## **K**

**KLF4:** Kruppel Life Factor 4

**KO:** KnockOut

**KD-PMVK:** Kinase Dead-PhosphoMeValonate Kinase

## **L**

**LXR:** Liver X Receptor

**LTP:** Lipid Transfer Protein

## **M**

**MAMs:** Mitochondrial-Associated Membranes

**MCP:** Monocyte Chemoattractant Protein

**MCU:** Mitochondrial Calcium Uniporter

**MCS:** Membrane Contact Sites

**ME:** Malic Enzyme

**MEFs:** Mouse Embryonic Fibroblasts

**MEK:** MAP/ERK kinase 1

**MERCs:** Mitochondria-ER Contacts

**MeViS:** MeValonate-induced Senescence

**MFN:** MitoFusiNs

**MiDAS:** Mitochondria-Dysfunction Associated Senescence

**MKD:** Mevalonate Kinase Deficiency

**MM:** Mitochondrial Membrane

**MMPs:** Matrix MetalloProteinases

**MtMP:** Mitochondrial Membrane Potential

**MOMP:** Mitochondrial Outer Membrane Permeabilization

**MS:** Mass Spectrometry

**mPTP:** mitochondrial Permeability Transition Pore

**mtDNA:** mitochondrial DNA

**mTORC1:** mammalian Target Of Rapamycin Complex 1

**MVA:** Mevalonate

**MVAP:** Mevalonate-5-Phosphate

**MVAPP:** Mevalonate-5-PyroPhosphate

**MVD:** MeValonate Decarboxylase

**MVK:** MeValonate Kinase

**MYC:** MYeloCytomatosis

## **N**

**NAD:** Nicotinamide Adenine Dinucleotide

**NADP:** Nicotinamide Adenine Dinucleotide Phosphate

**NAFLD:** Non-Alcoholic Fatty Liver Disease

**NASH:** Non-Alcoholic Steato-Hepatitis

**NBS:** Nijmegen Breakage Syndrome

**NCX:** Na<sup>+</sup>/Ca<sup>2+</sup> eXchangers;

**NCKX:** K<sup>+</sup>-dependent Na<sup>+</sup>/Ca<sup>2+</sup> eXchangers

**NFAT:** Nuclear Factor of Activated T-cells

**NF-κB:** Nuclear Factor-Kappa B

**NK:** Natural Killer

**NLRP3:** NLR Family Pyrin Domain Containing 3

**NRF:** Nuclear Respiratory Factor

## **O**

**OiS:** Oncogene-Induced Senescence

**OMM:** Outer Mitochondrial Membrane

**OSKM:** Oct4, Sox2, Klf4, c-Myc

**OXPHOS:** OXidative PHOSphorylation

## **P**

**PD:** Parkinson Disease

**PDGF-AA:** Platelet-Derived Growth Factor AA

**PDH:** Pyruvate DeHydrogenase



**PERK:** PKR-Related Endoplasmic Reticulum Kinase

**PGC1- $\alpha$ :** Peroxisome proliferator-activated Receptor Gamma Coactivator 1- $\alpha$

**PI3K:** Phosphoinositide 3-Kinase

**PIP<sub>2</sub>:** Phosphatidylinositol-4,5-bisPhosphate

**PK:** PoroKeratinosis

**PKA/B/C:** Protien Kinase A/B/C

**PLC:** PhosphoLipase C

**PM:** Plasma Membrane

**PMCA:** Plasma Membrane Ca<sup>2+</sup> ATPase

**PMVK:** PhosphoMeValonate Kinase

**POT1:** Protection Of Telomeres 1

**PRC:** Polycomb-Repressive Complex

**PTEN:** Phosphatase and TENsin homolog

**PUFA:** Poly Unsaturated Fatty Acid

## **R**

**RACK:** Receptor for Activated C Kinase

**RAS:** RAt Sarcoma

**RAF:** Rapidly Accelerated Fibrosarcoma

**Rb:** Retinoblastoma

**ROC:** Receptor-Operated Channels

**ROS:** Reactive Oxygen Species

**RS:** Replicative Senescence

**RXRA:** Retinoid X Receptor Alpha

**RYR:** Ryanodine Receptor

## **S**

**SA- $\beta$ -gal:** Senescence-Associated  $\beta$  galactosidase

**SAHF:** Senescence-Associated Heterochromatin Foci

**SASP:** Senescence-Associated Secretory Phenotype

**SDB:** Sodium DiButyrate

**SERCA:** SarcoEndoplasmic Reticulum Ca<sup>2+</sup> ATPase

**SIPS:** Senescence-Induced Premature Senescence

**SOC:** Store-Operated Channels

**SMOC:** SPARC-related MOdular Calcium binding protein

**SPCA:** Ca<sup>2+</sup>/Mn<sup>2+</sup> ATPases

**SQLE:** SQualene Epoxidase

**STAR:** Steroidogenic Acute Regulatory protein

**STING:** STimulator of INterferon response cGAMP interactor

**SRE:** Sterol Regulatory Element

**SREBP:** Sterol Regulatory Element-Binding Protein

**SSB:** Single-Strand Breaks

**S6K1:** S6 Kinase 1

## **T**

**TAF:** Telomere-Associated Foci

**TAT-IDP:** TAT fused IP3R-derived Peptide

**TASCC:** TOR-Autophagy Spatial Coupling Compartment

**TCA:** TriCarboxylic Acids

**TGF- $\beta$ :** Transforming Growth Factor- $\beta$

**TIF:** Telomere dysfunction-Induced Foci

**TIMP:** Tissue Inhibitor Of Metalloproteinases 1

**TIS:** Therapy-Induced Senescence

**TNF- $\alpha$ :** Tumor Necrosis Factor- $\alpha$

**TRF2:** Telomeric Repeat-binding Factor 2

**TRP:** Transient Receptor Potential

**TSA:** Trichostatin A

**T2D:** Type 2 Diabetes

## **U**

**UPR:** Unfolded Protein Response

**UPS:** Ubiquitine Proteasome System

**UQ:** UbiQuinone

**UV:** UltraViolet

## **V**

**VDAC:** Voltage Dependent Anion Channel 1

**VEGF:** Vascular Endothelial Growth Factor

## **0-9**

**4-HNE:** 4-OH-2-nonenal

**4-PBA:** 4-PhenylButyric Acid

**4EBP1:** 4E-Binding Protein 1

**53BP1:** p53-Binding Protein 1

**8-oxodG:** 8-oxo-2'-deoxyGuanosine



## - SUMMARY / RESUME -

**MOTS-CLES :** Métabolisme, sénescence cellulaire, flux calciques, contacts mitochondrie-endoplasmic reticulum, mévalonate, cholestérol, mitochondrie, vieillissement.

**RESUME:** La sénescence cellulaire est un programme intrinsèque à chaque cellule, pouvant être activée par une multitude de stress et conduisant à un arrêt de la prolifération cellulaire associé à la sécrétion de nombreux facteurs. La sénescence contribue à différents processus physio-pathologiques, allant du développement au cancer en passant par le vieillissement. Bien que de nombreux inducteurs et effecteurs de la sénescence aient été caractérisés, les mécanismes intrinsèques les liant et les régulant sont actuellement un sujet de recherche active. De récentes données suggèrent un métabolisme dérégulé au sein des cellules sénescents. Néanmoins, la contribution de l'homéostasie métabolique dans la régulation de la sénescence, n'est que peu comprise. Afin de mieux caractériser cette contribution, mon projet de thèse vise à étudier le rôle de deux voies métaboliques, incluant les flux calciques médiés par ITPR2 et la voie de synthèse lipidique du mévalonate. Mon travail de thèse a confirmé le rôle pro-sénescence d'ITPR2 dans un modèle *in vivo* impactant le vieillissement. De plus, il a révélé le rôle d'ITPR2 dans la régulation de contacts intracellulaires entre le réticulum endoplasmique et la mitochondrie, facilitant ainsi les échanges de calcium entre ces deux organites et accélérant la sénescence. Ensuite, mon travail de thèse a démontré que la voie lipidique du mévalonate stimule une sénescence médiée par l'axe cholestérol-ERR $\alpha$ -mitochondrie. En résumé, ces résultats ont permis de mieux caractériser l'importance des flux calciques et lipidiques, et plus généralement de l'homéostasie métabolique et de sa dérégulation, dans la mise en place de la sénescence cellulaire.

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**KEY WORDS:** Metabolism, cellular senescence, calcium fluxes, mitochondria-endoplasmic reticulum contacts, mevalonate, cholesterol, mitochondria, aging.

**SUMMARY:** Cellular senescence is an intrinsic cell program, elicited by myriad of stress and resulting in a permanent cell proliferation arrest associated with a particular secretome. Senescence program contributes to various physio-pathological contexts, ranging from development to cancer and aging. Deciphering how senescence is regulated is thus crucial to better understand these physio-pathological responses. While several inducers and effectors of senescence are characterized, the intrinsic subcellular mechanisms linking and regulating them are still a matter of active research. Recent evidences report a global dysregulated intracellular metabolism associated to senescence phenotype. Nonetheless, the functional contribution of metabolic homeostasis in regulating senescence is barely understood. In order to better characterize this contribution, my PhD project aims to investigate the role of two metabolic pathways, ITPR2-mediated calcium fluxes and lipid synthesis mevalonate pathway, in regulating senescence. Firstly, my PhD work confirmed the pro-senescence and aging role of ITPR2 in an *in vivo* model. Furthermore, it revealed the role of ITPR2 in regulating endoplasmic reticulum/mitochondria membranes contacts, thus facilitating calcium fluxes between these two organelles and subsequent senescence. Secondly, my PhD work demonstrated that the lipid-derived mevalonate pathway promotes senescence through a cholesterol-ERR $\alpha$ -mitochondria axis. Altogether, these results shed light on the importance of calcium fluxes/lipid metabolism, and more broadly on metabolic homeostasis and consequences of its deregulation, in the implementation of cellular senescence.

« Celui qui sait, qu'il ne sait pas, sait beaucoup. » Anonyme

# **- INTRODUCTION -**





## **- CHAPTER I -**

### **- Cellular Senescence: inducers, features, regulation and biological relevance -**

In 1961, Leonard Hayflick discovered that multiple serial passages of human diploid fibroblasts leads to an enlarged cell morphology and to an irreversible cell proliferation arrest (Hayflick and Moorhead, 1961). Though not defined as cellular senescence at that time, this discovery was the starting point of a field of research entirely dedicated to the study of this “new” cell fate.

Considered to be a stress response, cellular senescence is an orchestrated intrinsic cell program involving many actors, ultimately triggering two main features, namely a stable cell cycle arrest and a pro-inflammatory secretome. Since it is a coordinated cellular response, several distinct actors interact to regulate senescence. Inducers of the program initiate senescence upstream, while the regulators integrate and modulate this response, and the effectors activate the downstream cellular program. Beyond the mechanisms of its regulation, cellular senescence is involved in a myriad of pathophysiological conditions, from embryonic development to tissue regeneration, cancer and aging.

In this first part, the cellular senescence process will be described. Firstly, I will focus on the inducers of this cellular program. Secondly, I will highlight the experimental key features of cellular senescence, permitting its empirical study. Then, I will delineate the role of effectors and regulators of this cellular process. Finally, cellular senescence will be assessed in both tissue and organismal contexts for a better understanding of its biological relevance and involvement in pathophysiological conditions.

#### **1.1. Main inducers of the senescence program**

After World War II, advances in *in vitro* cell culture led to the establishment of an ever-growing number of cell lines (Landecker, 2007). In 1961, Leonard Hayflick, who had previously isolated the WI-38 fibroblast cell line from human lung, observed an arrest of proliferation of multiple cell lines after several passages (Hayflick and Moorhead, 1961), a replicative limit known as the “Hayflick limit” and more generally defined as cellular lifespan. This long-term replicative senescence (RS) is distinct from stress-induced premature senescence (SIPS), since the latter is promoted by multiple stresses that prematurely induce the onset of senescence.

### **1.1.1. Telomere shortening**

Articles describing molecular events underlying the “Hayflick limit” were first published twenty years after the initial observation of RS. This limited lifespan is directly linked to telomeres. At each S-phase, cells undergo asymmetric DNA replication and the attrition of DNA extremities called telomeres. Each cell division thus leads to a loss of 50 to 200 bp in telomeres. In humans, the length of telomeres is approximately 10-15 kb, formed by the iteration of the (TTAGGG) motif and shaped in a protective T-loop via protein complexes (Doksani et al., 2013). The chronic “end replication” telomere attrition results in a T-loop disruption, and a persistent DNA damage response (DDR), ultimately triggering a permanent cell cycle arrest and senescence (Allsopp et al., 1992; Bodnar et al., 1998; Harley et al., 1990; Martens et al., 2000) (**Figure 1**). The exogenous addition of telomerase, the enzyme responsible for elongating telomere extremities, is thus able to radically extend the “Hayflick limit” to give rise to immortalized cells (Bodnar et al., 1998). For all these reasons, telomeres were considered to be replicometers of cells and the cellular event resulting from their attrition was termed RS.

While persistent DDR in RS constitutes a chronic stress signal for cells, a myriad of other stresses, such as oxidative or oncogenic stresses, may also induce premature senescence that is thus called SIPS (He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014a) (**Figure 1**). It is thus essential to delineate the different stresses inducing SIPS, in order to understand the subsequent complexity of senescence phenotypes.

### **1.1.2. Oxidative stress**

Attempts to modulate the cellular lifespan did not initially focus on genetic manipulations, due to the lack of robust tools, but on variations of culture parameters, the most prominent being oxygen concentration in incubators (Balin et al., 1984; Honda and Matsuo, 1983; Michiels et al., 1990; von Zglinicki et al., 1995). While hyperoxia (>20% oxygen) tends to lower the ‘Hayflick limit’ and accelerate senescence (Honda and Matsuo, 1983; von Zglinicki et al., 1995), normoxia (~3% oxygen) leads to an extended cellular lifespan (Balin et al., 1984; Michiels et al., 1990; Parrinello et al., 2003). This effect of oxidative stress has been further linked to the generation of reactive oxygen species (ROS), highly reactive molecules that accumulate within cells, and are mostly fueled by cellular availability and consumption of oxygen (Chen and Ames, 1994; Chen et al., 1995; Dumont et al., 2000). Sources of ROS are numerous within the cell and can be generated in various subcellular compartments, including mitochondria through electron transport chain (ETC), endoplasmic reticulum (ER) through oxidative folding, peroxysomes through fatty acids  $\beta$ -oxidation, or lysosome membranes through enzymes NADPH oxidase (Gorrini et al., 2013). Both exogenous and endogenous ROS thus promote senescence both *in vitro* and *in vivo* (Chen and Ames, 1994; Dumont et al., 2000; Velarde et al., 2012) (**Figure 1**).



Beyond its role as an inducer of senescence, it should be mentioned that enhanced ROS generation accompanies senescence and the use of antioxidants functionally rescues cells from senescence (Lu and Finkel, 2008; Hernandez-Segura et al., 2018a).

### **1.1.3. Oncogenic stress**

Technological advances in genetic tools, growing interest in cancer biology, and emerging concepts of transformation and genes regulating it, led the cancer research community to decipher the *in vitro* steps of cellular transformation. Among these transforming genes, oncogenes trigger uncontrolled proliferation and tumor formation when these are mutated and aberrantly activated. Surprisingly and paradoxically, the *in vitro* aberrant activation of these pro-proliferative genes, such as the ones included in the MAPK/ERK pathway (RAS, RAF, MEK and ERK), stops cellular proliferation inducing premature cellular senescence (Serrano et al., 1997; Zhu et al., 1998), termed oncogene-induced senescence (OIS). Similarly, inactivation of tumor suppressor genes, such as PTEN, leads to an oncogenic stress triggering a similar senescence response (Chen et al., 2005). These first *in vitro* results shed light on an original role for the senescence program in tumor suppression. Aside from these *in vitro* results that were attributed to culturing artefacts by some scientists, physiological evidence of this tumor suppressor role of senescence was first observed *in vivo* in 2005 (Braig et al., 2005; Chen et al., 2005; Denchi et al., 2005; Michaloglou et al., 2005; Roberson et al., 2005) (*See INTRODUCTION - Chapter I. - Section 1.4.4.*). Various other known oncogenes, including PI3K/AKT or MYC, are also able to induce OIS (**Figure 1**), highlighting senescence as a universal tumor suppressive response.

### **1.1.4. Genotoxic stress**

Whereas RS has been directly linked to persistent DDR at the level of telomeres, genotoxic stress also induces senescence. Indeed, the occurrence of DNA strand breaks following  $\gamma$ -irradiation or via chemical agents, such as bleomycin, actinomycin D, topoisomerase inhibitors or cisplatin A, promotes DDR and subsequent cellular senescence *in vitro* (Leonardo et al., 1994; Robles and Adami, 1998; Shapiro et al., 1998) (**Figure 1**). Mechanistically, these genotoxic agents generate DNA damage, triggering a DNA damage response (d'Adda di Fagagna, 2008). The increasing use of these genotoxic agents, notably in chemo- (e.g. bleomycin, etoposide, doxorubicin) and radio-therapeutic contexts raised the question of their organismal impact beyond their oncolytic actions *in vivo*. As expected, at sublethal doses, these agents elicit senescence *in vivo* (Schmitt et al., 2002; Roninson, 2003; Le et al., 2010), termed therapy-induced senescence (TIS). The effects of this TIS in the context of tumoral microenvironment remains a matter of active research in the field of senescence (*See INTRODUCTION Chapter I. - Section 1.4.4.*).

### **1.1.5. Cellular senescence: beyond the stress response**

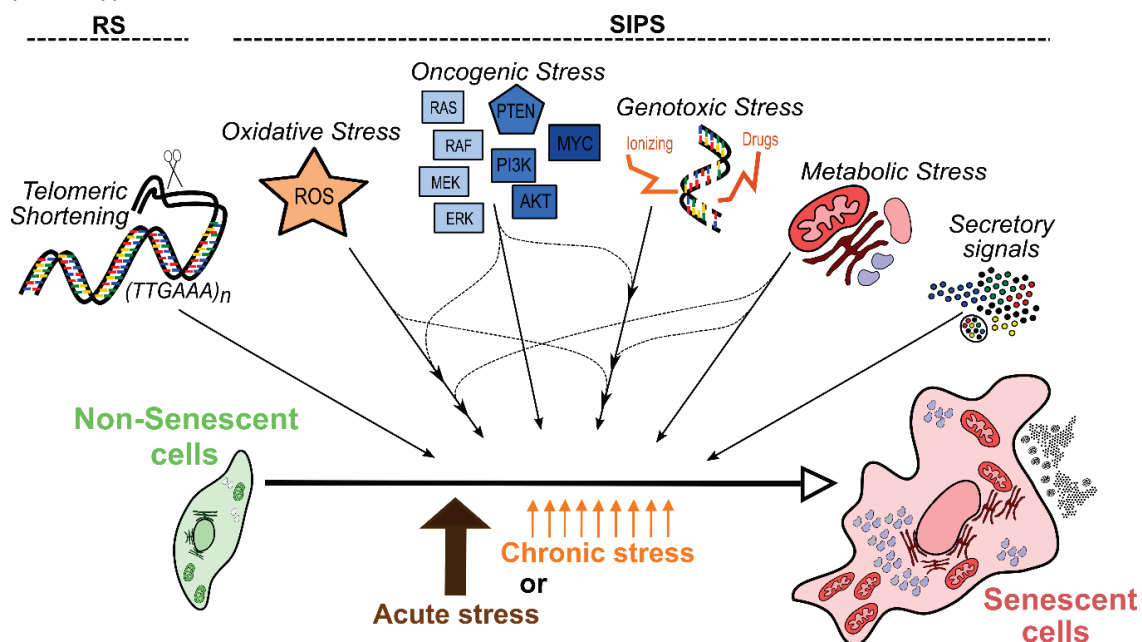
Telomeric, genotoxic, oxidative and oncogenic stresses have been widely described in the literature. Nonetheless, other inducers, mostly cross-talking with these former stresses, are currently investigated and augment the number of inducers promoting senescence. This field of research recently focused on secretory, reprogramming and metabolic factors and their role in promoting senescence.

Cellular senescence can be elicited by external stimuli, driven by secretory TGF- $\beta$  (Frippiat et al., 2001) or cytokines (Braumüller et al., 2013). These molecules are sensed by cells and may either initiate a senescence response (paracrine senescence) (Acosta et al., 2013; Hoare and Narita, 2013; Chen et al., 2015; Bird et al., 2018) or reinforce the senescence phenotype (autocrine senescence) (Acosta et al., 2008a; Kuilman et al., 2008; Wajapeyee et al., 2008). Secretion-mediated senescence (**Figure 1**) is thus integrated in the numerous cell-cell interactions occurring between senescent and non-senescent cells and arise for example during development (Muñoz-Espín et al., 2013; Storer et al., 2013)

In 2006, the discovery of reprogramming factors Oct3/4, Sox2, Klf4 and c-Myc (OSKM) and the generation of induced-pluripotent stem cells (iPSCs), important breakthroughs for regenerative medicine, paved the way for studies on the induction of reprogramming (Takahashi and Yamanaka, 2006). In 2009, multiple genetic manipulations of iPSCs highlighted the relationship between reprogramming and senescence. Indeed, the four OSKM factors, aside from generating iPSCs, favor the establishment of cellular senescence leading to poor reprogramming efficiency *in vitro* (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009b; Utikal et al., 2009), demonstrating that reprogramming elicits senescence response in a cell-autonomous manner. It should be noted that OSKM-induced senescence may be considered as a subtype of OiS (with c-Myc induction). Nonetheless, the interplay between reprogramming and senescence *in vivo* is still controversial (*See INTRODUCTION Chapter I. - Section 1.4.3.*)

Finally, a metabolic alteration appears to be an interesting candidate to explore the induction of senescence. This can be attributed to a change in the metabolism homeostasis, modifying crucial biochemical reactions involved in synthesis/degradation of metabolites and energy within the cell. Metabolic homeostasis is a dynamic equilibrium, consisting in a self-regulating process which controls intracellular biochemical reactions in order to maintain cell stability and cell survival. In the middle 1990's, metabolic alterations were suspected to play a role in senescence (Toussaint et al., 1995). More recently, mitochondrial dysfunction or even ER stress were found to promote it (Kim et al., 2019; Wiley et al., 2016), generating multiple intracellular responses such as increased oxidative stress, and opening new perspectives in this specific research area.

Since their discoveries *in vitro*, RS and SIPS have been both described in multiple *in vitro* and *in vivo* contexts (He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014a). As most inducers constitute cellular stresses, senescence appears to be a programmed stress response and there are virtually as many senescence inducers as there are stresses (**Figure 1**). One inducer may characterize one subtype of senescence such as RS for telomere shortening, OIS for oncogenic stress, TIS for genotoxic stress, mitochondrial-dysfunction associated senescence (MiDAS) for mitochondrial metabolic stress. More importantly, these stresses can be chronic and/or acute depending on their nature, frequency and intensity (**Figure 1**). Furthermore, since there are a variety of stresses and since these are mostly non-exclusive, cross-talks between these inducers exist (Lee et al., 1999a; Di Micco et al., 2006) (**Figure 1**), all converging to a senescence phenotype. Let us delineate the key features of this phenotype.



**Figure 1: Cellular senescence: a cellular stress response driven by multiple inducers.** Whether they are acute or chronic, various stresses induce a senescence response. This includes telomeric shortening, oxidative stress (ROS), oncogenic activation, genotoxic stress (DNA strand breaking agents, such as ionization or drugs), metabolic stress (mitochondria and ER dysfunction), and secretory signals. Dashed lines highlight cross-talks between these main senescence inducers. RS: Replicative Senescence. SIPS: Stress-induced premature senescence.

## 1.2. Key features of the senescence program

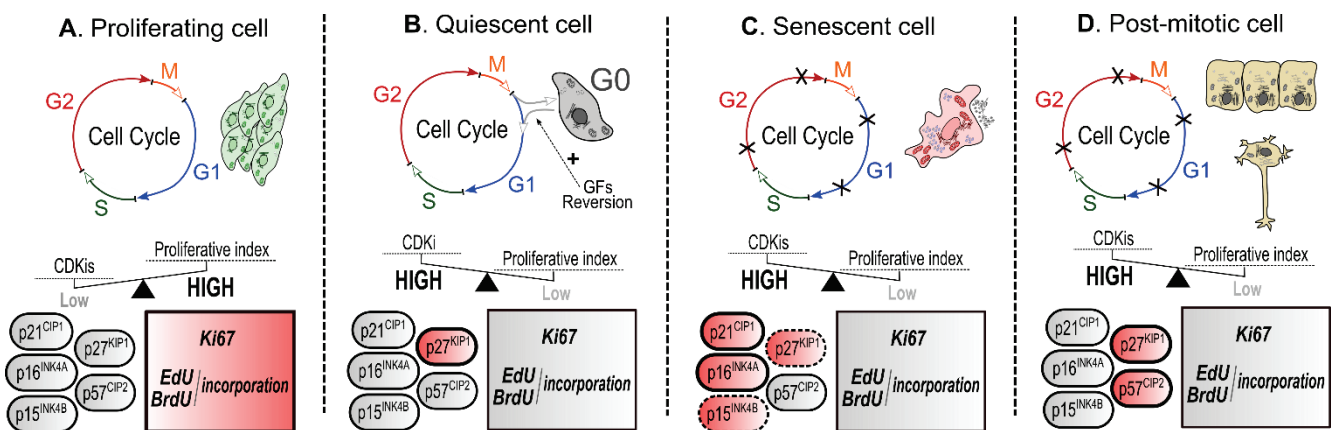
Experimental characterization of cellular senescence is a pre-requisite in order to investigate its process. The following senescence key features are present in most senescence subtypes and will be mentioned in this part according to a chronological order of discovery: (1) Permanent cell cycle arrest, (2) Cellular and subcellular morphological changes, (3) Resistance to apoptosis, (4) Senescence-associated- $\beta$ -galactosidase activity, (5) Macromolecular damage, (6) Specific epigenetic landscape and

(7) Secretory phenotype. Of note, some key features may not always be present, their combination thus being crucial to recognize senescent cells among others (Gorgoulis et al., 2019).

### 1.2.1. Permanent cell cycle arrest

In mitotic cells, cellular senescence was initially characterized by an enlarged morphology and a permanent cell proliferation arrest in 1961 (Hayflick and Moorhead, 1961). The latter is nowadays considered to be a canonical senescence feature, though other cellular fates, the most obvious being terminally differentiated post-mitotic cells, can share this feature.

Senescent cells are generally arrested in the G1 or occasionally in G2/M phases of their cycle (Gire and Dulić, 2015) (**Figure 2**). For cells in culture, cell cycle analysis by flow cytometry can be used to detect increased proportions of cells in these phases. Additional experimental evidence may be added such as a reduction of EdU/BrdU incorporation during S-phase (**Figure 2**). Furthermore, accounting for both *in vitro* and *in vivo* characterization, decreased *Ki67* mRNA/protein levels is widely used (**Figure 2**). In mitotic cells, cell cycle progression is controlled by various temporary cyclin/cyclin-dependent kinase (CDK) complexes. These complexes are blocked by cyclin-dependent Kinase inhibitors (CDKis) during cell cycle arrest. Pro-senescent signals enhance the expression of two main CDKis, namely  $p21^{CIP1}$  and  $p16^{INK4A}$  (See *INTRODUCTION - Chapter I. - Section 1.3.1.*). Less frequently, another CDKi,  $p15^{INK4B}$ , can be elicited by paracrine stress such as TGF- $\beta$  for example (Muñoz-Espín et al., 2013; Storer et al., 2013; Gibaja et al., 2019) (**Figure 2**).



**Figure 2: Cell cycle progression in proliferating and non-proliferating cells.** Cell cycle progression involves spatio-temporal checkpoints controlled by cyclin/cyclin-dependent kinase inhibitors (CDKi). Proliferating cells (**A**) express *Ki67*, incorporate EdU/BrdU, and display low CDKis levels. Quiescent cells (**B**), senescent cells (**C**), and post-mitotic cells (**D**) have a low proliferative index, with decreased *Ki67* and EdU/BrdU incorporation. While quiescence (**B**) in G0 phase is preferentially  $p27^{KIP1}$ -dependent (red) and is reversible following the addition of growth factors (GFs), senescence (**C**) and post-mitotic fate (**D**) are considered to be irreversible. Senescent cells preferentially upregulate CDKis  $p21^{CIP1}$ ,  $p16^{INK4A}$  or eventually  $p15^{INK4B}$  and  $p27^{KIP1}$ , while post-mitotic cells upregulate mostly  $p27^{KIP1}$  or  $p57^{CIP2}$ .

Cell cycle arrest is not a unique feature of senescing cells as it is also shared by mitotic quiescent cells and post-mitotic terminally-differentiated cells. In contrast to the quiescence state in G0 phase, the senescent state remains irreversible following the addition of exogenous growth factors (Terzi et al., 2016) (**Figure 2**). Nonetheless, post-mitotic differentiated cells are permanently arrested in G1 or G2 and raise the question of the exclusivity of this canonical characteristic (**Figure 2**). Permanent cell cycle is thus necessary but not sufficient to identify cellular senescence, and especially in post-mitotic cells.

### **1.2.2. Morphological cellular and subcellular changes**

In 1961, Leonard Hayflick observed morphological changes and enlarged morphology in senescing cells. This first observation is still commonly used to suspect a senescence phenotype *in vitro* but has been further described.

#### **a. Cytoplasmic morphological changes**

Increased cellular size and enlarged morphology are shared by almost all senescent subtypes (with an exception for Raf-induced senescence which often displays a spindle-like morphology in fibroblasts for example) and can be useful in *in vitro* characterization of senescence using any phase contrast and/or fluorescent microscope (**Figure 3**). Nonetheless, its use *in vivo* remains limited.

This enlarged morphology is frequently accompanied by increased cytoplasmic volume, vacuolization and multiple organelle network modifications. Indeed, senescence is mostly accompanied by an increase in both lysosomal and mitochondrial masses (Kurz et al., 2000; Lee et al., 2002; Moiseeva et al., 2009; Stab et al., 2016). While lysosome biology remains largely elusive in the context of senescence, several studies on mitochondria report an imbalanced fusion/fission process and an accumulation of elongated hyperfused dysfunctional mitochondria in senescing cells (Korolchuk et al., 2017; Ziegler et al., 2015). Overall, these cytoplasmic alterations underline a global dysregulated metabolism in senescent cells.

#### **b. Nuclear morphological changes**

Nuclear alterations are frequently associated with senescence. Indeed, most senescent nuclei display an increase in size, which can be used as a simple marker for high-throughput screening. In some cases, nuclear shape can be further altered. The inner nuclear envelope is lined with structural proteins, called lamins. All lamin subtypes, respectively A, B and C, are essential for nuclear architecture (morphology, size) and function (stability, expression) (Dechat et al., 2008). Lamin B1 loss is considered to be a common marker of senescence, associated with different senescence subtypes,

and validated *in vitro* and in an *in vivo* model of photoaged skin (Freund et al., 2012; Wang et al., 2017a).

Another specific lamin modification is found in a well-established model of senescence: progerin-induced senescence. Lamins are post-translationally modified and defects in lamin processing lead to severe pathologies, called laminopathies, that include Hutchinson-Gilford Progeroid Syndrome (HGPS) (Dobrzynska et al., 2016). This progeroid syndrome is caused by the synthesis of a truncated form of LaminA/C, called progerin. The maturation of normal Lamin A/C includes a farnesyl-group excision signal, which is absent in the truncated form, leading to an accumulation of farnesylated Lamin A/C and a global disorganization of the nucleus of HGPS cells (Eriksson et al., 2003). Misshapen nuclei display various genetic alterations, from shortened telomeres to genomic instability (Allsopp et al., 1992; Liu et al., 2005) and reorganized heterochromatin (Chandra et al., 2015), ultimately promoting premature cellular senescence.

Last but not least, senescent cells can present nuclear blebbing, found in cytosol and targeted by the p62-autophagosomal/lysosomal pathway leading to progressive depletion of histones (H2A, H2B and H3) within the senescing nucleus (Ivanov et al., 2013). Interestingly, this blebbing leads to the degradation of nuclear lamina and lamin B1 depletion (Dou et al., 2015). As a consequence of blebbing, extranuclear chromatin fragments can be found and observed in the cytosol of senescent cells (Dou et al., 2017; Glück et al., 2017).

Overall, if enlarged cytoplasmic morphology and altered nuclear architecture suggest a senescent phenotype *in vitro* enabling rapid high-throughput screening for instance, its use *in vivo* remains very limited.

### **1.2.3. Resistance to apoptosis**

The inability of senescent cells to commit cellular suicide has been extensively investigated (Wang, 1995). Indeed, senescent cells escape apoptosis induced by various stimuli such as serum starvation (Wang, 1995), ceramide (Hampel et al., 2004), UV light (Chaturvedi et al., 1999, 2004) and oxidative stress (Naderi et al., 2006). Multiple hypotheses were proposed to explain this resistance.

Mechanistically, senescent cells fail to stabilize p53, which is an essential step to engage the apoptotic process (Seluanov et al., 2001). The role of p53 has recently been revisited, with a study demonstrating an elevated level of the transcription factor FOXO4 associated with p53 sequestration in the nucleus and resistance to apoptosis (Baar et al., 2017). Besides, senescent cells may upregulate various anti-apoptotic members of the Bcl-2 family, that includes Bcl-2 itself (Crescenzi et al., 2003;

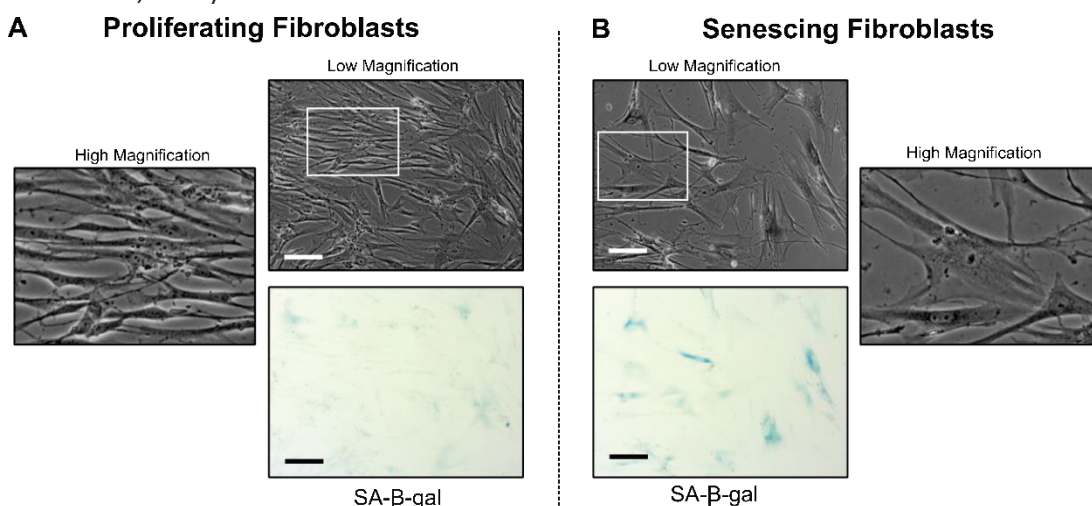
Ryu et al., 2007) or Bcl-xL and Bcl-W (Yosef et al., 2016). Furthermore, senescent cells display downregulation of apoptosis effectors such as Caspase-3 (Rebbaa et al., 2003; Marcotte et al., 2004). Overall, these results underline the link between senescence and apoptosis

As a translational approach, resistance to apoptosis was further investigated in order to develop drugs specifically killing senescent cells and thus called “senolytics”. (Deursen, 2019). Based on previous reports, either stabilizing p53 in the cytoplasm (with FOXO4 peptide) or inhibiting Bcl-2 family proteins (with ABT-737 or ABT-263) have emerged as the two major strategies developed to switch cells from a state of senescence to apoptosis (Baar et al., 2017; Bussian et al., 2018; Chang et al., 2016; Victorelli et al., 2019; Yosef et al., 2016).

Aside from studies investigating the potent effects of new senolytic compounds, the resistance of senescent cells to cellular suicide is not sufficiently evaluated *in vitro* and remains limited *in vivo*.

#### 1.2.4. Senescence-associated- $\beta$ -galactosidase activity (SA- $\beta$ -gal)

Lysosomes are intracellular detoxifying organelles relying on an acidic pH to catabolize macromolecules (nucleic acids, lipids, proteins) and participate in the final steps of autophagy (Kaushik and Cuervo, 2015; Maria Cuervo, 2004). In the context of senescence, the activity of a specific enzyme of this subcellular compartment has been linked to senescence: the  $\beta$ -galactosidase. Better known as senescence associated- $\beta$ -galactosidase (SA- $\beta$ -gal), its activity at pH 6.0 increases in senescent cells (Dimri et al., 1995), but is not necessary for the induction of the senescent phenotype (Lee et al., 2006). This SA- $\beta$ -gal activity is currently believed to reflect the increase in lysosomal mass in senescent cells (Kurz et al., 2000) and can be observed *in vitro* (Figure 3) and *in vivo* (Dimri et al., 1995; Debacq-Chainiaux et al., 2009).



**Figure 3: Morphology and SA- $\beta$ -galactosidase activity in senescent fibroblasts.** While proliferating fibroblasts (A) display a classical fibroid thin morphology and weak SA- $\beta$ -galactosidase activity, senescing fibroblasts (B) harbor an engulfed shape associated with an increase in SA- $\beta$ -galactosidase activity (blue). Scale bars: 5  $\mu$ m.

Of note, though SA- $\beta$ -gal remains a gold-standard for the detection of cellular senescence, it should be noted that some non-senescent cells, such as serum-starved or sub-confluent cells, may present increased lysosomal SA- $\beta$ -gal activity (Yang and Hu, 2005). Nonetheless, SA- $\beta$ -gal activity and permanent cell cycle arrest remain the two primary markers of senescent cells (Gorgoulis et al., 2019).

### **1.2.5. Macromolecular damage**

Various physical/chemical agents known to cause intracellular damage can trigger senescence (X-ray irradiation, genotoxic drugs). Aside from these exogenous agents, other endogenous products, such as ROS, may damage multiple biomolecules, including DNA, lipids, carbohydrates and proteins.

#### **a. DNA damage**

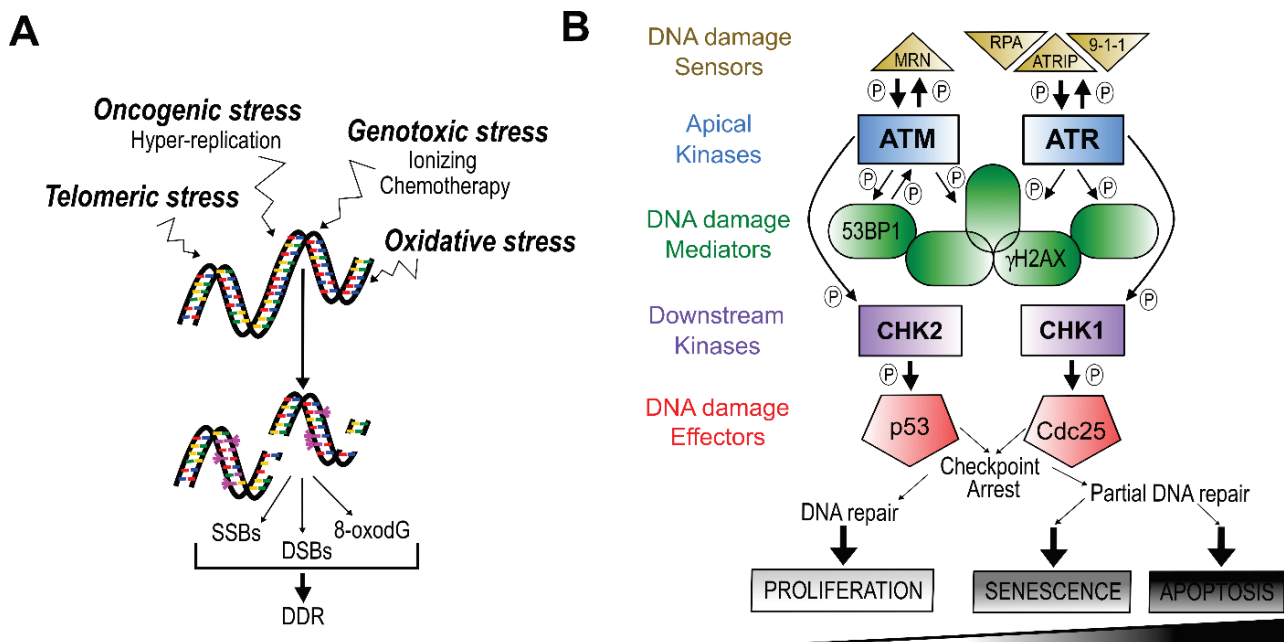
DNA damage is the most frequent intracellular damage investigated in the context of cellular senescence. As nuclear DNA constitutes one of the most precious contents within the cell, each cell may elicit upon DNA alteration a DDR, which is a well-known checkpoint for cell fate (d'Adda di Fagagna, 2008). DNA damage includes both minor (base oxidation) and major (single-strand breaks (SSB) and double-strand break (DSB)) lesions that can occur throughout the life of a cell (d'Adda di Fagagna, 2008) (**Figure 4.A**). Oxidative stress constitutes an enhancer of DNA damage, as the main oxidative DNA lesion, 8-oxo-2'-deoxyguanosine (8-oxodG) accumulates in senescent cells (Chen et al., 1995) and is sufficient to elicit pro-senescence signals (Rai et al., 2009) (**Figure 4.A**).

DNA damage is sensed by apical kinases ataxia-telangiectasia mutated (ATM) and/or ataxia telangiectasia and Rad3-related (ATR). These apical kinases recruit and target among others  $\gamma$ H2Ax and p53-binding protein 1 (53BP1), that are common markers of DNA damage. ATM and ATR further phosphorylate downstream kinases (respectively CHK2 and CHK1) (**Figure 4.B**). These downstream kinases then initiate cellular response through the respective phosphorylation of p53 and CDC25. Depending on the nature, intensity and duration of the lesions, the DDR either initiates apoptosis or cell cycle checkpoint arrest, which in turn activates either DNA repair and resumed proliferation or senescence (**Figure 4.B**).

As end-replication problems occur during serial cell culture, telomere shortening has been extensively studied in the field of senescence (Harley et al., 1990; Shay and Wright, 2019). Telomere extremities form a T-loop and are closely associated with shelterin proteins, such as telomeric repeat binding factor 2 (TRF2) and protection of telomeres 1 (POT1). The latter protect single-strand DNA from the activation of apical kinases, ATM and ATR, respectively abrogating subsequent DDR and further p53-dependent senescence response (van Steensel et al., 1998; Denchi and de Lange, 2007; Fujita et al., 2010). Progressive telomere disruption and uncapping induce DDR-dependent senescence



(d'Adda di Fagagna et al., 2003) (**Figure 4.A**), and generate telomere dysfunction-induced foci (TIFs) (Kaul et al., 2012; Takai et al., 2003). These TIFs can be prematurely induced by modulating key components of telomere maintenance (TRF2 or POT1) and promoting subsequent senescence (Denchi and de Lange, 2007). Moreover, overexpression of telomerase, the enzyme involved in telomere elongation, reduces TIFs and drastically extends replication potential in multiple cell types *in vitro* (Herbig et al., 2004). Interestingly, in old aged mice or baboons, senescent cells accumulate (Herbig et al., 2006; Jeyapalan et al., 2007; Yousefzadeh et al., 2020a) and DDR foci highlighted by  $\gamma$ H2AX and 53BP1 foci co-localize with telomere DNA (Herbig et al., 2006), reinforcing the hypothesis of telomere attrition and replicative exhaustion (Harley et al., 1990). Telomere attrition is not the only inducer of DDR, as enhanced genotoxic stress, oxidative stress or hyper-replication stress caused by aberrant oncogene activation can also initiate a robust DDR (Shapiro et al., 1998; Robles and Adami, 1998; Di Micco et al., 2006, 2011; Velarde et al., 2012).



**Figure 4: Induction by pro-senescent signals and main actors of DDR.** **A.** Multiple pro-senescent stress signals activate DDR through the generation of single-strand breaks (SSBs), double-strand breaks (DSBs), or DNA oxidation marks such as 8-oxo-2'-deoxyguanosine (8-oxodG). **B.** Main actors of the DDR involving the kinase signaling cascade from DNA damage sensors MRE11-RAD50-NBS1 (MRN), replication protein A (RPA), ATRIP, RAD9-RAD1-HUS1 (9-1-1); apical kinases ATM and ATR; DNA damage mediators including 53BP1 and  $\gamma$ H2Ax; downstream kinases CHK2 and CHK1, to effectors such as p53 and Cdc25. According to the duration and/or intensity (represented by a gradient) of the DDR, and the possibility of DNA repair, effectors initiate apoptosis, proliferation or senescence programs. (Adapted from Sulli et al., 2012)

Finally, senescing cells may display a size-independent alteration of telomeres. This includes the implementation of telomere-associated-foci (TAFs), resulting from oxidative DNA damage in

telomere G-rich repeats (Anderson et al., 2019; Hewitt et al., 2012; Kaul et al., 2012) and reinforced by oxidative stress.

#### **b. Lipid-, carbohydrate- and protein-associated damage**

Though largely understudied compared to DNA in the context of cellular senescence, other essential macromolecules such as lipids, carbohydrates and proteins may also be damaged.

Owing to their essential role as structuring and signaling molecules in both cytosol and membranes, lipids are frequently altered by oxidation. Of note, lipid membranes constitute a reservoir of harmful secondary oxidative products called advanced lipid peroxidation end products (ALEs) and products of peroxidation by ROS of poly-unsaturated fatty acids (PUFAs). These aldehydes (e.g. 4-OH-2-nonenal (4-HNE), malondialdehyde,...) can cause covalent adducts when they interact with side chains of cysteine, histidine and lysine residues (Baraibar and Friguet, 2013; Esterbauer et al., 1991). Functionally, 4-HNE is able to mediate both DNA damage-driven senescence (Flor et al., 2016, 2017) and paracrine senescence (Riahi et al., 2015).

It is worth mentioning that according to the literature, only one study has so far linked damaged carbohydrates, such as advanced glycation end products (AGEs), to premature senescence (Liu et al., 2014), while AGEs are frequently associated with senescence-associated pathophysiological contexts, such as aging (Ramasamy et al., 2005; Chaudhuri et al., 2018).

Proteostasis is necessary to maintain cell survival, as proteins are a constant target of a myriad of intracellular damage (oxidation, nitration, carbonylation, glycation, breaks,...) (Kaushik and Cuervo, 2015). ROS generated by senescing cells can oxidize amino acid residues, cysteines being preferentially targeted, and crosslink molecules. In replicative senescence, oxidized proteins accumulate in the cytosol (Ahmed et al., 2010; Sitte et al., 2000) and some specific oxidized proteins, such as oxidized vimentin in the plasma membrane, are proposed to be markers of senescence (Frescas et al., 2017). The overall accumulation of damage can be easily monitored by the evaluation of lipofuscin (mix of cross-linked and oxidized carbohydrates/lipids/proteins), accumulating in various senescent subtypes *in vitro* and *in vivo* (Georgakopoulou et al., 2012).

Observed at an early stage, macromolecular damage is shared by all senescent cells and most senescent subtypes (telomeric, oxidative, oncogenic, genotoxic, metabolic) (**Figure 5**). This damage might functionally elicit the induction of other key features of senescence, such as cell cycle arrest, and persists in senescent cells. Overall, this accumulation of damage can also be seen as a consequence of increased harmful by-products, such as ROS, and/or defective repair/recycling machinery.

### **1.2.6. Specific epigenetic landscape**

Like all cellular fates, cellular senescence is accompanied by changes in chromatin structure and architecture, impacting gene expression.

The formation of senescence-associated heterochromatin foci (SAHF) is associated with senescence, particularly in OIS (Narita et al., 2003). SAHF are areas of heterochromatin, enriched in H3K9me3 and macroH2A, driven by HMGA1/ASF1/HIRA complex, localized at E2F loci (Narita et al., 2003; Zhang et al., 2005), and that can be observed by DAPI staining. Nonetheless, SAHF are not universally observed in other senescent subtypes (upon telomere shortening and genotoxic stress) and highly depend on cell type (Kosar et al., 2011), raising the question of using SAHF as a universal senescence marker.

Several studies reported changes in histone variants or post-translational modifications of histones during senescence. Only a few are conserved between senescent subtypes and senescent inducers, including an increase in H3.3 and H2AJ variants, and enhanced HK20me3 (Paluvai et al., 2020). Of note, loss of linker histone H1 and other histone variants, or HMGB1 depletion were also observed in various senescent contexts (Funayama et al., 2006; O'Sullivan et al., 2010; Ivanov et al., 2013; Davalos et al., 2013). Finally, the re-activation of transposable elements can take place during senescence (De Cecco et al., 2019).

Gene promoters can be methylated and switched off through the process of CpG island methylation. Compared to proliferating normal cells, cells in replicative senescence display a global hypomethylation of CpG islands, with some exceptions of local hypermethylation (Cheng et al., 2017; Cruickshanks et al., 2013). This latter pattern resembles that of transformed cells, suggesting that cancer cells and senescent cells share common epigenetic features (Cruickshanks et al., 2013; Xie et al., 2018). Conversely, cells undergoing OIS and other stress-induced senescence responses (doxorubicin or ionizing-induced) do not display any global hypomethylation pattern (Bielak-Zmijewska et al., 2014; Koch et al., 2013; Xie et al., 2018), highlighting differences in epigenetics modification in various senescent subtypes (Paluvai et al., 2020).

Overall, though the epigenetic landscape is modified during the senescence, the nature and intensity of these changes vary depending on cell type and senescence inducer (**Figure 5**).

### **1.2.7. Senescence-associated secretory phenotype (SASP)**

Having characterized senescence by its permanent cell proliferation arrest and described a plethora of cell-autonomous effects, senescing cells are also characterized by non-cell autonomous effects, through a secretory phenotype.

Senescence is associated with a particular secretome, called senescence-associated secretory phenotype (SASP) (Acosta et al., 2008a; Coppé et al., 2008; Kuilman et al., 2008) (**Figure 5**). SASP contains a myriad of factors, including lipids but mostly proteins including interleukins, chemokines, and growth factors (GFs) or matrix metalloproteases (MMPs) (**Table 1**). Other factors strongly impacting neighboring cells, such as ROS, should also be mentioned. The effects of SASP are multiple through autocrine, paracrine and endocrine pathways, affecting tissue homeostasis (*See INTRODUCTION - Chapter I. - Section 1.4*). Pathophysiological roles of the senescence program). Of note, the SASP varies greatly, as (i) single-cell analysis revealed a strong heterogeneity in the SASP of different cell subpopulations (Wiley et al., 2017), (ii) it is highly dependent on cell type and senescence inducers (Hernandez-Segura et al., 2017; Wiley et al., 2016), and (iii) its dynamics alter throughout senescence, as evidenced by the emerging concept of early and late stage SASPs (De Cecco et al., 2019; Hoare et al., 2016).

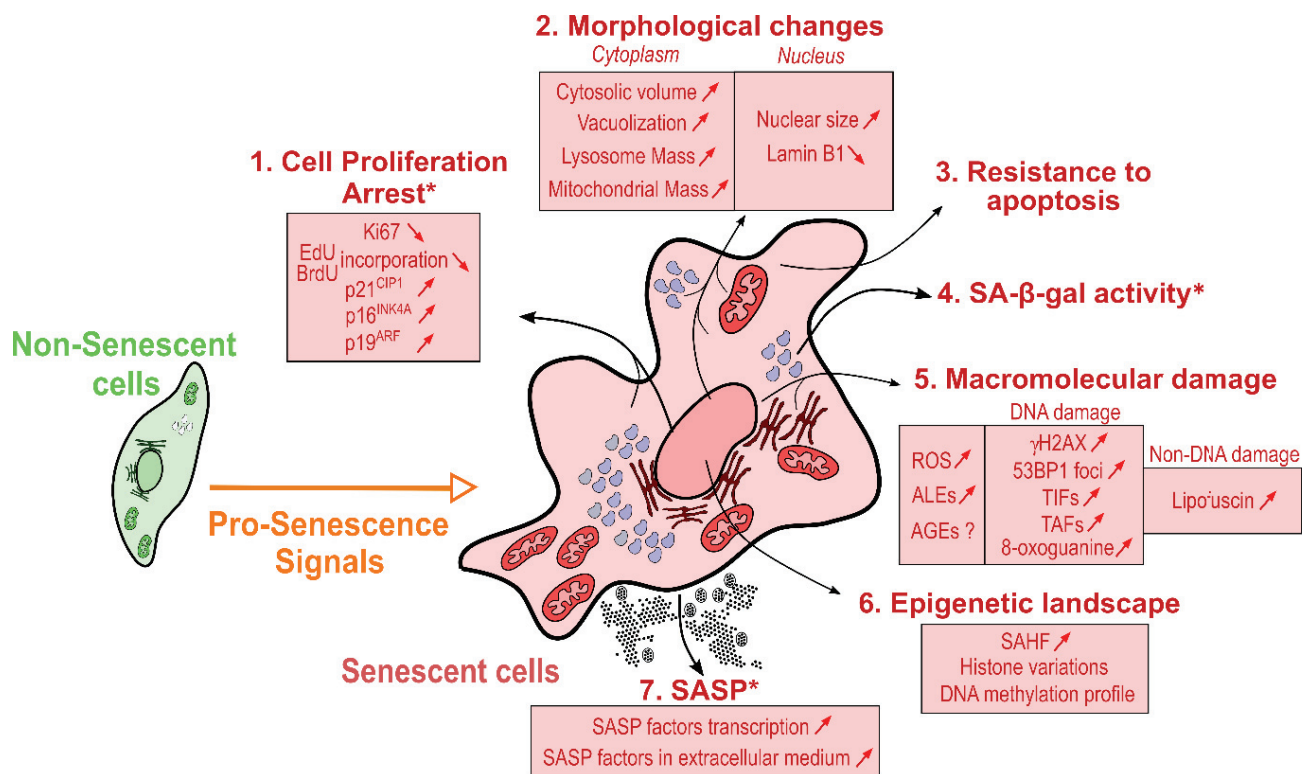
Nature	Biomolecules	Class	Example of factors
Soluble factors	Proteins	Interleukins	IL-6, IL-7, IL-1 $\beta$ , IL-1 $\alpha$
		Chemokines	IL-8, CXCL1, CXCL2, Ccl2, Ccl3, Ccl20, Eotaxin
		Growth Factors	EGF, bFGF, HGF, VEGF, CSF2, NGF, Angiogenin, IGFBPs
		Proteases	MMP-1, MMP-3, MMP-9, TIMP-1, Cathepsin B
		ECM	Fibronectin, Collagens, Laminin
		Receptors / Ligands	ICAM-1, EGF-R, CXCR2, NKG2D
		Others	TGF- $\beta$ , IFN- $\gamma$ , Vinculin, Serpin, FLNA
	Metabolites (non-protein)	Lipids-derived : Prostaglandine E2 (PGE2), 4-OH-2-nonenal (4-HNE)	
		Reactive Oxygen Species (ROS)	
		Nitric Oxide (NO)	
Vesicles	Proteins / Lipids / Nucleic acids (DNA, RNA and miRNA)		

**Table 1: Heterogeneity of SASP components.** The senescence-associated secretory phenotype (SASP) largely includes multiple biomolecules. Proteins, lipids, or ROS may either be soluble (red/blue) or “packaged” in extracellular vesicles (green). The SASP includes, among others, interleukins, chemokines, growth factors, proteases, extracellular matrix (ECM) proteins, receptors/ligands. Of note, SASP *sensu stricto* may refer strictly to the red part of this table, i.e. proteins secreted as soluble factors by senescent cells. Non-exhaustive list. (Adapted from Gorgoulis et al., 2019)

More recently, multiple analysis highlighted the role not only of soluble factors, but also of exosomes released by senescent cells. Formerly described (Lehmann et al., 2008), these extracellular vesicles (EVs) have recently been better characterized and can contain multiple biomolecules, including classical SASP soluble factors (e.g. IL-6, IL-8 ), and lipids, metabolites such as NAD<sup>+</sup>, or nucleic

acids that may participate in the various non-cell autonomous effects of senescence (Alibhai et al., 2020; Borghesan et al., 2019; Jeon et al., 2019; Takasugi et al., 2017; Terlecki-Zaniewicz et al., 2018) (**Table 1**). Interestingly, blocking the exosome machinery within normal cells leads to intracellular accumulation of cytoplasmic DNA, that is sensed by the cGAS-STING pathway, triggering a ROS/DDR-dependent senescence response (Takahashi et al., 2017). This observation reinforces the importance of this process in normal cells and suggests a role in maintaining senescence.

In summary, SASP is a specific secretome that encompasses various molecules and exosomes, including core molecules (e.g. IL-1 $\beta$ , IL-6, IL-8) and additional highly heterogeneous factors. The discovery of the SASP improved our understanding of the myriad of actions that senescent cells could mediate non-cell autonomously in tissues and organs.



**Figure 5: Snow White and the Seven Dwarves - An overview of the main features of cellular senescence.** Summary of the 7 main features of the senescence phenotype, contributing to its characterization *in vitro* or *in vivo*. The most frequent used features are indicated by a star. Boxes recapitulate markers for each feature that can be used experimentally to detect them.

Taken separately, none of the previously mentioned key features are sufficient to ascertain senescence, and permanent cell cycle arrest and SA- $\beta$ -galactosidase activity remain an absolute necessity for its confirmation. Conversely, when combined to other markers, they strongly indicate a senescent phenotype, though variability and intensity of some features (notably morphological

changes, epigenetic landscape and SASP) may remain dependent on cell types, stress inducers and tissue environment (Hernandez-Segura et al., 2018b) (Figure 5).

As a cellular fate, the senescence program and more precisely its main associated outcomes (including cell proliferation arrest and SASP) are finely regulated upstream.

### 1.3. Molecular effectors and regulators of the senescence program

An increasing number of functional studies report the regulation of the senescent key features (previously described) by signaling proteins, called in this section effectors or regulators, through genetic and/or pharmacological approaches. We distinguish (1) effectors as proteins downstream and directly inducing key features of senescence from (2) regulators as proteins modulating these latter effectors. This section first deals with the effectors of the two main features that drive pathophysiological effects of senescence, namely cell cycle arrest and SASP. I will then report different well-known regulators of the senescence program that I have grouped in three main interconnected categories: damage-related, epigenetic-related and metabolism-related regulators (See overview in Figure 8).

#### 1.3.1. Downstream effectors of permanent cell cycle arrest

As previously mentioned, cell cycle arrest is mediated by cyclin-dependent kinases inhibitors (CDKIs). CDKIs represent a family of nuclear proteins, that competitively interfere with CDKs and cyclins to stop cell cycle progression (Pavletich, 1999). In humans, CKIs are divided into two subtypes depending on targeted CDKs: Ink4 or Cip/Kip (Pavletich, 1999). The Ink4-ARF locus encodes three proteins p15<sup>INK4B</sup>, p16<sup>INK4A</sup>, and ARF (p19<sup>ARF</sup> in mouse / p14<sup>ARF</sup> in mouse), which target cyclin D-CDK4/CDK6 complexes mainly blocking the cells in the G1 phase (Pavletich, 1999; Serrano et al., 1993). The Cip/Kip family includes three proteins (p21<sup>WAF1/CIP1</sup>, p27<sup>KIP1</sup> and p57<sup>KIP2</sup>) and counteracts a broader spectrum of cyclin/CDK resulting in proliferation arrest in the different phases of the cell cycle (Pavletich, 1999). Among cyclin/CDK targets, the retinoblastoma (Rb) protein is phosphorylated, releasing the E2F transcription factor, which activates E2F-target genes encoding proteins involved in DNA synthesis and necessary for cell cycle progression through S-phase (Chicas et al., 2010). When CDKIs are upregulated, Rb is hypophosphorylated downstream and subsequently sequesters E2F, triggering cell cycle arrest in senescence (Lukas et al., 1995; Brugarolas et al., 1999) (Figure 6).

In humans, p21<sup>CIP1</sup> and p16<sup>INK4A</sup> are the two most frequent CDKIs upregulated during cellular senescence. Contributions of p21<sup>CIP1</sup> and/or p16<sup>INK4A</sup> to senescence depend on species, cell types and

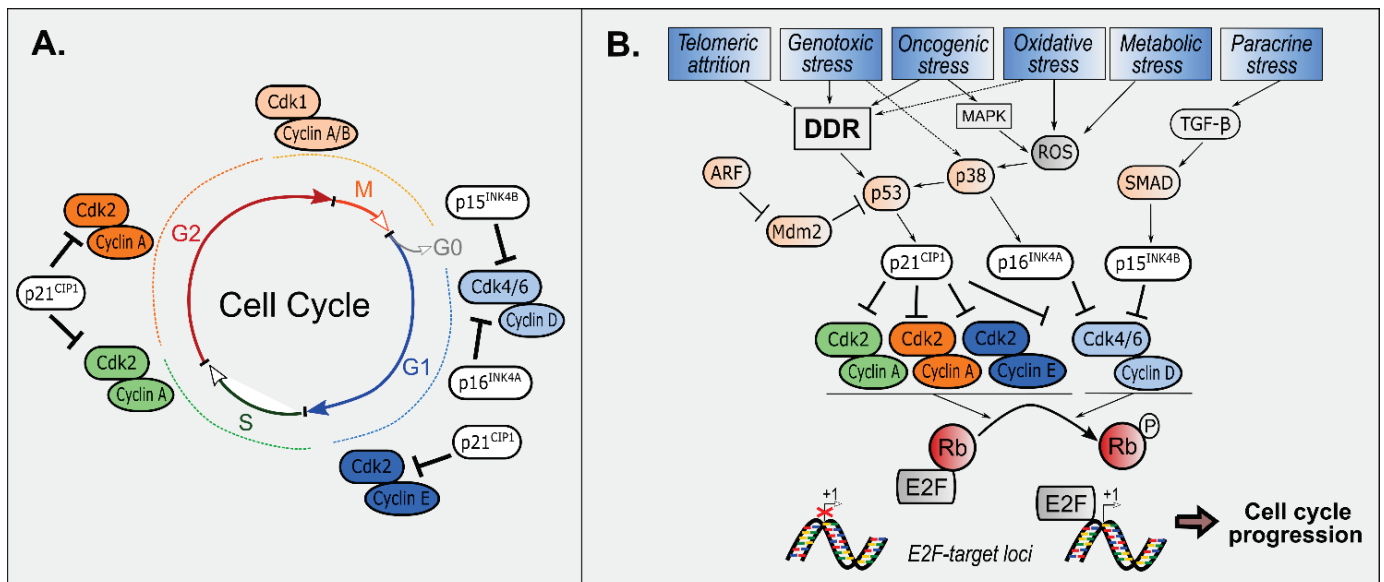
senescence inducers. For example, while p16<sup>INK4A</sup> is crucial for human and mouse OIS (Brookes et al., 2002; Drayton et al., 2003; Serrano et al., 1996), p21<sup>CIP1</sup> does not appear to be essential in murine cells (Pantoja and Serrano, 1999). Overall, the cooperative action of p16<sup>INK4A</sup> and p21<sup>CIP1</sup> appears to be necessary to ultimately trigger permanent cell cycle arrest in senescence. Indeed, according to various kinetic studies, a brief, rapid peak in p21<sup>CIP1</sup> expression, decreasing in “deep” senescent cells, is opposed to a continuous increase in p16<sup>INK4A</sup> expression (Alcorta et al., 1996; Robles and Adami, 1998; Stein et al., 1999). Current hypotheses propose that p21<sup>CIP1</sup> is firstly involved in a checkpoint-reversible cell cycle arrest, while p16<sup>INK4A</sup> elicits a later phase to lock and maintain senescence-associated cell cycle arrest.

Direct upstream regulations occur in the activation of p21<sup>CIP1</sup> and p16<sup>INK4A</sup>. Indeed, p53 is a master regulator of cell cycle arrest and senescence, and its loss is a powerful mechanism for escaping senescence, as it directly induces p21<sup>CIP1</sup> transcription to block cell cycle (**Figure 6**). p53 is mostly stabilized through post-translational modifications. For example, it is phosphorylated following DDR (d’Adda di Fagagna, 2008) (**Figure 3.B**) or acetylated (Peason et al., 2000) during OIS. This p53 stabilization may be DDR-independent and involve the ROS/p38 pathway (Xu et al., 2014) (**Figure 6**). p53 can be targeted by various ubiquitin-protein ligases E3, including double-minute 2 (Mdm2) (Brooks and Gu, 2006). Mdm2 triggers p53 nuclear export and its subsequent proteosomal degradation. Blocking Mdm2, through pharmacological inhibitors such as Nutlin 3-a stabilizes p53 and induces senescence (Efeyan et al., 2007). Endogenously, the ARF protein, encoded by the INK4A/ARF locus, also promotes Mdm2 degradation (Zhang et al., 1998) (**Figure 6**). Of note, the contribution of ARF (human p14<sup>ARF</sup> and mouse p19<sup>ARF</sup>) in regulating senescence seems to be species-specific and is more predominant in mice than in humans.

The INK4/ARF locus encodes another crucial CDKi involved in permanent cell cycle arrest: p16<sup>INK4A</sup>. In proliferating cells, this locus is usually repressed by two crucial polycomb complexes: EZH2-containing polycomb-repressive complex 2 (PRC2) (catalyzing H3K27 trimethylation) and -polycomb-repressive complex 1 (PRC1) (Bracken et al., 2007; Kotake et al., 2007). Following a pro-senescent signal, downregulation of histone methyltransferase EZH2 and recruitment of histone demethylase JMJD3 opens chromatin for transcription of the INK4/ARF locus (Bracken et al., 2007; Agger et al., 2009). Besides, strong senescent signals, including oncogenic/oxidative/metabolic stresses, elicit the activation of the ROS/p38 pathway and p16<sup>INK4A</sup> upregulation through the high mobility group box-containing protein (HBP1) (Xu et al., 2014) (**Figure 6**).

The terminal role of Rb in repressing E2F-target genes is dual. First, as previously mentioned, Rb interacts with E2F and inhibits transcription of E2F-target genes (Figure 6). Moreover, Rb initiates the formation of SAHF, that colocalize with E2F-targeted loci and lock their expression (Narita et al., 2003). In effect, Rb recruits H3K9 methyltransferase SUV39H1 and HP1 (Nielsen et al., 2001) in SAHF. These SAHF are then accompanied by the formation of promyelocytic leukemia (PML) bodies, later recruiting HIRA and ASF1A chromatin remodelers and the chromatin reader BRD4 super-enhancer (Rai et al., 2014; Ye et al., 2007).

Overall, the two main CDKs mediating cell cycle arrest in senescence, p21<sup>CIP1</sup> and p16<sup>INK4A</sup> are finely regulated. Upon pro-senescent signaling, p21<sup>CIP1</sup> is directly regulated by p53 stabilization mediated by ARF or DDR activation (Figure 6). In the meantime, p16<sup>INK4A</sup> is mostly regulated by chromatin remodeling. Remarkably, the INK4A-ARF locus is crucial in regulating cell cycle arrest during senescence.



**Figure 6: Cell cycle regulators during cellular senescence.** **A.** Checkpoints of cell cycle are found in each phase, and involve CDKs and upstream CDKIs in cellular senescence: p21<sup>CIP1</sup>, p16<sup>INK4A</sup>, p15<sup>INK4B</sup>. **B.** Multiple pro-senescent signals (telomere attrition, genotoxic / oncogenic / oxidative / metabolic / paracrine stresses) elicit various responses, through DNA damage response (DDR), ROS or TGF-β and activate CDKIs p21<sup>CIP1</sup>, p16<sup>INK4A</sup>, p15<sup>INK4B</sup>. Downstream, inhibition of Rb phosphorylation blocks the transcription of E2F-target genes, necessary for cell cycle progression.

### 1.3.2. Downstream effectors of SASP

The secretion of SASP factors is regulated by effectors at least at four different levels: transcription, translation, post-translation and secretion.



### **a. Transcriptional regulation of SASP**

So far, two main transcription factors are known to regulate the levels of SASP components through transcription: NF- $\kappa$ B and C/EBP $\beta$  (Acosta et al., 2008a; Kuilman et al., 2008; Chien et al., 2011). These two transcription factors integrate signals to mainly induce transcription of pro-inflammatory genes (*e.g.* IL-1 $\beta$ , IL-6, IL-8) (**Figure 7**). Directly upstream, post-translational stabilization of the transcription factor GATA4 and the DDR-dependent p38MAPK pathway were linked to senescence and more specifically SASP activation, through NF- $\kappa$ B (Freund et al., 2011; Kang et al., 2015). Moreover, SASP dynamics was recently found to be regulated by the Notch pathway. The latter regulates SASP through a bimodal mode during senescence induction, firstly repressing C/EBP $\beta$  to generate a first TGF- $\beta$ -rich secretome, secondly by activating C/EBP $\beta$  to promote the pro-inflammatory arm of SASP (Hoare et al., 2016) (**Figure 7**). Aside from Notch signaling, paracrine activation and/or autocrine reinforcement of NF- $\kappa$ B may also be enhanced via IL-1 $\alpha$  receptor and IL-8 receptor (CXCR2) (Acosta et al., 2008a; Orjalo et al., 2009) (**Figure 7**).

Beyond these main transcription factors involved in SASP regulation, some upstream and downstream regulators modulate SASP. This regulation involves the three categories previously mentioned: (1) damage-related, (2) epigenetic-related and (3) metabolism-related regulators.

### **b. Pre-transcriptional regulation of SASP transcription**

Multiple signals, directly upstream of NF- $\kappa$ B, C/EBP $\beta$ , GATA4 and p38MAPK transcription factors, modulate SASP and have been extensively investigated.

Firstly, the DDR was one the first damage-related SASP regulators described. DNA damage can form DNA segments with chromatin alterations reinforcing senescence, or DNA-SCARS, necessary for the activation of the pro-inflammatory SASP response (Rodier et al., 2009). Overall, the DDR machinery including ATM, ATR, NBS1 and CHK2, is required not only for the initiation but also for the maintenance of the SASP (Chen et al., 2015; Kang and Elledge, 2016; Kang et al., 2015; Rodier et al., 2009) (**Figure 7**). Recent evidence highlighted the importance of cytoplasmic chromatin fragments (CCFs), resulting from persistent DNA damage and nuclear blebbing targeted by the p62-autophagosome/lysosome (Ivanov et al., 2013; Dou et al., 2015). Accumulation of CCFs in senescent cells is sensed by cGAS, further activating STING, and ultimately triggering either IFN or NF- $\kappa$ B-dependent regulation of SASP (Dou et al., 2017; Glück et al., 2017; Ivanov et al., 2013; Takahashi et al., 2018; Vizioli et al., 2020) (**Figure 7**).

Secondly, epigenetic regulators are also included in SASP regulators. Indeed, the promoters of two main SASP genes, IL-6 and IL-8, are regulated by epigenetic changes. For example, degradation or downregulation of two histone writers, histone methyltransferase G9a and histone deacetylase Sirtuin

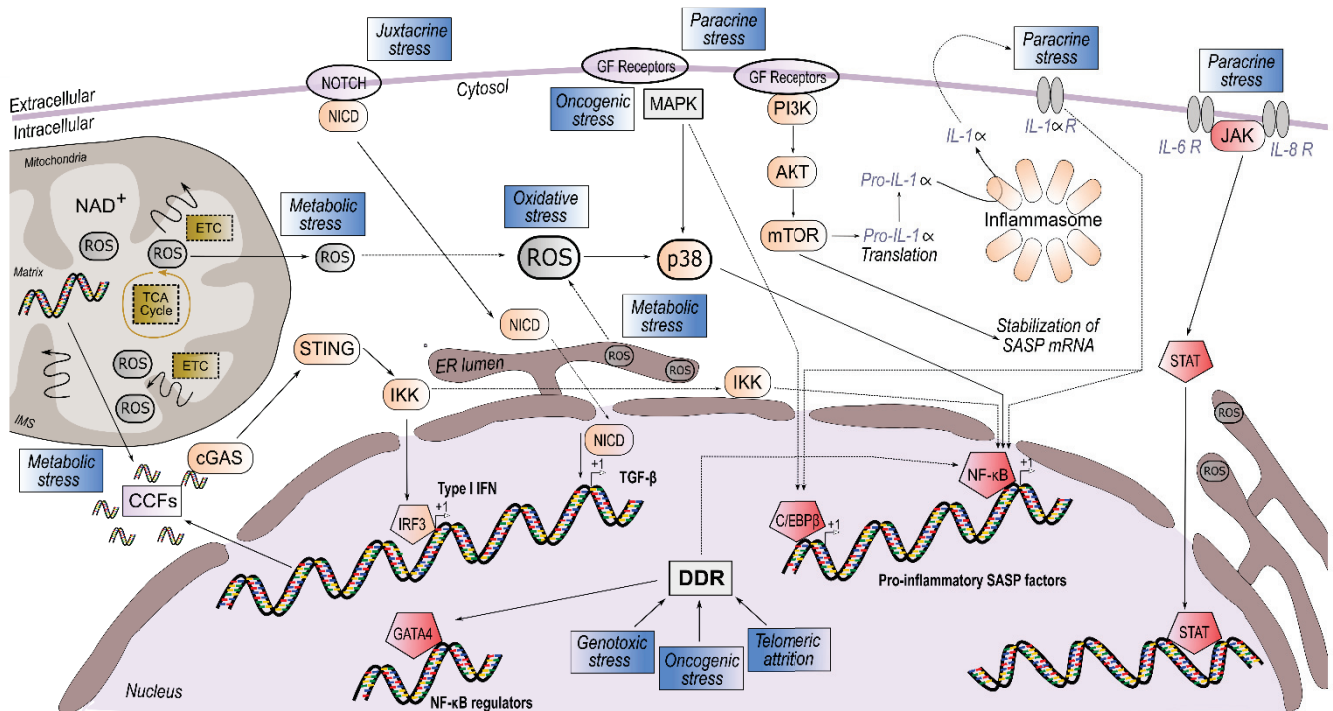
1 (SIRT1), are associated with decreased repressive marks H3K9me2 and increased acetylation in IL-6 and IL-8 gene promoters, respectively, subsequently upregulating their expression during senescence (Takahashi et al., 2012; Hayakawa et al., 2015). Other epigenetics regulators present on SASP gene loci include HMGB2 (Aird et al., 2016), BRD4 (Tasdemir et al., 2016), histone variant H2AJ accumulation (Contrepois et al., 2017), macroH2A1 loss (Chen et al., 2015) or L1 retrotransposons derepression (De Cecco et al., 2019).

Finally, metabolic features recently provided new insights into the potent role of metabolism in the upstream regulation of SASP transcription. Mitochondria, and especially their associated dysfunction, are necessary to drive SASP (Correia-Melo et al., 2016; Birch and Passos, 2017). Recently, mitochondrial dysfunction was shown to reinforce CCF accumulation in the nucleus, through a mitochondria-to-nucleus retrograde signaling (Vizioli et al., 2020). Moreover, cytoplasmic chromatin fragments (CCF) driving cGAS-STING-dependent SASP, can arise directly from mitochondrial dysfunction and mtDNA leakage (Chung et al., 2019; Maekawa et al., 2019). Besides, ER stress and ER-mediated oxidative stress may also contribute to SASP activation (Chen et al., 2015) (**Figure 7**), though this is currently under investigated.

### c. Post-transcriptional regulation of SASP

NF- $\kappa$ B, C/EBP $\beta$ , GATA4 and p38MAPK, SASP can be further post-transcriptionally regulated. One study demonstrated the role of inflammasome through maturation and cleavage of pro IL-1 $\alpha$  into its active form (Acosta et al., 2013). Among other regulators, the autophagy process and the metabolic mTOR pathway were studied in the context of SASP regulation (Herranz et al., 2015; Laberge et al., 2015; Narita et al., 2011). Indeed, mTOR activates IL-1 $\alpha$  translation (Laberge et al., 2015) and inhibits degradation of SASP mRNA transcripts (Herranz et al., 2015) (**Figure 7**). Finally, mTOR is located in special subcellular compartments, termed TOR-autophagy spatial coupling compartments (TASCC), necessary to initiate the SASP (Narita et al., 2011).

Overall, pro-senescent signals elicit cell cycle arrest and SASP via multiple regulators including on the one hand ARF/p53/p21<sup>CIP1</sup> and/or p16<sup>INK4A</sup> and on the other hand NF- $\kappa$ B and C/EBP $\beta$ . Induction of pro-senescent signals may be modulated by upstream regulators, including damage-related, epigenetic-related and metabolism-related regulators. Epigenetic regulators having previously been described in the regulation of cell cycle arrest and SASP, let us now focus on two other interconnected main regulators: damage- and metabolic-related regulators.



**Figure 7: SASP regulators during cellular senescence.** Pro-senescent signals (blue) induce various signals to activate the SASP. The two transcription factors NF- $\kappa$ B and C/EBP $\beta$  integrate multiple signals from MAPK, p38, DDR, and IKK signaling pathways to ultimately induce transcription of pro-inflammatory SASP factors. Upstream, DDR-activated transcription factor GATA4 induces transcription of NF- $\kappa$ B activators. Cytosolic chromatin fragments (CCFs) are sensed by cGAS-STING pathway that activates IKK inducing both NF- $\kappa$ B and Type I interferon (IFN) expression. The notch signaling pathway, through NICD, enhances TGF- $\beta$  expression. Post-transcriptional regulation involves the mTOR pathway and inflammasomes. While, mTOR induces translation of pro-IL-1 $\alpha$  and stabilization of SASP mRNA transcript, inflammasomes cleave pro-IL-1 $\alpha$  into its active form, that can reinforce through a positive feedback loop the activity of NF- $\kappa$ B.

### 1.3.3. Damage-related regulators of the senescence program

As mentioned previously, macromolecular damage is a key feature of senescent cells and is also implicated in signaling pathways involved in the regulation of senescence. Indeed, evidence indicates that telomere, oncogenic, oxidative or genotoxic stresses initiate DDR and senescence both *in vitro* and *in vivo* (Schmitt et al., 2002; d'Adda di Fagagna et al., 2003; Di Micco et al., 2006, 2011; Velarde et al., 2012) (Figure 3). While multiple pro-senescent signals activate DDR and subsequently stabilize p53, further inducing p21<sup>CIP1</sup> transcription (Leonardo et al., 1994) or activate p16<sup>INK4A</sup> (Shapiro et al., 1998), oxidative stress triggers p16<sup>INK4A</sup>/Rb pathway activation, clearly demonstrating links between macromolecular damage and cell cycle arrest.

Accumulation of macromolecular damage can be regulated through at least two distinct paths: production/scavenging of harmful molecules (like ROS) and modulation of repair/degradation machineries. Experimental manipulation of these biological parameters definitely modulates macromolecular damage and senescence. With regards to harmful molecules, endogenous ROS or lipid aldehydes (ALE) increase during senescence and might accelerate the process of macromolecular damage in senescing cells. Furthermore, while suppressing detoxifying enzymes like MnSOD2 promotes onset of senescence (Velarde et al., 2012), antioxidants are known to reverse many senescence phenotypes (Chen and Ames, 1994; Haendeler Judith et al., 2004).

For degradation/repair paths, it is necessary to subdivide them according to the macromolecule subtype. For instance, in the case of DNA, following DDR, DNA repair is initiated. The DNA repair machinery is functionally involved in regulating senescence, since the repression of DNA repair genes induces senescence (Collin et al., 2018), through increased DDR and potent genomic instability.

For lipids, carbohydrates and proteins, degradation/recycling paths rely on two independent pathways: (1) the ubiquitin proteasome system (UPS) and (2) the autophagy/lysosome pathway. Functionally, decline of either UPS or autophagy accelerates accumulated macromolecular damage and senescence *in vitro* (Chondrogianni et al., 2008; Song et al., 2017) and *in vivo* (Min et al., 2008). In senescent cells, the UPS and lysosome activity display an opposite regulation. (1) As a result of accumulated oxidized proteins, high levels of ERK enhance a protein degradation program through the UPS, termed senescence-associated protein degradation (SAPD), taking place in OIS and replicative senescence (Deschênes-Simard et al., 2013). (2) Senescence is accompanied by both increased lysosome content (Kurz et al., 2000) and defective lysosomal activities (Sitte et al., 2000), resulting respectively in increased SA- $\beta$ -gal detection and accumulation of lipofuscin (cross-linked and oxidized proteins). Because senescent cells accumulate damaged biomolecules, and autophagy is altered during senescence (Kang and Elledge, 2016; Young et al., 2009), SA- $\beta$ -gal activity could actually reflect stress-induced activation of autophagy. Overall, autophagy both promotes and inhibits senescence (Young et al., 2009; Narita et al., 2011; Dou et al., 2015; Kang et al., 2015; Kang and Elledge, 2016), highlighting a complex regulatory role in senescence that needs to be further deciphered (Leidal et al., 2018).

Degradation systems, such as autophagy, are considered to be metabolic features, as suggested by their regulation for example by the metabolic mTOR pathway (Young et al., 2009; Narita et al., 2011). Remarkably, cellular senescence is accompanied by multiple metabolic reprogramming programs that pave the way for further investigations into the role of metabolism in regulating senescence.

#### **1.3.4. Metabolic regulators of senescence program**

It should be noted that even though senescent cells present a specific metabolism (Gorgoulis et al., 2019), most of the senescent metabolic features reported herein are so far not univocally described as hallmarks of senescence, as they are currently subjected to intense research.

**CYTOPLASM.** Similarly to cancer cells, senescent cells may present a shift to a glycolytic state (James et al., 2015; Wiley et al., 2016) and an increase in glucose consumption (Dörr et al., 2013), reflecting for instance a different metabolic profile from quiescent cells. Moreover, lipid metabolism is modified during senescence (Cadenas et al., 2012; Flor et al., 2017; Inoue et al., 2017; Maeda et al., 2009; Ogrodnik et al., 2017; Song et al., 2017; Venable et al., 1995). Indeed, clues indicate that lipids like ceramide can induce senescence (Venable et al., 1995). In hepatocytes (Inoue et al., 2017; Ogrodnik et al., 2017), and as well as in fibroblasts (Flor et al., 2016), senescent cells display accumulation of lipid droplets. This accumulation of intracellular lipids includes free fatty acids (Song et al., 2017) or cholesterol (Cadenas et al., 2012; Maeda et al., 2009), and is associated with altered phospholipid synthesis or fatty acid processing (Cadenas et al., 2012; Gey and Seeger, 2013). Interestingly, some oxidized lipids, such as 4-HNE, promote a senescent phenotype (Riahi et al., 2015; Flor et al., 2016, 2017).

**LYSOSOMES.** Replicative senescence is accompanied by defective lysosomal activity (Sitte et al., 2000), resulting for example in the accumulation of lipofuscin (cross-linked and oxidized proteins) throughout cellular senescence. Nonetheless senescence is accompanied by an increased lysosomal mass, allowing SA- $\beta$ -gal detection (Kurz et al., 2000), suggesting abnormal lysosomal activity. Among the other marks of abnormal autophagy/lysosomal activities, senescent cells can display nuclear blebbing, accounting for CCFs and further targeted by the p62-autophagosome/lysosome pathway that can be activated during senescence (Kang and Elledge, 2016; Young et al., 2009). Nonetheless, the exact contribution of autophagy and lysosomes to senescence are still a matter of debate and need to be further investigated.

**MITOCHONDRIA.** Mitochondria are highly dynamic organelles, having various biological roles ranging from metabolism to apoptosis or thermogenesis. An increasing number of studies either report mitochondrial abnormalities in senescent cells, or demonstrate that mitochondrial dysfunction induces cellular senescence (Ziegler et al., 2015).

With regards to metabolism, dysregulation of malic enzyme (ME2) (Jiang et al., 2013) and pyruvate dehydrogenase (PDH) (Kaplon et al., 2013) are associated with senescence. Furthermore,

electron transport chain (ETC) efficiency and  $\text{NAD}^+/\text{NADH,H}^+$  redox balance, largely regulated by mitochondrial metabolism, are both decreased in senescent cells (Moiseeva et al., 2009; Wiley et al., 2016; Ziegler et al., 2015). Concomitantly, endogenous ROS can be generated as by-products of inefficient ETC and promote various senescent subtypes (Ziegler et al., 2015).

Abnormalities in mitochondrial dynamics were suggested to link mitochondrial dysfunction to senescence, through an imbalanced fusion/fission process and an accumulation of elongated hyperfused dysfunctional mitochondria in senescent cells (Korolchuk et al., 2017; Ziegler et al., 2015). Indeed, increased mitochondrial mass in senescence was reported (Lee et al., 2002; Moiseeva et al., 2009; Stab et al., 2016; Ziegler et al., 2015). Interestingly, these mitochondrial dysfunctions were particularly investigated for their role in promoting SASP (Correia-Melo et al., 2016; Birch and Passos, 2017; Vizioli et al., 2020). Importantly, retrograde signals through dysfunctional mitochondria or mtDNA leakage into the cytosol may activate nuclear DDR or cGAS-STING pathway, respectively, necessary to maintain the SASP in senescent cells (Correia-Melo et al., 2016; Vizioli et al., 2020). However, mitochondrial biogenesis and degradation (called mitophagy) are poorly understood in the context of senescence. Paradoxically, several recent studies indicate that increased mitochondrial biogenesis through peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1- $\alpha$ ) or estrogen-related receptor-alpha (ERR- $\alpha$ ), and defective mitophagy could promote cellular senescence (Korolchuk et al., 2017; Summer et al., 2019; Takenaka et al., 2020).

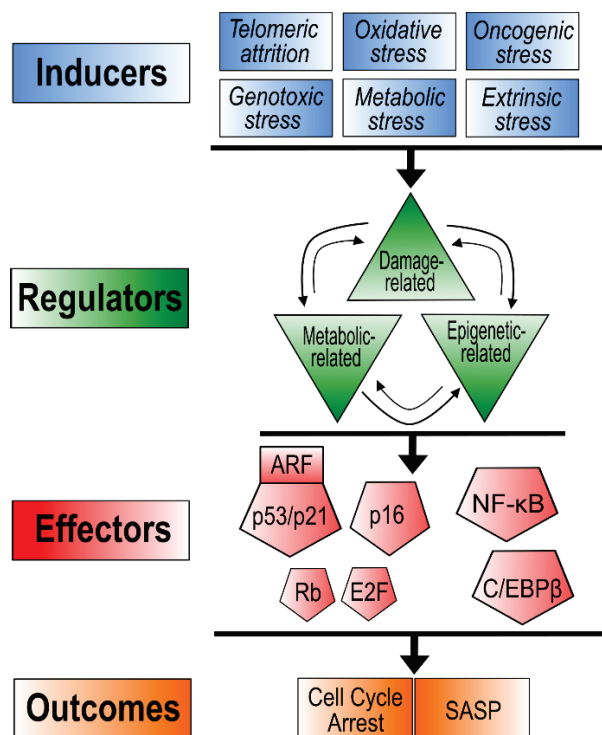
Metabolites, such as  $\text{NAD}^+$  or calcium, regulate mitochondrial functions and impact senescence. The constant generation of  $\text{NAD}^+$  is for example necessary to promote mitochondrial respiration in senescent cells and to sustain the SASP (Nacarelli et al., 2019), unveiling a distinct role for mitochondria in regulating cellular senescence outcomes. Last but not least, mitochondrial calcium regulates mitochondrial bioenergetics (Cárdenas et al., 2010; Rizzuto et al., 2012) and is accumulated during senescence, leading to mitochondrial membrane permeabilization and subsequent ROS generation (Ma et al., 2018; Martin and Bernard, 2018; Wiel et al., 2014).

**ENDOPLASMIC RETICULUM.** The endoplasmic reticulum (ER) forms an interconnected tubular network within the cell, particularly involved in protein/lipid synthesis and calcium exchanges. Because senescence is a stress response, deciphering the role of ER stress in this context is of interest. ER stress is an integrated cellular response, resulting for example from a persistent unfolded protein response (UPR) or from a luminal calcium depletion. Several branches compose ER stress including three main sensors : IRE1, ATF6 and PERK (Hetz, 2012). ER expansion and prolonged UPR were originally identified as mediating OIS in melanocytes (Denoyelle et al., 2006). These ER stresses were then found in various

senescence contexts *in vitro*, such as OIS (Blazanin et al., 2017; Chen et al., 2015; Drullion et al., 2018; Kim et al., 2019), UV- or X-ray-induced senescence (Kim et al., 2019; Panganiban et al., 2013). More interestingly, these data were confirmed and extended to pathophysiological senescence contexts, including therapy-induced senescence (TIS) in lymphomas (Dörr et al., 2013), diabetic nephropathy (Liu et al., 2015a), age-related sarcopenia (Tezze et al., 2017), and in a model of osteoarthritis (Liu et al., 2017). Whether global ER stress can be considered a marker of senescence or the individual contribution of each ER stress pathways (IRE1, ATF6 and PERK) to senescence, are both still under debate.

Altogether, these data indicate that senescent cells present metabolic changes. Unlike quiescent cells, they can be defined by an active metabolic profile (Kwon et al., 2019). As senescent cells are active secretory cells through SASP, this senescent profile might sustain both bioenergetics and substrate availability.

In summary, the two outcomes of senescence, cell cycle arrest and SASP, are tightly regulated upstream and initially induced by various stimuli (Figure 8). Between pro-senescent signals and effectors, numerous regulators modulate senescence and are (1) damage-related, (2) epigenetic or (3) metabolic (Figure 8). Of note, multiple cross-talks between these regulators are possible and necessary, for example between damage-related and epigenetics (Di Micco et al., 2011), or damage-related and metabolic pathways (Aird et al., 2015; Kang et al., 2017) (Figure 8).



**Figure 8: An integrative view of senescence regulation.** Within pre-senescent cells, multiple stress signals arising from inducers (blue) are sensed by regulators (green) that include damage-related, metabolic-related and epigenetic-related regulators. These interconnected regulators integrate, modulate and transmit senescent signals to the downstream effectors that include p53, p21, p16, Rb, E2F, NF-κB and C/EBPβ (red). These effectors ultimately trigger the main outcomes of cellular senescence (cell cycle arrest and SASP) (orange).

The finely regulated senescence phenotype is involved in various pathophysiological conditions.

#### **1.4. Pathophysiological roles of the senescence program**

The regulation of senescence takes place at the level of tissues, and reveals pathophysiological roles of the senescence program. Following pro-senescent signals, some independent cells of a tissue undergo senescence. Within the tissue, the two main outcomes of the senescence program affect tissue homeostasis: through (1) cell autonomous effects and cell proliferation arrest, and (2) non-cell autonomous effects due to the secretion of the SASP. Tissue homeostasis is partly defined by cell-cell communications that are crucial to regulate broader physiological processes.

##### **1.4.1. Cellular senescence and cell-cell communications**

Senescent cells are able to communicate within the tissue with other cells through multiple non-cell autonomous signals that include: SASP autocrine signals (Acosta et al., 2008a; Kuilman et al., 2008; Wajapeyee et al., 2008), SASP paracrine signals (Acosta et al., 2013; Hoare and Narita, 2013; Chen et al., 2015; Bird et al., 2018), cytoplasmic bridges (Biran et al., 2015) and juxtacrine signals (Parry et al., 2018). Paracrine senescence is thought to be one of the main mechanisms to locally spread senescence within tissues (Acosta et al., 2013; Chen et al., 2015; da Silva et al., 2019) (**Figure 8**). More importantly and as suggested by recent studies, parabiosis and potent endocrine or systemic effects of SASP through blood circulation may also alter distant tissue homeostasis (Alibhai et al., 2020; Barinda et al., 2020; Yousefzadeh et al., 2020b).

Aside from the secretion of various interleukins and chemokines, senescent cells upregulate the expression of transmembrane ligands interacting with key immune cells. Together, they create a local pro-inflammatory environment which favors chemoattraction of immune cells and immune surveillance of senescent cells. The cooperating action of chemokines (e.g. CCL2) and ligands of natural killer (NK) cell receptors (e.g. NKG2D ligand) leads to the recruitment of NK cells, neutrophils or macrophages, resulting in senescent cells clearance (Iannello and Raulet, 2014; Sagiv et al., 2016). Deficiencies in clearing senescent cells by the immune system are thus one of the main drivers of senescent cell accumulation (Ovadya et al., 2018). Finally, escape from immune surveillance by senescent cells appears to amplify chronic senescence (Pereira et al., 2019) (**Figure 9**).

Overall, the senescence program appears to be not only an isolated cellular stress response but also a dynamic and integrative signal within tissues. Cross-talks between senescent cells and



surrounding cells that include normal cells, immune cells or cancer cells, underlie the multiple pathophysiological roles of senescence.

#### **1.4.2. Cellular senescence and development**

Cellular senescence was initially considered solely to be a response to cellular stresses. Consistently, the concept of non-stress-mediated programmed cellular senescence was under-investigated until recently.

In 2013, programmed cellular senescence was discovered in embryonic development (Muñoz-Espín et al., 2013; Storer et al., 2013). This process takes place at multiple stages and is determinant for the development of the inner ear, neural roof plate or mesonephros (Gibaja et al., 2019; Muñoz-Espín et al., 2013; Rhinn et al., 2019; Storer et al., 2013). This senescence can also accompany apoptosis in the formation of digits (Lorda-Diez et al., 2015). Independent of the DDR and p53-driven pathway, senescence here is mechanistically mediated by TGF- $\beta$ /SMAD and PI3K/FOX, and ultimately triggered by p21<sup>CIP1</sup> (Lorda-Diez et al., 2015; Muñoz-Espín et al., 2013; Rhinn et al., 2019; Storer et al., 2013). Remarkably, this developmental programmed senescence is conserved across vertebrates, and found in other rodents and amphibians (Davaapil et al., 2017; Zhao et al., 2018). Furthermore, it is not restricted to the embryonic development. Indeed, late puberty leads to the implementation of programmed cellular senescence for example in bone (Li et al., 2017a; Rhinn et al., 2019), reinforcing the importance of senescence in the regulation of both embryonic and post-embryonic development (**Figure 9**).

If inefficiently cleared in the embryo, the accumulation of senescent cells may have detrimental effects (Keyes et al., 2005), suggesting a necessary spatial and temporal regulation of senescence during development.

#### **1.4.3. Cellular senescence and tissue regeneration**

As a damage-induced response, senescence is involved in wound repair and antifibrotic responses (**Figure 8**) in various mouse organs, including liver (Krizhanovsky et al., 2008), skin (Demaria et al., 2014a; Jun and Lau, 2010), lung (Schafer et al., 2017a), lymph node (Maarouf et al., 2018) and heart (Feng Teng et al., 2019; Sarig Rachel et al., 2019). SASP activation through the induction of CC motif chemokine ligand 1 (CCN1) (Feng Teng et al., 2019; Jun and Lau, 2010), platelet-derived growth factor AA (PDGF-AA) (Demaria et al., 2014a) and hemostasis factors (Wiley et al., 2019), leads to an efficient platelet activation and the recruitment of immune cells (NK cells and macrophages) surrounding senescent cells, triggering fibrosis resorption. Interestingly, this damage-induced senescence is found in other vertebrates, especially in salamander during limb amputation (Yun et al.,

2015) or zebrafish after fin injury (Da Silva-Álvarez et al., 2020). Nonetheless, the accumulation of chronic senescent cells may promote fibrosis in various models such as idiopathic pulmonary fibrosis (IPF) (Schafer et al., 2017a; Kato et al., 2020) or renal fibrosis (Miao et al., 2019), suggesting once again necessary spatial and temporal regulation of senescence during tissue regeneration (**Figure 9**).

Furthermore, as tissue regeneration is closely linked to reprogramming, it should be noted that senescence opposes reprogramming in two apparent ways. Firstly, as previously mentioned, OSKM factors, aside from iPSCs generation, favor the establishment of cellular senescence drastically reducing reprogramming efficiency *in vitro* (Banito et al., 2009) and the deletion of senescence effectors such as p53, p16<sup>INK4A</sup> or ARF drastically improves reprogramming efficiency (Banito et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009b; Marión et al., 2009; Utikal et al., 2009). Overall, these *in vitro* studies suggest a cell autonomous effect of senescence as an anti-reprogramming barrier and may add another layer of complexity to its tumor suppressive role. Nonetheless, no *in vivo* study so far has been conducted to confirm this particular role. Secondly, and beyond OSKM-driven senescence, injury-induced senescence and persistent SASP activity (particularly IL-6) (Mosteiro et al., 2016) promote *in vivo* reprogramming of neighboring cells, through a non-cell autonomous effect (Mosteiro et al., 2016; Ritschka et al., 2017; Chiche et al., 2017; Rhinn et al., 2019). Taken together, cell autonomous and non-cell autonomous effects of senescence appear to mainly have anti and pro-reprogramming effects, respectively, though further works are required to clarify their importance and contribution *in vivo*.

#### **1.4.4. Cellular senescence and cancer**

The impact of senescence in cancer has been extensively studied. Firstly, telomere attrition and replicative senescence block the immortalization of normal cells, a key feature of cancer cells (Kiyono et al., 1998). Secondly, activated oncogenes or loss of tumor suppressors activate OIS (Serrano et al., 1997; Zhu et al., 1998). Based on these two independent *in vitro* observations, cellular senescence appears to be an anti-tumoral barrier blocking immortalization of normal replicative and hyper-replicative cells. Nonetheless, the tumor suppressive role of OIS *in vivo* was confirmed only in 2005. At that time, multiple independent experiments demonstrated the presence of senescence in various pre-malignant lesions from lymphoma to pituitary and lung or skin lesions (Braig et al., 2005; Denchi et al., 2005; Michaloglou et al., 2005; Roberson et al., 2005). Escape from senescence, through genetic loss of key senescence effectors, such as p53, p19<sup>ARF</sup> or p16<sup>INK4A</sup> in these models, contributes to tumor progression. Overall, local and acute senescence is a powerful tumor suppressor in replicating or hyper-replicating cells (**Figure 9**).

However, chronic and systemic accumulation senescing cells could have detrimental effects and promote cancer initiation, progression or resistance. Indeed, as observed during aging (Herbig et al., 2006; Jeyapalan et al., 2007; Yousefzadeh et al., 2020a), accumulation senescing cells could be due to increased intracellular damage and/or decline in the senescence immune surveillance (Kang et al., 2011; Krizhanovsky et al., 2008; Lujambio et al., 2013). This accumulation increases the risk for cells to naturally escape senescence and favors tumor initiation and progression. Moreover, while cancer treatments (chemotoxic agents or irradiation) can induce cell proliferation arrest and senescence in cancer cells (Schmitt et al., 2002), persistent senescent cells display chemoresistance and are the source of cancer recurrence (Demaria et al., 2017) (**Figure 9**). Moreover, persistent senescent cells and associated SASP have numerous pro-tumorigenic non-cell autonomous effects on surrounding cancer cells, including cell growth, angiogenesis or even EMT (Krtolica et al., 2001; Coppé et al., 2006; Laberge et al., 2012; Liu et al., 2015b; Li et al., 2020) (**Figure 9**), as recently demonstrated *in vivo* in a HCC model (Li et al., 2020).

In line with this latter effect, the development of senolytics, drugs specifically clearing senescent cells, are highly interesting. Indeed, the synergic action of drugs killing firstly cancer cells and secondly therapy-induced senescent (TIS) cells in an increasing number of pre-clinical studies show promising results, thus extending in the future the possibility of using senolytics in combination with standard chemotherapy in cancer treatment (Dörr et al., 2013; Levenson et al., 2015; Deursen, 2019; Triana-Martínez et al., 2019; Guerrero et al., 2019; González-Gualda et al.; Wakita et al., 2020; Li et al., 2020).

#### **1.4.5. Cellular senescence and aging**

Senescent cells accumulate during aging in various animal models (Herbig et al., 2006; Jeyapalan et al., 2007; Wang et al., 2009), in spite of the heterogeneity in the speed of accumulation depending on organs (Tuttle et al., 2020), individual predisposition and exposomes for example. This chronic and systemic accumulation of senescence has detrimental effects and generates tissue dyshomeostasis (Muñoz-Espín and Serrano, 2014a), through both autonomous and non-autonomous effects. While cell autonomous effects lead to stem cell exhaustion (Castilho et al., 2009; Liu et al., 2007) and/or aberrant cellular function (Helman et al., 2016), non-cell autonomous effects through SASP mediate among others fibrosis and/or inflammation (Schafer et al., 2017a; Miao et al., 2019) (**Figure 9**).

Liver senescence has been particularly studied in the context of senescence. Intrahepatic cells including stellate cells and hepatocytes may undergo senescence. However, this senescence results in paradoxical beneficial and detrimental effects. While stellate cell senescence allows immune cell

recruitment and fibrosis clearance (Krizhanovsky et al., 2008), hepatocyte senescence drives age-dependent steatosis (Ogrodnik et al., 2017) and is thought to be an important factor of non-alcoholic fatty liver disease (NAFLD). Besides, as the pancreas controls insulin and glucagon secretions, pancreatic senescence and glucose homeostasis were recently explored in the context of senescence. While pancreatic  $\beta$  cell senescence enhances insulin secretion (Helman et al., 2016), its chronic accumulation during aging critically alters glucose homeostasis and confers insulin resistance to cells, similar to type 2 diabetes (T2D) (Aguayo-Mazzucato et al., 2019).

Immunosenescence is linked to a decline in the immune repertoire and is characterized by the exhaustion of naïve T cells (Flach et al., 2014; Yahata et al., 2011) and a chronic enrichment in memory T cells, that are CDKi positive (Signer et al., 2008) and secrete pro-inflammatory factors reflecting SASP composition (including IL-6 and TNF- $\alpha$ ) (Vicente et al., 2016). Together, immunosenescence is associated with “inflammaging”, and positively correlated with dementia, Parkinson’s disease, atherosclerosis, T2D, sarcopenia and a high risk of morbidity and mortality (Ventura et al., 2017) **(Figure 9)**.

Multiple other age-related pathologies have been functionally linked to senescence, including atherosclerosis, osteo-arthritis, sarcopenia, neurodegenerative disorders (He and Sharpless, 2017) **(Figure 9)**. As a proof-of-concept, multiple genetic and pharmacological approaches to selectively target and eliminate senescent cells in mouse models have resulted in delaying aging, improving both lifespan and healthspan (Baker et al., 2011; Ogrodnik et al., 2017; Schafer et al., 2017a; Jeon et al., 2017; Bussian et al., 2018; Xu et al., 2018; Zhang et al., 2019; Aguayo-Mazzucato et al., 2019). This cumulative evidence led the field of senescence to focus on new potent drugs selectively killing senescent cells, termed senolytics (Deursen, 2019), or to a lesser extent drugs reducing SASP secretion, termed senostatics. Multiple screens are currently investigating senolytic effects of compounds. So far and by chronological order of discovery, senolytics include Dasatinib/Quercetin (DQ) combination (Zhu et al., 2015; Ogrodnik et al., 2017; Schafer et al., 2017a), anti-apoptotic Bcl-2 family inhibitors (e.g. ABT-263 or ABT-767) (Chang et al., 2016; Childs et al., 2016; Yosef et al., 2016), Fisetin (Zhu et al., 2017), FOXO4 peptide (Baar et al., 2017), heat shock protein (HSP) 90 inhibitors (Fuhrmann-Stroissnigg et al., 2017), and more recently, cardiac glycosides (Guerrero et al., 2019; Triana-Martínez et al., 2019) **(Table 2)**.

While multiple senolytic drugs are being discovered, the efficiency of drug delivery and specific targeting of senescent cells remains challenging. This is why recent investigations report the growing interest in galactose-conjugated senolytics, promoting the release of senolytics in contact with

senescent cells thus improving the efficacy of senescence clearance and limiting cytotoxic side-effects during drug delivery (Guerrero et al., 2020; González-Gualda et al.)

Class of compounds	Mechanism of action	Molecules	Major senescence models	Major reported effects	References
<b>Tyr. kinase inhibitor</b> + <b>Flavonoids</b>	Inhibition of SRC, c-KIT, ephrin receptors  + Inhibition of PI3K/AKT signalling pathway (+ serpins)	<b>Dasatinib</b>  + <b>Quercetin</b>	<i>In vitro</i> HUVECs, Preadipocytes Irradiation-induced senescence  <i>In vivo</i> - Progeroid Ercc1 <sup>-Δ</sup> mice - Normally aged mice - Bleomycin-injury mouse model (lung) - Obese mouse model/ HFD-fed mice - AD mouse mode	- Enhance apoptosis in HUVECs, Preadipocytes, fibroblasts  - Reduced p16INK4A and SA-β-gal in irradiated limb - Improved cardiac functions/atherosclerosis/mobility - Reduce IPF - Reduce age-dependent hepato-steatosis - Improved obesity-induced metabolic dysfunction - Reduced systemic pro-inflammatory cytokines - Alleviates AD-associated cognitive deficits	(Zhu et al., 2015) (Roos et al., 2016) (Ogrodnik et al., 2017) (Schafer, 2017) (Palmer et al., 2019) (Xu et al., 2018) (Ogrodnik et al., 2019) (Zhang et al., 2019)
<b>Bcl-2 inhibitors</b>	Inhibition of upregulated anti-apoptotic proteins Bcl-2	<b>ABT-263</b>  <b>ABT-767</b>  A-1155463 A-1331852	<i>In vitro - In vivo</i> - Sub-lethal irradiated HSC - Anterior cruciate ligament transection - Normally aged mice - Tau-dependent neurodegenerative model - Insulin-resistant model (HFD-fed mice)	- Bone marrow and muscle haematopoietic stem cells rejuvenation - Reduction of post-traumatic osteoarthritis - Reduced levels of SASP factors and p16 <sup>INK4A</sup> in aged murine lungs - Reduction of senescent glial cells and inhibition of the degeneration of cortical and hippocampal neuron - Improved glucose metabolism and β cell function	(Chang et al., 2016) (Chikis et al., 2016) (Jeon, 2017) (Bussian et al., 2018) (Aguayo et al., 2019)  (Yosef et al., 2017) (Leverson et al., 2015)  (Zhu et al., 2017)
<b>Flavonoids</b>	?	Fisetin	<i>In vitro</i> HUVECs, IMR90, PreAdipocytes (Irradiation-induced senescence)  <i>In vivo</i> Progeroid Ercc1 <sup>-Δ</sup> mice Chronological aging WT mice	HUVECs → caspase-3 mediated apoptosis No effect on senescent IMR90 or Preadipocytes  Fat, Liver, Spleen, Kidney → reduced CDKIs and SASP Reduction of p16-positive cells (immune, endothelial cells) + Extending lifespan	(Zhu et al., 2017)  (Yousefzadeh, 2018)
<b>Modulatory Peptide</b>	Inhibits p53/FOXO4 interaction → nuclear export of p53	FOXO4	<i>In vitro - In vivo</i> IMR90 (Irradiation-induced senescence + Doxo-induced senescence) WT mice – Naturally aged or Doxo-induced senescence + Progeroid Xpd <sup>TID/TID</sup> mouse mode	Enhance p53-mediated apoptosis in IMR90 Reduces dox-induced senescence and chemotoxicity Reduces age-related kidney natural defects Alleviates age-related features (frailty, kidney,...)	(Barr et al., 2017)
<b>HSP90 inhibitors</b>	Inhibition of HSP90 ?	17-DMAG	<i>In vitro - In vivo</i> Progeroid Ercc1 <sup>-Δ</sup> mice	(1) In MEFs → Reduced cell volume + IL6 + p16 <sup>INK4A</sup> expression (2) In mice → Reduced frailty + p16 <sup>INK4A</sup> expression in liver/kidney	(Fuhrmann-Stroissnigg et al., 2017)
<b>Cardiac Glycosides</b>	Inhibit Na <sup>+</sup> /K <sup>+</sup> ATPase (ATP1A1 subunit)	Digoxin  Oubain	<i>In vitro - In vivo</i> A549 xenograft PDX xenograft Lung fibrosis	Synergize with Gemcitabine effects  Reducing p16 <sup>INK4A</sup> and fibrosis	(Triana-Martinez et al., 2019)  (Guerrero et al., 2019)

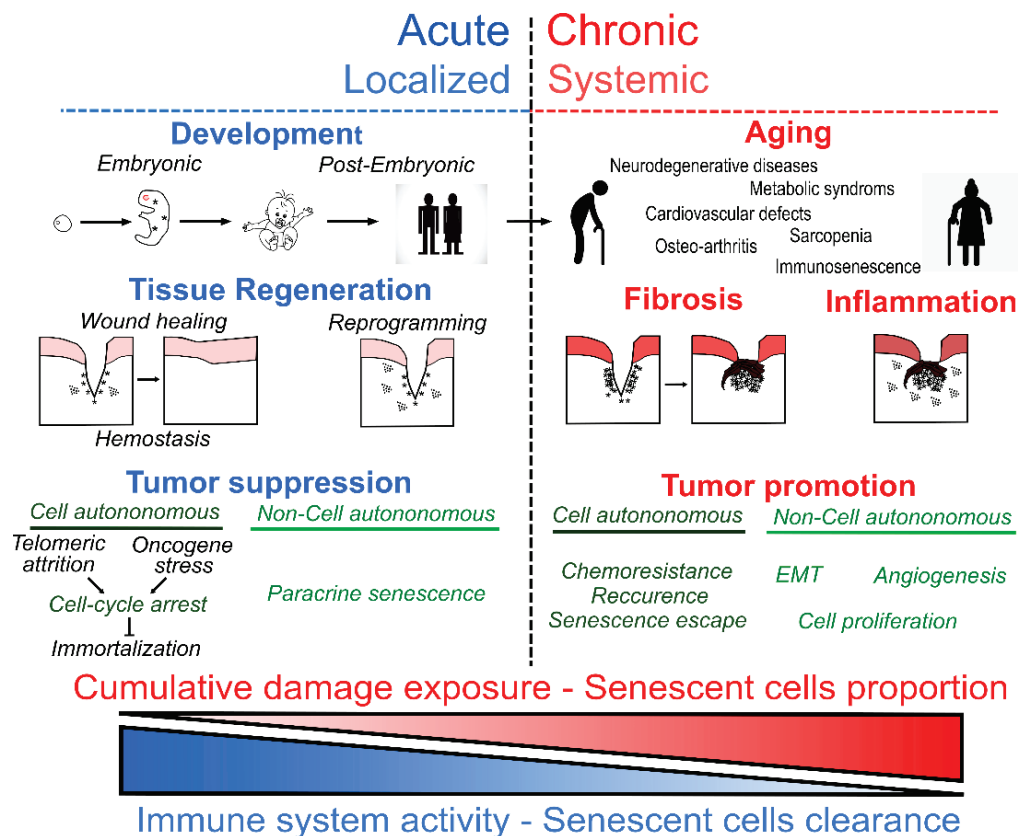
**Table 2: List of the main known compounds with senolytic actions.** First discovered (red), inhibition of various tyrosine kinases (Dasatinib + Quercetin) and pro-survival pathways (Bcl-2 inhibitors) are amongst the two main senolytics reported in literature. Flavonoids, modulatory peptide-FOXO4 and HSP90 inhibitors (blue) display evidence of senolytic effects though they have not been further investigated so far. Cardiac glycosides (green) account for the latest class of senolytics discovered acting on ion channels Na<sup>+</sup>/K<sup>+</sup> ATPase. Of note, this list is non-exhaustive and other compounds including azithromycin, roxithromycin (Ozsvari et al., 2018), piperlongumine (Wang et al., 2016c), or more recently BET family protein degrader (BETd) (Wakita et al., 2020) have senolytic effects as well.

#### 1.4.6. The two facets of cellular senescence: acute and chronic contexts

The multiplicity of roles for senescence in pathophysiological contexts illustrate the complexity of this phenotype. While acute and local short-lived senescence is beneficial, contributing to wound healing, tissue regeneration or tumor suppression, chronic and systemic long-lived senescence is detrimental, leading to tumor promotion, aging and age-related pathologies (**Figure 9**). Although numerous studies are focusing on discovering senolytics to delay aging, it is crucial to keep in mind the benefits of cellular senescence.

The origin of chronic accumulation of senescent cells might be dual as suggested by recent reports. (1) The accumulation of damage associated with decreased efficiency in repair/degradation programs is thought to be one of the main drivers of pro-senescent stimuli (Ogrodnik et al., 2019;

Yousefzadeh et al., 2020a). (2) Deficiencies in clearing senescent cells by the immune system, probably linked to immunosenescence, constitute another promoter of senescent cell accumulation during aging (Ovadya et al., 2018), immune surveillance escape mechanisms by senescent cells further amplifying chronic senescence (Pereira et al., 2019) (**Figure 9**).



**Figure 9: Physiological and pathological roles of cellular senescence.** When acute and localized, cellular senescence participates in development, tissue regeneration (wound healing, hemostasis, reprogramming), and tumor suppression. All these beneficial effects are mediated through cell autonomous and non-cell autonomous effects (driven by the SASP). When chronic and systemic, accumulation of senescent cells participates to aging and various age-related pathologies, fibrosis, systemic inflammation and tumor promotion. EMT: epithelial-mesenchymal transition.

To summarize this first chapter, cellular senescence is a complex phenotype induced by multiple stresses, thus constituting an integrative cellular stress response. These multiple stresses lead to some heterogeneity in senescence phenotypes. Nonetheless, some cellular key features are shared by senescent cells that facilitate delineation of senescence both *in vitro* and *in vivo*. This diversity in inducers and effectors mirrors the multiplicity of regulators, adding another layer of complexity to the senescence program. In spite of major heterogeneity, senescence is not an isolated cellular response but is involved in key biological processes, both physiological and pathological, reinforcing the importance to dissect mechanisms regulating this orchestrated senescence program.

While multiple inducers and effectors of cellular senescence are well-characterized, intrinsic subcellular mechanisms linking and regulating them remain a matter of active research. I will now present two important pathways in the context of senescence, namely the calcium signaling pathway through the inositol triphosphate receptor 2 (ITPR2), and the mevalonate pathway, both of which were the subject of my PhD.

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## **- CHAPTER II -**

### **-The ER calcium-release channel ITPR2: features, interactions and biological roles -**

As previously noted, while senescent cells display a dysregulated metabolism, multiple metabolic regulators also influence the fate of senescing cells. Regulation of calcium homeostasis is a prerequisite for normal cell functioning. Interestingly, calcium metabolism was recently shown to be linked to cellular senescence (Yu et al., 2013; Wiel et al., 2014; Farfariello et al., 2015; Borodkina et al., 2016; Ma et al., 2018; Martin and Bernard, 2018), opening new exciting perspectives to better understand how calcium homeostasis affects senescence and subsequent pathophysiological contexts.

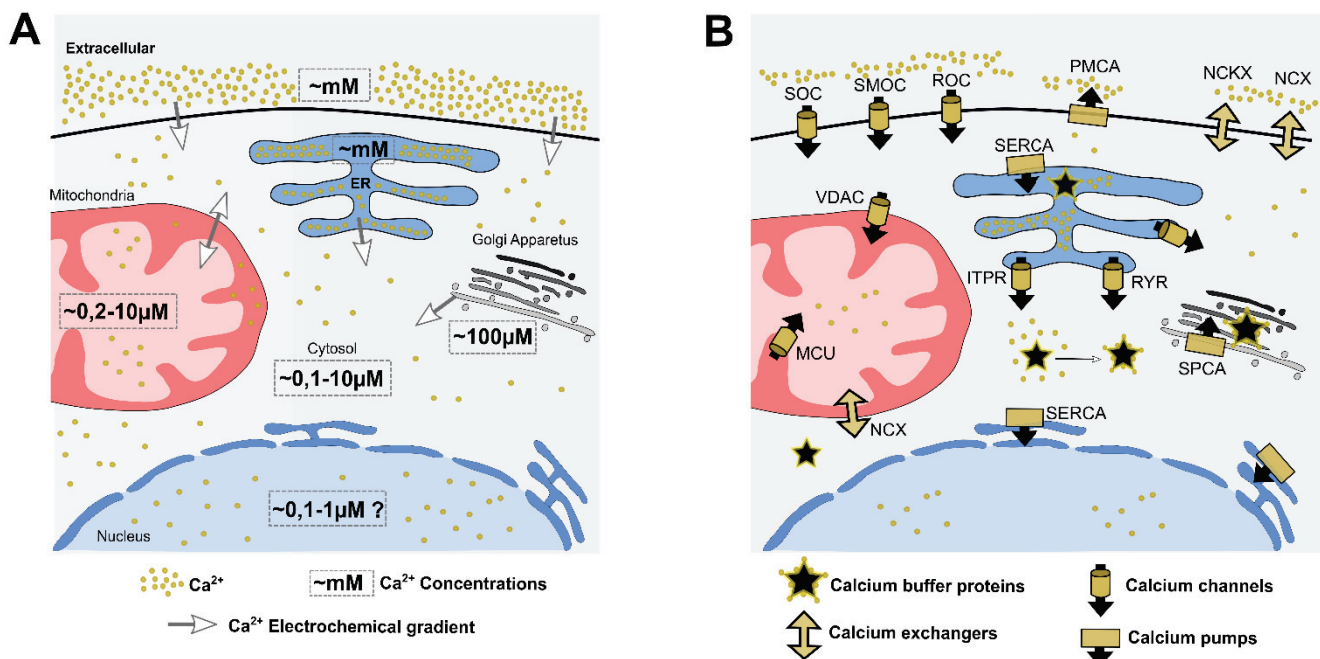
#### **2.1. Calcium homeostasis and cellular senescence**

##### **2.1.1. Calcium within the cell: a necessary balance**

Calcium, and especially its dynamics within the cell, participates in various crucial cellular processes that include among others proliferation, secretion, migration, metabolism or apoptosis (Clapham, 2007). Being less soluble in H<sub>2</sub>O than other cellular ions such as magnesium, calcium in its cationic form Ca<sup>2+</sup> is likely to precipitate with phosphate, and becomes cytotoxic at high concentrations (Clapham, 2007). For this reason, every cell maintains a low Ca<sup>2+</sup> concentration in the cytoplasm (~100 nM) releasing Ca<sup>2+</sup> from the cell (mM concentrations), though some intracellular tanks, such as ER and to a lesser extent mitochondria, store it efficiently (Clapham, 2007). These reservoirs thus create multiple electrochemical gradients (**Figure 10.A**).

Beyond this potential toxic effect, as a cation, Ca<sup>2+</sup> alters local electrostatic fields especially in amino acids, thus modulating protein conformation and acting as a powerful signaling molecule. To balance the signaling and cytotoxic effects of Ca<sup>2+</sup>, multiple channels (passive Ca<sup>2+</sup> transfer according the electrochemical gradient), exchangers and ATPases pumps (active Ca<sup>2+</sup> transfer against the electrochemical gradient requiring energy) regulate calcium homeostasis and fluxes inside the cell (Clapham, 2007) (**Figure 10.B**). ITPR2 is part of this regulatory machinery acting as a channel anchored to the ER membrane.





**Figure 10: Calcium homeostasis within the cell.** **A.** Heterogeneity of calcium contents in cellular and extracellular compartments. Differences in calcium concentration create a local electrochemical gradient from either side of membranes. **B.** Calcium fluxes between intracellular compartments are regulated either by channels (passive fluxes) according to the gradient, or by exchanger and pump (active fluxes) requiring energy, depending on other electrochemical gradients or ATP, respectively. Other proteins may buffer and store calcium locally. SOC: store-operated channels; SMOC: SPARC-related modular calcium binding protein; ROC: receptor-operated channels; PMCA: plasma membrane  $\text{Ca}^{2+}$  ATPase; NCX:  $\text{Na}^+/\text{Ca}^{2+}$  exchanger; NCKX:  $\text{K}^+$ -dependent  $\text{Na}^+/\text{Ca}^{2+}$  exchanger; SERCA sarcoendoplasmic reticulum  $\text{Ca}^{2+}$  ATPase; SPCA:  $\text{Ca}^{2+}/\text{Mn}^{2+}$  ATPase; ITPR: inositol triphosphate receptor; RYR: ryanodine receptor; MCU: mitochondrial calcium uniporter; VDAC: voltage dependent anion channel.

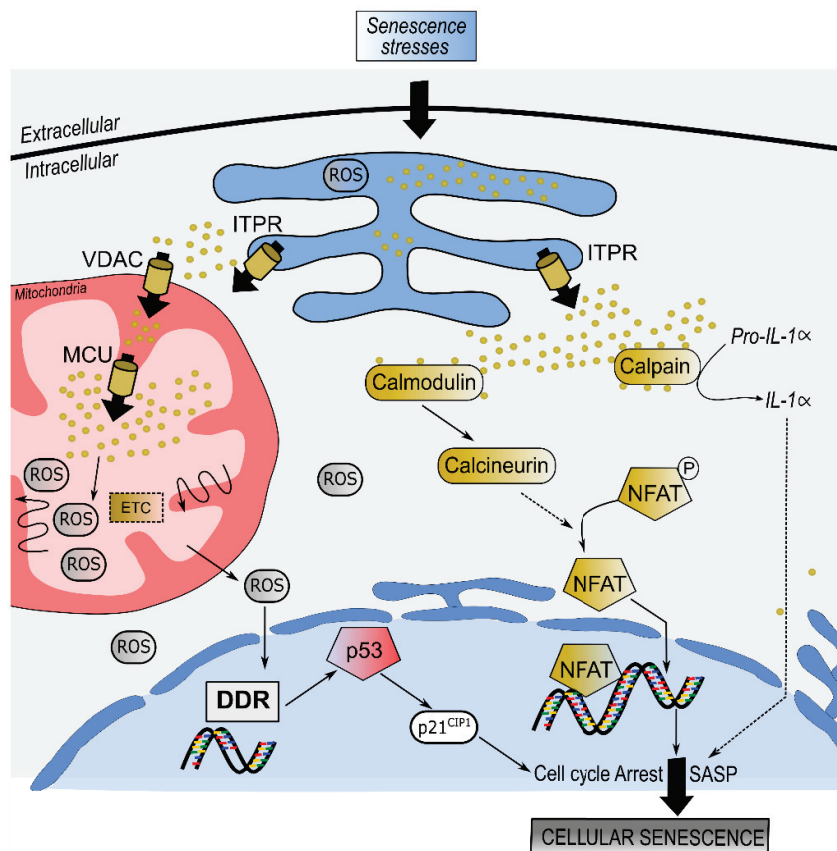
### 2.1.2. Clues as to the involvement of calcium in the regulation of senescence features

Calcium is involved in numerous senescence-associated pathophysiological contexts such as cancer progression, wound healing or development (Wu et al., 2010; Enyedi and Niethammer, 2015; Webb and Miller, 2003). Moreover, calcium modulation during cell cycle progression and secretion (Martin and Bernard, 2018) both suggest a role for calcium in regulating cellular senescence

The first clue as to the involvement of calcium signaling pathways in senescence came from research on the calmodulin/calcineurin/NFAT pathway. This pathway is activated following a rise in cytosolic  $\text{Ca}^{2+}$  (**Figure 11**) (Crabtree and Olson, 2002). Elevated cytosolic  $\text{Ca}^{2+}$  activates this pathway upstream, and was shown to mediate a p53-dependent senescence (Wu et al., 2010). This role for intracellular calcium fluxes in regulating senescence was further evidenced, as a rise in intracellular calcium was shown to accompany senescence (Borodkina et al., 2016; Wiel et al., 2014; Yu et al., 2013). This increased cytosolic calcium is mediated either by extracellular (Yu et al., 2013) and/or intracellular

stocks (Borodkina et al., 2016; Wiel et al., 2014), such as ER calcium stocks (Wiel et al., 2014). Further studies have reinforced the importance of calcium in specifically modulating the activity of key actors of SASP, such as IL-1 $\alpha$  or NLRP3 (Acosta et al., 2013). Calcium induces calpain activation to cleave pro-IL-1 $\alpha$  in IL-1 $\alpha$  and regulates SASP initiation (McCarthy et al., 2013). In addition calcium is necessary to independently activate the NLRP3 inflammasome (Murakami et al., 2012), which has been linked in other studies to SASP onset (Acosta et al., 2013).

In order to extend these first observations on the role of calcium fluxes and calcium signaling pathways in regulating senescence, deciphering the role of main calcium channels remains necessary. In a senescence bypass screen using an shRNA library targeting the whole genome, our lab identified one of these channels, IP3 receptor 2, or ITPR2, as a positive regulator of senescence *in vitro* (Wiel et al., 2014) (**Figure 11**) (See *INTRODUCTION - Chapter II. - Section 2.3.5.*). The family of IP3Rs, or ITPRs, includes three members ITPR1, ITPR2 and ITPR3. These channels are ubiquitous ER-anchored calcium-releasing channels involved in multiple cellular signaling pathways (Foskett et al., 2007).



**Figure 11: Calcium fluxes in cellular senescence.** Calcium fluxes mediate senescence signaling within the cell. To the left, the ITPR-dependent rise in the mitochondria through MCU and VDAC channels leads to ROS generation and subsequent activation of p53/21<sup>CIP1</sup> pathway. To the right, increased cytosolic calcium (that can be mediated among others by ITPRs) promotes calmodulin/calcineurin/NFAT pathway and/or cleavage of pro-IL-1 $\alpha$  into mature IL-1 $\alpha$ . (Adapted from Martin and Bernard, 2018)

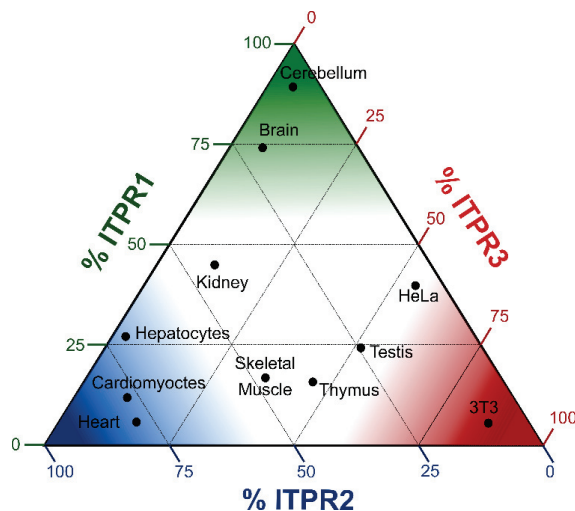
Though ITPR2 has been the subject of fewer investigations than ITPR1, a growing number of studies have revealed the specificities of ITPR2 and paved the way to further study its role in cellular signaling and pathophysiological conditions.

## 2.2. Characteristics of the ITPR2 calcium channel

### 2.2.1. ITPR2: an ITPR channel

In the ITPR family, the three members ITPR1, ITPR2 and ITPR3 share 60 to 80 % homology in their amino acid sequences. ITPRs are conserved among vertebrates and were fully cloned in the 1990s. ITPR2 was the second to be discovered (Südhof et al., 1991), and the literature has since then mostly covered the role and functions of ITPR1, while most cells express one or more ITPR isoforms according to cell type and differentiation level (Mountian et al., 1999; Newton et al., 1994; Sugiyama et al., 1994; Wojcikiewicz, 1995). Moreover, this expression is cellular context-dependent as exogenous factors, such as high glucose levels, can modulate ITPR transcript levels (Lee et al., 1999b).

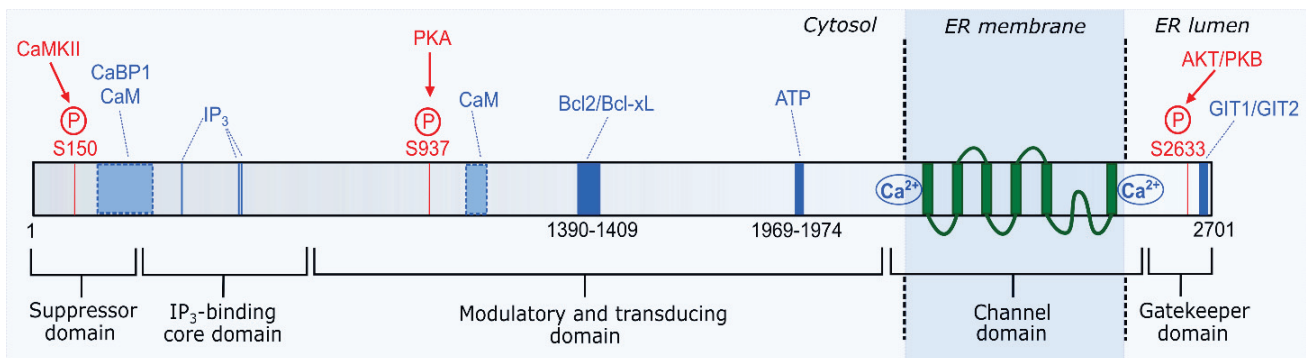
Due to this high heterogeneity and the fact that ITPRs can form heterotetramers (Monkawa et al., 1995), analyzing subtype-specific regulation of individual ITPR isoforms in native tissue remains hampered. To overcome these limitations, functional features of specific homotetrameric receptors, such as ITPR2, were mostly studied in a defined system based on the generation of a null background in the DT40 chicken B-cell precursor (DT40-3KO cells) following the deletion of all three ITPR isoforms by homologous recombination (Alzayady et al., 2013; Sugawara et al., 1997). Interestingly, the reintroduction of concatenated dimers demonstrated that some ITPR isoforms have a predominant activity over others, namely concatenated forms of ITPR1/ITPR2 are dominated by ITPR2, which is of utmost importance for our study (Alzayady et al., 2013). Nevertheless, and beyond this artificial DT40-3KO model, the ITPR2 isoform is abundant in various types of native cells, that include astrocytes, atrial cardiomyocytes, hepatocytes, and secretory cells (**Figure 12**), thus constituting cells of interest to investigate the specific role of ITPR2.



**Figure 12: Relative ITPR expression in different tissues and cell lines.** According to transcriptomic analyses, the expression of ITPR isoforms reflects a heterogeneity in their transcriptional regulation. Hepatocytes, cardiomyocytes and astrocytes constitute three cell types in which ITPR2 is more expressed than other ITPR isoforms. (Adapted from Ivanova et al., BBA, 2014)

### 2.2.2. Structure of ITPR2

Like all ITPRs, the structure of ITPR2 has been characterized and contains five different domains: a N-terminal coupling domain (suppressor domain), an IP<sub>3</sub>-binding core domain, an internal coupling domain (modulatory or transducing domain), a channel domain and a C-terminal coupling domain (gatekeeper domain) (Südhof et al., 1991). Moreover, ITPR2 displays numerous binding sites (IP<sub>3</sub>, ATP, Bcl2, GIT1/GIT2) and phosphorylation sites (Betzenhauser et al., 2009a; Maxwell et al., 2012; Vanderheyden et al., 2009), that contribute to modulating its activity (**Figure 13**).



**Figure 13: Structure of the ITPR2 protein.** ITPR2 is divided into five subdomains. These domains contain a transmembrane domain (green), various supposed (light blue) or established (blue) interaction sites, and numerous phosphorylation sites (red). (Adapted from Vervloessem et al., 2015)

### 2.2.3. Mechanism of action

ITPRs are activated by different ligands. IP<sub>3</sub> is the canonical activator of ITPRs, the name IP<sub>3</sub>-receptor given to the channel. IP<sub>3</sub> is a diffuse cytoplasmic molecule arising from the cleavage at the plasma membrane of PIP<sub>2</sub> by phospholipase C (PLC). PIP<sub>2</sub> cleavage leads to the generation of Diacylglycerol (DAG) which remains within the plasma membrane and IP<sub>3</sub> that diffuses into the cytosol. IP<sub>3</sub> binds directly to ITPRs and allows the opening of the channel and a passive release of Ca<sup>2+</sup> from the ER to the cytosol leading to subsequent biological effects (**Figure 15.A**). Remarkably, ITPR2 displays a specific feature concerning IP<sub>3</sub> affinity. Indeed, among ITPR isoforms, ITPR2 presents the highest affinity for IP<sub>3</sub> (Iwai et al., 2007; Südhof et al., 1991). This highest affinity of IP<sub>3</sub> for ITPR2 does not depend on the IP<sub>3</sub>-binding core domain, but only on the suppressor domain (Iwai et al., 2007).

Beyond this IP<sub>3</sub>-canonical activation, Ca<sup>2+</sup> and ATP are able to modulate ITPR2 opening. Since it is activated by IP<sub>3</sub> through the IP<sub>3</sub>-binding core domain, ATP may potentiate this IP<sub>3</sub>-activation (Bezprozvanny and Ehrlich, 1993) while Ca<sup>2+</sup> acts as a competitive agonist (Bezprozvanny et al., 1991; Finch et al., 1991). Indeed, ITPRs present an activity depending on calcium, as low Ca<sup>2+</sup> concentration potentiates their activity, while high Ca<sup>2+</sup> concentration inhibits their activation, giving rise to a bell-

shaped curve response (Bezprozvanny et al., 1991; Finch et al., 1991). Of note, this bell-shaped curve is flattened for ITPR2 compared to other ITPRs (Ramos-Franco et al., 1998, 2000), providing ITPR2 with a broader range of calcium concentrations that can induce its opening (**Figure 14**). It is worth mentioning that beyond cytoplasmic calcium, luminal ER calcium also modulates ITPR2 opening, preventing excessive ER calcium depletion (Missiaen et al., 1992) that could be detrimental for cells. With regards to the regulation ITPR2 by ATP, it appears that ITPR2 is the only ITPR isoforms that does not require ATP for opening under optimal conditions, i.e. optimal  $\text{Ca}^{2+}$  and  $\text{IP}_3$  concentrations (Betzenhauser et al., 2008, 2009b) (**Figure 14**).

#### **2.2.4. Subcellular localization of ITPR2**

Unlike other ITPR isoforms, ITPR2 is present in different subcellular localizations, not only in the ER (Vervloessem et al., 2015). Indeed, aside from its main and well reported localization at the ER, ITPR2 can be located at the nuclear envelope, plasma membrane, or in secretory vesicles and Golgi apparatus, depending mostly on cell type (Vermassen et al., 2004; Kaja et al., 2011; Ivanova et al., 2014; Vervloessem et al., 2015) (**Figure 14**).

Moreover, within the cell, ITPR isoforms assemble as tetramers and are known to be spatially regulated, forming clusters (Alzayady et al., 2013; Monkawa et al., 1995). Nevertheless, while ITPR1 and ITPR3 are spread homogeneously across the ER membrane, the localization of ITPR2 is scattered (Alzayady et al., 2013). This punctuated localization can be linked to a lower intracellular mobility, as evidenced by biochemical diffusion experiments (Alzayady et al., 2013) (**Figure 14**).

#### **2.2.5. Mechanisms of regulation of ITPR2**

ITPR2 is regulated at multiple levels, that include transcriptional, translational and post-translational regulation, the latter being the most extensively studied.

##### **a. Transcriptional regulation**

The study of the ITPR2 locus and its promoter are prerequisites to decipher its transcriptional regulation. The ITPR2 promoter is GC rich, without displaying a conventional TATA box (Morikawa et al., 1997). 300 bp upstream of the first ATG codon, the promoter contains 7 transcript initiation sites, flanked by CpG islands (Morikawa et al., 1997). Three transcription factors (TFs) regulating *Itpr2* transcription have so far been identified. The ITPR2 promoter presents five potent c-AMP response elements (CRE), and cAMP/CREB enhances ITPR2 transcript levels in hepatocytes (Kruglov et al., 2017). The two other transcription factors include NFATc1 in cardiac and ETS1 in dendritic cells (Sankar et al., 2014; Yang et al., 2014). Our lab identified another transcriptional regulator, acting as an ITPR2

repressor: the retinoid X receptor alpha (RXRA) (Ma et al., 2018). Interestingly, exogenous stresses, like oxidative- or hydrogen sulfide-mediated stress, enhance ITPR2 expression in neurons and HeLa cells, respectively (Kaja et al., 2011; Lencesova et al., 2013), through a yet unknown mechanism.

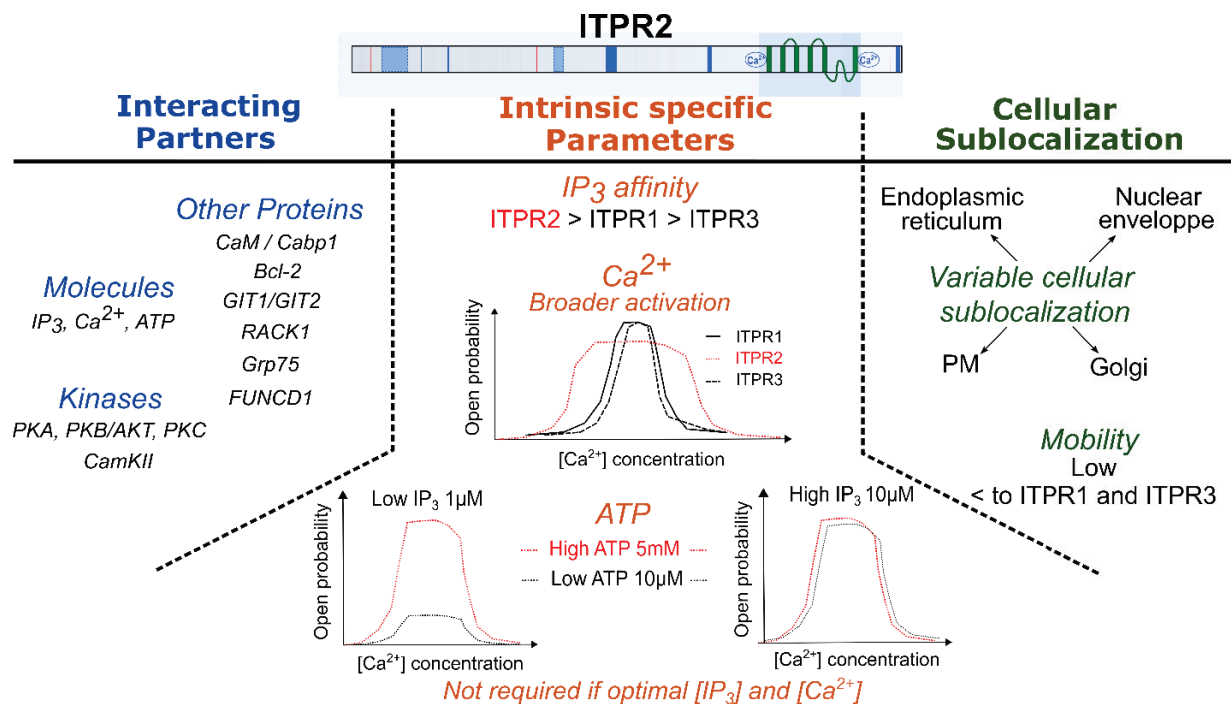
#### **b. Post-transcriptional regulation**

Most of the described regulations of ITPR2 take place after transcription. For instance, ITPR2 mRNA transcripts may be degraded by miRNA-133a, acting as a negative regulator in rat neonatal myocytes (Drawnel et al., 2012). Nevertheless, extensive studies were conducted to decipher the post-translational regulation of ITPR2.

Firstly, ITPR2 activity is modulated by interactions with other proteins. Indeed, more than 50 proteins were shown to interact with ITPR isoforms (Ivanova et al., 2014). Those includes among others Ca<sup>2+</sup>-binding proteins (e.g. calmodulin, Cabp1), apoptotic proteins of Bcl-2 family, scaffold proteins such as G-protein-coupled receptor kinase-interacting protein (GIT) or receptor for activated C kinase (RACK) 1, and adenylyl cyclase (Rong et al., 2008; Zhang et al., 2009; Patterson et al., 2004; Tovey et al., 2008). Furthermore, the activity of ITPR2 largely depends on its phosphorylation status. As IP3Rs are direct substrates for serine/threonine or tyrosine kinases (Vanderheyden et al., 2009) (**Figure 13**). Among them, protein kinase A (PKA) was the first characterized and most extensively studied in the field of ITPRs and particularly in ITPR2. This ubiquitous cAMP-dependent protein kinase PKA phosphorylates the serine 937 of ITPR2, a frequent phosphorylation site found in the liver, to facilitate its opening in the DT40-3KO cell model (Villén et al., 2007; Betzenhauser et al., 2009a), suggesting its importance in ITPR2-rich tissues. Beyond PKA activation, ITPR2 can also be repressed by other kinases, such as calmodulin kinase II (CaMKII) or protein kinase C (PKC). Indeed, a rise in intracellular calcium activates the multifunctional serine/threonine protein kinase CaMKII, which is thus able to phosphorylate serine 150 of ITPRs in cardiomyocytes and kidney cell models (Bare et al., 2005; Maxwell et al., 2012). Less investigated in the context of ITPR2, PKC could indirectly phosphorylate ITPR2 participating in its inhibition (Arguin et al., 2007). Overall, CaMKII and PKC appear to mediate a negative feedback after ITPR2 opening. Furthermore, ITPR2 can be phosphorylated by protein kinase B (PKB)/AKT on serine 2633 notably inhibiting its activity, ER-mitochondrial calcium fluxes and subsequent mechanisms of intrinsic cell death (Szado et al., 2008), linking modulation of ITPR2 activity with its biological roles.

Secondly, several studies reported the role of oxidation on ITPR isoforms and a variation in their activity, by modifying the thiol reaction of cysteinyl residues. Indeed, IP3Rs contain numerous reactive thiol groups, accounting for 56 residues in amino acid sequences of human and mouse (NCBI

Reference Sequence - NP\_002214.2 and NP\_064307.2). These thiol groups are common molecular targets of ROS. Oxidative stress, and more specifically superoxide anion ( $O_2^-$ ), is able to sensitize ITPR2 to boost the  $IP_3$ -induced calcium release (IICR) (Bánsághi et al., 2014). Moreover, the use of thimerosal, eliciting thiol groups, enhances ITPR2 activity at low concentrations, while it inhibits it at high concentration (Khan et al., 2013). Overall, post-translational modifications by oxidative stress and thiol modification modulate ITPR2 sensitivity and subsequent  $IP_3$ -induced calcium release (IICR).



**Figure 14: Properties and specificities of the ITPR2 protein.** ITPR2 interacts with numerous partners, including molecules, kinases and various other cellular proteins (blue). Compared to other ITPR isoforms, ITPR2 displays the highest  $IP_3$  affinity, a larger scale of cytosolic  $[Ca^{2+}]$  variations, and no ATP requirement with optimal  $[IP_3]$  and  $[Ca^{2+}]$ . The subcellular localization of ITPR2 varies, as although it is mainly found at the ER membranes, it may be present in the nucleus, Golgi apparatus, or plasma membrane with the lowest mobility within the cell.

Hence, ITPR2 is mostly regulated at transcriptional and post-translational levels based on current literature. Through various binding partners and as a kinase substrate, ITPR2 is dynamically regulated within cells. This finely-tuned regulation is crucial and takes place in different biological contexts.

### 2.3. Role of ITPR2 in the regulation of biological processes

Under optimal conditions,  $IP_3$ -induced calcium release (IICR) regulates ER-cytosolic and ER-mitochondrial calcium fluxes, which are subsequently upstream of multiple cascade signaling

pathways within the cell. Here are some of the cellular roles involving ITPR2-dependent cellular calcium fluxes. Notably, in order to assess the role of ITPR2 in physiological processes, the study of the two *Itpr2* knockout mice models developed by Chen's team (Li et al., 2005) and Mikoshiba's team (Futatsugi et al., 2005) is a prerequisite and will be discussed in following sections. These two mice models were generated by disrupting the genes in their first codons, and no embryonic lethality or severe post-birth phenotypes were observed (Li et al., 2005; Futatsugi et al., 2005).

### **2.3.1. Secretion**

ITPR2 is highly expressed in numerous secretory cells, including in cells of exocrine gland pancreas, salivary glands, lacrimal glands, olfactory glands, liver or goblet cells of the small intestine (Vervloessem et al., 2015). All these epithelial cells are polarized and involved in the secretion of various fluids from the apical membrane of cells into the lumen. As ITPR2 partly regulates cytosolic calcium, it participates in the promotion of secretion. Indeed, the rise in intracellular calcium is accompanied by enhanced secretion in multiple secretory cells, including neurons, especially in the fusion process of endomembranes (Neher and Zucker, 1993; Sullivan et al., 1993) (**Figure 15.D**). The sole role of ITPR2 seems to be modest in exocrine secretion as suggested by the mild reduction of secretion of salivary, lacrimal and mucus glands in *Itpr2* KO mice. On the contrary, double *Itpr2/Itpr3* KO mice present a severe dry phenotype, with simultaneously dry mouth, dry eyes and pancreatic insufficiency (Futatsugi et al., 2005; Inaba et al., 2014; Park et al., 2008), suggesting a synergistic role for ITPR2 and ITPR3 in secretion. This hypothesis on the role of ITPR2 in physiological secretion is confirmed by the fact that sweat glands of *Itpr2* KO mice display lower  $Ca^{2+}$  associated with sweat secretion defects (Klar et al., 2014).

### **2.3.2. Contraction**

ITPR2 is the most highly expressed ITPR isoform in two subtypes of cardiomyocytes: atria and ventricle cardiomyocytes (Vervloessem et al., 2015). Hence, an extensive research is into its role in modulating cardiac functions. Firstly, ITPR2 is highly expressed in models of heart failure in mice and humans (Harzheim et al., 2009). Indeed, ITPR2 is upregulated in human hearts following ischemic dilated cardiomyopathy, as well as in aortic-banded hypertrophic murine heart (Harzheim et al., 2009, 2010). Surprisingly, the study of *Itpr2* KO models demonstrated that no cardiac dysfunction was observed under basal conditions. Nevertheless, the arrhythmic events driven by ionotropic stimulation of Endothelin 1 (Edn1) are abrogated in *Itpr2* KO mice, suggesting a protective role for ITPR2 against ionotropic-stimulated arrhythmia (Li et al., 2005). Of note, another study demonstrated that FUN14 domain containing 1 (FUNCD1) interacts with ITPR2 to inhibit calcium transfer from the ER to the



mitochondria and prevents cardiac dysfunction *in vivo* (Wu, 2017) (**Figure 15.D**). Besides, normal cardiogenesis partly relies on ITPR2, as double *Itpr1/Itpr2* KO mice die *in utero* at embryonic stage E11.5 with major heart disorders, especially around ventricles (Uchida et al., 2010).

### **2.3.3. Migration**

While it has been largely reported that intracellular calcium pikes regulate cell movement and especially cellular migration (Stossel, 1993; Lee et al., 1999c), the specific role for ITPR- (and ITPR2-) mediated calcium fluxes in cellular migration appears to mediate some of these effects. Broadly, ITPRs and IICR were initially shown to promote migration of endothelial and immune cells, such as neutrophils (Pettit and Hallett, 1998; Singleton and Bourguignon, 2002). Later, the migratory process in WI-38 fibroblasts was thoroughly shown to rely on spatio-temporal organization of calcium microdomains, called flickers (~5  $\mu\text{m}$  in diameter and 10–2000 ms in duration) and generated by both transient receptor potential (TRP) and ITPR2 channels (Wei et al., 2009). According to this major discovery, ITPR-dependent migration of fibroblasts was recently shown to enhance skin wound healing (Belkacemi et al., 2018). The role of ITPR2 in migration has been depicted in other normal cells, such as dendritic cells (Yang et al., 2014), as well as in a tumoral context. Indeed, in human lung cancer cells, the endoplasmic reticulum resident protein 44 (ERP44) abrogates cellular migration through repression of ITPR2 (Huang et al., 2016). Overall, ITPRs- and more specifically ITPR2-calcium mediated fluxes are crucial for cell migration (**Figure 15.D**).

### **2.3.4. Apoptosis**

The roles of ITPR-calcium mediated fluxes in the regulation of cell survival and death have been extensively described (Ivanova et al., 2014) (**Figure 15.D**). Early evidence arose from studying ITPR1-deficient T-cells and their resistance to various apoptosis-triggering stresses, including FAS ligands or exposure to corticoids (Jayaraman and Marks, 1997). Later, ITPR-mediated apoptosis was linked to both increased cytosolic and mitochondrial calcium. Indeed, a rise in cytosolic calcium can activate calcineurin/NFAT and is necessary for ITPR-dependent apoptosis (Jayaraman and Marks, 2000). Calcineurin dephosphorylates the pro-apoptotic BH3-only protein Bad, the latter further translocating to the outer mitochondrial membrane to form a heterodimer with the anti-apoptotic Bcl-xL protein (Wang et al., 1999). Moreover, as mitochondrial calcium enhances outer mitochondrial membrane permeabilization, cytochrome c release, and initiation of apoptosis, the ER-mitochondria calcium fluxes were investigated in the context of cell death and the subsequent role of ITPRs was deciphered. Indeed, pro-survival signals mediated by Akt/PKB induce ITPR phosphorylation, further inhibiting calcium fluxes from ER to mitochondria (Szado et al., 2008). Other proteins can also limit these ER-

mitochondria calcium fluxes. For example, the anti-apoptotic Bcl-2 interacts with ITPRs (Rong et al., 2008, 2009). Remarkably, some cancer cell lines (such as B-cell lymphomas) displaying a high level of ITPR2 (Akl et al., 2014) are addicted to this Bcl-2/ITPR2 interaction, limiting ER-mitochondria calcium transfers and subsequent apoptosis (Rong et al., 2009). An engineered peptide inhibiting this interaction, termed stabilized TAT fused IP3R-derived peptide (TAT-IDP), disrupts this Bcl2/ITPR2 interaction, re-establishing ER-mitochondria calcium transfers, thus sensitizing lymphoma cells to apoptosis (Rong et al., 2008; Zhong et al., 2011; Akl et al., 2013). Of relevance in the field of senescence, interfering with Bcl-2 interactions is one of the paths investigated to sensitize senescing cells to apoptosis (notably through ABT-737) (*See INTRODUCTION - Chapter I. - Section 1.2.3.*).

Importantly, various stresses, such as chronic oxidative stress, are able to upregulate ITPR2 expression at mRNA levels (Kopacek et al., 2009; Kaja et al., 2011; Lencesova et al., 2013) or boost its activity (Bánsághi et al., 2014), suggesting that ITPR2 is regulated via stress responses.

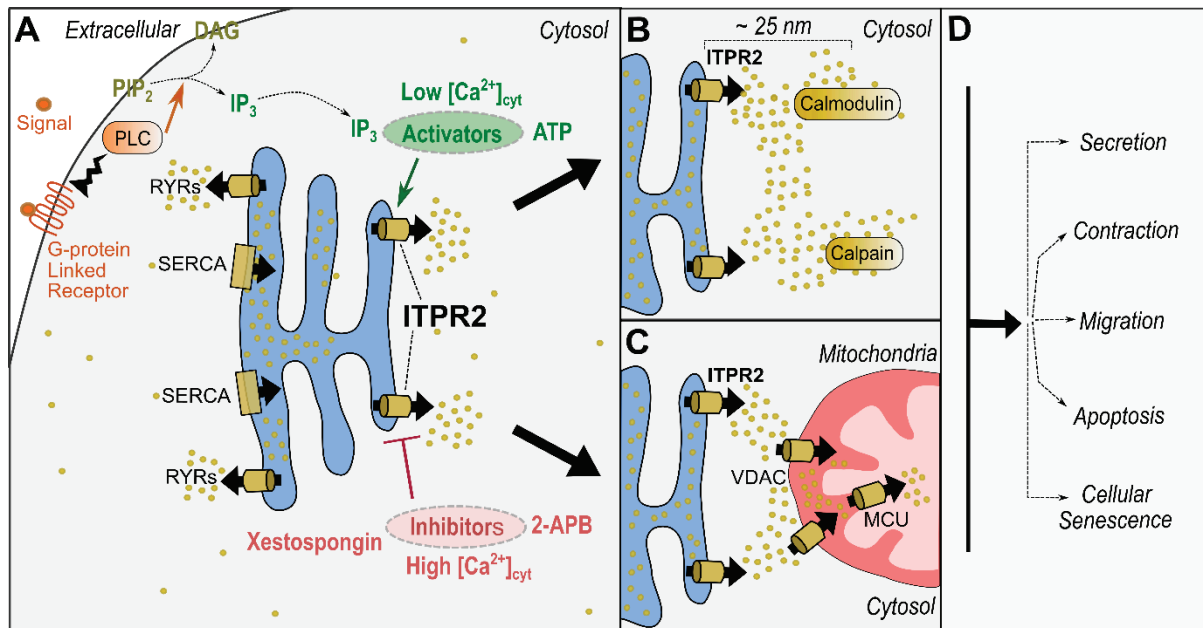
As earlier mentioned, apoptosis shares common inducers and effectors with cellular senescence, suggesting that ITPR-calcium mediated fluxes could be involved in regulating cellular senescence.

### **2.3.5. Cellular senescence**

If the importance of intracellular calcium has been largely depicted in contexts of secretion, contraction, migration or apoptosis, more recent work unveiled the role of calcium fluxes (Martin and Bernard, 2018) and more particularly ITPR2-mediated fluxes in regulating senescence (Ma et al., 2018; Wiel et al., 2014).

Previously, our lab screened for positive regulators of OIS, investigating the effect of a whole-genome shRNA library in an OIS-escape model. Among them, several ion channels were identified, including ITPR2 and the mitochondrial calcium uniporter (MCU). The MCU is located in the inner mitochondrial membrane (IMM) and participates in the loading of calcium into the mitochondrial matrix (Rizzuto et al., 2012). Further investigations demonstrated that ITPR2, in cooperation with MCU, was necessary to mediate OIS in hMEC and RS in fibroblasts (Wiel et al., 2014). This first ITPR2/MCU-mediated model was later validated using RXRA-deficient cells which present abnormal upregulation of ITPR2 expression (Ma et al., 2018). *In vitro*, the mechanism underlying the induction of senescence through ITPR2/MCU axis was linked to the mitochondrial calcium accumulation in the matrix, a

decreased MMP and a subsequent generation of ROS, leading to a p53/p21-dependent senescence (Ma et al., 2018; Wiel et al., 2014) (Figure 15.C).



**Figure 15: Activation and biological importance of ITPR2.** **A.** Extracellular signals (such as growth factors) may trigger the G-protein linked receptor-dependent activation of phospholipase C (PLC). PLC cleaves PIP<sub>2</sub> into DAG and IP<sub>3</sub>. While DAG stays in the plasma membrane, soluble IP<sub>3</sub> diffuses into the cytosol to activate ITPR2. Other activators such as low [Ca<sup>2+</sup>]<sub>cyt</sub> or ATP potentiate the activation of ITPR2. Among the inhibitors, high [Ca<sup>2+</sup>]<sub>cyt</sub> or chemical inhibitors Xestospongin inhibit ITPR2 activity. **B.** ITPR2 opening leads to increased local [Ca<sup>2+</sup>]<sub>cyt</sub> that may activate calcium-binding proteins (e.g. calmodulin, calpain) locally (around 25nm around). **C.** Enhanced local [Ca<sup>2+</sup>]<sub>cyt</sub> at the proximity of mitochondria leads to its uptake by the latter through VDAC at the OMM and MCU at the IMM. **D.** The combination of these two effects leads to fundamental biological processes such as secretion, contraction, migration, apoptosis and cellular senescence.

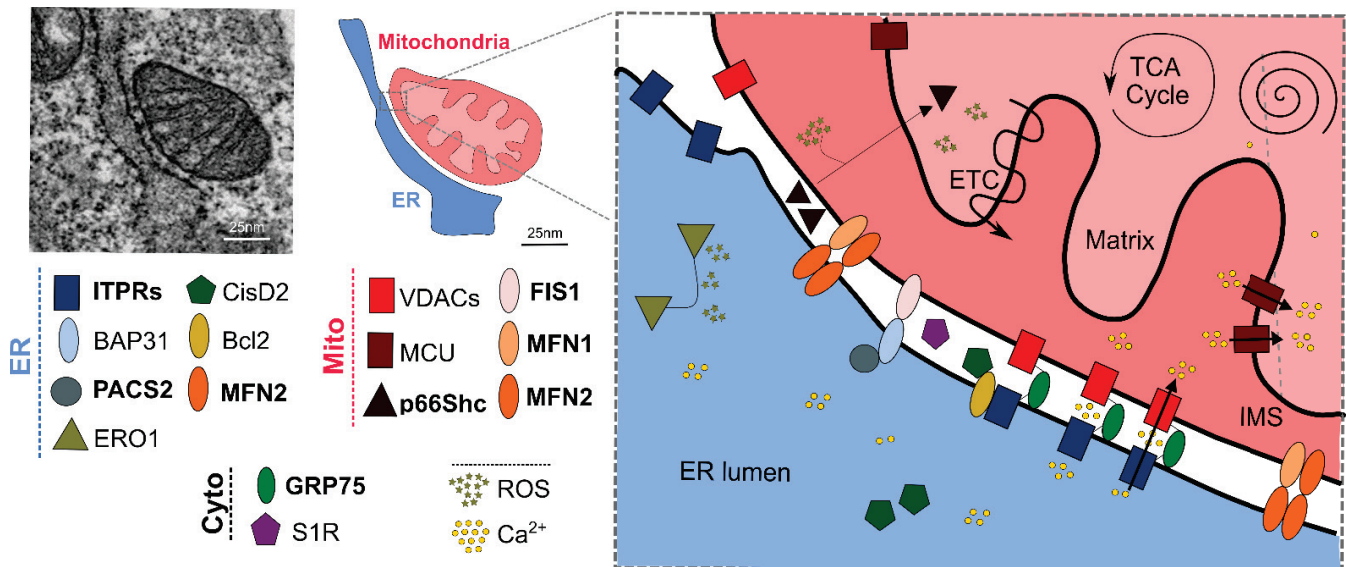
## 2.4. Mitochondria-ER contacts: a localization of ITPR2

### 2.4.1. MERCs: an intracellular signaling platform between two organelles

Mitochondrial-endoplasmic reticulum contacts (MERCs), also termed mitochondria-associated membranes (MAMs) in the literature, are tight contacts (less than 50 nm) that allow a local communication between endomembranes of the mitochondria and ER (Csordás et al., 2018). The identification of proteins present at MERCs was determined using proximity biotinylation by mass spectrometry (MS), and revealed the presence of ITPRs, including ITPR2, in this fraction (Cho et al., 2017; Hung et al., 2017).

MERCs could contain up to 800 proteins (Cho et al., 2017; Hung et al., 2017), including fusion/fission proteins (MFN1/MFN2) (de Brito and Scorrano, 2008), scaffold proteins (GRP75), ion channels (ITPRs/VDAC/SERCA), enzymes (FACL4, PSS1) and various signaling proteins (CisD2, PERK,

IRE1, NLRP3, mTOR, SigmaR1, p66Shc) (Moltedo et al., 2019) reinforcing their roles as signaling platforms within the cell (**Figure 16**). The structures of MERCs are highly dynamic and known to be physiologically present in every eukaryotic cell, accounting for approximately 10 to 15% of all mitochondrial membranes (Csordás et al., 2018). The importance of MERCs has been emphasized in the past years, as they are involved in metabolites fluxes (including calcium, ROS, phospholipids, cholesterol,...) between endoplasmic reticulum and mitochondria (Marchi et al., 2014; Missiroli et al., 2018a; Pinton, 2018; Moltedo et al., 2019).



**Figure 16: ITPR2 can be localized at mitochondria-ER contacts (MERCs).** **A.** Electron micrographs of MERCs. Scale bar: 25 nm. **B.** MERCs are dynamic and contain more than 600 hundred proteins. ITPR2 (dark blue rectangle) is one of the components of MERCs. Other ER-resident and mitochondria-resident proteins are closely associated through MERCs. Cytosolic proteins may punctually be part of MERCs. Tethers allow the establishment of MERCs and include ITPR-GRP75-VDAC, MFN1/MFN2, FIS1/BAP31. ROS and calcium are generated by both organelles and participate in the regulation of MERC functions.

#### 2.4.2. Importance of MERCs in cellular responses and pathophysiological contexts

Within the cell, MERCs have been more broadly depicted in the regulation of (1) ER and mitochondrial functions (Marchi et al., 2014) and (2) cellular processes such as autophagy, inflammation or apoptosis (Csordás et al., 2018; Marchi et al., 2014; Moltedo et al., 2019; Pinton, 2018).

MERCs, as an interface between ER and mitochondria, modulate mitochondrial functions and bioenergetics through the regulation of ER-mitochondrial calcium fluxes, ROS generation and phospholipid synthesis (Marchi et al., 2014; Lewis et al., 2016; Galmes et al., 2016; Carreras-Sureda et al., 2019). As a consequence of enhanced ER-mitochondrial calcium fluxes, MERCs may promote apoptosis (Hayashi and Su, 2007; Li et al., 2009a; Iwasawa et al., 2011; Doghman-Bouguerra et al.,

2016; Marchi et al., 2018). Of note, MERCs act as modulators of mitochondrial dynamics allowing ER tubules to physically wrap themselves around mitochondria subsequently promoting mitochondrial local fragmentation (Friedman et al., 2011).

Aside from their role in modulating ER/mitochondrial functions or apoptosis, MERCs act as a signaling platform containing key proteins involved in autophagy, metabolism or inflammation, such as mTOR (Betz et al., 2013) or the NLRP3 inflammasome (Zhou et al., 2011), this latter constituting a regulatory checkpoints for inflammation (Missiroli et al., 2018a). The role of various ITPRs in ER-mitochondrial calcium fluxes notably in the regulation of apoptosis and senescence argues in favor of a role for MERCs in regulating senescence, although no study has so far focus on this role.

As MERCs are dynamic, disruption or enrichment of MERCs, trigger several pathological conditions, and have been explored notably in the brain and liver in different contexts related to neurodegeneration and metabolic disorder, respectively (Arruda et al., 2014; Lee et al., 2018; Pinton, 2018; Rieusset, 2018; Hernández-Alvarez et al., 2019).

To summarize this chapter, ITPR2, being part of ITPRs family, is essential for calcium efflux from the ER. Regulated at multiple levels, this efflux from the ER mediates numerous functions, such as migration, contraction or apoptosis, within the cell. As part of MERCs, it actively contributes to modify their features, modifying calcium exchanges and calcium content of both ER and mitochondria compartments. After discovering its role in cellular senescence *in vitro* (Wiel et al., 2014), other investigations need to be further conducted regarding its possible involvement in senescence *in vivo* and physiological aging.

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## **- CHAPTER III -**

### **-The mevalonate pathway: description, regulation and biological importance-**

Senescent cells harbor drastic metabolic changes affecting both catabolism and anabolism. Catabolism of senescent cells has been well described. While some studies suggest a shift to glycolytic state coupled to an increased glucose consumption (Dörr et al., 2013; James et al., 2015), numerous works established mitochondrial dysfunction associated with an elevated AMP/ATP ratio (Wang et al., 2003; Zwerschke et al., 2003; Ziegler et al., 2015; Wiley et al., 2016). Conversely, anabolism remains understudied, though several recent studies have highlighted modifications of proteome biogenesis or lipid anabolism in senescing cells (Kim et al., 2010a; Flor et al., 2017; Lessard et al., 2018). Indeed, unlike quiescent cells, senescent cells should display an active anabolism in order to synthesize and secrete SASP factors.

Recently, constitutive expression of a kinase library in fibroblasts allowed us to identify pro-senescent kinases (Ferrand et al., 2015). Among these pro-senescent kinases, we uncovered the mevalonate kinase (MVK) and the phosphomevalonate kinase (PMVK), two enzymes involved in the lipid anabolic pathway: the mevalonate (MVA) pathway.

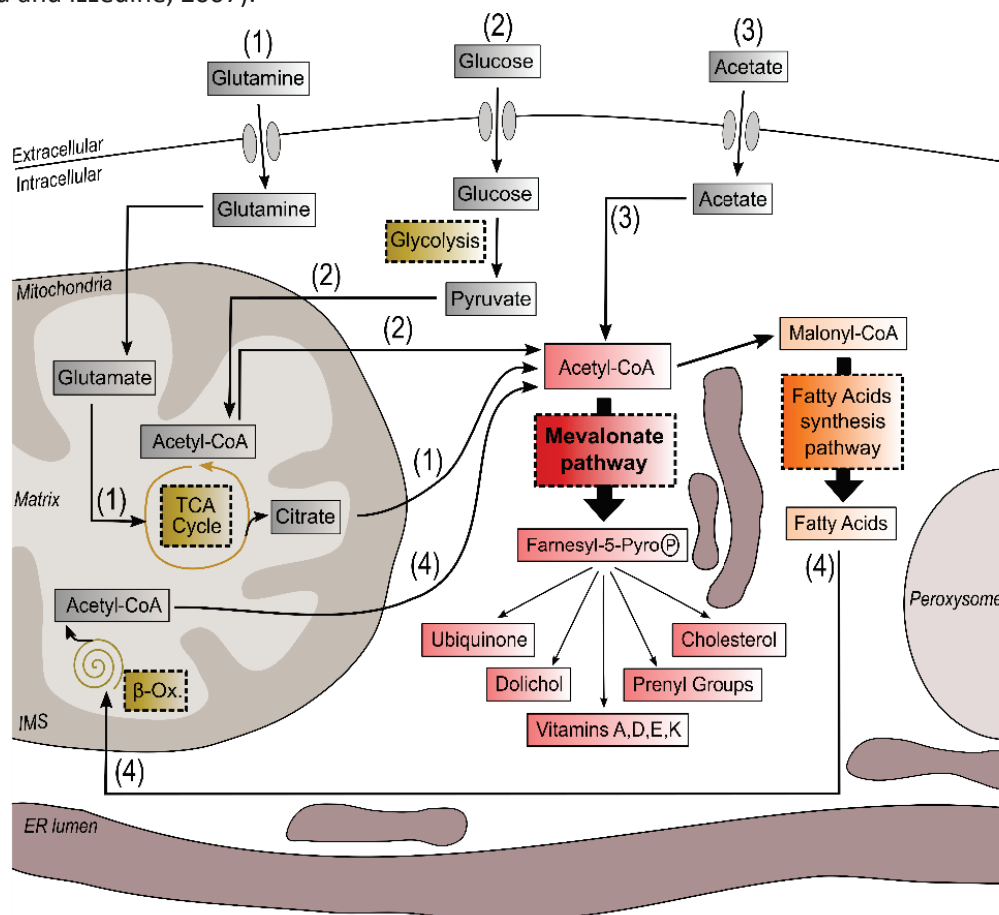
#### **3.1. A conserved lipid anabolic pathway generating isoprene units**

The MVA pathway is at the crossroads of lipid metabolism and fueled by acetyl-CoA. This acetyl-CoA is itself produced by various nutrient sources, that include (1) glucose, (2) glutamine, (3) acetate, and (4) fatty acid (**Figure 17**). Also termed isoprenoids pathway, the MVA pathway generates isoprene units, as elementary bricks to subsequently generate isoprenoids macromolecules, such as ubiquinone, dolichol and cholesterol in downstream biosynthetic subbranches (**Figure 17**).

##### **3.1.1. A main backbone of 5 consecutive reactions**

In the mid-1950s, while deciphering the biosynthesis pathway of cholesterol, Konrad Bloch and Feodor Lynen described for the first time the chemical reactions involved in the MVA pathway. A few years later, in 1964, they shared the Nobel Prize in Physiology or Medicine for their discoveries (Bloch, 1965).

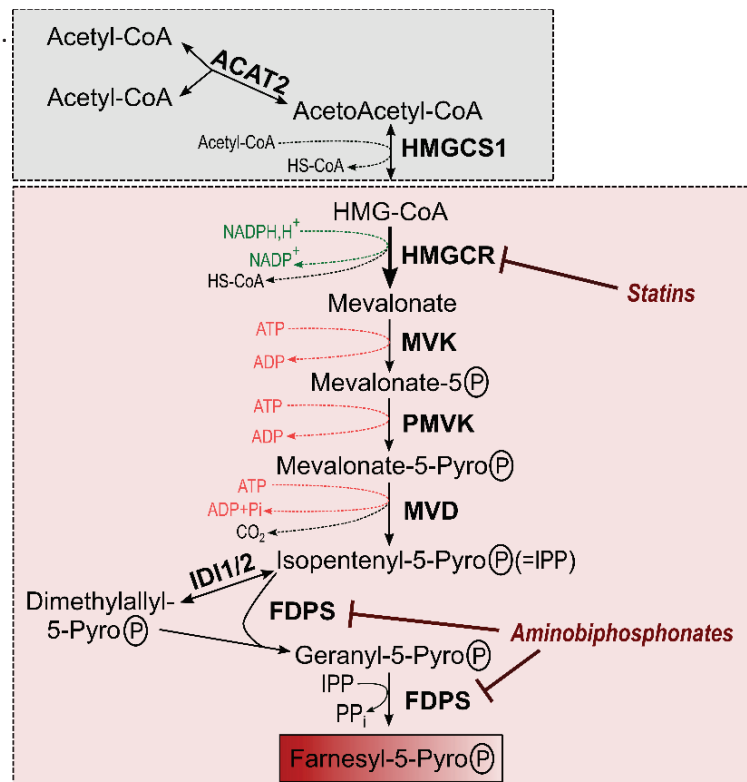
The two first transformations of 3 molecules of acetyl-CoA into acetoacetyl-CoA and subsequent 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) are necessary to fuel the MVA pathway. These two reactions are reversible and catalyzed by acetyl-CoA acetyl transferase 2 (ACAT2), also known as thiolase, and HMG-CoA synthase 1 (HMGS1), respectively. Further transformation of HMG-CoA in mevalonate constitutes the first irreversible rate-limiting step of the pathway, thus initiating *sensu stricto* the MVA pathway. This oxide-reduction reaction involves the HMG-CoA reductase (HMGR) that relies on  $\text{NADPH}^+$  (Miziorko, 2011) (Figure 18). HMGR is a glycoprotein anchored to the ER membrane with a C-terminal catalytic domain in the cytosol (Liscum et al., 1985). As first rate-limiting enzyme, HMGR has been widely studied (Istvan and Deisenhofer, 2001) and can be targeted through the use of statins including, among others, pravastatin, lovastatin, atorvastatin or lovastatin (Buhaescu and Izzedine, 2007).



**Figure 17: The mevalonate pathway and lipid homeostasis within the cell.** Acetyl CoA fuels the mevalonate pathway (red). Endogenous sources of acetyl-CoA are diverse: (1) from glucose and glycolysis-generated pyruvate as a result of mitochondrial pyruvate dehydrogenase (PDH) activity, (2) to glutamine through the transformation of glutamate and citrate in tricarboxylic acids (TCA) cycle (3) to acetate, (4) to fatty acids *via* the mitochondrial  $\beta$ -oxidation ( $\beta$ -Ox). Of note, even if most of the steps in the mevalonate pathway are cytosolic, some of them are located in the ER or peroxisome membranes. The final product of mevalonate pathway is farnesyl-5-pyrophosphate. Metabolic pathways (yellow) and complementary lipid anabolic pathways (orange) are also depicted (Adapted from Mullen et al., 2016).

The two further chemical transformations of mevalonate constitute the second and third steps of the pathway and require two consecutive kinases: the mevalonate kinase (MVK) and the phosphomevalonate kinase (PMVK). These two kinases use two consecutive ATP molecules and energize mevalonate substrate through the double addition of a phosphate group, in the form of pyrophosphate (PP), to obtain mevalonate-5-pyrophosphate (MVAPP) (Miziorko, 2011) (**Figure 18**).

The fourth step requires the mevalonate-diphosphate decarboxylase (MVD) enzyme and converts mevalonate-5-PP into isopentenyl-5-PP (IPP) (**Figure 18**). The fifth step further involves two enzymes, isopentenyl diphosphate isomerase (IDI) and the farnesyl diphosphate synthase (FDPS). The first IDI isomerizes isopentenyl-5-PP in dimethylallyl-5-PP. The condensation of these two molecules by FDPS form a geranyl-5-PP. The addition of another IPP to geranyl-5-PP completes the mevalonate pathway by generating Farnesy-5-PP (Miziorko, 2011). The FDPS enzyme is targeted by a class of molecules, termed aminobiphosphonates, including among others alendronate, zoledronate, zarogonate (**Figure 18**).



**Figure 18: Chemical reactions of the mevalonate pathway.** The two previous steps (blue rectangle) of the MVA pathway (red rectangle) lead to the production of HMG-CoA. Name of required enzymes are indicated in bold. ACAT2, acetyl-CoA acetyltransferase 2; HMGCS1, HMG-CoA synthase 1; HMGCR, HMG-CoA reductase; MVK, mevalonate kinase; PMVK, phosphomevalonate kinase; MVD, mevalonate-diphosphate decarboxylase; IDI, isopentenyl diphosphate isomerase; FDPS, farnesyl diphosphate synthase. Some chemical reactions require either the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH, H<sup>+</sup>), or ATP. ATP-dependent decarboxylation through MVD releases CO<sub>2</sub>. Two classes of inhibitors target the MVA pathway: statins inhibiting HMGCR and aminobiphosphonates blocking FDPS.



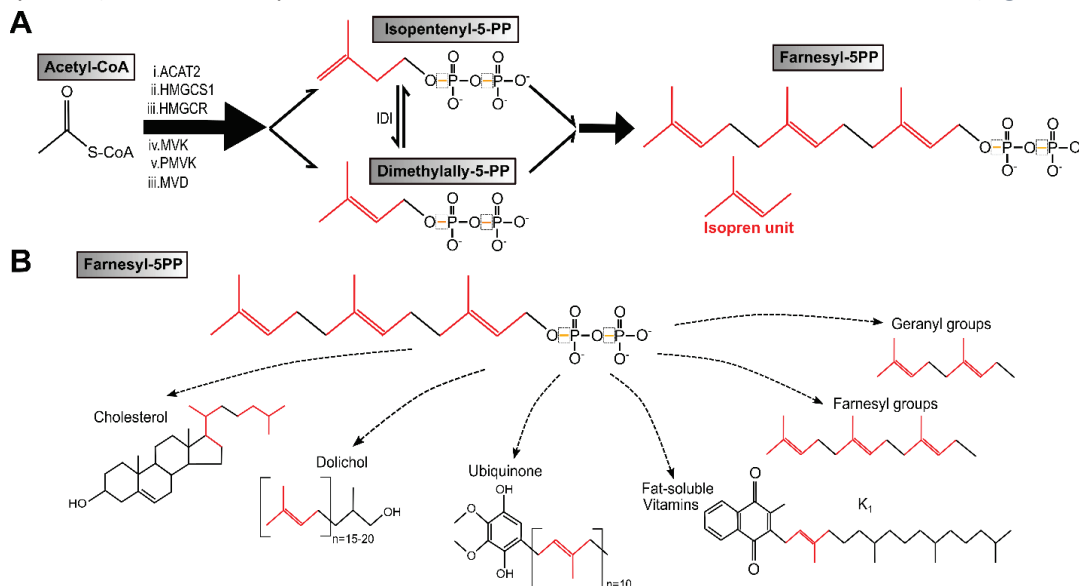
### 3.1.2. Conservation and variation between species and tissues

The MVA pathway is present in most eukaryotes (fungi, plants, animals), archaea and some rare eubacteria. Mostly conserved in eukaryotes, it constitutes an important part of the lipid anabolism within the cells, aside from and complementary to fatty acid biosynthesis (Miziorko, 2011). In non-eukaryote cells, and especially in archaea, the second and third steps catalyzed respectively by MVK and PMVK are replaced by alternative steps (Vinokur et al., 2014). Of note, MVA pathway is not the only anabolic pathway to generate polyisoprenoids, as an alternative non-MVA pathway is used by plants and eubacteria (Eisenreich et al., 2004).

In eukaryotes, the MVA pathway seems to be ubiquitous while it is largely studied in a hepatocyte context, as liver constitutes an important organ for systemic lipid homeostasis (Musso et al., 2009). Other studies report the importance of the MVA pathway in phenotypes of immune cells (notably monocytes, macrophages or T-lymphocyte) (Sene et al., 2013; Bekkering et al., 2018; Larson-Casey et al., 2019a; Thurnher and Gruenbacher, 2015), neurons (Moutinho et al., 2017) or adipocytes (Yeh et al., 2018), and to a lesser extent mouse embryonic (Clendening et al., 2010) and human fibroblasts (Appelkvist et al., 1999).

### 3.1.3. The farnesyl-5-pyrophosphate: an isoprene reservoir and crossroad molecule

The farnesyl-5-pyrophosphate (F5PP) is the end-product of the MVA pathway *sensu stricto* (Figures 17 and 18). F5PP contains three isoprene units (Figure 19.A), elementary units being further condensed to form more complex isoprenoids biomolecules, that include prenyl groups (farnesyl and geranyl ones), dolichol, ubiquinone, cholesterol and non-sterol vitamins A, D, E, and K (Figure 19.B).



**Figure 19: Structure and fates of the isoprene-carrier farnesyl-5-pyrophosphate. A.** Production of farnesyl 5-PP. Isoprene units are labelled in red. Farnesyl-5-PP contains 3 isoprene units. **B.** Farnesyl-5-PP is used in different biosynthetic pathways, and isoprene units are incorporated in numerous lipid-

derived molecules that include cholesterol, dolichol, ubiquinone, fat-soluble vitamins, and prenyl (farnesyl and geranyl) groups.

### **3.2. Regulation of the mevalonate pathway**

The dysregulation of the MVA pathway has been largely described in both metabolic disorder and cancer (Ohara et al., 1993; Kim Jay H. et al., 1994; Favier and Schulert, 2016; Mullen et al., 2016). In cancer cells, to sustain their high metabolic profile, the MVA pathway is upregulated by oncogenes also known to trigger cellular senescence through OIS, including Ras or Myc (Hofmann et al., 2015; Mullen et al., 2016; Wang et al., 2017b). This MVA pathway is necessary for the cell survival in many cancer subtypes (Hart et al., 2015; Hashimoto et al., 2016a; Laezza et al., 2015; Liang et al., 2018; Moon et al., 2019; Kaymak et al., 2020), rendering the use of inhibitors including statins and aminobiphosphonates an interesting targeted therapeutic approach (Buhaescu and Izzedine, 2007a; Goulitquer et al., 2018).

#### **3.2.1. Metabolite regulation of mevalonate enzymes**

The MVA pathway is regulated by its own metabolites. Firstly, limiting acetyl-CoA availability reduces the production of FPP. Secondly, the products of some reactions can also act as negative feedback regulators of the enzyme, to avoid accumulation of intermediates and regulate metabolite flux. For example, MVK was reported to be subject to a feedback inhibition by downstream intermediates (at micromolar doses), such as FPP and GPP (Dorsey and Porter, 1968). More recently, some downstream products of the MVA pathway, such as the endogenous sterol intermediate lanosterol, were found to promote HMGCR degradation (Chen et al., 2019). Of note, substrate analogs can be used *in vitro* to block the incorporation of “normal” metabolites and the subsequent metabolite flux (**Figure 21**). This is the case for 6-fluoro-mevalonate-5-pyrophosphate or diphosphoglycolylproline which act both as competitive inhibitors with mevalonate-5-pyrophosphate of the MVD enzyme, blocking sterol production (Nave et al., 1985; Voynova et al., 2008).

#### **3.2.2. Transcriptional regulation of mevalonate enzymes: importance of SREBP**

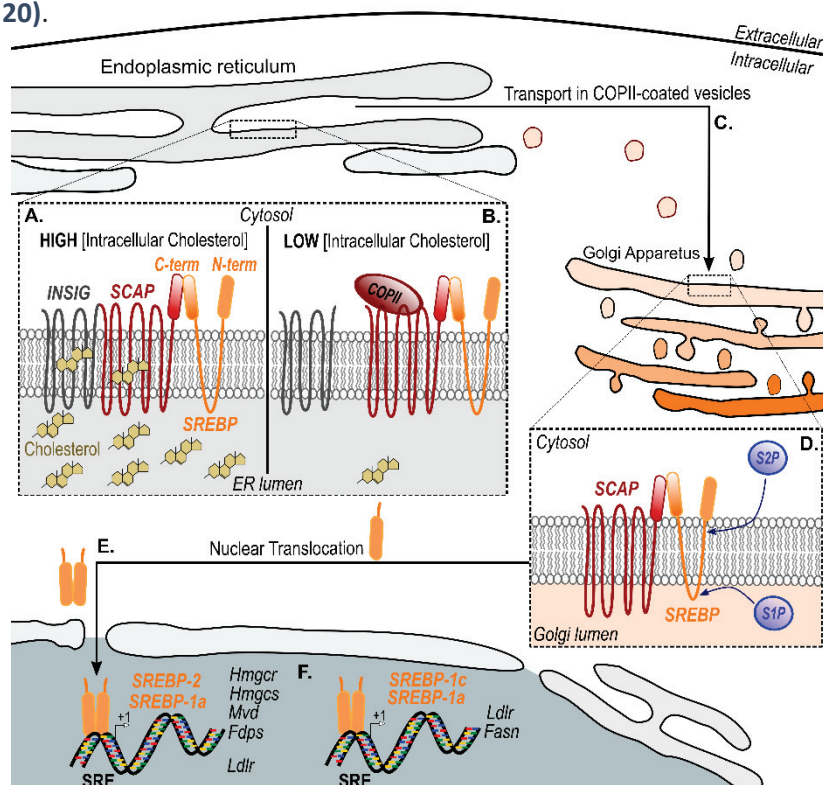
The second regulation, and probably the most investigated one, is the role of the sterol regulatory element-binding protein (SREBP) family of transcription factors. This family of helix-loop-helix leucine zipper (HLH-LZ) proteins contains three different proteins (SREBP1a, SREBP1c and SREBP2), encoded by two different loci (respectively *Srebf1* and *Srebf2*) (Hua et al., 1993; Yokoyama et al., 1993). These proteins regulate the transcription of lipid metabolism genes, including genes of the MVA pathway (for SREBP2 and SREBP1a) and genes involved in fatty acid synthesis (for SREBP1a,

SREBP1c) (Amemiya-Kudo et al., 2002) (**Figure 20**). MVA pathway target genes include *Hmgcs*, *Hmgcr* and *Fdps*, all of these genes presenting sterol regulatory elements (SREs) (ATCACCCAC) within their promoter (Sato et al., 1996; Amemiya-Kudo et al., 2002) (**Figure 20**).

### a. The SREBP-regulated sterol feedback response

Prior to discovering the chemical reactions of the mevalonate pathway, the first clue to a feedback regulation of cholesterol was reported in 1933 with Schoenheimer's work. After he had placed mice in sealed bottles and fed them a cholesterol-free diet, he observed a *de novo* production of cholesterol inside the bottle, this production being stopped by a cholesterol-containing diet (Goldstein and Brown, 2015). The mechanism underlying this sterol feedback response occurring at the ER membrane was deciphered in the mid-1990s.

SREBP proteins are anchored to the ER membrane. While the cytosolic N-terminal domain of SREBPs contains a basic HLH-LZ sequence that can bind to DNA *via* to SREs, the cytosolic C-terminal regulatory domain interacts with the ER-anchored SREBP cleavage-activating protein (SCAP) (Hua et al., 1996) (**Figure 20**).



**Figure 20: SREBP-regulated sterol feedback response.** SREBP interacts with SCAP and INSIG at the ER membrane. **A.** Upon high sterol content, SCAP and INSIG sense cholesterol through ER-lumen domains and maintain SREBP at the ER membrane. **B.** Upon sterol depletion, INSIG dissociates from this SCAP/SREBP complex and a COPII binding site is unveiled. **C.** COPII-coated vesicles bud from ER membranes to be transported to the Golgi apparatus where cleavage by S2P and S1P release N-terminal bHLH-LZ domain of SREBP (**D**). **E.** The nuclear translocation and the binding to SREs upstream

of mevalonate pathway genes (left panel) or upstream fatty acid synthesis genes (right panel) enhance their transcription **(F)**.

If a sufficient quantity of cholesterol is detected, SCAP binds to cholesterol in the ER lumen, acting as a sterol sensor (Radhakrishnan et al., 2004), and retains SREBPs protein at the ER membrane. This SREBP/SCAP-cholesterol complex is locked by another ER-resident protein insulin-induced gene 1 protein (INSIG1) (Yang et al., 2002) **(Figure 20.A)**. Upon cholesterol depletion, SCAP sensor conformation is modified and unveils a COPII site of interaction (Radhakrishnan et al., 2007) **(Figure 20.B)**. The SCAP/SREBP cluster complex is further packaged into COPII-coated vesicles that bud from ER membranes and are addressed to the Golgi apparatus (Antonny and Schekman, 2001) **(Figure 20.C)**. In the Golgi, the site-2 protease (S2P) cleaves the N-terminal half of SREBP, releasing the transcriptionally active HLH-LZ segment (Rawson et al., 1997) **(Figure 20.D)**. Release and nuclear translocation of the N-terminal part of SREBP allow the transcription of genes containing SREs on their promoter (Sato et al., 1996; Amemiya-Kudo et al., 2002) **(Figure 20.E-F)**.

#### **b. Oncogene-mediated interactions with SREBP**

Owing to their strong demand for biomolecules in order to sustain their proliferation, cancer cells and especially the transformation process were extensively studied for their ability to regulate the mevalonate pathway (Mullen et al., 2016). Indeed, many oncogenic signals promote transcriptional regulation of MVA pathway, some through the interaction with SREBP.

The PI3K/AKT pathway is crucial in cell survival and cell proliferation, and its aberrant activation (for example through loss of its upstream inhibitor PTEN or hyperactivation of upstream growth factor tyrosine kinase) is frequently observed in cancers (Engelman, 2009). Firstly, PI3K/AKT pathway activation increases transcription of *Srebf1* and *Srebf2* (Fleischmann and Iynedjian, 2000; Porstmann et al., 2005; Luu et al., 2012). Secondly, PI3K/AKT pathway stabilizes nuclear SREBP1a and SREBP2 by limiting their proteosomal degradation (Sundqvist et al., 2005) **(Figure 21)**. Overall, the global upregulation of MVA pathway through PI3K/AKT/SREBP is functionally involved in the progression of different tumor models, including hepatocellular carcinoma (HCC), melanoma, and breast cancer *in vitro* and *in vivo* (Calvisi et al., 2011; Yamauchi et al., 2011; Ricoult et al., 2016).

Furthermore, downstream of the PI3K/AKT signaling, the mTOR complex 1 (mTORC1) is also a powerful regulator of the MVA pathway. mTORC1 is a master coupler of cellular growth and metabolism, activating global mRNA translation by phosphorylating ribosomal S6 kinase 1 (S6K1) and inhibiting negative regulator of cap-dependent translation 4E-binding protein 1 (4EBP1) (Shimobayashi and Hall, 2014). Beyond this canonical role, mTORC1 was shown to activate SREBPs and subsequently induce *de novo* lipogenesis that is required for cell growth (Porstmann et al., 2008; Düvel et al., 2010).

Moreover and in accordance with these observations, a positive correlation between a high level of phosphorylated S6K1 and transcription of MVA pathway genes, such as *Mvk*, was obtained from patients with primary breast cancer (Ricoult et al., 2016). Finally and unlike mTORC1, AMP kinase (AMPK) restricts anabolic pathways when intracellular ATP levels are low, and can inhibit proteolytic processing activity of hepatic SREBP2 and subsequent transcriptional expression of *Hmgcs* and *Hmgcr* genes (Li et al., 2011) (**Figure 21**).

p53 gain-of-function mutations (p53<sup>MUT</sup>) and Rb loss are two frequent alterations in cancers. While WT p53 is able to downregulate MVA genes in HCC, accounting for one of its multiple tumor suppressor roles (Moon et al., 2019), the role of the gain-of-function mutation R273H (TP53<sup>R273H</sup>) is not completely clear and could be tissue specific. Indeed, in a breast cancer model, R273H mutation enhances nuclear p53<sup>MUT</sup>/SREBP2 interaction and boosts the transcription of MVA pathway genes in breast cancer (Freed-Pastor et al., 2012). Conversely, the same gain-of-function mutation R273H in glioblastoma models represses transcription of MVA genes (Laezza et al., 2015), suggesting a tissue-specific role for p53 in regulating MVA pathway genes. Although less investigated so far in normal cells, WT p53 represses transcription of MVA pathway genes as suggested by their downregulation in MEFs upon nutlin-3a treatment (Moon et al., 2019). Besides, pRB negatively regulates the MVA pathway by both inhibiting E2F1/E2F3 interactions on *Srebf1* or *Fdps* promoters and SREBP1/SREBP2 direct interactions with *Fdps* promoter (Shamma et al., 2009). Overall, p53<sup>MUT</sup> and Rb loss are two other oncogenic pathways enhancing MVA through SREBP2 interaction (**Figure 21**).

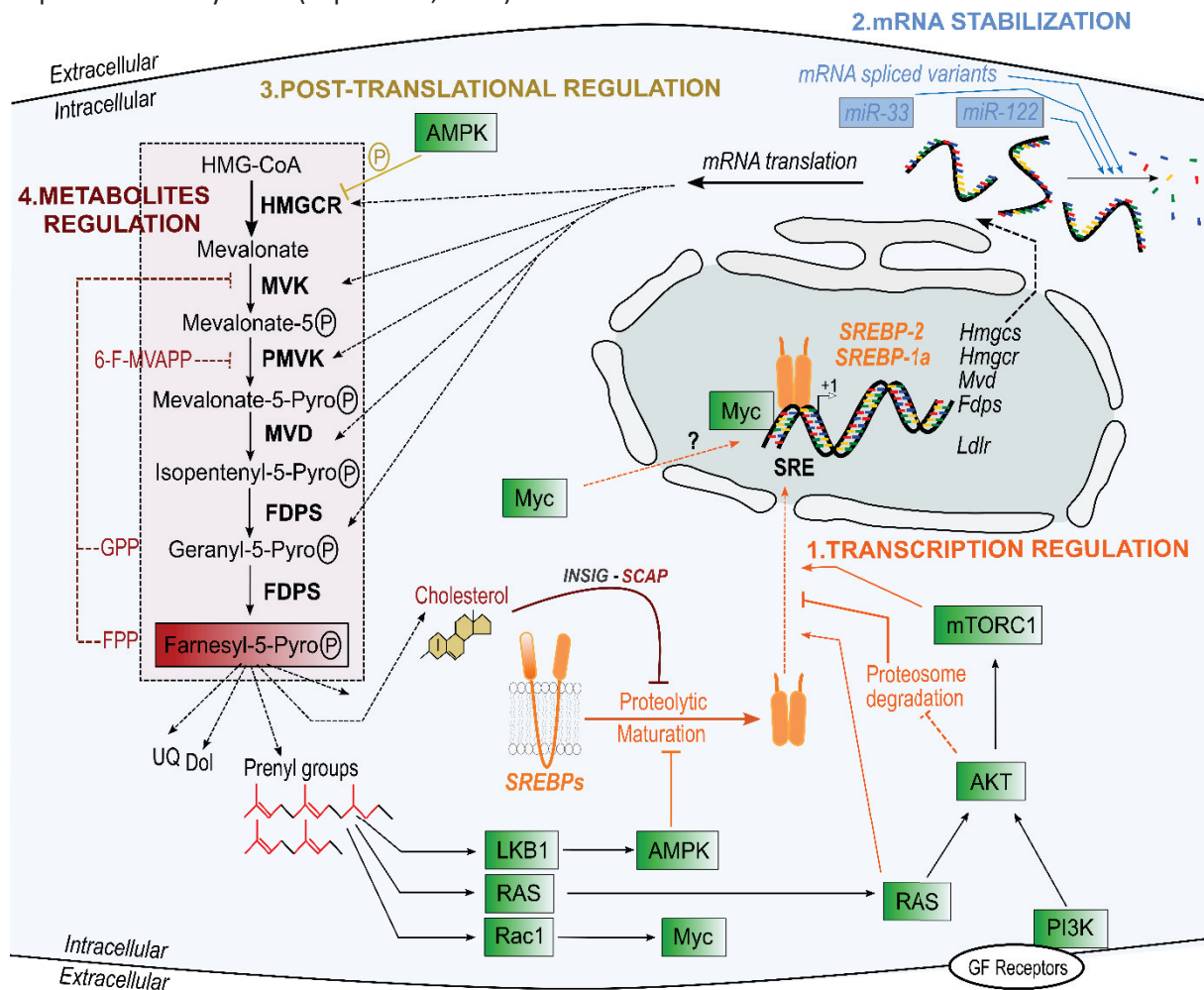
Other known oncogenes such as Myc (Dang, 2012) were found to potentially regulate the MVA pathway, independently of SREBP2 activity. The encyclopedia of DNA elements (ENCODE) project shows that MYC is able to bind to the promoters of MVA pathway genes (Dunham et al., 2012) (**Figure 21**). Moreover, a haplo-insufficient mouse model of *Myc* +/- displays increased lifespan, that is correlated with a decreased expression of various genes of the MVA pathway including *Hmgcs1*, *Hmgcr*, *Mvd* and *Srebf2* (Hofmann et al., 2015).

### **3.2.3. Post-transcriptional regulation of mevalonate enzymes**

Expression of MVA pathway genes is modulated by the generation of alternative splice variants (Medina et al., 2011). Indeed, HepG2 cells upon sterol loading or liver biopsies of monkeys fed with cholesterol-supplemented diet display an alternative splicing of HMGCS1, HMGCRCR and MVK transcripts, ultimately reducing their half-life (Medina et al., 2011). Moreover, MVA enzyme transcripts are targeted by microRNA. The contribution of miRNAs in regulating the MVA pathway is also

important, as evidenced by miR-122 or by miR-33, intronic miRNAs encoded in the SREBP genes, acting as a negative feedbacks for cholesterol synthesis and mevalonate pathway activation in the liver (Fernández-Hernando Carlos et al., 2013) (**Figure 21**).

Besides, MVA enzymes are targeted by post-translational regulation. For example in the liver, AMP-activated protein kinase (AMPK), upon glucagon stimulation, can phosphorylate HMGCR at Ser872 site of the C-terminus part to rapidly inhibit its activity (Beg et al., 1978; Clarke and Hardie, 1990) (**Figure 21**). In addition to HMGCR, HMGCS1 may be targeted in *C. elegans* through an age-dependent sumoylation (Sapir et al., 2014).



**Figure 21: Regulation of the mevalonate pathway.** 1. Transcription regulation of MVA pathway genes through SREBP interactions (orange) is the main pathway affected by various signaling proteins (green). Other regulations of the MVA pathway happen post-transcriptionally through mRNA stabilization (2) (blue), post-translational regulation (3) (yellow), or metabolite regulation (4) (red).

### 3.3. Biological importance of the mevalonate pathway

As part of anabolism, neo-synthesis of isoprene-derived molecules through MVA pathway is necessary for fundamental biological processes. In addition to terminal end-products of downstream

branches, including sterol and non-sterol end-products, some metabolite intermediates participate in numerous cellular functions.

### **3.3.1. Mevalonate pathway intermediates**

Even if not extensively reported, some MVA pathway intermediates, such as mevalonate itself, can influence phenotypes of immune cells (Thurnher and Gruenbacher, 2015; Bekkering et al., 2018). Remarkably, MVK deficiency leads to hyper immunoglobulin D syndrome (HIDS) and has been studied in the context of immune cell training. Accumulation and excessive secretion of mevalonate in blood leads to the activation of IGF1-R and subsequent mTOR activation in myeloid cells, ultimately triggering a constitutive trained immunity phenotype, which could explain some of the inflammatory defects of HIDS patients (Bekkering et al., 2018).

### **3.3.2. Mevalonate pathway and non-sterol end products**

#### **a. Biosynthesis of prenyl groups: implications in farnesylation/geranylation**

As their name suggests it, farnesyl-PP and geranyl-PP carry one farnesyl group (three isoprene units) and one geranyl group (two isoprene units), respectively. These prenyl groups can be transferred to the C-terminal extremity of proteins presenting a -CAAX domain, C representing a cysteine residue, AA two aliphatic residues, and X a various C-terminal amino acid (Berndt et al., 2011). Small GTPases of the Rho subfamily (RhoA, Rac1 et Cdc42), RAS, liver kinase B1 (LKB1), are part of the numerous -CAAX proteins regulated by farnesylation and geranylation. Through fueling of prenyl groups, the MVA pathway thus participates in the direct regulation of various signaling pathways, such as RAS/PI3K/AKT (Kusama et al., 2002), or LKB1/AMPK (Houde et al., 2014). Furthermore, prenylation of small GTPases such as Rac1 and RhoA enhances other pro-oncogenic pathways, such as the Myc pathway in HCC (Cao et al., 2011) and YAP/TAZ pathway in breast cancer cells (Sorrentino et al., 2014).

Aside from its role in regulating cell survival and proliferation, prenylation is more broadly necessary for protein-membrane interactions and protein-protein interactions modulating various other cell signaling pathways (Berndt et al., 2011) (**Figure 22**).

#### **b. Biosynthesis of dolichol: implications in glycosylation**

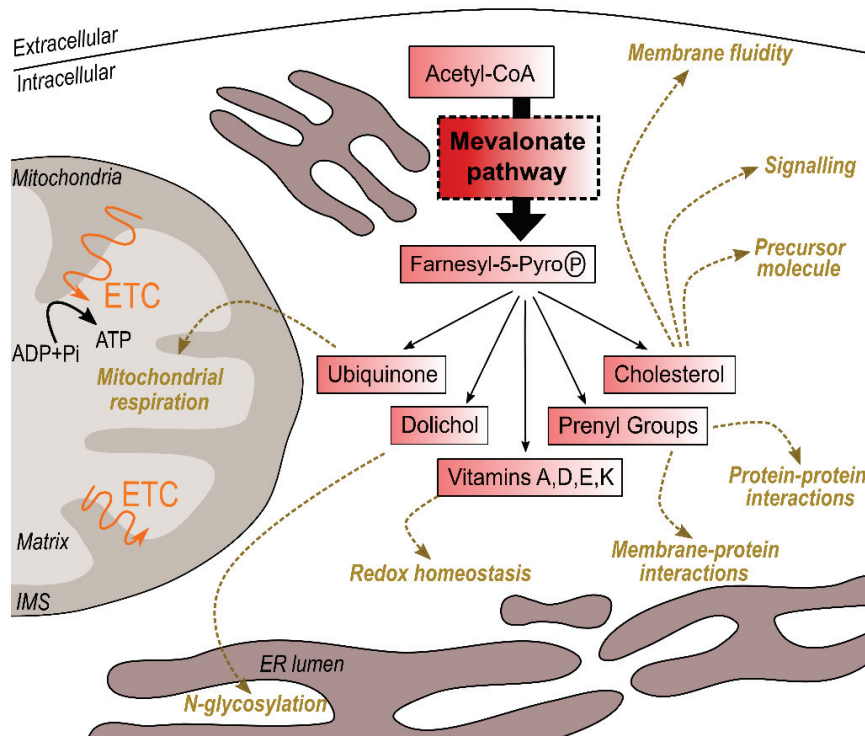
A *cis*-prenyltransferase, the dehydrodolichyl diphosphate synthase (DDS) complex, catalyzes the addition of IPP to farnesyl-5PP (FPP) and generates dehydrodolichyl pyrophosphate (Dedol-PP), a precursor of dolichol. Dolichol is a glycosyl lipid carrier required for the biosynthesis of several classes

of glycoproteins in the lumen of the ER (Chojnacki and Dallner, 1988). Dehydrodolichyl diphosphate synthase (DDS) combines two catalytic subunits encoded by DHDDS1 and NUS1 (Grabińska et al., 2016) (Figure 20).

Dolichol-mediated N-glycosylation in the ER lumen, coupled to O-glycosylation in the Golgi apparatus, participate in crucial post-translational modifications regulating numerous fundamental biological processes, including among others cell signaling, cell-cell communication and cell-matrix interactions (Pinho and Reis, 2015) (Figure 22).

### c. Biosynthesis of ubiquinone: implications in mitochondrial function

The polyisoprenoid side-chain of ubiquinone (UQ), also termed quinone coenzyme Q (CoQ), arises from farnesyl-5-PP and condensation of multiple IPP. UQ biosynthesis is associated with the inner mitochondrial membrane (Wang and Hekimi, 2016). Indeed, end-product UQ localizes to the electron transport chain in the inner membrane of the mitochondria, where the quinone group acts as a electron shuttle from complex I or II to complex III, subsequently necessary to generate the proton H<sup>+</sup> gradient and ATP during oxidative phosphorylation (Wang and Hekimi, 2016) (Figure 22).



**Figure 22: Importance of the mevalonate pathway in cell biology.** Farnesyl-5-pyrophosphate is the end product of the MVA pathway (red). Its transformation participates in the production of multiple polyisoprenoids, including ubiquinone, dolichol, non-sterol vitamins A, D, E, K, prenyl groups, and cholesterol. Ubiquinone is a component of the electron transport chain (ETC) in the mitochondria. Dolichol is a precursor used for efficient N-glycosylation in the ER lumen. Some vitamins, like vitamin E, play a role in redox homeostasis. Prenyl groups are involved in membrane- or protein-interactions. Cholesterol is involved in membrane fluidity, signaling, and acts as a precursor molecule.



### **3.3.3. Mevalonate pathway and sterol end product: cholesterol**

Chemical reactions of the MVA pathway were first described with regards to investigations on the cholesterol biosynthetic pathway (Bloch, 1965).

#### **a. Biosynthesis of cholesterol**

The biosynthesis of cholesterol involves a dozen chemical reactions (**Figure 22**). Farnesyl-5-pyrophosphate (F5PP) is initially transformed into squalene by the first specific enzyme of the cholesterol biosynthetic subpathway, the farnesyl-diphosphate farnesyltransferase 1 (FDFT1) (Bloch, 1965), another well-known enzymes being squalene epoxidase (SQLE). The cholesterol biosynthetic pathway is ubiquitous and conserved among tissues, though some cells, like hepatocytes or neurons, produce more cholesterol than others (Moutinho et al., 2017).

#### **b. Biological roles of cholesterol**

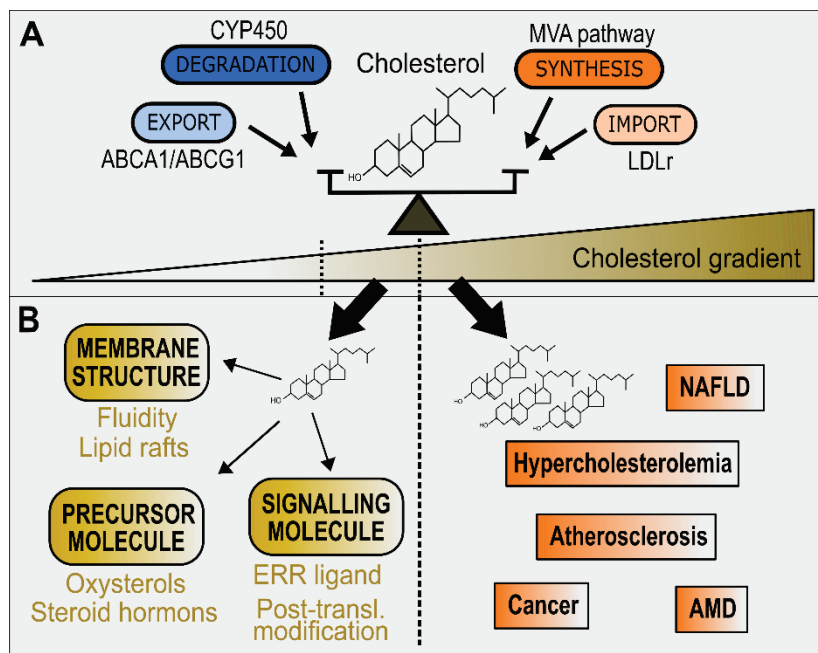
Cholesterol acts as a structural molecule in membranes, modulating their fluidity (Cooper, 1978). Cholesterol is also more abundant in special plasma membrane microdomains, called lipid rafts and including sphingolipid and proteins, to form signaling platforms at the interface between the intra- and extracellular matrix (Lingwood and Simons, 2010).

Beyond its structural role, cholesterol is involved in multiple other processes. First, the addition of a cholesterol group to a protein is a specific post-translational modification, that has been described to regulate sonic hedgehog (Shh) during development (Eaton, 2008). Second, cholesterol may act as a signaling molecule, being a ligand for the estrogen-related receptor  $\alpha$  (ERR $\alpha$ ) (Wei et al., 2016). Finally, cholesterol may be catabolized and serve as a substrate for the generation of subsequent molecules. The first enzymatic step of cholesterol catabolism involves the family of cytochrome P450 enzymes, and occurs in the mitochondrial matrix, generating oxysterols (Jusakul et al., 2011). These oxysterols can also be generated *via* non-enzymatic reactions and auto-oxidation of cholesterol, involving oxygen or nitrogen reactive species (Jusakul et al., 2011). As highly reactive molecules, oxysterols are not only mutagens, but are involved in pathophysiological conditions, regulating for example liver X receptor alpha (LXR $\alpha$ ) (Janowski et al., 1996) in normal, inflammatory and cancer contexts (Lin and Gustafsson, 2015). In the liver, excessive cholesterol may ultimately lead to the formation of bile acid through additional steps and to its secretion (Russell and Setchell, 1992).

Finally, steroid hormones, such as testosterone and estrogens, are also derivatives of cholesterol, and display crucial roles for example in the regulation of development and cancers (especially hormone-dependent breast cancer and prostate cancers).

### c. Pathological contexts of cholesterol

Cholesterol, and more specifically its metabolic dysregulation, is linked to multiple pathological contexts. Intracellular cholesterol content is governed by four complementary mechanisms: (i) synthesis (through MVA pathway) and (ii) import on the one hand, and (iii) degradation and (iv) export on the other hand (**Figure 23.A**). Following a dysfunction in these mechanisms, cholesterol may either accumulate or be reduced. Cholesterol serum level is used to evaluate this homeostasis and is altered in specific genetic diseases such as familial hypercholesterolemia (FH) (**Figure 23.B**). This recessive genetic disorder affected 10 million people worldwide in 2004 and the frequency of heterozygotes is high with approximately one per 500 individuals in most countries (Civeira, 2004). A high incidence of premature cardiovascular events, such as coronary heart disease (CHD) (<55 years in men and <65 years in women), reduces life expectancy observed in heterozygous FH families (Civeira, 2004; Mabuchi H et al., 1989). For thirty years, statins, MVA inhibitors, have been widely used to reduce this incidence (Shepherd et al., 1995), increasing life expectancy of FH patients by 10-30 years (Civeira, 2004).



**Figure 23: Pathophysiological involvement of cholesterol homeostasis.** **A.** Cholesterol homeostasis within the cell is regulated by export (*via* the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1), degradation (*via* cytochrome P450 enzymes family), synthesis (*via* the MVA pathway) and import (*via* low density lipoproteins receptor, LDLr). **B.** Cholesterol is required for membrane structure but is also a precursor for oxysterols and steroid hormones, and signaling molecules, acting as ligands or used for post-translational (post-transl.) modifications. A dysregulation of cholesterol homeostasis may lead to excessive cholesterol and is linked with non-alcoholic fatty liver disease (NAFLD), hypercholesterolemia, atherosclerosis, cancer and age-related macular degeneration (AMD).

Aside from hypercholesterolemia, excessive cholesterol was associated with various other pathologies, such as atherosclerosis (Kim Jay H. et al., 1994), NAFLD (Min et al., 2012), or even macular degeneration (van Leeuwen et al., 2004; Sene et al., 2013) (**Figure 23.B**). Remarkably, aging constitutes one of the main drivers of these later pathologies and cellular senescence has functionally been associated with them (Childs et al., 2016; Roos et al., 2016; Ogradnik et al., 2017; Blasiak, 2020).

#### **3.3.4. Clinical relevance: the pharmacological inhibition of the mevalonate pathway**

In order to assess the different biological roles of the MVA pathway, their inhibitors, such as statins and aminobiphosphonates, were largely used (Assmus Birgit et al., 2003; Kim et al., 2011; Mullen et al., 2016) and are of therapeutic interest in numerous diseases, such as atherosclerosis or cancer (Shepherd et al., 1995; Buhaescu and Izzedine, 2007a; Mullen et al., 2016). Aside from these studies and from the pharmacological use of these inhibitors, known to have pleiotropic effects (Liao and Laufs, 2004), only a few genetic studies have so far been conducted to determine the precise role of the genes implicated in the MVA pathway in normal cells.

To summarize this chapter, the MVA pathway is crucial in lipid synthesis and participates in the production of essential cellular polyisoprenoids, including dolichol, ubiquinone and cholesterol. Nonetheless and as suggested by the growing use of MVA pathway inhibitors to treat pathologies such as atherosclerosis, osteoporosis or cancer, its dysregulation and particularly its upregulation may be detrimental for cells and the organism.

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« L'ennui dans ce monde, c'est que les idiots sont sûrs d'eux et les gens sensés pleins de doutes »  
Bertrand Russell - XXème siècle

## **- OBJECTIVES -**



Cellular senescence displays a high diversity in inducers, regulators and effectors. Cellular senescence is crucially involved in key biological processes, both physiological and pathological, reinforcing the importance to dissect mechanisms regulating this orchestrated senescence program. While multiple inducers and effectors of cellular senescence are well-characterized, intrinsic subcellular mechanisms linking and regulating them remain a matter of active research. Metabolic alteration appears to be an interesting candidate to explore the regulation of senescence.

Metabolic homeostasis is a dynamic equilibrium controlling intracellular biochemical reactions in order to maintain optimal cell functions and survival. In the middle 1990's, metabolic alterations were suspected to play a role in senescence (Toussaint et al., 1995). More recently, mitochondrial dysfunction or even ER stress were found to promote it (Kim et al., 2019; Wiley et al., 2016), by generating perturbations of metabolic homeostasis. This opened new perspectives in this specific research area.

In this context, the general aim of my PhD work was to decipher the role of metabolic alterations in the regulation of cellular senescence. To do so and based on previous results from the lab, I focused on two crucial metabolic paths involved in cell functions: calcium and lipid homeostasis. Calcium and lipids are crucial in cellular biology, thus constituting critical fields to study in the context of senescence.

The first part of my work was focused on the role of the ER-calcium channel ITPR2 in regulating senescence and physiological aging *in vivo*. This investigation on the physiological relevance of ITPR2-mediated senescence *in vivo* was conducted taking advantage of the *Itpr2* *-/-* mouse model, which is viable and fertile (Futatsugi et al., 2005). Based on strong previous *in vitro* results from the lab (Wiel et al., 2014; Ma et al., 2018), this work combined *in vivo* and *in vitro* approaches to further elucidate the role of ITPR2 in regulating senescence.

The second part of my work aimed at tackling the role of MVA pathway in modulating cellular senescence. Based on a screen approach, our lab previously identified numerous pro-senescent kinases (Ferrand et al., 2015) and included MVK and PMVK, two enzymes of the MVA pathway. This work was dedicated to explore molecular mechanisms behind the MVA pathway, deciphering its role in regulating cellular senescence.

Aside from these projects, I participated in the study of other senescence regulators we are interested in, including phospholipase A2 receptor 1 (PLA2R1) (*SEE ANNEXES PART*).





# - RESULTS -



The results I have obtained during my PhD are presented in the following part, in forms of manuscripts. While ITPR2 manuscript has been submitted and is under revision in *Nature Communications*, MVA manuscript will be submitted in the next few months. Each manuscript will be preceded by a short summary of the study.



## **- CHAPTER I -**

### **1. - Roles of ITPR2 and MERCs in the regulation of cellular senescence –**

#### **1.1. Summary of the study**

The ER calcium-release channel ITPR2 and ER-mitochondrial calcium fluxes are known to be involved in regulation of cell fate, such as apoptosis. Our lab extended the role of ITPR2 in the regulation of another cell fate: cellular senescence (Ma et al., 2018; Wiel et al., 2014). The two latter works emphasize the role of ITPR2 in promoting cellular senescence *in vitro* in OIS in hMEC, RS in fibroblasts (Wiel et al., 2014) and in premature senescence induced by RXR knockdown in fibroblasts (Ma et al., 2018). These two works converge into a model where ITPR2 enhances mitochondrial calcium accumulation to drive subsequent ROS accumulation and senescence (Ma et al., 2018; Wiel et al., 2014).

My PhD study investigated the physiological relevance of ITPR2-mediated senescence *in vivo*. For this purpose, we took advantage of the *Itpr2*<sup>-/-</sup> mouse model, which is viable and fertile (Futatsugi et al., 2005). As senescent cells accumulate during aging and participates in shortening lifespan (Herbig et al., 2006; Jeyapalan et al., 2007; Baker et al., 2011, 2016), we monitored aging and followed up lifespan and healthspan through weight and resistance to metabolic stress in *Itpr2*<sup>-/-</sup>. As ITPR2 promotes senescence *in vitro*, we assessed in old mice (23-month-old) (1) senescence markers notably in liver, lung and skin and (2) histological age-related alterations such as steatosis and fibrosis in liver.

*Itpr2*<sup>-/-</sup> mice displayed improved aging such as increased lifespan coupled to a better response to metabolic stress, less immunosenescence, as well as less liver steatosis and fibrosis. Cellular senescence, which is known to promote these alterations, was decreased in both *Itpr2* KO mice and *Itpr2* KO embryo-derived cells. Interestingly, loss of ITPR2 *in vivo* and *in vitro* decreased the number of contacts between the ER and the mitochondria, while forced contacts between these two organelles induced premature senescence in normal cells. Taken together, these original findings shed light on the role of membrane contacts sites and facilitated exchanges between the ER and the mitochondria through ITPR2 in regulating senescence and physiological aging.

- 1.2. Article: “Calcium channel ITPR2 and mitochondria-ER contacts promote cellular senescence and aging”

# **Calcium channel ITPR2 and mitochondria-ER contacts promote cellular senescence and aging**

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The authors have declared that no conflict of interest exists

## **Abstract**

Cellular senescence is induced by multiple stresses and results in a stable proliferation arrest accompanied by a pro-inflammatory secretome. Senescent cells accumulate during aging, promoting various age-related pathologies and thus limiting lifespan. The endoplasmic reticulum ITPR2 calcium-release channel and calcium fluxes from the ER to the mitochondria have been identified as drivers of cellular senescence in human cells. Here we show that *Itp2* knockout mice display improved aging such as increased lifespan, a better response to metabolic stress, less immunosenescence, as well as less liver steatosis and fibrosis. Cellular senescence, which is known to promote these alterations, is decreased in both *Itp2* KO mice and *Itp2* KO embryo-derived cells. Interestingly, ablation of ITPR2 *in vivo* and *in vitro* decreases the number of contacts between the mitochondria and the ER and forced contacts between these two organelles induce premature senescence in normal cells. These new findings shed light on the role of contacts and facilitated exchanges between the ER and the mitochondria through ITPR2 in regulating senescence and physiological aging.

## **Keywords**

Aging, cellular senescence, mitochondria-endoplasmic reticulum contacts, ITPR2, calcium.

## Introduction

Cellular senescence is an important process regulating different pathophysiological processes from embryonic development to aging. In particular, it promotes various age-related diseases and shortens the lifespan (Baker et al., 2011, 2016; Childs et al., 2017). Cellular senescence is characterized by a stable cell cycle arrest and a pro-inflammatory senescent-associated secretory program (SASP), both involved in the pathophysiological effects of senescent cells (He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014b). Although downstream factors, such as p16, directly blocking cell cycle or activating the SASP are largely described, the upstream molecular and sub-cellular mechanisms controlling these factors are largely unknown. The network and activity of mitochondria are dysregulated during cellular senescence, though the characterization and cause of these alterations are largely unknown (Ziegler et al., 2015).

Calcium critically regulates many cellular and molecular processes including but not limited to secretion, autophagy, migration, proliferation and cell death (Berridge et al., 2000). More recently calcium has been shown to be regulated during cellular senescence and to impact its outcome (Farfariello et al., 2015; Martin and Bernard, 2018). We and others have recently shown that transfer of calcium from the endoplasmic reticulum (ER) through inositol 1,4,5-trisphosphate receptor (ITPR or IP<sub>3</sub>R) ER channels to the mitochondria and its subsequent mitochondrial accumulation leads to cellular senescence in normal human cells (Borodkina et al., 2016; Ma et al., 2018; Martin and Bernard, 2018; Wiel et al., 2014). Contact sites between mitochondria and ER, also called Mitochondria-ER Contacts (MERCs) or Mitochondria-Associated ER Membranes (MAMs), have emerged as hotspots for calcium transfer and signaling (Brito and Scorrano, 2010; Marchi et al., 2014; Raturi and Simmen, 2013; van Vliet et al., 2014). ITPR2 can be part of MERC sites, coordinating among others calcium transfer

(Marchi et al., 2014). Potential role of these MERC sites on cellular senescence are currently unknown.

In this study, we investigated the physiological role of ITPR2 and MERCs in the regulation of cellular senescence and aging by using *Itpr2* knockout mice and their derived primary cells as well as human normal cells.

## Results

***Itpr2* knockout increases lifespan and limits age-related phenotypes in mice.** In order to investigate the importance of ITPR2 on physiological aging, we studied and monitored cohorts of *Itpr2* knockout mice (KO) (Li et al., 2005) up to their death. Remarkably, *Itpr2* KO enhanced the median and maximum lifespan of female mice by 23% and 39%, respectively (Fig. 1A). Loss of *Itpr2* did not affect the survival of male mice (Supplementary Fig. 1A). The differences in lifespan between males and females are well described. Indeed, many studies report striking gender-associated lifespan differences following a modification in regimen or gene expression, though the mechanisms underlying such differences remain unclear (Ali et al., 2006; Austad and Fischer, 2016; Kane et al., 2018; Lamming et al., 2014). According to our results, WT males lived longer than WT females as already reported (Kunstyr and Leuenberger, 1975), suggesting that *Itpr2* could contribute to the intrinsic lifespan difference between male and female mice.

Aging is a complex systemic process involving numerous vital organs. Several aging-related features were monitored: immunosenescence designated as immune aging (Pawelec, 2014), liver alterations, response to metabolic challenge, bone density and tumor lesions. Immunosenescence, essentially characterized by the exhaustion of naïve T cells and accumulation of memory T cells, was examined by quantifying the differences between these two populations (Shanley et al., 2009) in the spleens of 23-month old WT and *Itpr2* KO mice.



*Itp2* KO mice displayed a decreased number of both memory CD4<sup>+</sup> T cells and effector memory CD8<sup>+</sup> T cells compared to control littermates (Fig. 1B). This decrease was associated with an increased proportion of both naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and an overall increase in naïve/memory T cells (Supplementary Fig. 1B), strongly supporting that ITPR2 promotes immunosenescence in aged mice.

Since ITPR2 is highly expressed in the liver (Vervloessem et al., 2015), we examined the structure and function of the liver in aged mice. Compared to WT mice, the livers of old *Itp2* KO mice displayed no marks of macroscopic steatosis (Fig. 1C) and fewer lipid droplets (Supplementary Fig. 1C). Accumulation of lipid is a key feature of liver steatosis (Nagle et al., 2009), mainly through increased triacylglycerol (TAG) synthesis. Whole-genome transcriptome analysis revealed that loss of *Itp2* reduced the cellular lipid metabolic Gene Ontology signature and the fatty acid metabolism Gene set in the liver of old mice (Supplementary Fig. 1D-E). More specifically, mRNA levels of the fatty acid biosynthesis enzymes *Elovl1* (Elongation of very long chain fatty acids protein 1) and *Fabp7* (Free fatty acid binding protein 7) were significantly reduced in the liver of old *Itp2* KO female mice (Fig. 1D). Moreover, *Itp2* KO mice displayed decreased liver fibrosis (Fig. 1E) and presented a decreased blood Aspartate Aminotransferase (AST) level (Fig. 1F), a marker of damaged liver (Frith et al., 2009).

We next investigated the ability of old WT and *Itp2* KO mice to respond to a metabolic challenge, since during aging, liver dysfunction contributes to an altered response (Jackson et al., 1988). Although no difference between the two genotypes was observed in the regulation of glycaemia during a glucose-tolerance test in 20-month-old mice, 26-month-old WT male mice had lost their ability to regulate their glycaemia, whereas KO littermates responded normally to glucose injection (Fig. 1G), suggesting that *Itp2* KO mice were protected from age-induced glucose intolerance. Furthermore, *Itp2* KO resulted in the abolition of age-related

increased basal blood glycaemia (Supplementary Fig. 1F). This parameter could not be assessed in females as most of the females were already dead at this age (Fig.1A). Besides, no significant differences were observed between old WT and *Itpr2* KO mice for the following parameters: bone mineral density and content (Supplementary Fig. 1G), weight (Supplementary Fig. 1H), and tumor lesions (Supplementary Fig. 1I).

Together, these results support that ITPR2 promotes some alterations associated with physiological aging.

**Loss of *Itpr2* reduces cellular senescence in mice and their derived cells.** As increased cellular senescence is known to promote all of the age-related alterations described above (He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014b) and as the ITPR2 calcium channel is a positive regulator of cellular senescence in human cells (Wiel et al., 2014), we wondered whether loss of *Itpr2* decreased cellular senescence in mice and in mice-derived cells. Transcriptomic analyses revealed that liver from 23-month-old *Itpr2* KO mice presented fewer markers of cellular senescence, as evidenced by a decrease in the inflammatory response signature, a signature that can be related to pro-inflammatory SASP (Salminen et al., 2012) and includes *ccl3*, *ccl4*, *ccl12*, *cxc15* and *cxc110*, and *p16<sup>ink4a</sup>* mRNA levels (Fig. 2A and Supplementary Fig. 2A). These results were confirmed in a larger group of mice by RT-qPCR on *Ccl3* and *p16<sup>ink4a</sup>* (Fig. 2B) and by the immunohistochemical analysis of the *p16<sup>ink4a</sup>* protein (Fig. 2C).

Reminiscent of *Itpr2* loss extending lifespan of female animals only, *Ccl3* and *p16* mRNA levels were markedly reduced in the liver of old *Itpr2* KO females compared to livers of old WT females. A similar but weaker trend towards a reduction in *Ccl3* and *p16* expression was observed in the liver of old *Itpr2* KO males compared to liver of old WT males (Supplementary Fig. 2B). Additionally, *p16* mRNA levels were two-fold higher in the liver of

old WT females compared to WT males, consistent with the difference in lifespan observed above between the sexes (Fig. 1A and Supplementary Fig. 1A). In line with a role for cellular senescence in promoting liver aging and dysfunction (Aravinthan and Alexander, 2016; Ogrodnik et al., 2017; Palmer et al., 2019), intrahepatic fibrosis level as well as blood AST levels and lipid droplets quantity were positively correlated with the p16<sup>ink4a</sup> senescence marker in the liver of old mice (Fig. 2D-F).

To further confirm a link between the loss of *Itpr2* and decreased levels of senescence, beyond the liver, we explored the replicative potential of *Itpr2* KO- and WT-derived mouse embryonic fibroblasts (MEFs). Late passaged *Itpr2* KO MEFs showed a decreased level of cellular senescence, as illustrated by a decrease in the level of several cellular senescence markers, namely the Senescence Associated- $\beta$ -Galactosidase activity (SA- $\beta$ -Gal) (Fig. 2G and Supplementary Fig. 2C), cell proliferation (Supplementary Fig. 2D), p16<sup>ink4a</sup> mRNA (Fig. 2H) and protein (Fig. 2I) levels and finally a reversion of the enhanced inflammatory response as evidenced by transcriptomic data (Fig. 2J and Supplementary Fig. 2E).

Because ITPR2 can mediate calcium efflux from the ER to the mitochondria and that mitochondrial calcium can mediate senescence (Ma et al., 2018; Wiel et al., 2014), we determined calcium levels in the late passaged *Itpr2* KO and WT MEFs, using ratiometric genetic reporters. *Itpr2* KO MEFs displayed higher ER calcium levels and lower mitochondrial calcium levels when compared to the WT late passaged MEFs (Supplementary Fig. 2F and G), as well as decreased mitochondrial depolarization and mitochondrial ROS production (Supplementary Fig. 2H and I). These results strongly suggested that old *Itpr2* KO MEFs, which undergo less senescence, have moderated calcium fluxes from the ER to mitochondria and attenuated mitochondrial dysfunction.

In conclusion, these results support that loss of *Itp2* reduces the level of cellular senescence, limits ER-mitochondrial calcium fluxes, and is correlated with an improvement in the structure and function of the liver of old mice.

**Loss of *Itp2* diminishes Mitochondria-ER Contacts.** ITPR2 can be part of and promotes the Mitochondria-ER Contacts (Bartok et al., 2019; Marchi et al., 2014), which are hotspots for calcium exchange between the ER and the mitochondria (Marchi et al., 2017; van Vliet et al., 2014). Moreover, ITPR2 promotes cellular senescence in human cells by favoring calcium transfer to the mitochondria (Wiel et al., 2014), and we observed here that late passaged senescence-resistant *Itp2* KO MEFs showed decreased levels of mitochondrial calcium (Supplementary Fig. 2F). We thus evaluated whether cellular senescence regulated by ITPR2 could involve MERCs.

Assessment of the number of MERCs was performed by examining close proximity between ER, using ITPR1, and mitochondria, using VDAC1, both proteins being components of MERCs, as previously described and validated in the liver of mice using proximity ligation assay (PLA) (Tubbs et al., 2014a). The number of MERCs was 2-fold lower in the liver of old *Itp2* KO mice compared to WT littermates (Fig. 3A and Supplementary Fig. 3A) and these changes were not due to changes in *Itp1* and *Vdac1* levels (Supplementary Fig.3B-C). The number of MERCs was also correlated with the level of the senescence marker p16<sup>Ink4a</sup> (Fig. 3B and Supplementary Fig. 3D). Remarkably the number of MERCs in mice was positively correlated with the relative level of intrahepatic fibrosis (Supplementary Fig. 3E). Further supporting a link between ITPR2 levels, senescence and the number of MERCs, late passage *Itp2* KO MEFs displayed a decrease in the number of MERCs compared to old and senescent WT MEFs, according to both proximity ligation assays between ITPR1 and VDAC1 (Fig. 3C), without any change in ITPR1 and VDAC1 levels (Supplementary Fig. 3F-G), and transmission

electronic microscopy (TEM) (Fig. 3D and Supplementary Fig. 3H). Beyond the number of MERCs, the distance between the ER and the mitochondria in MERCs increased in late passage *Itp2* KO MEFs (Fig. 3E and Supplementary 3I). Perimeter and number of mitochondria were not decreased in old *Itp2* KO MEFs (Supplementary Fig. 3J), excluding this bias in the analysis of MERCs. As observed *in vivo*, the number of MERCs was also positively correlated with the senescence marker SA- $\beta$ -Gal in MEFs (Fig. 3F), substantiating once again a link between MERCs and cellular senescence.

Hence, these results support that fewer and more relaxed MERCs in *Itp2* KO mice and their derived MEFs are correlated with lower levels of cellular senescence.

**Increasing MERCs induces premature senescence.** Loss of *Itp2* decreases cellular senescence and this effect is correlated with a lower number of MERCs raising the possibility that MERCs directly promote cellular senescence. In order to test this hypothesis, we used a synthetic linker containing a domain anchored to the ER membrane and another one anchored to the mitochondrial outer membrane, as previously described (Csordás et al., 2006), to force the interaction between mitochondria and the ER (Fig. 4A and Supplementary Fig. 4A). TEM analysis showed that constitutive expression of MERC linkers increased the number of MERCs (Fig. 4B), increased the total length of the ER-mitochondria interface, and brought mitochondria and ER membranes closer according to distances measured between ER and mitochondria in MERCs, probably also tightening some pre-existing MERCs (Fig. 4C, 4D and Supplementary Fig. 4B) without increasing the perimeter and the number of mitochondria (Supplementary Fig. 4C). These MERCs were functional as they favored calcium transfer from the ER to the mitochondria, reducing calcium in the ER while promoting its accumulation in mitochondria (Fig. 4E and Supplementary Fig. 4D). Importantly, increasing MERCs via the constitutive expression of linkers led to premature senescence in cells as shown by their

decreased ability to proliferate and incorporate EdU (Fig. 4F-G), an increased proportion of SA- $\beta$ -Gal-positive cells (Fig. 4H) and an increased expression of p16<sup>INK4A</sup> and various SASP components, including CCL3, IL-8 and IL1-Beta (Fig. 4I).

Mechanistically, the linker induced mitochondrial depolarization and mitochondrial ROS accumulation (Fig. 4J-K), which can be induced by increased mitochondrial calcium and also are well-known senescence inducers (Lemasters et al., 2009; Ma et al., 2018; Wiel et al., 2014). Accordingly, NAC anti-oxidant treatment (Fig. 4L-N) as well as knockdown of p53 (Supplementary Fig. 4E-G), a downstream effector of ROS (Acosta et al., 2008b; Augert et al., 2009), prevented linker-induced senescence. Activation of the pro-inflammatory arm of the SASP can be NF- $\kappa$ B-dependent (Acosta et al., 2008b; Chien et al., 2011; Ferrand et al., 2015). Knockdown of RelA, a member of the NF- $\kappa$ B family of transcription factor, did not revert proliferation arrest (Supplementary Fig. 4H), yet reverted the induction of IL-8 and IL1-Beta but not of CCL3 in linker-expressing cells (Supplementary Fig. 4I), indicating that the pro-inflammatory SASP induced by linkers is partly regulated by NF- $\kappa$ B activation.

In conclusion, forcing contacts between ER and mitochondrial membranes is sufficient to trigger premature cellular senescence, involving a mitochondrial ROS/p53 pathway and a partial NF- $\kappa$ B-dependent SASP induction.

## Discussion

In this study, we unraveled a role for the calcium channel ITPR2 in the regulation of lifespan, physiological aging and cellular senescence. During these last few years, the functional link between cellular senescence, age-related alterations and lifespan has been demonstrated by eliminating the senescent cells using genetic or pharmacological tools (Childs et al., 2017; Deursen, 2019). In our study, we observed that loss of *Itpr2* decreases the level of senescence *in vitro* and *in vivo* and improves aging, suggesting that ITPR2 regulates aging by impacting cellular senescence.

We mainly studied the impact of *Itpr2* in old mice by focusing on the liver for several reasons. ITPR2 belongs to a family of proteins comprising two other members, ITPR1 and ITPR3, which are known to exert some redundant activities on cancer sensitivity (Ouyang et al., 2014) or on the regulation of senescence (Wiel et al., 2014). In the bone, where the loss of *Itpr2* does not have any significant beneficial impact, its mRNA level is less abundant than *Itpr1* (Supplementary Fig. 5). Of note, in the liver, *Itpr2* is known to be highly expressed compared to the two other *Itprs* (Supplementary Fig. 5) (Vervloessem et al., 2015). Moreover, cellular senescence is involved in age-related liver alterations (Aravinthan and Alexander, 2016; Ogrodnik et al., 2017). Consequently, the liver appears to be a perfect candidate organ to examine the impact of *Itpr2* deletion on cellular senescence and organ aging and its associated alterations. In line with this hypothesis, *Itpr2* KO delays age-related features of the liver, including both steatosis and fibrosis. Moreover, decreased circulating AST levels as well as a better response to a metabolic challenge observed in *Itpr2* KO are expected to depend, at least in part, on a better liver function during aging (Asrih and Jornayvaz, 2015). Altogether, our work unravels that liver ITPR2, while it is the most abundant ITPR in this organ, participates actively to its chronological decline.

Cellular senescence induced by short term injury stimulates wound healing (Demaria et al., 2014b), protects from fibrosis especially in the liver (Kong et al., 2012; Krizhanovsky et al., 2008; Nishizawa et al., 2016) and enhances stemness and regeneration (Milanovic et al., 2018), whereas long-term accumulation of senescent cells promotes fibrosis and degeneration in various contexts (Schafer et al., 2017b). Accordingly, liver regeneration of young *Itpr2* KO mice after hepatectomy is reduced compared to their control littermates (Khamphaya et al., 2018). In this latter study, senescence was not examined but we can speculate that it might contribute to liver regeneration. Interestingly, another *in vivo* study in the brain reported the beneficial effect of *Itpr2* loss in persistent damage-induced phenotype (Li et al., 2015). Once again, senescence has not been evaluated in this photothrombosis-induced cerebral ischemia, but ischemia injuries are known to induce senescence in other models (Li et al., 2017b; Maarouf et al., 2018). Overall, ITPR2, as a regulator of cellular senescence, could exert both beneficial effects after short-term injury in young individuals but detrimental effects after chronic injuries such as during aging.

Aside from the role of ITPR2 in cellular senescence *in vivo* and age-related physiological declines, our work also shed light on its role as a potent regulator of the formation and/or maintenance of MERCs. Of note, in a previous study, we did not observe any changes in the MERCs during oncogene-induced senescence in immortalized human mammary epithelial cells according to co-immunofluorescence experiments (Wiel et al., 2014). Discrepancies between these results might be due to the difference in the senescence system used or/and to the fact that our current study used more sensitive approaches to evaluate MERCs. *Itpr2* KO mice liver or MEFs display a decreased number of MERCs. This decrease occurred only in senescent MEFs when compared to non-senescent ones. How ITPR2 regulates MERCs remains unclear but it has recently been identified as required to maintain MERC (Bartok et al., 2019). In addition, as it can link GRP75 and VDAC with the ER and the



mitochondria (Brito and Scorrano, 2010; Paillusson et al., 2016), its loss might destabilize this ER-mitochondrial tethering system. Interestingly, ITPR1, which can regulate senescence similarly to ITPR2 (Wiel et al., 2014), and the linker, which induces MERCs, have also been shown to promote steatosis and to alter glucose homeostasis in obese mice (Arruda et al., 2014), reinforcing our current observations in *Itp2* KO mice during aging. Obesity is known to promote cellular senescence, which mediates organismal dysfunctions during obesity including steatosis and glucose homeostasis alterations (Ogrodnik et al., 2017; Palmer et al., 2015, 2019). Even though cellular senescence was not investigated in the study by Arruda (Arruda et al., 2014), we can speculate that the effects of ITPR1 and MERCs are mediated, at least in part, by cellular senescence. ITPR1 and ITPR2 may thus promote senescence and subsequent key drivers of age-related defects in the liver, including steatosis and alterations of glucose homeostasis.

Structural changes at the interface between the ER and the mitochondria induced by ITPR2 likely mediate part of the effect of ITPR2 on senescence. Indeed, we have shown that inducing MERCs by constitutively expressing a synthetic linker leads to premature senescence. MERCs are known to be involved in multiple cellular signaling processes, including calcium transfer to the mitochondria (Brito and Scorrano, 2010; Marchi et al., 2017; Patergnani et al., 2011; Raturi and Simmen, 2013). Mitochondrial calcium contributes to mitochondrial bioenergetics regulation (Giorgi et al., 2018), and its rise can contribute to cellular senescence by inducing ROS production (Borodkina et al., 2016; Ma et al., 2018; Martin and Bernard, 2018; Wiel et al., 2014). We observed that forced contacts between mitochondria and ER induce mitochondrial calcium accumulation, mitochondrial ROS accumulation and p53-dependent senescence, in line with our previous results deciphering that ITPR2 regulates senescence in a ROS-dependent manner (Borodkina et al., 2016; Ma et al., 2018; Martin and Bernard, 2018; Wiel et al., 2014). As an association had been reported between mitochondria, ROS, NF- $\kappa$ B

transcription factors and SASP (Birch and Passos, 2017; Nelson et al., 2018), we decided to investigate this relationship. We demonstrated the involvement of the RelA NF- $\kappa$ B transcription factor in regulating some SASP factors during linker-induced senescence, and observed a decrease in NF- $\kappa$ B activity in old *Itp2* KO liver and MEFs according to GSEA analysis (Supplementary Fig. 6). MERCs also constitute signaling platforms involved in pro-inflammatory responses, notably via both formation and regulation of the inflammasome NLRP3 (Zhou et al., 2011; Missiroli et al., 2018b). In addition, NLRP3 has been associated with the production of SASP components (Acosta et al., 2013). Interestingly, *Itp2* KO liver and MEFs display a dampened inflammatory response whereas inducing MERCs enhances the inflammatory response, as evidenced by IL8 and CCL3 expression. This inflammatory response can promote paracrine senescence and mediate part of the pro-aging effects of cellular senescence (Baker et al., 2016; Childs et al., 2017). We can thus speculate that ITPR2, through higher amounts of functional and closer MERCs, could contribute to cellular senescence and aging by two complementary and synergistic mechanisms, namely i) the increased mitochondrial calcium level and the subsequent ROS production which can induce a p53-dependent cell cycle arrest and a p53- and NF- $\kappa$ B-dependent SASP, as suggested by our results, and ii) by other mechanisms, for instance inflammasome activation which also triggers pro-inflammatory SASP production known to mediate autocrine senescence. Beyond MERCs and calcium transfer from the ER to the mitochondria, cytosolic calcium and its signaling could also contribute to the senescence phenotype (Martin and Bernard, 2018), in line with the decrease in NFAT signaling observed in *Itp2* KO liver and MEFs according to GSEA analysis (Supplementary Fig. 7).

Overall our study demonstrates that ITPR2 calcium channel and ER-mitochondria contacts promote cellular senescence and physiological aging. How organelles integrate and

communicate signals during cellular senescence is unknown. Our study unveils this new exciting field of investigation as it paves the way to future studies investigating the roles of organelles contacts in controlling pathways and actors of cellular senescence and aging.

## Methods

**Cell culture and reagents.** MRC5 normal embryonic human fibroblasts (ATCC, Manassas, VA, USA), kidney 293T or 293 GP cells (Clontech, Mountain View, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, USA) with GlutaMax and supplemented with 10% FBS (Sigma-Aldrich, Saint-Louis, USA) and 1% penicillin/streptomycin (ThermoFisher Scientific). Mouse Embryonic Fibroblasts were prepared with embryos at E12.5 and cultured in Dulbecco's modified Eagle's medium with GlutaMax, 10% FBS, 1% penicillin/streptomycin and 1% of Gibco™ MEM Non-Essential Amino Acids Solution (ThermoFisher Scientific). N-Acetyl-Cysteine (NAC) (A9165, Sigma-Aldrich) has been used directly after infection at a final concentration of 1 mM, and renewed every two days.

**Vectors, transfection and infection.** Linker and control sequences were described in (Csordás et al., 2006) and cloned in the lentiviral vector pLV[Exp]-Hygro-CMV by Vectorbuilder. 293T or 293GP virus producing cells were transfected using the GeneJuice reagent according to the manufacturer's recommendations (Merck Millipore). Two days after transfection, the viral supernatant was harvested, combined with fresh medium (1/20 for 293T cells 1/2 for 293GP cells) and hexadimethrine bromide (8 µg/mL; Sigma-Aldrich), and used to infect targeted cells. One day later, infected cells were selected with Hygromycin B (ThermoFisher Scientific) at 15 µg/mL or Neomycin (ThermoFisher Scientific) at 75 µg/mL for 6 days, split and seeded back for 2 or 6 days.

**siRNA transfection.** MRC5 cells were infected (Ctrl/Linker), hygromycin-selected for 6 days. They were then seeded and transfected with small interference (si) RNA (against RelA or p53 ((Dharmacon)) previously incubated for 20 min with Dharmafect (Dharmacon) 0.6% in antibiotics and serum-free DMEM with Glutamax (Final siRNA quantity in the well: 15nmol). The day after transfection, medium was changed with DMEM with Glutamax (10% FBS and 1 % antibiotics).

**Calcium imaging.** For specific ER and mitochondrial ratiometric genetic reporter, MEFs or MRC5 were infected with pLNCX2 G-CEPIA1-ER (Suzuki et al., 2014) or pLNCX2-mito-GEM-GECO1 (Zhao et al., 2011) and neomycin-selected for 10 days. For MRC5, cells were then infected by Linker, hygromycin-selected for 6 days, and seeded in Lab-Tek Chambered Coverglass (ThermoFisher Scientific). Two days after, cells were washed with HBSS, without  $Ca^{2+}$ ,  $Mg^{2+}$  and phenol red, and observed at 37°C and 5%  $CO_2$  under a Zeiss LSM 780 confocal microscope. Excitation wavelength was monitored at 408nm and 565nm. For calcium reporters, detection of fluorescence was monitored for F(470-500nm) (Calcium-bound reporter) and F(>520nm) (Calcium-free reporter). Ratio  $F(470-500nm) / F(>520nm)$  was calculated. For Linker experiments involving Linker, detection of FRP was monitored for F(580 +/- 30nm). In order to proceed to single-cell analyses, LSM files were then converted to Columbus software. For experiments involving Linker, only RFP+ positive cells were analysed. Measurement of fluorescence intensity at single-cell level was evaluated with Columbus software.

**RNA extraction, reverse transcription, and real-time quantitative PCR.** RNA was extracted with phenol-chloroform using Upzol (Dutscher, Brumath, France). Synthesis of cDNA was performed using Maxima First cDNA Synthesis Kit (ThermoFisher Scientific) from

1 µg of total RNA. Generated cDNA (50 ng/µL) was used as a template for quantitative PCR (qPCR) run, and mixed with primers (200 nM), SYBR™ Green PCR Master Mix (ThermoFisher Scientific) or TaqMan mix (Roche) and Universal Probe Library probes (100 µM) (ThermoFisher Scientific) for the gene of interest. Reactions were performed in triplicate. qPCR analyses were carried out with the FX96 Thermocycler (Biorad, Hercules, USA). Relative mRNA levels were calculated using the Comparative Ct ( $\Delta\Delta CT$ ) method. mRNA levels of 2 (*Gapdh/Actb*) housekeeping genes were used for normalization. Primers sequences and housekeeping genes used are listed in Supplementary Table S1.

**Senescence associated- $\beta$ -Galactosidase analysis, Crystal violet, EdU Assay, mitochondrial JC1, and mitochondrial ROS quantification.** For SA- $\beta$ -Galactosidase assay, cells were washed with PBS 1X, fixed for 5 min in 2% formaldehyde / 0.2% glutaraldehyde, rinsed twice in PBS 1X, and incubated at 37°C overnight in SA- $\beta$ -Galactosidase staining solutions as previously described (Debacq-Chainiaux et al., 2009). For crystal violet assay, cells were washed with PBS 1X, fixed for 15 min in 3.7% formaldehyde and stained with crystal violet. For EdU Assay, Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit was used according to manufacturer's recommendations (ThermoFisher Scientific). For JC1, JC1-Mitochondrial Membrane Potential Assay Kit (ab113850, Abcam) was used. JC1 monomers and aggregates were both excited at 488 nm. Detection of fluorescence for JC1 monomers and aggregates were performed respectively at 530nm and 590nm. Ratio F(aggregate)/F(monomer) was subsequently evaluated. Cell Meter™ Mitochondrial Hydroxyl Radical Detection Kit (ATT Bioquest) allowed detection of mitochondrial ROS according to manufacturer's recommendations. Excitation was monitored at 488nm, and fluorescence emission was measured at 530 +/- 30 nm.

**Animals.** C57Bl/6 *Itp2* KO mice were described in and genotyped as explained in (Li et al., 2005). WT and KO littermates were used for different experiments. Mice were maintained in laminar-flow boxes under standard conditions (standard diet and water *ad libitum*) in the specific pathogen-free (SPF) animal facility Anican platform at the Cancer Research Center of Lyon. Experiments were conducted according to animal care guidelines of European Union and French laws. Protocols were authorized by the local Animal Ethic Evaluation Committee (CECCAPP:C2EA-15) and by the French Ministry of Education and Research (APAFIS#734-2015052915081986).

**Proximity ligation assay (PLA) and electron microscopy for MERC analysis.** For PLA, cells were washed with PBS 1X, and fixed for 10 min in 10% Formaldehyde. 1:1 volume of Glycine 1 M was added to the fixation solution and cells were washed with Glycine 100 mM for 15 min. Cells were permeabilized with 0.1% Triton 1X and next incubated overnight at 4 °C with primary antibodies. Primary antibodies and dilutions used are listed in Supplementary Table S2. Cells were washed with PBS-Tween 0.3% and incubated with PLA probes. Ligation and polymerization steps were performed according to manufacturer's recommendations (Sigma Aldrich). Acquisition of at least 30 fields per condition was performed using Operetta CLS High-Content Analysis System (PerkinElmer).

For transmission electron microscopy, 1:1 volume of glutaraldehyde 4% was added to the culture medium and cells were incubated 15 min at 4 °C. Glutaraldehyde/medium was discarded and 1:1 volume of glutaraldehyde 4 % / cacodylate 0.2 M pH 7.4 was added. Cells were then fixed in glutaraldehyde 2%, washed three times for 1 hr at 4°C, post-fixed with 2% OsO<sub>4</sub> 1 hr at 4°C, and dehydrated with an increasing ethanol gradient. Impregnation was

performed with Epon A (50%) plus Epon B (50%) plus DMP30 (1.7%). Inclusion was obtained by polymerisation at 60°C for 72 hr. Ultrathin sections (approximately 70 nm thick) were cut on a UCT (Leica) ultramicrotome, mounted on 200 mesh copper grids and contrasted with uranyl acetate and lead citrate. Acquisition of 4 to 10 fields per cell and 10-20 cells per condition was performed with a Jeol 1400JEM (Tokyo, Japan) transmission electron microscope equipped with an Orius 600 camera and Digital Micrograph at CIQLE platform (UCBL-Lyon). MERCs were determined as distance below 50 nm between ER and OMM membranes. Quantification of MERCs number per mitochondria, length of MERCs and mean/minimal distances <50nm between ER and OMM membranes per MERC, was determined using a macro Fiji software kindly provided by G. Hajnoczky (Weaver et al., 2017). Using this macro, the number of mitochondria per cell was calculated and the perimeter of each mitochondrion forming a MERC contact and associated with ER membrane in TEM micrographs was assessed.

**Immunoblot and Immunohistochemistry.** For immunoblot experiments, cells were lysed in RIPA buffer. After protein quantification, 30 µg of proteins were loaded and resolved by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with TBS Tween / Milk 5% for 1 hr and incubated at 4°C with primary antibodies overnight. Primary antibodies and dilutions used are listed in Supplementary Table S2. Membranes were then incubated with secondary antibody for 1 hr at room temperature. Detection was performed using ECL kit (Amersham).

For immunohistochemistry, organs were collected and snap-frozen in liquid nitrogen for RNA and protein extraction, or fixed in 10% formalin for 24 hr and then ethanol 70%, before processing and paraffin embedding. Paraffin-embedded murine tissues were serially sectioned at 3-mm thickness. After deparaffinization and rehydration, the slides were incubated in 3%



hydrogen peroxide in distilled water to block endogenous peroxidases. For heat-induced antigen retrieval, tissue sections were boiled in 10 mmol/L citrate buffer pH 6.0 in a microwave oven for 15 min. The slides were incubated for 30 min with low-background" antibody diluent (DAKO Real) and overnight at 4°C with the primary antibody (listed in Supplementary Table S2) diluted in the low-background antibody diluent (DAKO Real). After rinsing in PBS, the slides were incubated with a biotinylated secondary antibody bound to a streptavidin peroxidase conjugate (Dako E0468) for 1 hr at room temperature. Slides were treated with Streptavidin HRP (Vector) and then bound antibody was revealed with the DAB peroxidase substrate kit (Vector). Sections were counterstained with hematoxylin and the slides were finally dehydrated and mounted. At least 1,000 cells taken from 5 independent fields were quantified.

**Transcriptomic analysis.** Transcriptome analysis of liver tissue or MEFs derived from WT or *Itp2* KO mice were performed using Whole Mouse Genome Microarrays 4x44K v2 (Agilent Technologies) and one-color gene expression Agilent workflow. Total RNA were extracted with NucleoSpin® RNA according to the manufacturer's recommendations (Macherey-Nagel). cRNAs were synthesized and labelled with Cy3 dye starting from 100 ng of total RNA using one-color Low Input Quick Amp Labeling Kit (Agilent Technologies). After quality control validation, 1650 ng of Cy3-labeled cRNAs purified with RNeasy Mini-spin columns (Qiagen) were hybridized on the 4x44K arrays for 17 hr at 65°C. Microarrays were washed and scanned with an Agilent DNA microarray scanner G2565CA (Agilent Technologies). Fluorescence signals were extracted and normalized with Feature Extraction Software Version 10.5.1.1 (Agilent Technologies) and transferred to Genespring GX 12.6 software (Agilent Technologies) for data processing and data mining. Expression data were normalized in Genespring using 75th percentile method. Microarray probes were filtered using Agilent flag filter to remove probes

with raw signal below 20 in all the conditions tested. Transcriptomic analysis on livers was performed from 4 independent female mice for each genotype and differentially expressed genes between *Itpr2* KO versus WT with fold change cutoffs  $>$  or  $<$  2 were selected. Transcriptomic analysis on MEFs was performed from 3 independent mice for each genotype and differentially expressed genes between *Itpr2* KO and WT were selected using moderated t-test p value  $<$  0.01 with a Benjamini–Hochberg correction and fold change cutoffs  $>$  or  $<$  2. For data visualization, hierarchical clustering was performed with the Euclidian metric and complete linkage method. The Gene Ontology (GO) tool from GeneSpring software allowed determination of significant statistical enrichment of biological processes based on computation p-values described by standard hypergeometric distribution. Gene Set Enrichment Analysis (GSEA) were performed using the GSEA v2.0.13 software using default parameters. All gene set files for this analysis were obtained from GSEA website ([www.broadinstitute.org/gsea/](http://www.broadinstitute.org/gsea/)). Datasets are available in GEO GSE139982 for MEF analyses and GSE139967 for liver analyses.

**Blood analysis and phenotyping.** Before sacrifice, intracardiac harvesting of blood was performed and measurement of blood Aspartate Aminotransferase (AST) level was evaluated using the Activated Aspartate Aminotransferase assay on the ARCHITECT c 16000 Systems™.

After sacrifice spleens from WT or *Itpr2* KO mice were collected aseptically and single-cell suspensions were prepared in DMEM medium (Invitrogen) containing 2 mM glutamine, 100 mg/ml gentamicin, and 6% FCS. Splenocytes were stained for 30 min at 4°C with the appropriate mixture of mAbs diluted in staining buffer (PBS supplemented with 1% FCS [Life Technologies] and 0.09% NaN<sub>3</sub> [Sigma-Aldrich, Saint Quentin-Fallavier, France]). The following Abs (clones) were used: CD4 (RM4.5), CD8 (53-6.7), CD44 (IM7.8) (all BD

PharMingen). CD4<sup>+</sup>/CD44<sup>high</sup> and CD4<sup>+</sup>/CD44<sup>low</sup> were considered, respectively, as memory and naïve CD4 T cells. CD8<sup>+</sup>/CD44<sup>high</sup> and CD8<sup>+</sup>/CD44<sup>low</sup> were considered respectively as effector/memory and naïve CD8 T cells. All analyses were performed on a Becton Dickinson FACS Fortessa LSRII and analyzed with FlowJo software (TreeStar, Asland, OR, USA).

**Measurements of bone mineral density and bone mineral content.** After sacrifice, femurs were harvested and stored in EtOH 70%. Bone mineral content and bone mineral density were determined by dual energy X ray absorptiometry (DXA) using a Lunar PIXImus densitometer (Wipro, GE Healthcare).

**Liver analyses.** For Sirius Red staining, livers were collected and fixed in 10% formalin for 24 hr and then ethanol 70%, before processing and paraffin embedding. Paraffin-embedded murine tissues were serially sectioned at 3-4 µm thickness, de-waxed and hydrated. Nuclei were stained with Weigert's haematoxylin for 8 min, and then the slides were washed for 10 min in running tap water. Picro-sirius red (10%) (Cat#365548 and Cat# P6744-1GA, Sigma Aldrich) was used for staining for 1 hr. Slides were washed in two baths of acidified water and Dehydrated in three baths of 100 % ethanol. Slides were cleared in xylene and mounted. Acquisition of at least 5 fields per mice has been performed. Quantification of stained area was performed using ImageJ software according to its website's recommendations.

For red oil staining, livers were collected, snap-frozen, frozen-sectioned at 8 µm thicknesses. Sections were fixed in formalin, washed with tap water and rinsed with 6 % isopropanol. Slides were stained with freshly prepared Oil Red O (0.5% of CI 26125 (Sigma-Aldrich) in 60% isopropanol) working solution 15 min, and immediately rinsed with 60%

isopropanol. Nuclei were stained with alum hematoxylin 5 dips before mounting. Acquisition of at least 5 fields per mice has been performed and red lipid droplets were counted.

**Intraperitoneal glucose tolerance test (IPGTT).** C57Bl/6 *Itp2* WT and KO male mice at the indicated ages were starved overnight. Blood tail glycemia was first measured at T0 using Abbott Freestyle Papillon Vision glucometer according to device manufacturer's recommendations (Abbott). Intraperitoneal glucose injection (2.5 mg/g) was performed and blood tail glycaemia was then measured as indicated.

**Statistical analysis.** Demographic data were graphed and processed using Statistica software to compute mean and maximum lifespans, and p values (log-rank test) for each cohort. Values represent mean  $\pm$  SD or SEM as indicated in the figure legend. Statistical analyses for groups were performed as indicated in the figure legend. Depending on size of sampling, D'agostini & Pearson or Shapiro-Wilk normality tests, were used before proceeding to any analyses. Parametric tests were two tailed, unpaired or paired: Student's t test (equal variance) or Welch's t-test (for non-equal variance). Mann-Whitney U Test was performed for non-parametric tests. For multiple comparisons (>3), one-way ANOVA was performed and subsequent Tukey Test, without Geisser-Greenhouse's correction. Spearman test was used for correlations analysis. All the statistical analyses were performed using GraphPad Prism 7 (\* P < 0.05; \*\* P < 0.01; \*\*\* P < 0.001).

## Data Availability

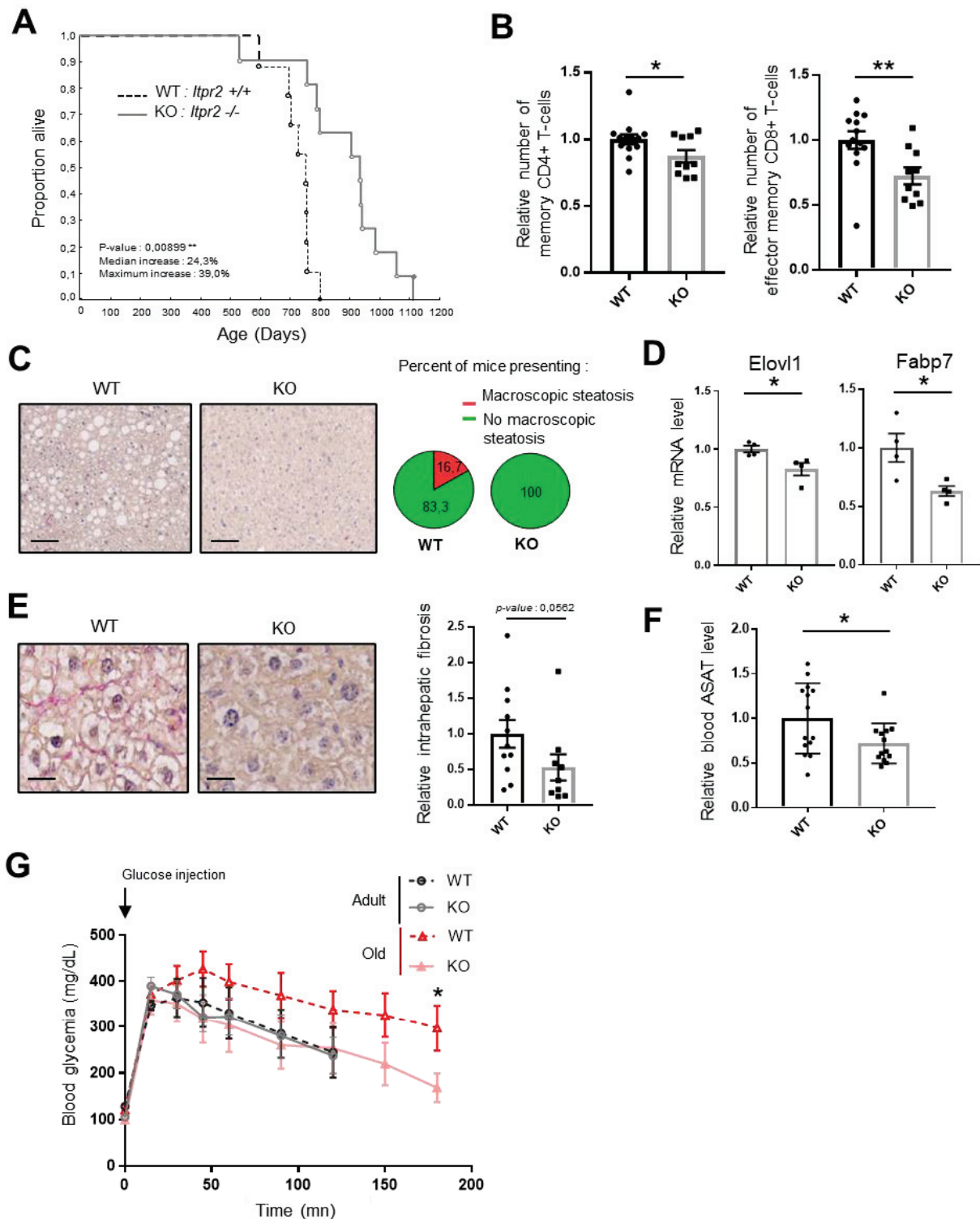
Datasets are available in GEO GSE139982 for MEF analyses and GSE139967 for liver analyses. The source data underlying the Figures 1(A,B,C,D,E,F,G), 2(B,C,D,E,F,G,H,I), 3(A,B,C,D,E,F), 4(B,C,D,E,G,H,I,J,K,M,N) and Supplementary Figures 1(A,B,C,F,G,H,I), 2(B,F,G,H,I), 3(A,B,C,D,E,F,G,I,J), 4(B,C,D,F,G,I), 5 are provided as a Source Data file.

## Author contributions

D.V., D.G., D.V.Z., G.C. and A.G. managed mice cohort, follow up, tissue processing and MEFs preparation. D.V.Z., S.J. and D.G. performed tissue staining and analysis. D.V.Z., G.C. perform *in vitro* senescence assays. C.W. analyzed structures. S.D. and J.M. perform immunophenotyping. F.V. and N.P. provided materials. L.P. analyzed blood samples. S.A. processed some murine tissues and performed some analysis. J.M.F and D.V.Z. performed transcriptomic experiments and analysis. N.B., J.R. and D.V.Z. perform proximity ligation experiments. D.V.Z. performed the other experiments. D.B., N.M. and D.V.Z designed the study and interpreted the overall results. D.B. and N.M. supervised the work. D.V.Z and D.B. wrote the manuscript with input from all authors.

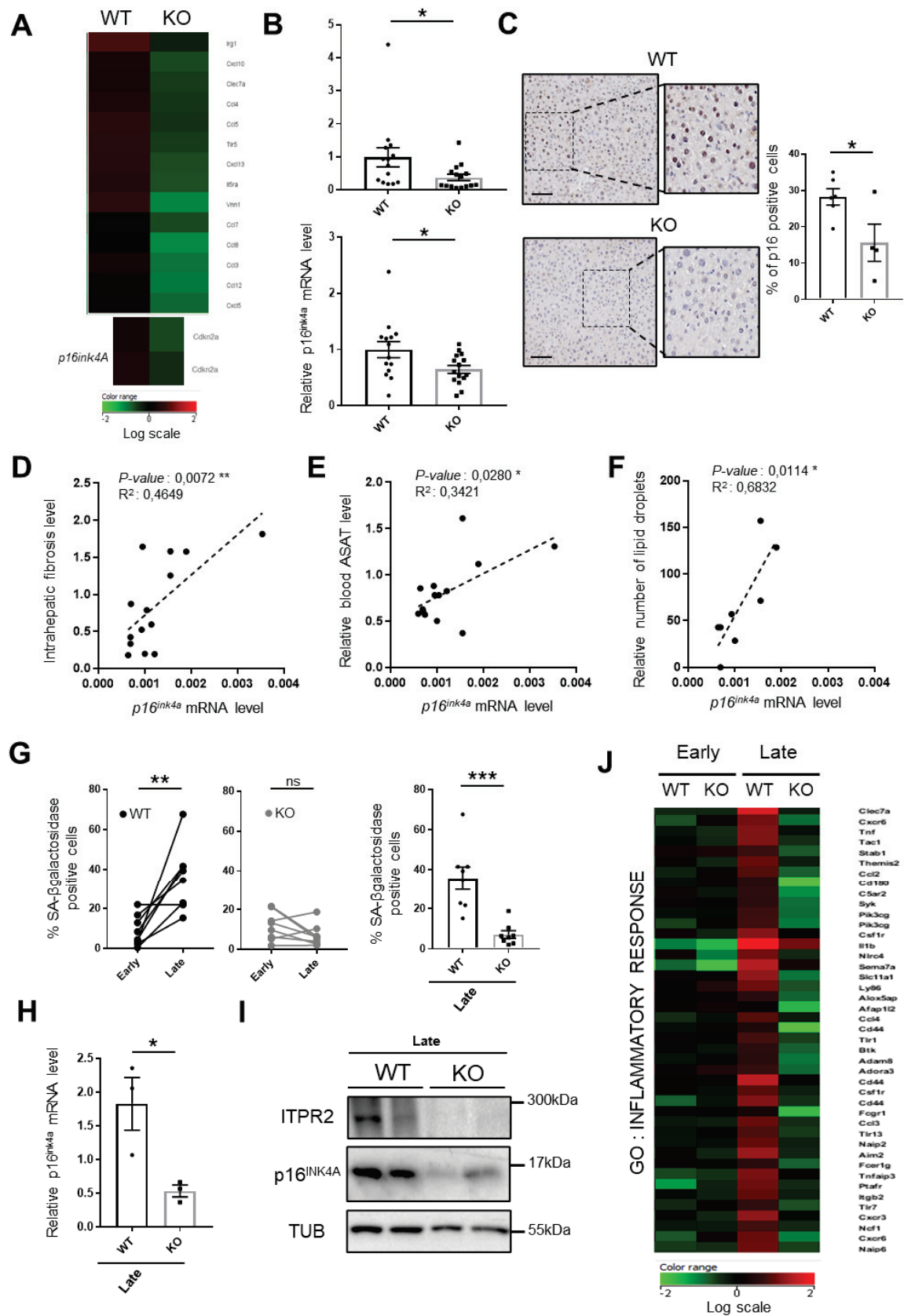
## Acknowledgements

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**Figure 1. *Itp2* knockout increases lifespan and limits age-related phenotypes in mice.** (A) Survival curves of *Itp2* WT (n=9) or KO (n=11) C57BL/6 female mice. Log-rank test. (B) Immunophenotyping of the spleens of 23-month-old mice displaying relative number of memory (CD44<sup>high</sup>) CD4<sup>+</sup> and CD8<sup>+</sup> T-cells in *Itp2* WT (n=13) and KO (n=10) female and male mice. Mean  $\pm$  SEM. Unpaired Student t-test. (C) Micrographs of liver sections stained with Sirius Red of 23-month-old male and female *Itp2* WT (n=12) and KO (n=10) mice. Scale bar: 25  $\mu$ m. Quantification of the percentage of mice presenting macroscopic steatosis. (D) mRNA level extracted from microarray data in the livers of 23-month-old WT (n=4) and KO

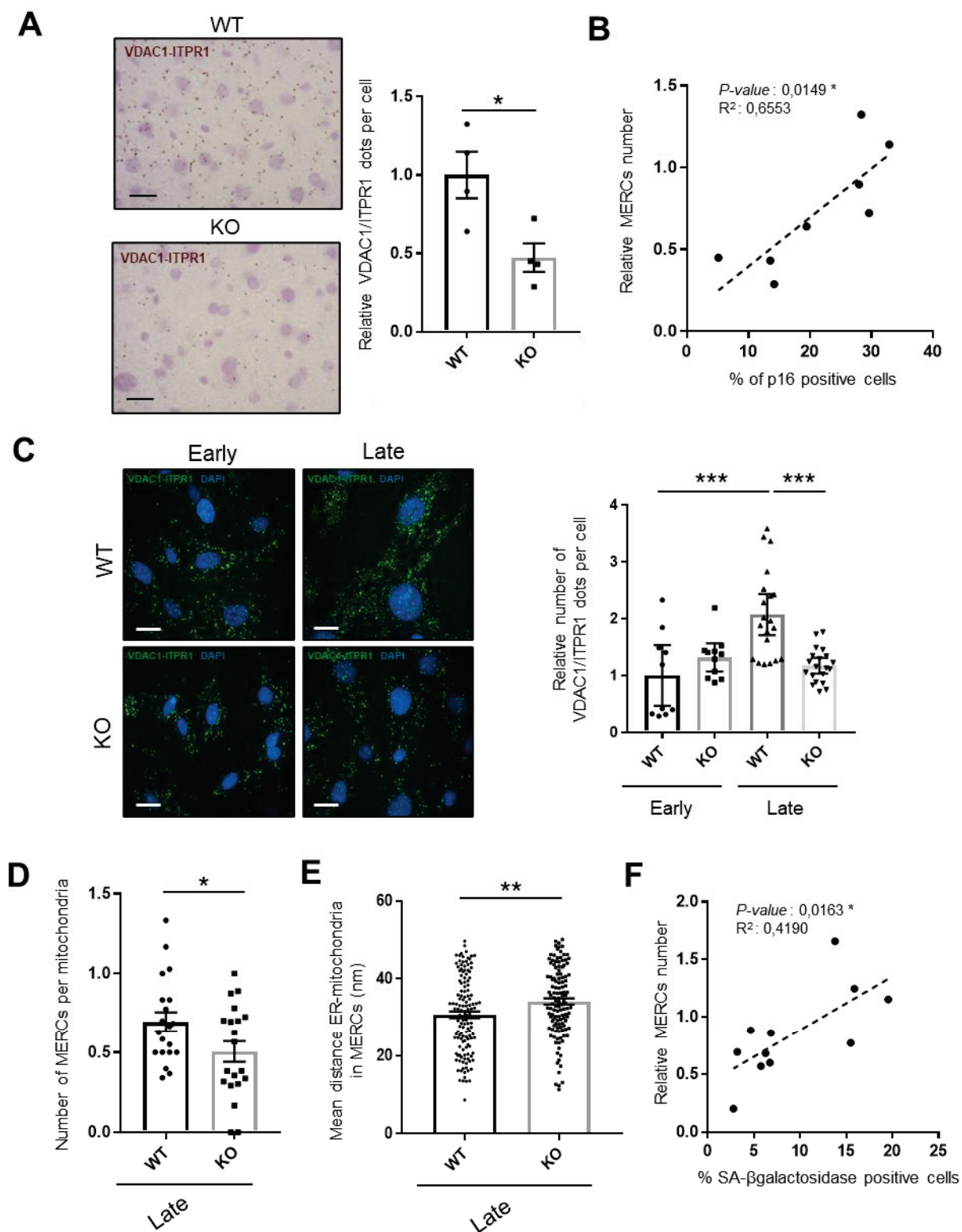
(n=4) female mice. Mean +/- SEM. Unpaired Student t-test. (E) Micrographs of liver sections stained with Sirius Red. Scale bar: 10  $\mu$ m. Quantification of relative intrahepatic collagen fibers according to Sirius Red staining in 23-month-old male and female *Itpr2* WT (n=12) and KO (n=10) mice. Mean +/- SEM. Mann-Whitney U Test. (F) Quantification of relative blood AST level of 23-month-old *Itpr2* WT (n=14) and KO (n=13) female and male mice. Mean +/- SEM. Unpaired Student t-test. (G) Intraperitoneal glucose-tolerance test of adult (20-month-old) and old (26-month-old) *Itpr2* WT (n=6) and KO (n=8) male mice. Mean +/- SEM. Unpaired Welch's t-test.



**Figure 2. Loss of *Itpr2* in mice reduces key features of cellular senescence.** (A) Inflammatory Response Gene Ontology obtained from the genes downregulated in the livers of 23-month-old *Itpr2* WT (n=4) and KO (n=4) female mice, according to transcriptomic

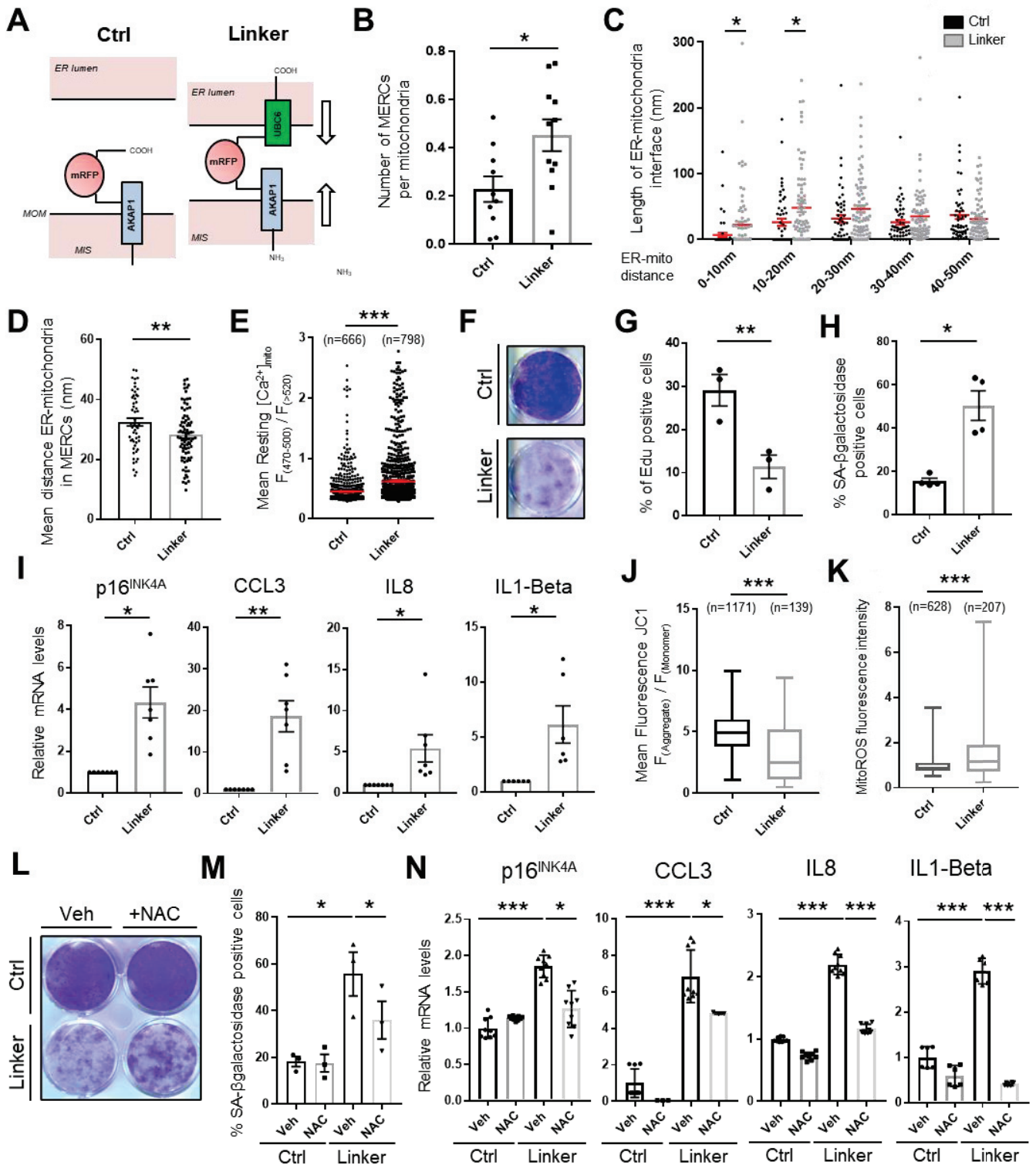


microarray analyses, corrected p-value=0.019. (B) Relative *ccl3* and *p16<sup>ink4a</sup>* mRNA levels in liver of 23-month-old *Itpr2* WT (n=14) and KO (n=15) female and male mice. Mean +/- SEM. Unpaired Student t-test. (C) Micrographs of liver sections and quantification of p16-positive cells, stained by immunohistochemistry (IHC) in the livers of 23-month-old mice *Itpr2* WT (n=4) and KO (n=4) female mice. Mean +/- SEM. Unpaired Student t-test. (D-E) Linear regression analyses between intrahepatic fibrosis level or blood AST level and *p16<sup>ink4a</sup>* mRNA levels in the livers of 23-month-old female and male mice (n=14). Spearman test. (F) Linear regression analyses between number of lipid droplets and *p16<sup>ink4a</sup>* mRNA levels in liver of 23-month-old female mice (n=7). Spearman test. (G) Quantification of SA- $\beta$ -galactosidase-positive cells in *Itpr2* WT (n=8) and KO (n=7) MEFs at early and late passage. Unpaired Student t-test. Mean +/- SEM. (H) *p16<sup>ink4a</sup>* mRNA level in WT (n=3) and KO (n=3) MEFs at late passage. Mean +/- SEM. Unpaired Welch's t-test. (I) *p16<sup>ink4a</sup>*, *Itpr2* and tubulin protein levels in *Itpr2* WT and KO MEFs at late passage. (J) Inflammatory Response Gene Ontology extracted from microarray of *Itpr2* WT (n=3) and KO (n=3) MEFs at early and late passage, corrected p-value=2,292<sup>-12</sup>.



**Figure 3. Loss of *Itp2* diminishes contacts between mitochondria and the ER.** (A) Proximity ligation assay (PLA) using VDAC1 (outer mitochondrial membrane) and ITPR1 (ER membrane) antibodies in the livers of 23-month-old *Itp2* WT (n=4) and KO (n=4) female mice. Dots are formed when distance between ITPR1 and VDAC1 is below 50 nm. Representative

pictures (Scale bar: 5  $\mu\text{m}$ ) and quantification shown as mean  $\pm$  SEM. Unpaired Student t-test. (B) Linear regression analysis between the number of MERCs and the percentage of p16-positive cells in the liver of 23-month-old *Itp2* WT (n=4) and KO (n=4) female mice. Spearman test. (C) PLA using Vdac1 and Itpr1 antibodies in early and late passage *Itp2* WT (n=4) and KO (n=4) MEFs. Representative pictures (scale bar: 5  $\mu\text{m}$ ) and quantification indicating mean  $\pm$  SEM. One-way ANOVA. Multiple comparisons test. (D) Quantification of the number of MERCs normalized to mitochondria number in late passage *Itp2* WT and KO MEFs. MERCs are defined by a distance  $< 50$  nm between ER and OMM membranes, evaluated by transmission electron microscopy. Mean  $\pm$  SEM of at least 20 cells. Unpaired Student t-test. (E) Mean ER-mitochondria distance in MERCs in late passage *Itp2* WT and KO MEFs, based on electron microscopy study. Mean  $\pm$  SEM of a least 20 cells. Mann-Whitney U Test. (F) Linear regression analysis between number of MERCs and percentage of SA- $\beta$ -galactosidase positive cells at different passages of *Itp2* WT (n=4) and KO (n=4) MEFs. Spearman test.



**Figure 4. Inducing MERCs promotes premature senescence.** (A) Graphical representation of the artificial genetic linker generated to induce MERCs formation. MOM=Mitochondrial Outer Membrane; MIS=Mitochondrial Intermembrane Space. (B) MRC5 cells were infected by a control (Ctrl) or linker (Linker) encoding lentiviral vector and hygromycin selected. Using transmission electron microscopy, the number of MERCs normalized against the number of mitochondria was calculated. MERCs are defined by a distance < 50 nm between ER and OMM membranes, evaluated by transmission electron microscopy. Mean +/- SEM of 10-12 cells. Unpaired Student t-test. (C) Measurement of mitochondrial membrane length associated with

ER, depending on the distance (0-50 nm) between mitochondria and ER in Ctrl/Linker-infected MRC5 cells. Mean +/- SEM of 100 fields per condition. Multiple t-tests. (D) Quantification of mean ER-mitochondria distance in Ctrl/Linker-infected MRC5 cells. Mean +/- SEM of 100 fields per condition. Mann-Whitney U Test. (E) MRC5 cells were infected with mito-GEM-GECO1 mitochondrial calcium reporter encoding retroviral vector, neomycin selected, and infected with Ctrl or Linker encoded lentiviral vector and next hygromycin selected. Single-cell analysis was performed on RFP-positive cells. n represents the number of analyzed cells. Mean +/- SEM. Mann-Whitney U Test. (F) Crystal violet staining for Ctrl/Linker-infected and selected MRC5 cells 12 days after infection. (G) Quantification of EdU-positive cells for Ctrl/Linker-infected MRC5 cells. Mean +/- SEM (n=3 independent experiments). Unpaired Student t-test. (H) Quantification of SA- $\beta$ -galactosidase-positive cells in Ctrl/Linker-infected MRC5 cells. Mean +/- SEM (n=4 independent experiments). Unpaired Welch's t-test. (I) RT-qPCR representing relative p16<sup>INK4A</sup>, CCL3, IL8 and IL1-Beta mRNA levels in Ctrl/Linker-infected and selected MRC5 cells. Mean +/- SEM (n=5 independent experiments). Unpaired Welch's t-test. (J) Single-cell analysis on RFP-positive Ctrl/Linker infected and selected cells, of mitochondrial membrane depolarisation using JC1 probe. Ratio between  $F_{\text{aggregate}}/F_{\text{monomer}}$  was determined, n representing the number of analyzed cells. Regular whisker plot (Median, Min and Max). Mann-Whitney U Test. (K) Single-cell analysis of RFP-positive Ctrl/Linker infected and selected cells of mitochondrial ROS fluorescence. n represents the number of analyzed cells. Regular whisker plot (Median, Min and Max). Mann-Whitney U Test. (L) Crystal violet staining for Ctrl/Linker-infected MRC5 cells treated with vehicle (Veh) or NAC (1 mM) 12 days after infection. (M) Quantification of SA- $\beta$ -galactosidase-positive MRC5 cells in Ctrl/Linker-infected and selected cells, treated with vehicle (Veh) or NAC (1 mM) (n=3 independent experiments). Mean +/- SEM. One-way ANOVA. Multiple paired t-tests. (N) RT-qPCR representing relative RelA, p16<sup>INK4A</sup>, CCL3, IL8 and IL1-Beta levels in Ctrl/Linker-infected and selected MRC5 cells treated with vehicle (Veh) or NAC (1 mM). Mean +/- SD. One-way ANOVA. Multiple paired t-tests.

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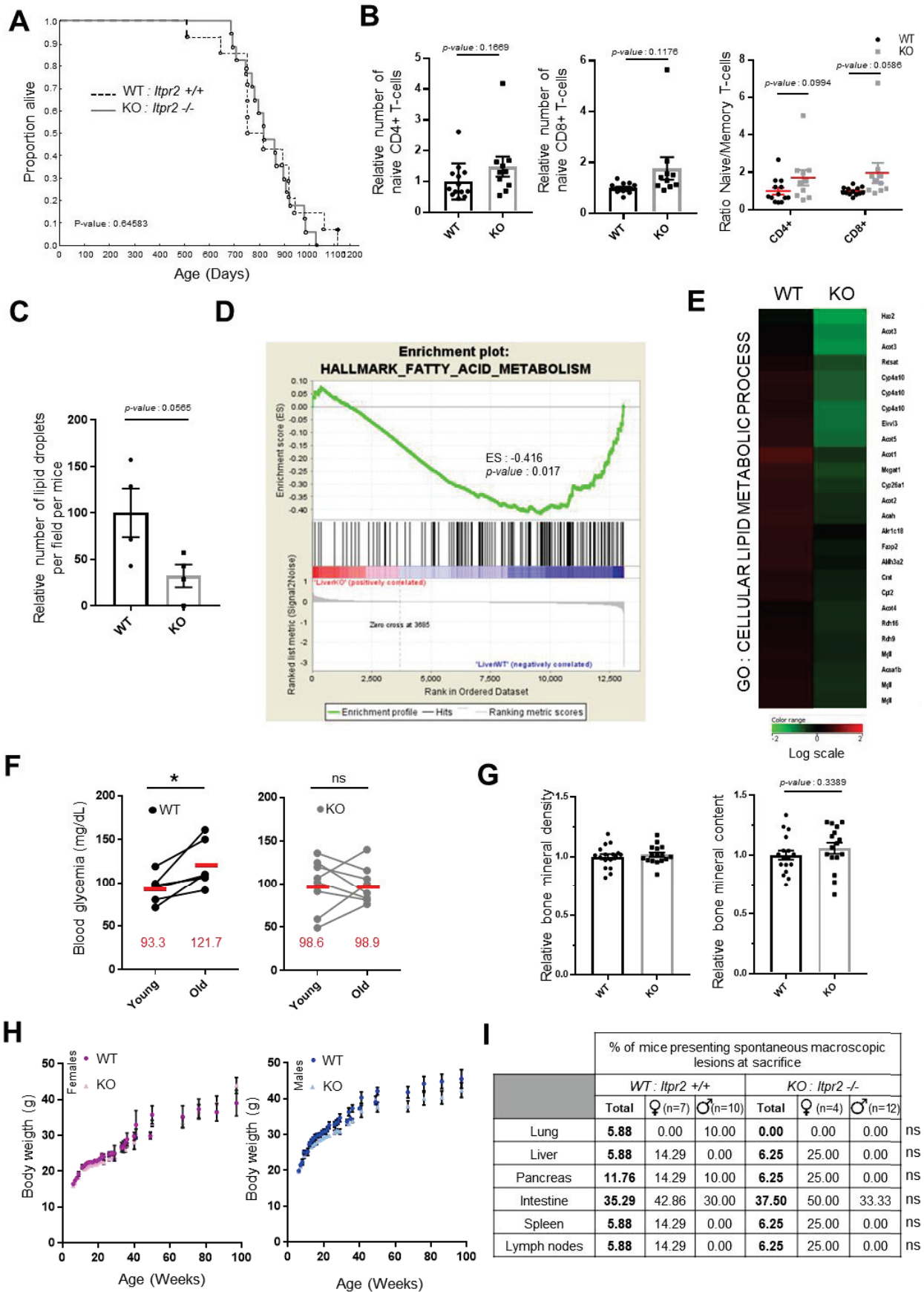


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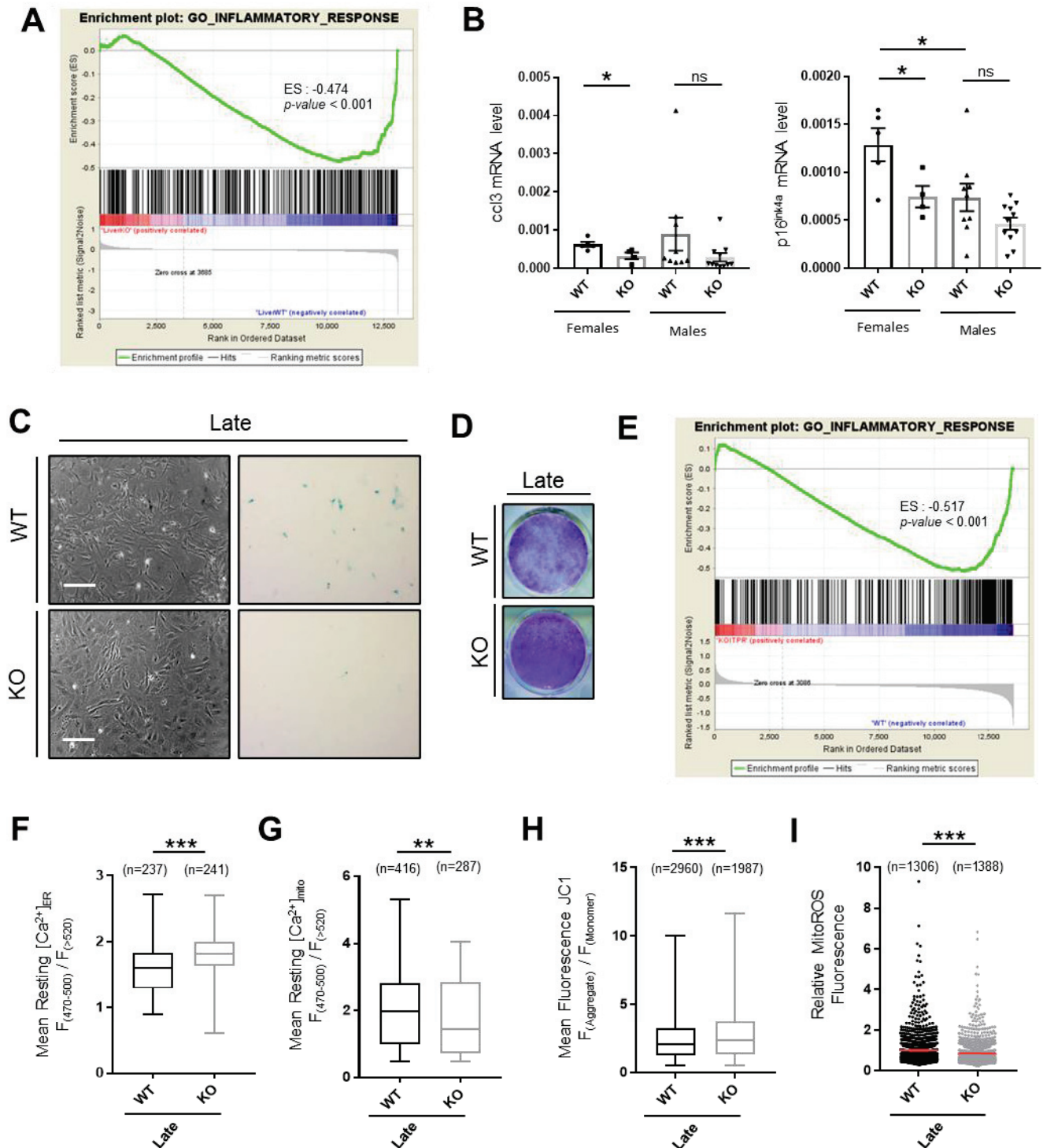
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**Supplemental Materials**



**Supplemental Figure 1.** (A) Survival curves of male *ltp2* WT (n=14) or KO (n=17) C57BL/6 mice. Log-rank test. (B) Immunophenotyping of the spleens of 23-month-old mice displaying

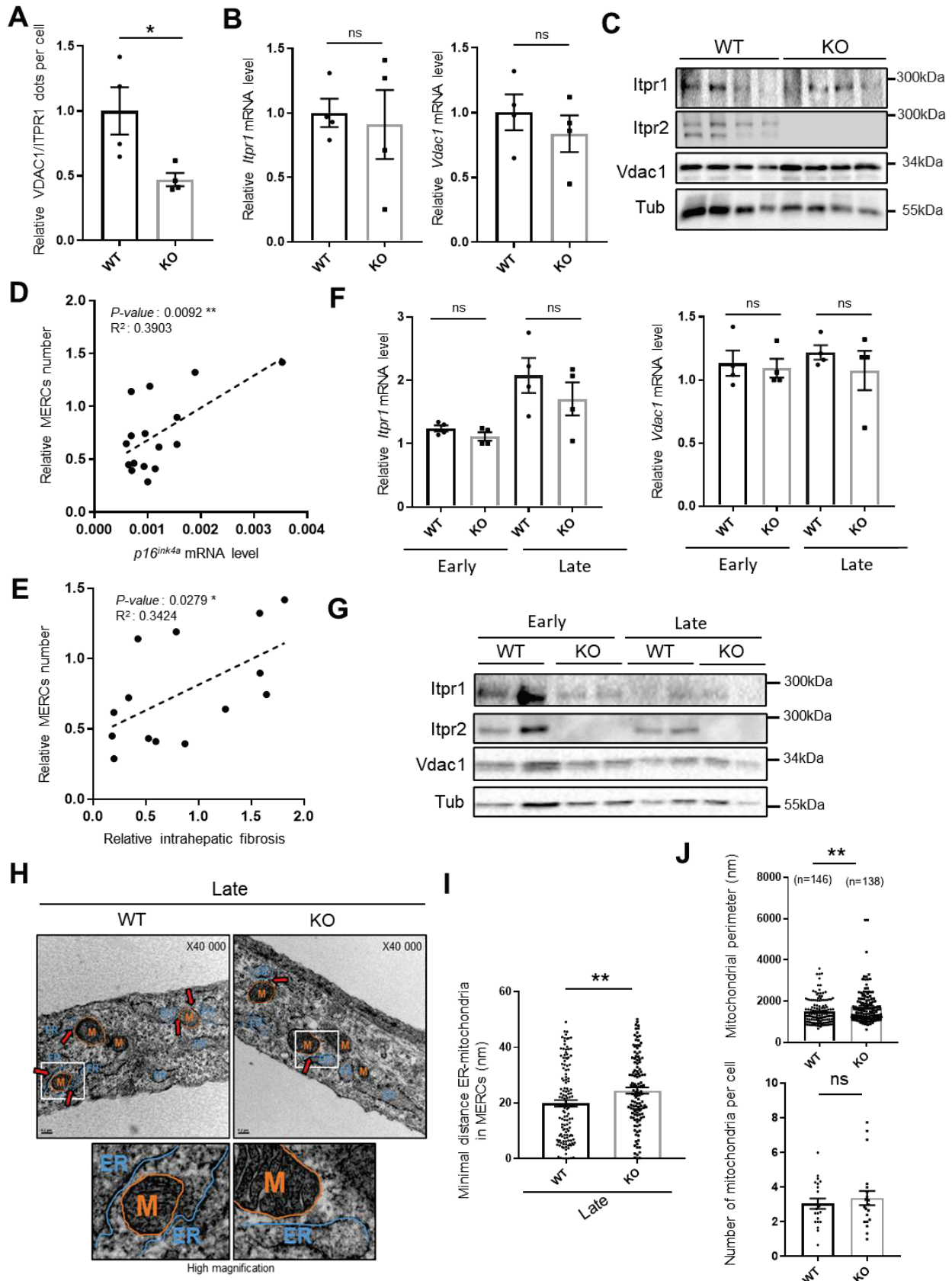
relative number of naïve (CD44<sup>low</sup>) CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and the ratio of naïve/memory T-cells in *Itp2* WT (n=13) and KO (n=10) in male and female mice. Mean +/- SEM. Unpaired t-test. (C) Quantification of the relative number of lipid droplets in liver slices stained with Red Oil of *Itp2* WT (n=4) and KO (n=4) female mice. Mean +/- SEM. Unpaired t-test. (D) GSEA showing decreased enrichment in gene set related to fatty acid metabolism of 23-month old females *Itp2* KO (n=4) compared to WT (n=4) female mice, according to transcriptomic analyses. (E) Cellular Lipid Metabolic Process Gene Ontology (obtained from the genes downregulated in the livers of 23-month-old females *Itp2* WT (n=4) and KO (n=4) female mice, according to transcriptomic analyses, corrected p-value: 0.0234. (F) Basal blood glycaemia of young (14-month-old) and old (26-month-old) *Itp2* WT (n=6) and KO (n=8) male mice. Paired t-test. (G) Quantification of bone mineral density and content of 23-month old *Itp2* WT (n=17) and KO (n=16) male and female mice. Mean +/- SEM. Unpaired t-test. (H) Body weight monitoring during aging of female *Itp2* WT (n=9) and KO (n=11) mice and male *Itp2* WT (n=14) and KO (n=17) mice. Mean +/- SEM. Unpaired t-test. (I) Quantification of macroscopic tumoral lesions in various organs of *Itp2* WT (n=17, 7 females and 10 males) and KO (n=16, 4 females and 12 males). Fisher's exact test.



**Supplemental Figure 2.** (A) GSEA for Inflammatory Response GO in liver of old *Itpr2* KO (n=4) versus WT (n=4) female mice, according to transcriptomic analysis. (B) *Ccl3* and *p16<sup>ink4a</sup>* mRNA levels in liver of *Itpr2* WT (n=14) and KO (n=15) female and male mice. Mean  $\pm$  SEM. Unpaired t-test. (C) Micrographs of SA- $\beta$ -galactosidase assay in late passage *Itpr2* WT and KO MEFs, representative of n=3 biological replicates. Scale bar: 20  $\mu$ m. (D) Crystal violet staining for late passage MEFs *Itpr2* WT and KO 5 days after seeding, representative of n=3 independent experiments. (E) GSEA for Inflammatory Response GO in late passaged MEFs *Itpr2* WT (n=3) versus KO (n=3), according to transcriptomic analyses. (F-G) MEFs were infected with pLNCX2 G-CEPIA1-ER or pLNCX2-mito-GEM-GECO1 retroviral vector and selected. Single-cell analysis of mitochondrial and ER calcium of late passage *Itpr2* WT and KO MEFs, n indicating the number of analyzed cells. Regular whisker plot (Median, Min and

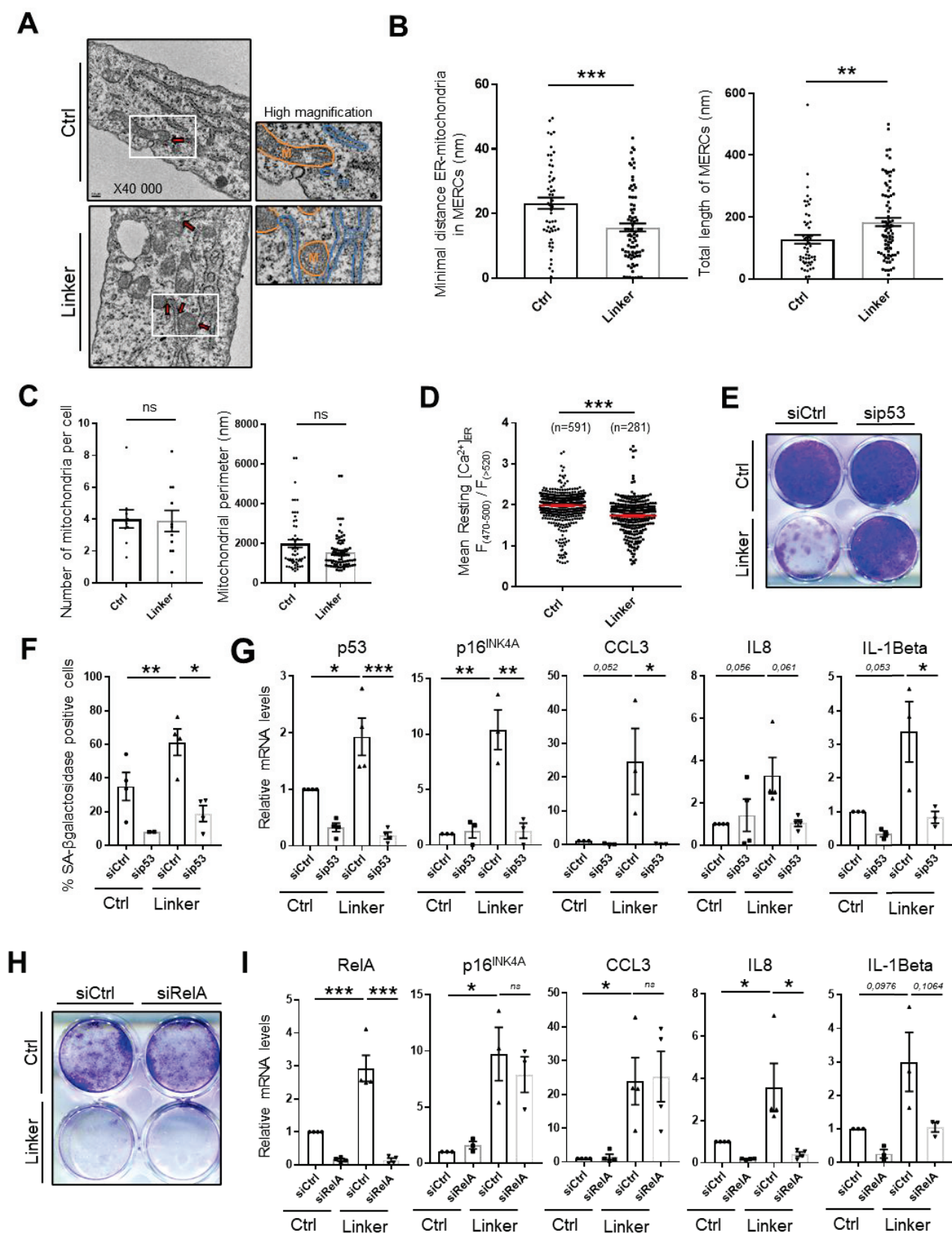
Max). Mann-Whitney U Test. (H) Single-cell analysis of mitochondrial membrane depolarization using JC1 probe, n representing the number of analyzed cells. Mean +/- SEM. Mann-Whitney U Test. (I) Single-cell analysis of mitochondrial ROS fluorescence in late passage *Itp2* WT and KO MEFs, n representing the number of analyzed cells. Regular whisker plot (Median, Min and Max). Mann-Whitney U Test.





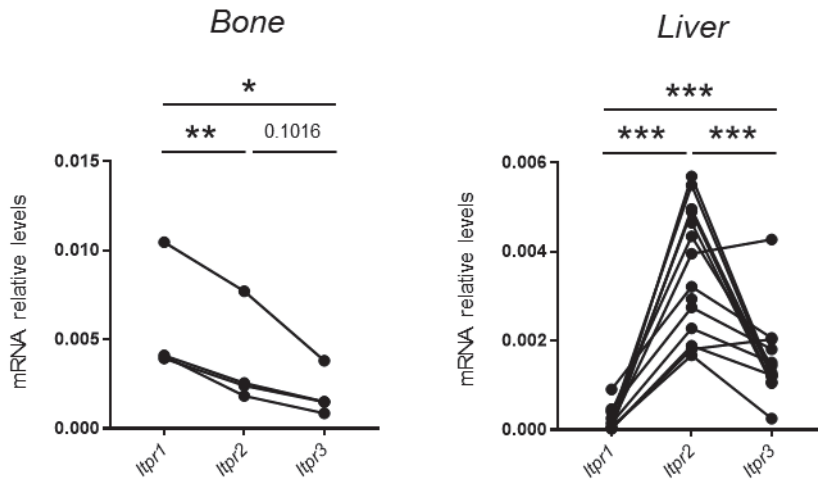
**Supplemental Figure 3.** (A) Quantification of PLA using VDAC1 and ITPR1 antibodies in the livers of 23-month-old *Itp2* WT (n=4) and KO (n=4) male mice. Mean +/- SEM. Unpaired t-test. (B) *Itp1* and *Vdac1* mRNA levels in livers of 23-month-old *Itp2* WT (n=4) and KO (n=4) female mice. Mean +/- SEM. Unpaired t-test. (C) *Itp1*, *Itp2*, *Vdac1* and tubulin protein levels in livers of 23-month-old *Itp2* WT and KO female mice. (D-E) Linear regression

analyses between the number of MERCs and p16<sup>ink4a</sup> mRNA levels in the livers or relative intrahepatic fibrosis in 23-month-old *Itpr2* WT (n=8) and KO (n=8) male and female mice. Spearman test. (F) *Itpr1* and *Vdac1* mRNA levels in early/late passaged *Itpr2* WT (n=4) and KO (n=4) MEFs. Mean +/- SEM. Unpaired t-test. (G) *Itpr1*, *Itpr2*, *Vdac1* and tubulin protein levels in early/late passage *Itpr2* WT and KO MEFs. (H) Representative electron micrographs of late passage *Itpr2* WT and KO MEFs. Magnification X 40 000. ER and mitochondria are artificially labelled respectively in blue and orange. (I) Minimal distance in MERCs of late passage *Itpr2* WT and KO MEFs. Mean +/- SEM of 120-140 fields, accounting for 20 cells (J) Number of mitochondria and perimeters of each mitochondrion associated to a MERC in late passage *Itpr2* WT and KO MEFs. Mean +/- SEM of 120-140 fields, accounting for 20 cells.



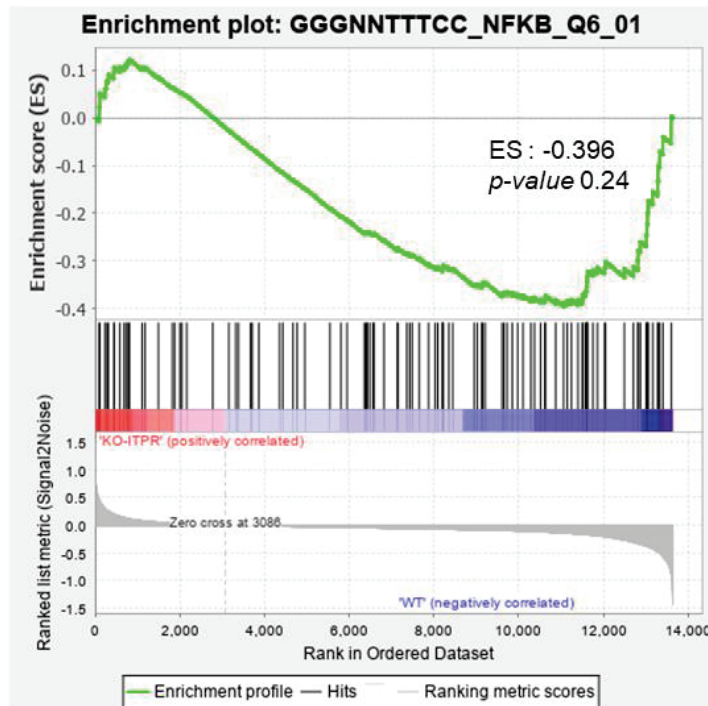
**Supplemental Figure 4.** (A) Representative electron micrographs of Ctrl and Linker-infected MRC5 cells. ER and mitochondria are artificially labelled respectively in blue and orange. Arrows indicate ER-mitochondria contacts. (B) Minimum ER-mitochondria distance in MERCs and total length of MERCs in Ctrl and Linker-infected MRC5 cells. Mean  $\pm$  SEM. Mann-Whitney U Test. (C) Number of mitochondria per cell and perimeters of each mitochondrion associated to a MERC in Ctrl- and Linker-infected MRC5 cells. Mean  $\pm$  SEM.

Mann-Whitney U Test. (D) MRC5 cells were infected with pLNCX2 G-CEPIA1-ER calcium reporter encoding retroviral vector, neomycin selected, infected with Ctrl-or Linker-encoding lentiviral vector and next hygromycin selected. Single-cell analysis of ER calcium on RFP positive cells was performed, n in the graph indicating the number of analyzed cells. (E) Ctrl or Linker-infected and selected MRC5 cells were transfected with control siRNA or an siRNA directed against p53. Crystal violet staining was performed 12 days after siRNA transfection and seeding of the same number of cells. (F) Quantification of SA- $\beta$ -galactosidase-positive cells in Ctrl/Linker-infected MRC5 cells, transfected with siCtrl or sip53. Mean  $\pm$  SEM (n=3 independent experiments). Paired t-tests. (G) RT-qPCR representing relative p53, p16<sup>INK4A</sup>, CCL3, IL8 and IL1-Beta levels in Ctrl/Linker-infected and selected MRC5 cells transfected with siCtrl or sip53. Mean  $\pm$  SEM (n=3 to 4 independent experiments). Paired t-test. (H) Crystal violet staining of Ctrl or Linker-infected and selected MRC5 cells, transfected with siCtrl or siRelA. Staining was performed 8 days after siRNA transfection. (I) RT-qPCR representing relative RelA, p16<sup>INK4A</sup>, CCL3, IL8 and IL-1Beta levels in Ctrl or Linker-infected and selected MRC5 cells next transfected with siCtrl or siRelA. Mean  $\pm$  SEM (n=3 to 4 independent experiments). Paired t-test.

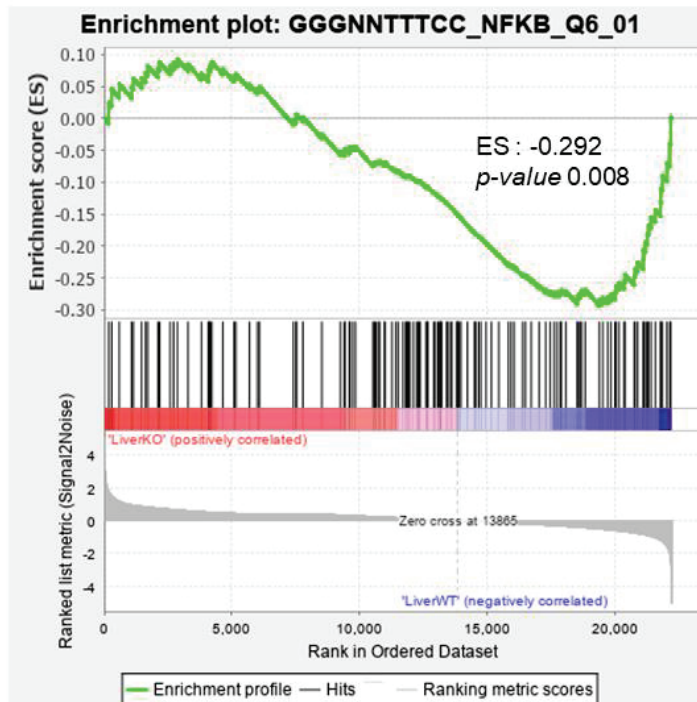


**Supplemental Figure 5.** *Itpr1*, *Itpr2* and *Itpr3* mRNA levels in bone and liver of *Itpr2* WT female and male mice. Paired t-test.

## MEF

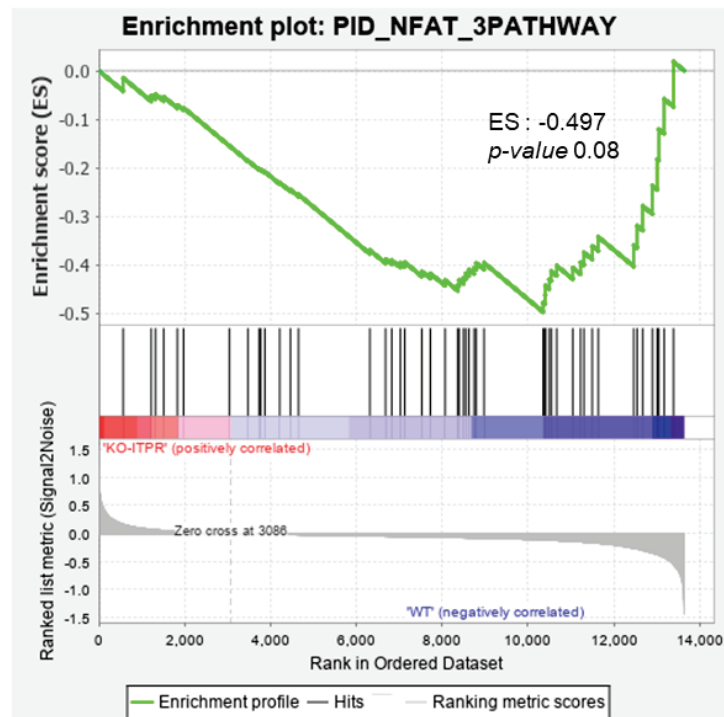


## Liver

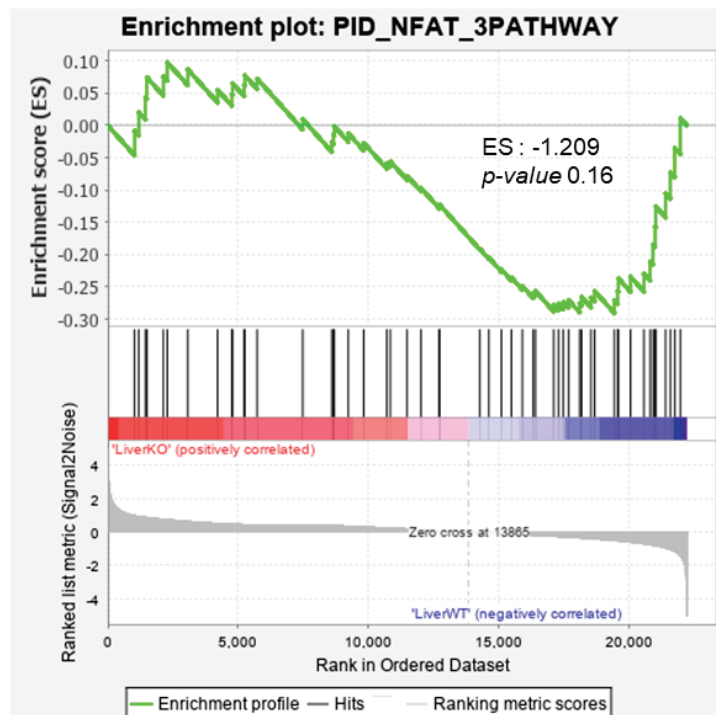


**Supplemental Figure 6.** GSEA showing decreased enrichment in gene set related to NF- $\kappa$ B pathway in both late passage MEFs *Itpr2* KO (n=3), compared to WT (n=3) (upper panel), and liver of *Itpr2* KO (n=4) compared to

## MEF



## Liver



**Supplemental Figure 7.** GSEA showing decreased enrichment in gene set related to NFAT pathway in both late passage MEFs *Itp2* KO (n=3), compared to WT (n=3) (upper panel), and liver of *Itp2* KO (n=4) compared to WT (n=4) female mice (lower panel), according to transcriptomic analyses.

## Supplemental Table 1

Gene	Sequence and UPL probes	
<b>Mouse - UPL</b>		
	Sequence	UPL
Gapdh-Forward	CTAAGGCCAACCGTAAAAAG	#80
Gapdh-Reverse	ACCAGAGGCATACAGGGACA	
ccl3-Forward	TGCCCTTGCTGTTCTCTCT	#40
ccl3-Reverse	GTGGAATCTCCGGCTGTAG	
Itpr1-Forward	ACCCTGAGGAAAGTTCTGC	#85
Itpr1-Reverse	GAAGGCATCTTTGGAGGAA	
Itpr2-Forward	TGGTGATGGGAGACCAAACCTG	#16
Itpr2-Reverse	GAAGATGTGCCGATGGT	
Itpr3-Forward	TGGTGATGGGAGACCAAACCTG	#80
Itpr3-Reverse	TCTAAGCGTACGTTCAAGATGA	
Vdac1-Forward	ACCTTTGATTCGTCTCTCG	#48
Vdac1-Reverse	TGCTCCCTCTGTACCCCTGT	
<b>Mouse - SYBR</b>		
Actb-Forward	GCCTCCTTCTGGGTATGG	
Actb-Reverse	AGGTCCTTACGGATGTCAACG	
p16 <sup>ink4a</sup> -Forward	GTGTGCATGACGTGCGGG	
p16 <sup>ink4a</sup> -Reverse	GCAGTTCGAATCTGCACCGTAG	
<b>Human - UPL</b>		
	Sequence	UPL
ACTB-Forward	ATTGGCAATGAGCGGTTC	#11
ACTB-Reverse	GGATGCCACAGGACTCCAT	
CCL3-Forward	CAGAATCATGCAGGTCTCCAC	#56
CCL3-Reverse	GCGTGTGACGAGCAAGTG	
IL8-Forward	AGACAGCAGAGCACACAAGC	#72
IL8-Reverse	ATGGTTCCTCCGGTGGT	
IL1-Beta-Forward	TACCTGTCCTGCGTGTGAA	#78
IL-1Beta-Reverse	TCTTTGGTAATTTTGGGATCT	
RELA-Forward	CTGGCTGGGGACAGAAG	#39
RELA-Reverse	TCATGAAGAAGAGTCTTTCAAGC	
TP53-Forward	AGGCCTTGGAACTCAAGGAT	#12
TP53- Reverse	CCCTTTTGGACTTCAGGTG	
<b>Human - SYBR</b>		
ACTB-Forward	CATGTACGTTGCTATCCAGGC	
ACTB-Reverse	CTCCTTAATGTCACGCAAGAT	
p16 <sup>INK4A</sup> -Forward	CGGTGCGAGGCCGATCCAG	
p16 <sup>INK4A</sup> -Reverse	GCGCCGTGGAGCAGCAGCAGT	

## Supplemental Table 2

Protein	Reference	Use	Dilution
p16 <sup>ink4a</sup>	sc-1207	IHC	1/1000
p16 <sup>ink4a</sup>	sc-468 (batch K0608)	WB	1/500
ITPR2	sc-398434 (Santa Cruz)	WB	1/500
Tub	T6199 (Sigma-Aldrich)	WB	1/5000
ITPR1	sc-28614 (Santa Cruz)	PLA	1/1000
	sc-271197 (Santa Cruz)	WB	1/500
VDAC1	ab-14734 (Abcam)	PLA	1/1000
		WB	1/500

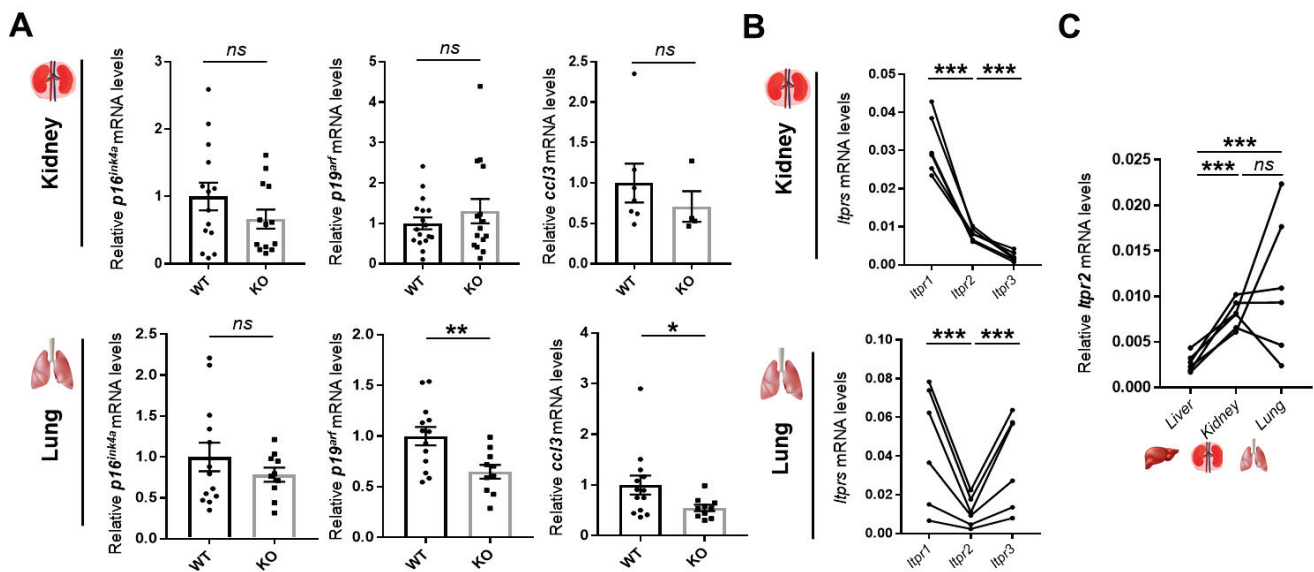


### 1.3. Additional results

#### The reduction of senescence markers is not restricted to liver in *Itpr2* $-/-$ mice

*Itpr2* loss reduces senescence markers and inflammation in liver and is also associated with reduced hepatic age-related alterations, such as steatosis, fibrosis and sensitivity to metabolic stress. Nevertheless, these improved liver functions are probably not the only reason of the enhanced lifespan we observed in *Itpr2* KO mice.

In addition to liver, we monitored the mRNA levels of three senescence markers, namely *p16<sup>ink4a</sup>*, *p19<sup>arf</sup>* and *ccl3* in kidney and lung of 23-month-old *Itpr2* KO mice and their control littermates. *Itpr2* deletion did not affect mRNA levels of *p16<sup>ink4a</sup>*, *p19<sup>arf</sup>* and *ccl3* in kidney (Figure 24.A). Nevertheless, *Itpr2* loss reduced significantly *p19<sup>arf</sup>* and *ccl3* mRNA levels in lung (Figure 24.A). Intriguingly, *Itpr2* was not the predominant form in terms of mRNA levels neither in kidney nor in lung (Figure 24.B). Nevertheless, *Itpr2* mRNA levels were higher in lung compared to liver, where previous phenotypes were observed (Figure 24.C).



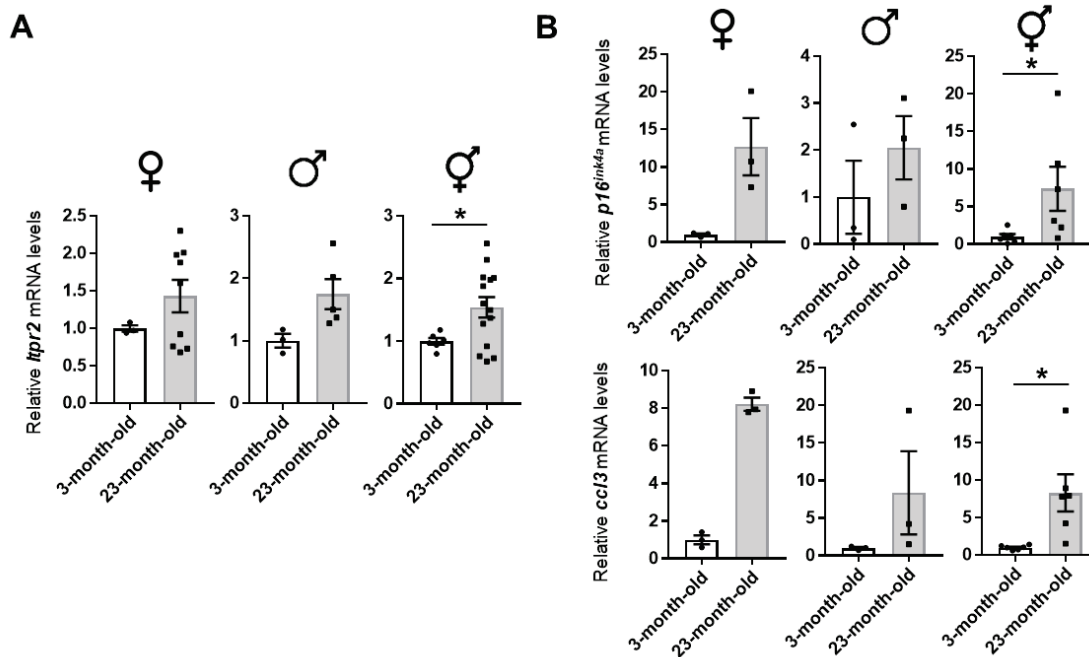
**Figure 24: *Itpr2* loss also reduces senescence markers in lung.** **A.** Relative mRNA levels of three senescence markers *p16<sup>ink4a</sup>*, *p19<sup>arf</sup>* and *ccl3* in kidney (upper panel) or lung (lower panel) of 23-month-old *Itpr2*  $+/+$  (WT) and *Itpr2*  $-/-$  (KO) mice. In kidney, data represent mean  $\pm$  SEM of  $n=17$  WT and  $n=14$  KO (*p16<sup>ink4a</sup>*, *p19<sup>arf</sup>*) and  $n=6$  WT and  $n=4$  KO (*ccl3*). In lung, data represent mean  $\pm$  SEM of  $n=13$  WT and  $n=10$  KO (*p16<sup>ink4a</sup>*, *p19<sup>arf</sup>*, *ccl3*). Unpaired normal T-test. **B.** mRNA levels of different ITPRs isoforms in kidney (upper panel) and lung (lower panel) ( $n=6$  WT). Paired t-test. **C.** Relative *Itpr2* mRNA levels in liver, kidney and lung ( $n=6$  WT). Paired t-test.

Taken together, these results suggest that *Itpr2* loss does not alleviate only senescence markers in liver, but also in lung, with reduction of *p19<sup>arf</sup>* and *ccl3* mRNA levels. Whether reduction of senescence marker is linked to the alleviation of age-related and senescence-associated lung defects,

such as fibrosis or emphysema (Schafer et al., 2017a; Triana-Martínez et al., 2019), needs to be investigated.

### **ITPR2 expression during aging**

Senescent cells accumulate during aging (Herbig et al., 2004; Jeyapalan et al., 2007; Tuttle et al., 2020; Yousefzadeh et al., 2020a), and this observation raises the question of a modulation of *Itpr2* gene expression and ITPR2 activity with age. Because liver is on one hand well studied in context of senescence and on the other hand an ITPR2-rich organ, and based on our results in *Itpr2* KO mice, we assessed mRNA levels of *Itpr2* in the liver of WT 3-month-old mice and 23-month-old mice. Interestingly, *Itpr2* mRNA levels were slightly increased during aging in liver (**Figure 25.A**), concomitantly with two markers of cellular senescence, namely CDKi  $p16^{\text{ink4a}}$  and SASP factor *ccl3* (**Figure 25.B**).



**Figure 25: *Itpr2* mRNA levels increase during liver aging.** **A.** Relative mRNA levels of *Itpr2* liver of females, males and both sexes for 3-month-old or 23-month-old WT mice. Data represent mean +/- SEM. n=3x3-month-old females / n=3x3-month-old males / n=5x23-month-old females / n=9x23-month-old males. Unpaired t-test. **B.** Relative mRNA levels of  $p16^{\text{ink4a}}$  and *ccl3* in liver of females, males and both sexes for 3-month-old or 23-month-old WT mice. n=3x3-month-old females / n=3x3-month-old males / n=3x23-month-old females / n=3x23-month-old males. Unpaired t-test.

Altogether, these results suggest an age-dependent regulation of transcription of *Itpr2* gene. As ITPR2 is also modulated at post-transcriptional level, ITPR2 protein level and stability could be further assessed to complete the study of ITPR2 expression during aging.



## **- CHAPTER II -**

### **2. - Roles of MVA pathway in the regulation of cellular senescence –**

#### **2.1. Summary of the study**

Senescing cells display a distinct metabolism from normal proliferating cells (Gorgoulis et al., 2019). These global metabolic rearrangements are not univocally expressed but account for a growing number of studies (Gorgoulis et al., 2019). Among these rearrangements, lipid metabolism is globally modified (Cadenas et al., 2012; Flor et al., 2017; Inoue et al., 2017; Maeda et al., 2009; Ogrodnik et al., 2017; Song et al., 2017; Venable et al., 1995). Observed in hepatocytes (Inoue et al., 2017; Ogrodnik et al., 2017) and also in fibroblasts (Flor et al., 2016), senescent cells accumulate lipid droplets. This accumulation of intracellular lipids includes free fatty acids (Song et al., 2017) or cholesterol (Cadenas et al., 2012; Maeda et al., 2009), and is associated with altered phospholipid synthesis or fatty acid processing (Cadenas et al., 2012; Gey and Seeger, 2013). Whether these senescence-associated alterations are functionally involved in regulation cellular senescence remains barely understood. Indeed, while early clues indicated that exogenous lipids like ceramide may induce senescence response (Venable et al., 1995), the particular role of endogenous bio-synthesized lipids in cellular senescence is currently unknown.

MVA pathway is part of lipid anabolism and involved in the endogenous biosynthesis of poly-isoprenoids, such as prenyl groups, ubiquinone, cholesterol or dolichol, being determinant for many cellular vital processes including respectively protein-protein interactions, mitochondrial respiration, membranes fluidity or glycosylation (Goldstein and Brown, 1990). Though MVA pathway is necessary for the survival of cancer cells (Mullen et al., 2016), its role in regulating cellular senescence in normal cells is still elusive. A previous screen approach identified 33 pro-senescent kinases promoting cellular senescence (Ferrand et al., 2015). Among these kinases, two kinases of the MVA pathway, the mevalonate kinase (MVK) and the phosphomevalonate kinase (PMVK), were found to be able to independently drive a senescence response (Ferrand et al., 2015). In this study, we explored how these kinases and more importantly whether the MVA pathway and the biosynthesis of poly-isoprenoids can regulate cellular senescence.

We observed that enhancing MVA pathway drives cellular senescence through the subbranch of cholesterol biosynthesis. In normal fibroblasts, the accumulation of cholesterol promotes an ERR $\alpha$ -

regulated mitochondrial biogenesis and subsequent ROS production, ultimately triggering a p53-dependent senescence. Taken together, these findings unravel MVA pathway and cholesterol accumulation as new pro-senescent signals that mediate a nuclear-encoded mitochondrial program. Overall, this work suggests the potential role of cellular senescence in regulating cholesterol-linked diseases.

2.2. Article: “Mevalonate pathway governs cellular senescence *via* a cholesterol / ER $\alpha$  / mitochondrial program”

**Mevalonate pathway governs cellular senescence via a  
cholesterol/ ERRA /mitochondrial program**

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The authors have declared that no conflict of interest exists

## **Abstract**

Cellular senescence is an intrinsic cell program induced by various stresses that leads to a stable proliferation arrest and to a senescence-associated secretory phenotype. Accumulation of senescent cells during aging participates in a myriad of age-related pathologies and regulates lifespan. Changes in the metabolism of senescent cells have been depicted and could participate to this phenotype. Regulation of catabolism in senescent cells has been mostly deciphered while regulation of anabolism is poorly understood. In this work, we describe the mevalonate pathway, an anabolic pathway leading to the endogenous biosynthesis of poly-isoprenoids, such as cholesterol, as a positive regulator of cellular senescence. Mechanistically, this mevalonate-induced senescence is accompanied by an intracellular cholesterol accumulation. Accumulation of cholesterol, which can be a ligand of  $ERR\alpha$  nuclear receptor, promotes  $ERR\alpha$  transcriptional activity and its subsequent mitochondrial program leading to ROS production, DNA damage and finally a p53-dependent senescence. Overall, this work unravels a new role of the mevalonate pathway and of cholesterol accumulation in regulating senescence through a nuclear-encoded mitochondrial program. Finally, it emphasizes the potential role of cellular senescence in regulating cholesterol-associated diseases.

## **Keywords**

Cellular senescence, mevalonate pathway, cholesterol,  $ERR\alpha$ , mitochondria

## Introduction

Cellular senescence is an intrinsic program promoted by a myriad of stresses (Gorgoulis et al., 2019). This program is canonically characterized by a permanent cell cycle arrest and a senescence associated secretory phenotype (SASP) (Gorgoulis et al., 2019), both implicated in the physio-pathological effects of senescent cells (He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014b). Senescence-associated physio-pathological contexts include development, tissue regeneration, cancer or aging (He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014b), mainly depending on clearance dynamics, time-life, and persistence of senescent cells. As a consequence of age, senescent cells accumulate within the organism (Herbig et al., 2006; Jeyapalan et al., 2007; Tuttle et al., 2020) and promote numerous age-related pathologies. Recently triggered a wide growing interest for findings drugs specifically inducing the death of these these “aged cells”, these drugs thus called senolytics (Childs et al., 2017). Mechanistically, while downstream factors, such as p21<sup>CIP1</sup> and p16<sup>INK4A</sup> or NF-κB and C/EBPβ, respectively blocking cell cycle progression or promoting SASP, were extensively studied (Gorgoulis et al., 2019; He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014b), upstream molecular and sub-cellular mechanisms controlling these factors are less understood.

Senescent cells harbour drastic metabolic changes concerning both catabolism and anabolism (Gorgoulis et al., 2019; Zwerschke et al., 2003; Dörr et al., 2013; James et al., 2015; Ziegler et al., 2015; James et al., 2016; Flor et al., 2017). In fact, senescent cells display major metabolic rearrangements as firstly evidenced by the interplay of main metabolic pathways including mTOR and AMPK in regulation of senescence fate (Herranz et al., 2015; Narita et al., 2011; Pan et al., 2020; Summer et al., 2019; Wiley et al., 2016; Zwerschke et al., 2003). Secondly, some catabolism aspects of senescent cells have been widely studied, displaying among others altered glycolytic state and glucose utilization (Dörr et al., 2013; James et al.,

2015; Gitenay et al., 2014), and a deregulated mitochondrial metabolism associated with an elevated AMP/ATP ratio and an altered NAD<sup>+</sup> metabolism (Zwerschke et al., 2003; Wiley et al., 2016; Jones et al., 2005; Wang et al., 2003; Nacarelli et al., 2019). On the contrary, only few studies highlighted the involvement of protein biogenesis or lipid anabolism in the regulation of senescence (Flor et al., 2017; Kim et al., 2010b; Lessard et al., 2018). Anabolism of senescent cells remains therefore puzzling.

Lipid metabolism is deeply modified in senescent cells (Cadenas et al., 2012; Flor et al., 2017; Inoue et al., 2017; Maeda et al., 2009; Ogrodnik et al., 2017; Song et al., 2017; Venable et al., 1995). In hepatocytes (Inoue et al., 2017; Ogrodnik et al., 2017) and fibroblasts (Flor et al., 2016), senescent cells particularly display an accumulation of lipid droplets. (Shimabukuro et al., 2016). This accumulation of intracellular lipids accounts for an increase of free fatty acids (Song et al., 2017) and free cholesterol (Cadenas et al., 2012; Maeda et al., 2009) and is seen as a consequence of reorganized metabolism of senescent cells. While early clues indicated that exogenous lipids like ceramide may induce senescence response (Venable et al., 1995), the particular role of endogenous bio-synthesized lipids in the regulation of senescence is currently unknown.

Mevalonate (MVA) pathway is part of lipid anabolism and involved in the endogenous biosynthesis of poly-isoprenoids, such as prenyl groups, ubiquinone, cholesterol or dolichol, being determinant for many cellular vital processes including respectively protein-protein interactions, mitochondrial respiration, membranes fluidity or glycosylation (Goldstein and Brown, 1990). While MVA pathway is necessary for the survival of cancer cells (Mullen et al., 2016), its role in regulating cellular senescence in normal cells is still elusive. A previous screen performed by our team identified 33 pro-senescent kinases promoting cellular senescence (Ferrand et al., 2015). Interestingly, these kinases included two kinases of the the MVA



pathway, namely the mevalonate kinase (MVK) and the phosphomevalonate kinase (PMVK) (Ferrand et al., 2015). In the present study, we explore how the mechanisms of action of these kinases in this context and more importantly whether the mevalonate pathway and the biosynthesis of poly-isoprenoids regulate cellular senescence. Unexpectedly, this work leads to the identification of a new mechanism regulating senescence: a cholesterol-dependent transcriptional program leading to mitochondrial metabolism dysregulation.

## Results

### Mevalonate pathway regulates cellular senescence

We previously identified two kinases belonging to the MVA pathway and acting as pro-senescent kinases : MVK and PMVK (Ferrand et al., 2015). In order to investigate the role and the relevance of the MVA pathway in regulating senescence, we either constitutively expressed or downregulated (through shRNA strategy) the central PMVK enzyme before to analyse premature senescence and replicative senescence in normal human fibroblasts (Fig. 1A). As expected, its constitutive expression (Supp. Fig. 1A) led to decreased cell proliferation and density (Fig. 1B-C) associated with the induction of key markers of cellular senescence, including senescence associated- $\beta$ -galactosidase (SA- $\beta$ -gal) activity (Fig. 1D) and mRNA levels of  $p16^{INK4A}$  and  $p21^{CIP1}$  CKI, as well as SASP marker *IL-8* (Fig. 1E and Supp. Fig.1A). To confirm that the pro-senescent role of PMVK is dependent of its kinase activity, we generated by single site-directed mutagenesis a kinase dead (KD) mutant by substituting a Lysine by a Methionine in position 22 (K22M) (Herdendorf and Miziorko, 2006). Constitutive expression of KD PMVK (Supp. Fig. 1B) did not reduce cell density (Supp. Fig. 1C), neither increased SA- $\beta$ -gal activity (Supp. Fig. 1D) or  $p16^{INK4A}$ ,  $p21^{CIP1}$  and *IL-8* mRNA levels (Supp. Fig. 1E), suggesting the importance of PMVK kinase activity in the induction of a senescent phenotype. Importantly, downregulation of PMVK through shRNA strategy (Fig. 1E) delayed replicative senescence of the normal human fibroblasts as it increased their replicative potential (Fig. 1B-C) and it reduced both SA- $\beta$ -gal activity and mRNA levels of  $p16^{INK4A}$ ,  $p21^{CIP1}$  and *IL-8* at late passage (Fig. 1D-E).

PMVK transfers a phosphate group from ATP to Mevalonate-5-Phosphate (MVAP) and generates Mevalonate-5-Pyrophosphate (MVA-PP) (Fig. 1A). Interestingly, long-term exogenous addition of MVA-PP, the metabolite product of PMVK, led to a premature

senescence as suggested by progressive cell proliferation arrest (Supp. Fig. 1F-G) and to increased SA- $\beta$ -gal activity coupled with elevated *p16<sup>INK4A</sup>* and *p21<sup>CIP1</sup>* mRNA levels (Supp. Fig. 1H-I).

To further prove that this observed senescence is mediated by the MVA pathway, we stably expressed a shRNA against PMVK and subsequently ectopically expressed MVK (Fig 1A). As expected, constitutive expression of MVK (Supp. Fig. 1J-K) decreased cell density (Supp. Fig. 1L) and elevated SA- $\beta$ -gal activity (Supp. Fig. 1M) and *p16<sup>INK4A</sup>*, *p21<sup>CIP1</sup>* and *IL-8* mRNA levels (Fig. 1N). To be noted, the knockdown of PMVK abolished MVK-induced premature senescence (Fig. 1F-I). These data demonstrate that MVK-induced senescence is rescued by knocking down PMVK, supporting that metabolites flux through MVA pathway play a pivotal role in the induction of senescence.

Overall, these results highlight the role of MVA pathway in promoting cellular senescence in normal human fibroblasts.

### **Mevalonate-induced senescence is mediated by the cholesterol biosynthetic branch**

Within the cell, the MVA pathway is the sole source of Farnesyl-5-Pyrophosphate (F5PP). F5PP is the end-product of the MVA pathway (Fig. 1A) and presents three isoprene units, elementary units further used either to be transferred as prenyl groups to proteins or to be condensated into more complex poly-isoprenoids, that include among others dolichol, ubiquinone, or cholesterol (Goldstein and Brown, 1990) (Supp. Fig. 2A). In order to dissect the role of downstream branches in the MVA-induced senescence, we knocked down the first enzyme of each specific branch and subsequently overexpressed PMVK (Supp. Fig. 2A). The knock down of FDFT1, the first enzyme of the cholesterol biosynthesis pathway, reverted by about 20% the effect of PMVK overexpression on cell number, an effect comparable to the one observed with FDPS knockdown (Sup Fig. 2B). Knockdown of the upstream enzymes

controlling the others branches did not rescued, even partially, the decreased cell number observed in PMVK-expressing cells (Supp. Fig. 2B). SiRNA-mediated *FDFTI* knockdown (Fig. 2C) reverted PMVK-induced decreased cell density (Fig. 2A and Supp. Fig. 2C), SA- $\beta$ -gal activity (Fig. 2B), and increased *p16<sup>INK4A</sup>*, *p21<sup>CIP1</sup>* and *IL-8* mRNA levels (Fig. 2C), without impacting *PMVK* mRNA level (Supp. Fig. 2D). To confirm and extend these results, stable knockdown of *FDFTI* by shRNA was performed. ShRNA-mediated *FDFTI* knockdown (Fig. 2F) in PMVK-overexpressing cells was able to partially rescue the decreased in cell density (Fig. 2D) and the induction of SA- $\beta$ -gal activity (Fig. 2E), and *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>*, and *IL-8* mRNA levels (Fig. 2F).

As our results suggest that cholesterol biosynthesis could be involved in PMVK-induced senescence, we investigated whether cholesterol accumulated in these senescent cells. PMVK-overexpressing cells displayed an elevated intracellular cholesterol content (Fig. 2G). Further confirming this observation, the expression of two genes known to be induced when cells accumulate cholesterol, *SREBF2* and *ABCA1*, was upregulated during PMVK-induced senescence (Fig. 2H). Accordingly, over expression of PMVK KD mutant did not elevate intracellular cholesterol content neither increase *SREBF2* and *ABCA1* mRNA levels (Supp. Fig. 2E-F). Moreover, *FDFTI* knock down in PMVK-overexpressing cells blunted this increase in *SREBF2* and *ABCA1* transcript levels (Supp. Fig. 2G) Remarkably, transcript levels of *SREBF2* and *ABCA1* positively and independently correlated with transcript levels of three senescence markers, namely *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* and *IL-8* (Supp. Fig. 2H), reinforcing this cholesterol/senescence interplay.

We next investigated whether an intracellular cholesterol accumulation could also be found in senescence induced by other stresses. Elevated cholesterol was found in senescent cells induced by p53-stabilizing drug Nutlin-3a and in replicative senescent cells (Supp. Fig. 2I). Interestingly, chemically-induced accumulation of intracellular cholesterol (Fig. 2I)

triggered premature senescence, as judged by the appearance of key features of senescence: decreased cell density (Supp. Fig. 2J), increased SA- $\beta$ -gal activity (Fig. 2J and Supp. Fig. 2K) and upregulation of p16<sup>INK4A</sup> mRNA levels (Fig. 2K)

Altogether these results indicate a role for cholesterol accumulation in the regulation of mevalonate-induced senescence.

### **Intracellular cholesterol accumulation drives an ERR $\alpha$ transcriptional program during mevalonate-induced senescence**

Cholesterol has recently been described to be the ligand of the Estrogen-Related Receptor alpha (ERR $\alpha$ ) (Wei et al., 2016), the latter being not only a key regulator of mitochondrial biogenesis and functions (Audet-walsh and Giguère, 2015) but also a mediator of cholesterol effects. As cholesterol accumulates within cells overexpressing PMVK, we assessed the expression of nuclear-encoded mitochondrial targets of ERR $\alpha$ , including *UQCRC1*, *NDUF5A*, *SDHA* and *SDHB* (Audet-walsh and Giguère, 2015). The constitutive expression of WT PMVK, but not of KD PMVK, upregulated the expression of ERR $\alpha$ -target genes *UQCRC1*, *NDUF5A*, *SDHA* and *SDHB* (Fig. 3A) and of *ESRRA*, gene encoding ERR $\alpha$ , through a positive auto-feedback loop (Laganière et al., 2004) (Fig. 3A).

To determine the consequences of the activation of ERR $\alpha$  transcriptional program in PMVK-expressing cells, we monitored the expression of mitochondrial proteins regulating mitochondrial functions (mtDNA replication and repair), including mitochondrial transcription factors A (TFAM), B1 and B2 (TFB1M and TFB2M, respectively) (Picca and Lezza, 2015). In PMVK-expressing cells, *TFAM*, *TFB1M* and *TFB2M* mRNA levels were all upregulated (Fig 3B and Supp. Fig. 3A). Once again, this upregulation is not observed in KD PMVK-expressing cells upregulated (Fig. 3B and Supp. Fig. 3A).

Importantly, the observed induction of  $ERR\alpha$  and  $ERR\alpha$  targets in PMVK-expressing cells is partially abrogated by the knockdown of *FDFT1* using either si- or shRNA strategy (Fig. 3C and Supp. Fig 3B). As a consequence of disrupted  $ERR\alpha$  transcriptional program, the increased *TFAM* mRNA level is also blunted by knock down against *FDFT1* (Fig. 3D). In line with the previous observations, mRNA levels of *SREBF2* were positively correlated with mRNA levels of the  $ERR\alpha$  target genes *UQCRFS1*, *NDUF5A*, *SDHA*, *SDHB*, and *TFAM* (Supp. Fig. 3C). Taken together, these results suggest that  $ERR\alpha$  activation and associated-mitochondrial biogenesis during MVA-induced senescence is mediated by cholesterol biosynthesis.

We next sought to determine whether cholesterol-dependent  $ERR\alpha$  program functionally mediate the MVA-induced senescence. Knockdown of  $ERR\alpha$  in PMVK-overexpressing cells (Fig. 3G), inhibits  $ERR\alpha$  transcriptional program (Supp. Fig. 3F) and partly rescued the decreased cell number (Fig. 3E and Supp. Fig 3D-E), the increased SA- $\beta$ -gal activity (Fig. 3F), and the elevated *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* and *IL-8* mRNA levels (Fig. 3G). Of note, this knockdown does not affect mRNA levels of PMVK (Supp. Fig 3G). In addition, pharmacological inhibition of  $ERR\alpha$  by XCT-790 (Supp. Fig. 3F), an  $ERR\alpha$  antagonist (Busch et al., 2004), reverted premature senescence-induced by PMVK overexpression according to cell proliferation assay (Fig. 3H-I), SA- $\beta$ -gal activity assay (Fig. 3J) and measurements of *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* and *IL-8* mRNA levels (Fig. 3K), without altering PMVK expression (Supp. Fig. 3G)

Overall, all these results highlight the importance of  $ERR\alpha$  activity in the establishment and maintenance of MVA-induced senescence.

## **Cholesterol-dependent $ERR\alpha$ transcriptional program enhances mitochondrial ROS generation, DNA damage and a p53-dependent senescence**

The increased  $ERR\alpha$ -mediated mitochondrial biogenesis raises the question of its involvement in MeViS. Interestingly, the mitochondrial biogenesis transcription factor A (TFAM) mRNA levels positively correlate with two on three senescence markers, including *p21<sup>CIP1</sup>* and *p16<sup>INK4A</sup>*, reinforcing an interplay between mitochondrial biogenesis and cellular senescence (Supp. Fig. 4A). As mitochondrial-associated genes,  $ERR\alpha$ -target genes encode for subunits of complexes of the electron transport chain (ETC), the latter I, III and IV generating an electrochemical proton gradient and an associated mitochondrial membrane potential (MMP or  $\Delta\Psi_m$ ). Because partial and mild depolarisation of MMP can be a prone inducer of cellular senescence (Wiel et al., 2014), we assessed the MMP value through the use of cationic dye JC-1, in order to determine whether the activation of  $ERR\alpha$ -mediated mitochondrial biogenesis could alter it. Importantly, MVA-induced senescence is accompanied by a depolarization of the MMP (Fig. 4A), and the expression of the KD-PMVK does not induce it (Fig. 4A).

Variation of MMP and associated mitochondrial dysfunction may induce the generation of reactive oxygen species (ROS) (Moiseeva et al., 2009), as mitochondria constitute an important source of ROS within the cell. Mitochondrial ROS were higher in PMVK-overexpressing cells compared to control cells and KD PMVK-overexpressing cells (Fig. 4B). This elevated mitochondrial level of mitochondrial ROS was rescued both by independent knockdown of *FDFT1* or *ESRRA* (Fig. 4C), demonstrating that the activation of cholesterol-mediated  $ERR\alpha$  program functionally triggers the generation of mitochondrial ROS. Total cytosolic ROS were also elevated in PMVK-overexpressing cells compared to control cells (Supp. Fig. 4B), and rescued either by *siFDFT1* and *siESRRA* (Supp. Fig. 4C). Besides, the use of antioxidant alleviated MVA-induced senescence, as suggested by the reduction of SA- $\beta$ -gal activity and cell proliferation arrest upon N-Acetyl-Cysteine (NAC) treatment (Supp. Fig. 4D-

E). Taken together, these data suggest that a cholesterol-dependent  $ERR\alpha$  program promotes an increased mitochondrial biogenesis, coupled with mitochondrial depolarisation and subsequent ROS production during MVA-induced senescence.

Oxidative stress is known to promote cellular senescence through DNA damage (Gorgoulis et al., 2019), DNA damage response (DDR) and p53 activation (He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014b). MVA-induced senescence, through constitutive overexpression of PMVK, resulted in increased DNA damage as measured by  $\gamma$ -H2AX and 53BP1 foci (Fig. 4D and Supp. Fig. 4F). Moreover, in addition to  $p21^{CIP1}$ , the expression of other p53 target genes, including *GDF15* and *GADD45A* increased during MVA-induced senescence (Fig. 4E and Supp. Fig. 4G). This p53-dependent upregulation was abrogated by siRNA against *FDFT1* and *ESRRA* (Fig. 4E and Supp. Fig. 4H), demonstrating that p53 transcriptional program relies on the activation of cholesterol- $ERR\alpha$  activity. Functionally, MVA-induced senescence is terminally driven by p53, as suggested by reversion of PMVK-induced SA- $\beta$ -gal activity (Fig. 4F) and increased  $p21^{CIP1}$ ,  $p16^{INK4A}$  and *IL-8* mRNA levels during the knock down of p53 (Fig. 4G). On the contrary, p53 knockdown was not able to rescue either the activation of  $ERR\alpha$  transcriptional program or the generation of mitochondrial ROS (Supp. Fig. I-J).

Taken together, these data support that p53 acts downstream of cholesterol-dependent  $ERR\alpha$  activity and subsequent ROS generation to terminally promote MVA-induced senescence.



## Discussion

In this study, we deciphered the role of the MVA pathway in cellular senescence. Its activation triggers premature senescence whereas its inhibition delays replicative senescence in normal human cells. MVA-induced senescence is mediated, at least partly, by the biosynthetic cholesterol pathway that leads to cholesterol accumulation and the activation of an ER $\alpha$  transcriptional program triggering mitochondrial ROS accumulation, DNA damage and a p53-dependent senescence (Fig. 5).

Based on a genetic approach, our study demonstrates a pro-senescent role of MVA pathway in normal fibroblasts. Other studies suggested a role of MVA pathway in cellular senescence by pharmacological approaches, using MVA pathway inhibitors: statins and aminobiphosphonates. Remarkably, these compounds are known to have pleiotropic effects (Liao and Laufs, 2004) and are therefore not the most appropriate tools to dissect the mechanisms beyond the MVA pathway. However and in line with our results, blocking mevalonate pathway with these inhibitors has been shown to delay senescence in human endothelial cells (Assmus Birgit et al., 2003), whereas it promotes senescence in oral keratinocytes and has no obvious effect in oral fibroblasts (Kim et al., 2011), suggesting a potent cell-specific role of the MVA pathway in the regulation of cellular senescence. Senescence is also characterized by its SASP, the later accounting for non-cell autonomous effects of the phenotype and impacting among others immune system recruitment, local inflammation, paracrine senescence, or tumorigenesis (He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014b). Our results demonstrate that MVA-induced senescence promotes pro-inflammatory SASP, while reducing MVA activity blunts it. MVA pathway inhibitors, such as statins or aminobiphosphonates, have been shown to blunt SASP (Griveau et al., 2018; Wang et al., 2020) and some of its effects, including pro-tumorigenic activity (Liu et al., 2015b). Dampening SASP

through statins could explain their numerous anti-inflammatory outcomes *in vivo* (Liou et al., 2014; Montecucco and Mach, 2009). Indeed, simvastatin treatment has been early found to reduce *in vitro* and *in vivo* the expression of main SASP pro-inflammatory cytokines including IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1 (Rezaie-Majd et al., 2002; Sakoda et al., 2016). Beyond these pharmacological studies, our results provide the first genetic dissection of this pathway in the context of senescence in normal fibroblasts and could explain some of the anti-inflammatory effects of MVA pathway inhibitors *via* a blunted SASP.

We also observed that the enhanced activity of MVA pathway, through constitutive expression of MVA enzymes, leads to accumulation of cholesterol within normal cells, through the biosynthetic pathway of cholesterol. This intracellular cholesterol accumulation is then necessary to functionally induce a senescence response. Even though the lipid metabolism is known to be modified during senescence (Cadenas et al., 2012; Flor et al., 2017; Inoue et al., 2017; Maeda et al., 2009; Ogrodnik et al., 2017; Song et al., 2017; Venable et al., 1995), only ceramide was earlier found to regulate and promote the onset of senescence (Venable et al., 1995). It was shown that senescent fibroblasts and hepatocytes display accumulation of lipid droplets (Cadenas et al., 2012; Flor et al., 2017; Inoue et al., 2017; Maeda et al., 2009; Ogrodnik et al., 2017), which is related to an increased free fatty acids (Song et al., 2017) and cholesterol (Cadenas et al., 2012; Maeda et al., 2009) content. Moreover, MVA pathway and cholesterol synthesis genes are upregulated in a model of oncogene-induced senescence (OIS) (Pantazi et al., 2019). However, these descriptive studies proposed cholesterol and/or lipid droplets accumulation as a consequence of senescence phenotype. Beyond these descriptive studies and in line with our results, it has been recently found that knockdown of MVA pathway genes (e.g. *MVD*) or cholesterol synthesis genes (e.g. *FDFT1*, *SQLE*, *DHCR7*) could contribute to a partial rescue of cell proliferation arrest in Ras-induced senescence (Pantazi et al., 2019). Taken together, all these data suggest that an enhanced activity of MVA pathway and subsequent

cholesterol pathway might thus (i) explain free cholesterol accumulation in senescent cells and (ii) propose a functional involvement of this cholesterol accumulation mediating senescence phenotype.

Our study reinforces the role of mitochondria in regulating cellular senescence, in our context through the promotion of  $ERR\alpha$ -regulated mitochondrial biogenesis and subsequent ROS production. Various mitochondrial metabolic rearrangements have been shown either to accompany or to promote cellular senescence (Ziegler et al., 2015; Wiley et al., 2016; Arnould et al., 2002; Hutter et al., 2004; Kaplon et al., 2013; Jiang et al., 2013; Stab et al., 2016). Largely described, abnormal mitochondrial dynamics accompanies cellular senescence, notably through the accumulation of hyperfused mitochondria (Gorgoulis et al., 2019; Ziegler et al., 2015; Hernandez-Segura et al., 2018a). Nonetheless, the contribution of mitochondrial biogenesis has been rarely described (Korolchuk et al., 2017; Summer et al., 2019; Takenaka et al., 2020). Mitochondrial biogenesis could improve mitochondrial functions, however these functions are known to decline in an age-dependent manner in multiple organs (Short et al., 2005) and inhibition or disruption of ETC complex induces premature senescence (Miwa et al., 2014; Ziegler et al., 2015). Accumulation of ETC components, through mitochondrial biogenesis may lead to reduced ETC activity through subsequent complexes disruption (Miwa et al., 2014). Cellular senescence participates in aging and various age-related pathologies (Gorgoulis et al., 2019; He and Sharpless, 2017; Muñoz-Espín and Serrano, 2014b), including osteoporosis. Noteworthy and in line with our results,  $ERR\alpha$   $-/-$  model mice display reduced osteoporosis in female mice (Delhon et al., 2009; Teyssier et al., 2009). While no studies on osteocytes have been performed in our hands in the context of senescence, we can speculate a reduction of senescent cells in bones of  $ERR\alpha$   $-/-$  during aging. Interestingly, this anti-osteoporosis effect can be mimicked with inhibitors of the MVA pathway, as demonstrated by the use of aminobiphosphonates in the management of osteoporosis in postmenopausal women (Kanis et

al., 2008). Besides,  $ERR\alpha$  knockout in human mesenchymal stem cells (hMSCs) reduces the expression of Osteopontin, another SASP factor. Alterations of TFAM expressions are seen during aging, with an upregulation in frontal cortex and inversely downregulation in liver or muscle (Picca and Lezza, 2015), suggesting a tissue specificity in modulating TFAM expression during aging.

While senescence has been related to both normal and physio-pathological conditions *in vivo*, major cell effectors triggering senescence *in vivo* remain to be elucidated. *In vivo* studies in worms and mouse suggest that the mevalonate pathway is modulated with aging. Age-dependent post-translational modifications have been proposed to regulate mevalonate metabolism during aging in *C. elegans* (Sapir et al., 2014). Furthermore, some final products of mevalonate pathway and subbranches, such as dolichol or cholesterol, have been shown to accumulate *in vivo* with aging in mouse and other mammals (Parentini et al., 2005), or to be linked to age-related pathologies (Wood et al., 2002) whereas other, such as ubiquinone, are reduced in age-tissues (Bentinger et al., 2010). More interestingly, inhibition of mevalonate pathway during physiological aging process has been as well studied in worms, mouse and even in human (Jacobs et al., 2013; Jahn et al., 2020; Varela et al., 2008). The first study showed that complementary use of statins and aminobiphosphonates alleviates premature age-related features and enhances lifespan of a murine model of premature aging (Varela et al., 2008). The second study has been conducted in patient over 70 years old, and demonstrate a decreased mortality in statin-treated cohorts (Jacobs et al., 2013). Finally, the third one showed that this statin-mediated lifespan extension is observed as well in *C.elegans* (Jahn et al., 2020). Of note, these three studies demonstrate a pro-longevity role of mevalonate pathway blocking, though suggest that this role is independent of cholesterol. Overall and in regards to our results, the dysregulation of mevalonate pathway with aging and the consequent accumulation or reduction

of final products (cholesterol among others) could be determinant to drive cellular senescence, aging and age-related pathologies *in vivo*.

## Material and Methods

**Cell culture and reagents.** MRC5 normal human fibroblasts (ATCC, Manassas, VA, USA), and kidney 293T or 293 GP cells (Clontech, Mountain View, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, USA) with GlutaMax and supplemented with 10% FBS (Sigma-Aldrich, Saint-Louis, USA) and 1% penicillin/streptomycin (ThermoFisher Scientific). Mevalonate-5-Pyrophosphate (Sigma-Aldrich, 94259) was added at 5 $\mu$ M final concentration in the complete medium. XCT-790 (Medchem, HY-10426/CS-2413) was used at 150 nM. N-Acetyl-Cysteine (NAC) (A9165, Sigma-Aldrich) has been used directly after infection at 1 mM. These compounds were renewed every two days. U18666A was provided by Cholesterol Assay Kit (Cell-Based) (Abcam, ab133116), used for a single treatment at 2,5  $\mu$ M.

**Vectors, transfection and infection.** Retroviral vectors were used to constitutively overexpress MVK or PMVK. Vector plasmids were provided by Addgene in the Myristoylated Kinase Library (Kit #1000000012 ) described in (Boehm et al., 2007). K22M kinase dead mutation of the kinase dead (KD) mutant PMVK was generated using QuickChange Site-Directed Mutagenesis Kit (Agilent, Catalog # 200518). Lentiviral particles were used to constitutively express shPMVK (pLV[shRNA]-Hygro-U6> hPMVK[shRNA#4], Target sequence: GAGAACCTGATAGAATTTATC) of shFDFT1 (pLV[shRNA]-Hygro-U6> hFDFT1[shRNA#2], Target sequence CAACGATCTC CCTTGAGTTTA and pLV[shRNA]-Hygro-U6> hFDFT1[shRNA#3], Target sequence: ACC ATTTGAATGTTTCGTAATA) were provided by VectorBuilder. 293T or 293GP virus producing cells were transfected using the GeneJuice reagent according to the manufacturer's recommendations (Merck Millipore). Two days after transfection, viral supernatant was collected, diluted with fresh medium (1/4 for 293T

cells and 1/2 for 293GP cells) and hexadimethrine bromide was added (final concentration 8 µg/ml; Sigma-Aldrich). Target cells were then infected, centrifugated with virus particles for 30 minutes at 2000 rpm and subsequently incubated during 6 hours at 37°C 5% CO<sub>2</sub>. Fresh medium was added after 6 hours incubation. One day later, infected cells were selected with Hygromycin B (ThermoFisher Scientific) at 15 µg/mL or Geneticin (ThermoFisher Scientific) at 75 µg/mL.

### **siRNA**

Fibroblasts cells were plated and reverse transfected with ON-TARGET plus SMART pool of small interference (si) RNAs: siCtrl (Catalog: D-001810-10-20 / Lot#2693147), siFDFT1 (Catalog: #L-009442-00-0005 / Lot#180518), siESRRA (Catalog: #L-003403-00-0005 / Lot#191211), sip53 (Catalog: #L-003329-00-0001) / Lot#180912) (Horizon Discovery) previously incubated for 20 min with Dharmafect 1 Transfection Reagent (Horizon Discovery) 0,6% in antibiotics- and serum-free medium DMEM with Glutamax. Final siRNA quantity in the well: 15nmol). The day after, cells were infected (with retroviral particles containing PMVK) as referenced previously.

**RNA extraction, reverse transcription, and real-time quantitative PCR.** Total RNAs were extracted with phenol-chloroform using Upzol (Dutscher, Brumath, France). Synthesis of cDNA was performed using Maxima First cDNA Synthesis Kit (ThermoFisher Scientific) from 1 µg of total RNA. cDNA (50 ng/µL) was used as a template for quantitative PCR (qPCR), and mixed with primers (200 nM), SYBR™ Green PCR Master Mix (ThermoFisher Scientific) or TaqMan mix (Roche) and Universal Probe Library probes (100 µM) (ThermoFisher Scientific) for the gene of interest. Reactions were performed in triplicate. qPCR analyses were carried out

with the FX96 Thermocycler (Biorad, Hercules, USA). Relative mRNA levels were calculated using the Comparative Ct ( $\Delta\Delta\text{CT}$ ) method. Normalization with Actb is graphed in figures. Primers sequences and housekeeping genes used are listed in Supplementary Table S1.

**Senescence-associated  $\beta$ -Galactosidase analysis and Crystal violet.** For SA- $\beta$ -Galactosidase assay, cells were washed with PBS 1X, fixed for 5 min in 2% formaldehyde / 0.2% glutaraldehyde, rinsed twice in PBS 1X, and incubated at 37°C overnight in SA- $\beta$ -Galactosidase staining solutions as previously described (Debacq-Chainiaux et al., 2009). For crystal violet staining, cells were washed with PBS 1X, fixed for 15 min in 3.7% formaldehyde and stained with crystal violet.

**ROS and JC1 quantification.** Total cytosolic ROS were assessed using CellROX™ Green Reagent according to manufacturer's recommendations. (ThermoFisher Scientific). Mitochondrial ROS were assessed using Cell Meter™ Mitochondrial Hydroxyl Radical Detection Kit (ATT Bioquest) according to manufacturer's recommendations. Excitation 488nm/Emission 530 +/- 30 nm. For JC1, JC1-Mitochondrial Membrane Potential Assay Kit (ab113850, Abcam) was used. JC1 monomers and aggregates were both excited at 488 nm. Detection of fluorescence for JC1 monomers and aggregates were performed respectively at 530nm and 590nm. Ratio F(aggregate)/F(monomer) was subsequently evaluated. All the quantifications of ROS and JC1 was performed using Columbus Software.



**Cholesterol Measurement.** Relative intracellular cholesterol concentration was assessed using Cholesterol Assay Kit (Cell-Based) (Abcam, ab133116) according to manufacturer's recommendations. Quantification was performed using ImageJ Software.

**Immunoblot and Immunofluorescence.** For immunoblot experiments, cells were lysed in RIPA buffer. After protein quantification using Bradford assay and DO 535nm, 30 µg of proteins were loaded and resolved by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with TBS Tween / Milk 5% for 1 hr and incubated at 4°C with primary antibodies overnight. Primary antibodies and dilutions used are listed in Supplementary Table S2. Membranes were then incubated with secondary antibody for 1 hr at room temperature. Detection was performed using ECL kit (Amersham). For immunofluorescence experiments, cells were washed with PBS 1X, fixed for 15 min in 3.7% formaldehyde, and permeabilized with Triton 100X 0,1% for 10 min. Blocking was performed using PBS with 20% FBS during 30 minutes and cells were then incubated at 4°C with primary antibodies overnight. Primary antibodies and dilutions used are listed in Supplementary Table S2. Cells were incubated with secondary antibody for 1hour, and washed before proceeding to image acquisition and analyses.

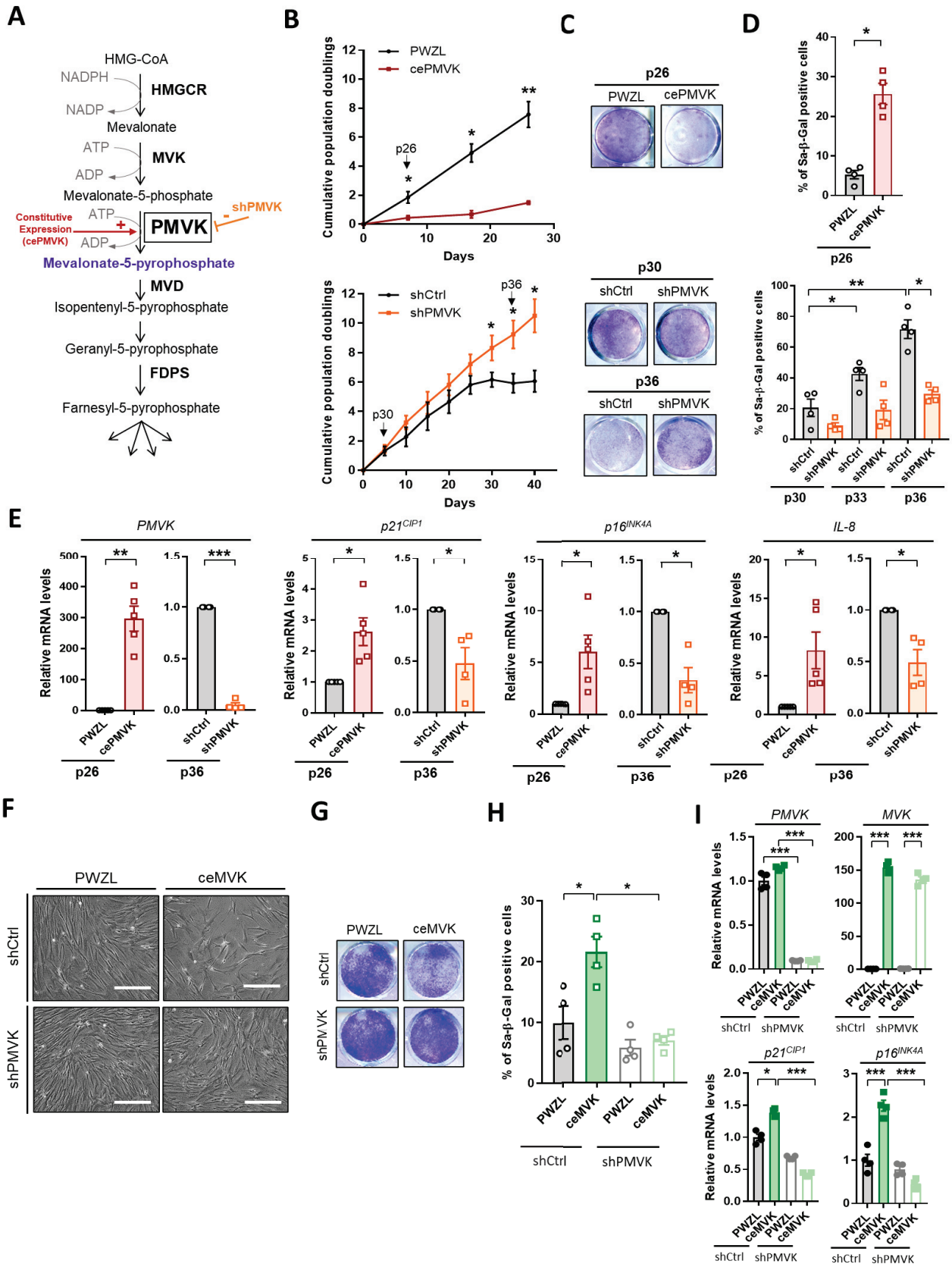
**Statistical analysis.** Values represent mean  $\pm$  SD or SEM as indicated in the figure legend. n represents the number of biological independent replicates. Statistical analyses for groups were performed as indicated in the figure legend. D'agostini & Pearson normality for big sampling or Shapiro-test for reduced sampling were used before proceeding to any analyses. Parametric tests were two tailed, unpaired or paired: Student's t test (equal variance) or Welch's t-test (for non-equal variance) as indicated in the figure legend. Mann-Whitney U Test was performed for

non-parametric tests. Spearman test was used for correlations analysis. All the statistical analyses were performed using GraphPad Prism 7 (\* P < 0.05; \*\* P < 0.01; \*\*\* P < 0.001).

## **Author contributions**

## **Acknowledgements**

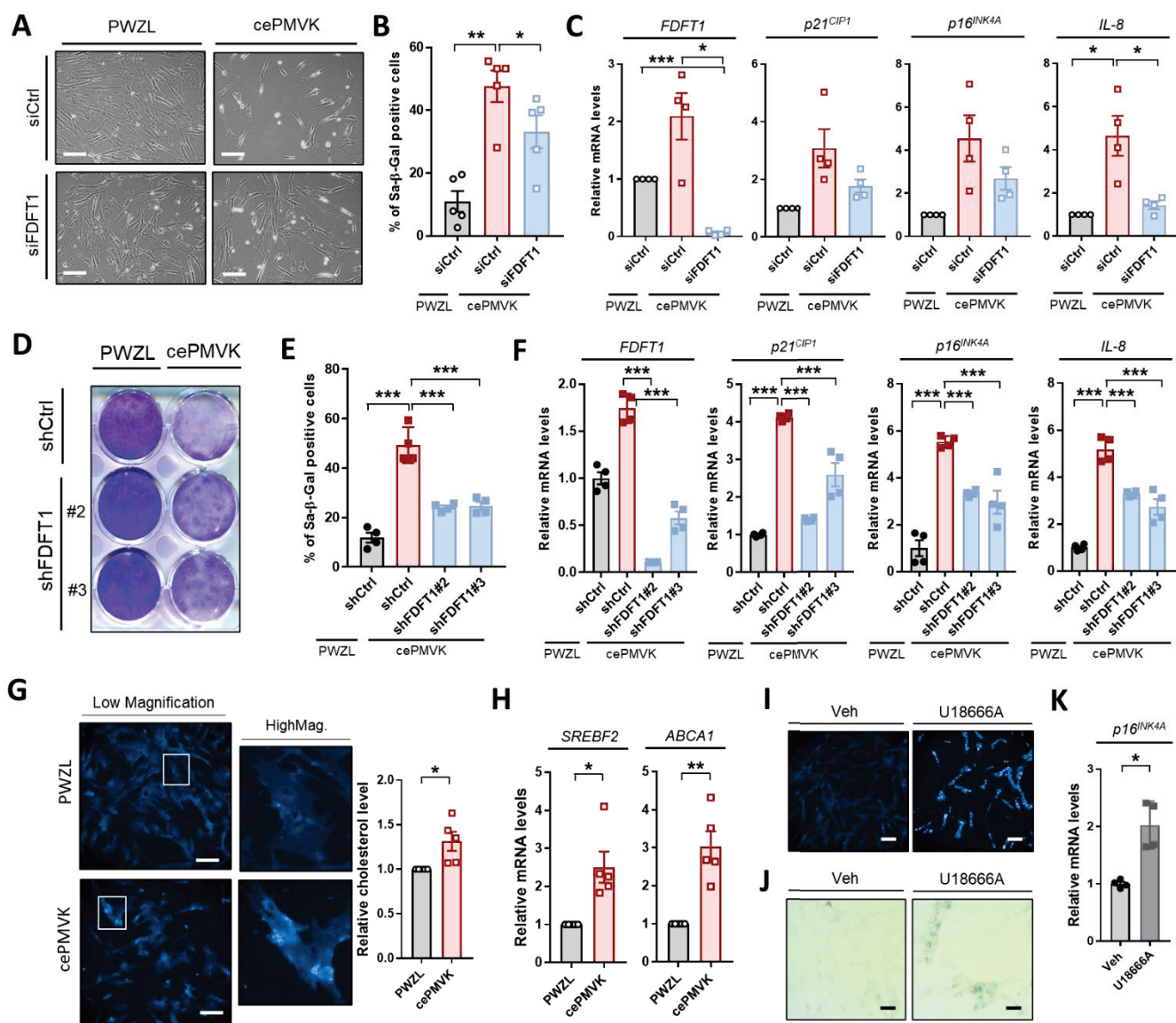
This work was supported by a grant of the Ligue Nationale contre le Cancer (Comité du Rhône) (NM). DZ is supported by the Fondation pour la Recherche Médicale FRM (FDT201904008259), NM by INSERM and DB by CNRS. We thank laboratory members for helpful suggestions and collaborations.



**Figure 1. The MVA pathway regulates senescence in normal cells**

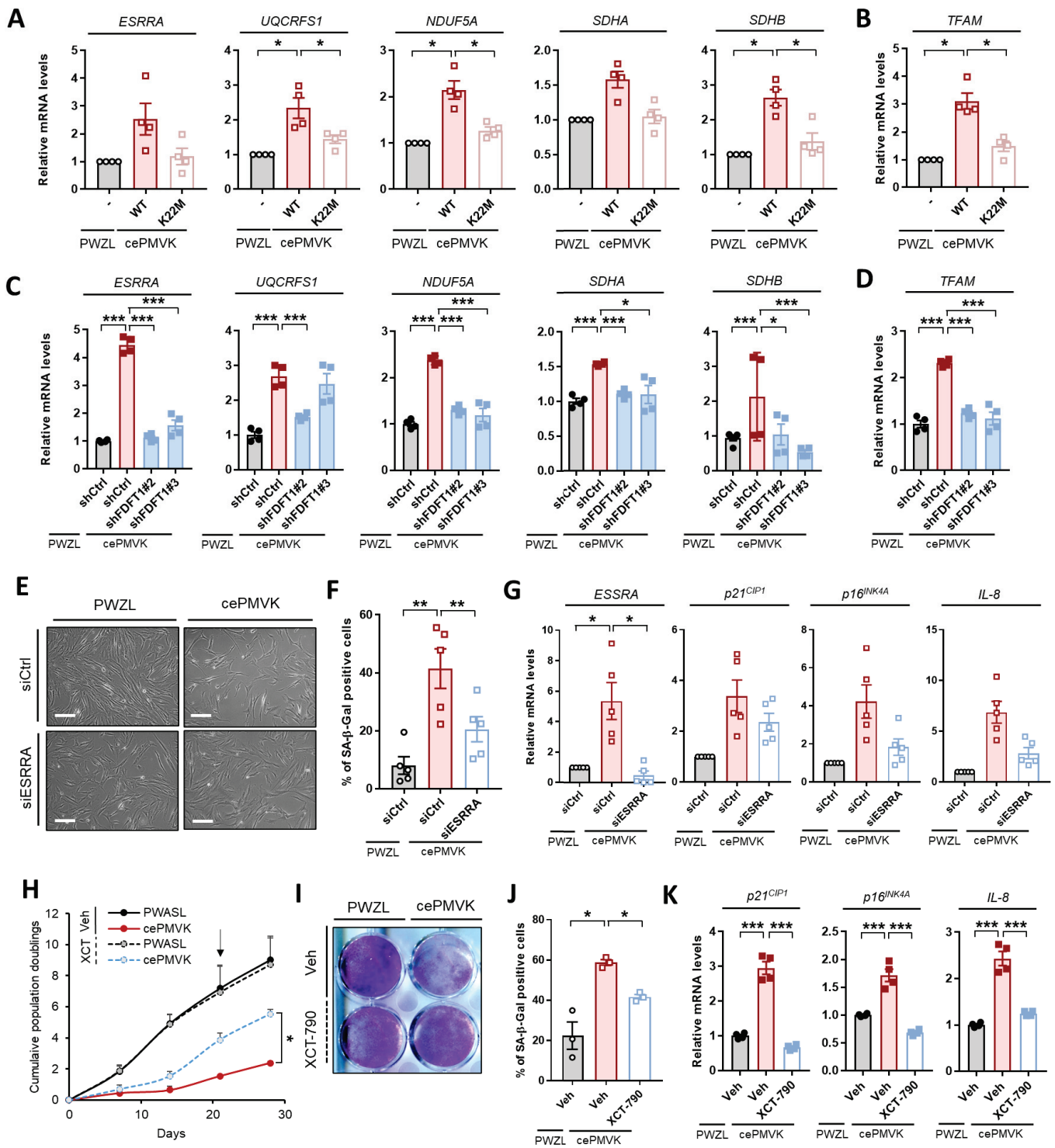
**A.** Schematic representation of the MVA pathway in eukaryotic cells. Enzymes are represented in bold. In our study, the PMVK enzyme is targeted *via* two complementary genetic approaches: constitutive expression (red) or knock down by shRNA (orange). **B.** Growth curves of MRC5 fibroblasts constitutively overexpressing PMVK (cePMVK) or empty vector (PWZL) (left panel), and shRNA against PMVK (shPMVK) or scramble shRNA (shCtrl) (right panel). Mean

+/- SEM of n=4-5 independent experiments. **C.** Crystal violet staining of PWZL or PMVK-overexpressing cells at early passage (left panel), and of scramble shCtrl-and shPMVK-expressing cells at early (p30) and late (p36) passage. Representative experiment (n=4). **D.** Quantification of SA- $\beta$ -gal positive cells in PMVK- (left panel) and shPMVK- (right panel) expressing cells. Mean +/- SEM of n=4 independent experiments. **E.** RT-qPCR of *PMVK*, *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>*, *IL-8* genes in PMVK- and shPMVK-expressing cells. Mean +/- SEM of n=5 biological replicates. **F.** Micrographs of MRC5 fibroblasts overexpressing MVK (ceMVK) and scramble shRNA (shCtrl) or shRNA against PMVK (shPMVK). Scale bar: 10 $\mu$ m. **G.** Crystal violet staining 8 days after expressing MVK in shCtrl or shPMVK cells. Representative experiment (n=3). **H.** Quantification of SA- $\beta$ -gal positive cells after constitutive expression of MVK in shCtrl or shPMVK cells. Mean +/- SEM of n=3 independent experiments. **I.** RT-qPCR of *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* genes in shCtrl or shPMVK cells expressing MVK. Mean +/- SD of one representative experiment (n=3).



**Figure 2. MVA-induced senescence is partly driven by the cholesterol biosynthetic pathway.**

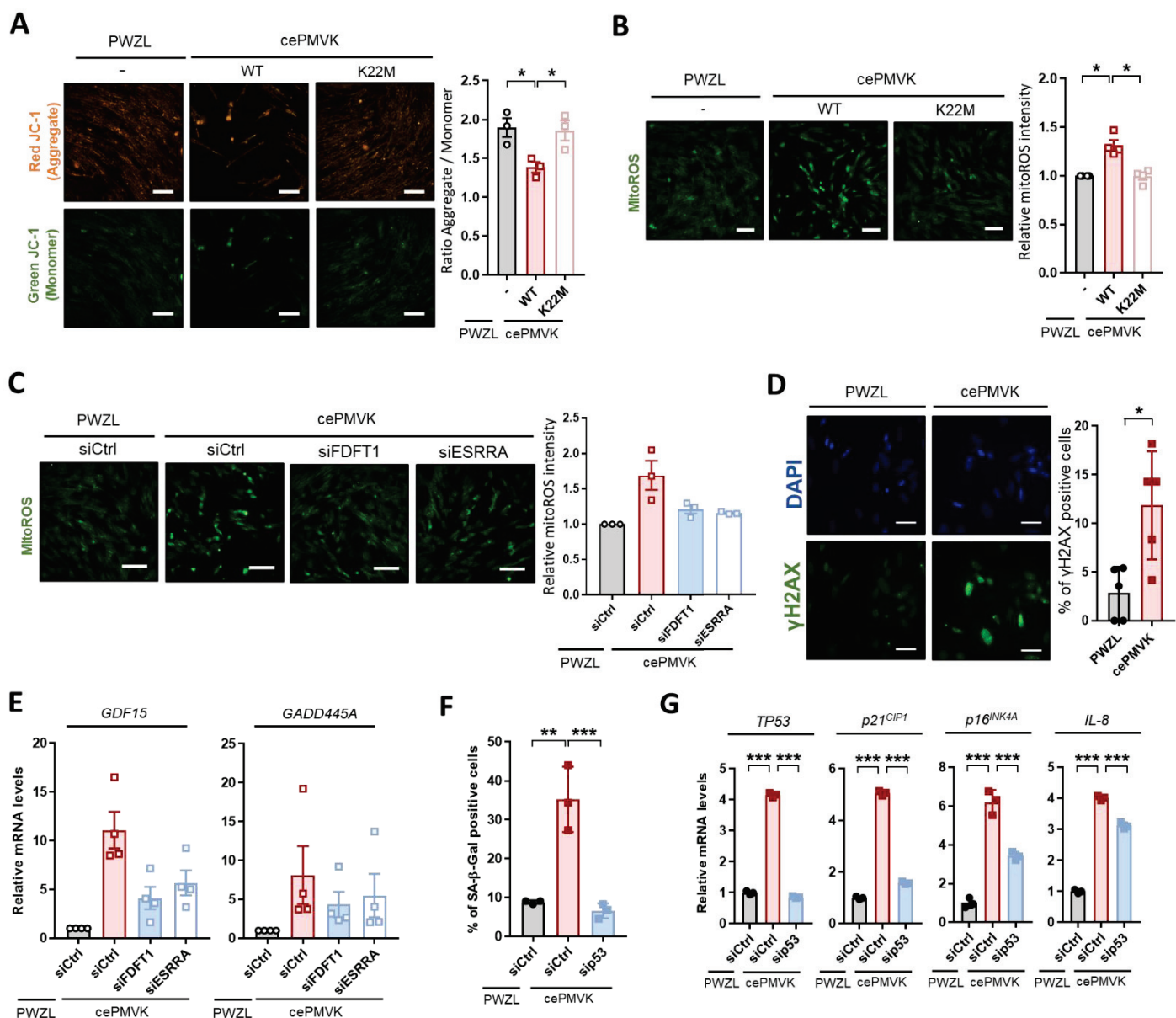
**A.** Micrographs of MRC5 fibroblasts transfected with siRNA against FDFT1 or siCtrl and constitutively overexpressing PMVK or empty vector PWZL. Scale bar: 10 $\mu$ m. **B.** Quantification of SA- $\beta$ -gal positive cells after transfection with siFDFT1 or siCtrl in PMVK-overexpressing cells. Mean  $\pm$  SEM of n=4 independent experiments. **C.** RT-qPCR of *FDFT1*, *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* and *IL-8* genes in cells constitutively overexpressing PMVK, and previously transfected with siFDFT1 or siCtrl. Mean  $\pm$  SEM of n=4 independent experiments. **D.** Crystal violet staining at day 12 after constitutive overexpression of PMVK in shCtrl or shFDFT1 cells. Representative experiment (n=3). **E.** Quantification of SA- $\beta$ -gal positive cells in cePMVK in shCtrl or shFDFT1 cells. Mean  $\pm$  SD of one representative experiment (n=3). **F.** RT-qPCR of *FDFT1*, *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* and *IL-8* genes in cells constitutively overexpressing PMVK-expressing shCtrl or shFDFT1 cells. Mean  $\pm$  SD of one representative experiment (n=3). **G.** Micrographs (left) and quantification of relative intracellular cholesterol level (right) after cholesterol assay in PMVK-overexpressing cells. Mean  $\pm$  SEM of n=4 independent experiments. Scale bar: 10 $\mu$ m. **H.** RT-qPCR of *SREBF2* and *ABCA1* cholesterol-regulated genes. Mean  $\pm$  SEM of n=5 independent experiments. **I.** Cholesterol Assay in U18666A-treated cells (2,5 $\mu$ M) 48 hours after treatment. Scale bar: 10 $\mu$ m. **J.** Micrographs of SA- $\beta$ -gal positive cells in U18666A-treated cells, 72 hours after treatment. Scale bar: 10 $\mu$ m. **K.** RT-qPCR *p16<sup>INK4A</sup>* gene U18666A-treated cells, 72 hours after treatment.



**Figure 3. MeViS requires a cholesterol-dependent  $ERR\alpha$  transcriptional program.**

**A-B.** RT-qPCR of *ESRRA* and  $ERR\alpha$ -target genes (including *UQCRFS1*, *NDUF5A*, *SDHA*, *SDHB*) in PMVK-overexpressing cells. Mean  $\pm$  SEM of  $n=5$  independent experiments. **C-D.** RT-qPCR of  $ERR\alpha$  and  $ERR\alpha$ -target genes in cells PMVK-overexpressing cells, expressing shFDFT1 or previously transfected by siFDFT1. Mean  $\pm$  SEM of  $n=3-4$  independent experiments. **E.** Micrographs of PMVK-overexpressing cells previously transfected by siERRA at day 6 after expression of PMVK. Scale bar: 10 $\mu$ m. **F.** Quantification of SA- $\beta$ -gal positive cells in PMVK-overexpressing cells after transfection with siERRA. Mean  $\pm$  SEM of  $n=4$  independent experiments. **G.** RT-qPCR of *FDFT1*,  $p21^{CIP1}$ ,  $p16^{INK4A}$  and IL-8 genes in PMVK-overexpressing cells, and previously transfected by siFDFT1. **H.** Growth curves of PMVK-

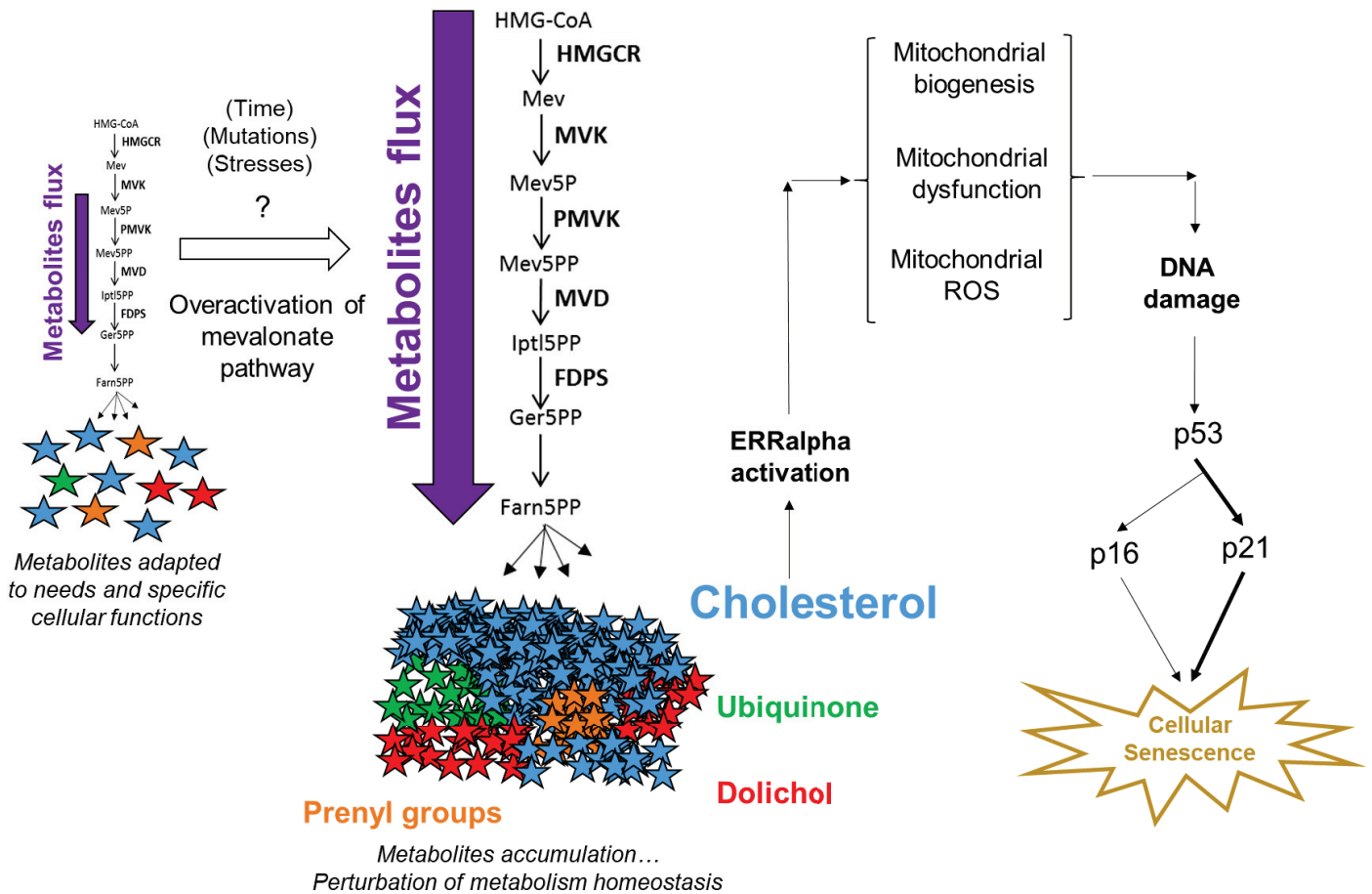
overexpressing cells and treated every 2 days with ERR $\alpha$  inhibitor (XCT-790). **I.** Crystal violet staining of PMVK-overexpressing cells treated or not with XCT-790 during 21 days. Representative experiment (n=4). **J.** Quantification of SA- $\beta$ -gal positive cells in PMVK-overexpressing cells treated or not with XCT-790 during 21 days. Mean +/- SEM of n=4 independent experiments. **K.** RT-qPCR of *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* and *IL-8* genes in PMVK-expressing cells treated or not with XCT-790 during 21 days. Mean +/- SEM of n=4 independent experiments.



**Figure 4. Cholesterol-dependent ERRA program promotes mitochondrial membrane depolarization, ROS generation, DNA damage and p53-dependent senescence**

**A.** Mitochondrial membrane potential measurement through JC-1. Ratio Aggregate/Monomer are represented. Mean +/- SEM of n=3 independent experiments. **B.** Mitochondrial ROS quantification in WT- and KD-PMVK-overexpressing cells. Mean +/- SEM of n=4 independent experiments. **C.** Mitochondrial ROS quantification in PMVK-overexpressing cells and previously transfected by siFDFT1 or siESRRA. Mean +/- SEM of n=3 independent experiments. **D.** Immunofluorescence micrographs of cells PMVK-expressing cells stained for GammaH2AX protein (left panel) and quantification of GammaH2AX positive cells (right panel). Mean +/- SD of one representative experiment (n=2). **E.** RT-qPCR of p53 target-genes (*GADD45A* and *GDF15*) in PMVK-overexpressing cells previously transfected by siFDFT1 or siERR $\alpha$ . Mean +/- SEM of n=4 independent experiments. **F.** Quantification of SA- $\beta$ -gal positive cells positive cells in PMVK-overexpressing cells and previously transfected with sip53. Mean +/- SD of one representative experiment (n=3). **G.** RT-qPCR of *TP53*, *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* and *IL-8* genes in PMVK-expressing cells, previously transfected with sip53. Mean +/- SD of one representative experiment (n=3).





**Figure 5: Mevalonate pathway acts as mediator of cellular senescence through cholesterol/ERR $\alpha$ /mitochondria axis.**

Mevalonate pathway is regulated by nutrient supplies or cell fate, and the metabolite flux coming from this pathway is adapted to the cellular needs. With aging and stress, heightened activity of the mevalonate pathway enhances this metabolic flux leading to increased end products, including cholesterol. Cholesterol accumulation triggers activation of ERR $\alpha$  transcriptional program and leads to a boost in mitochondrial activity and mitochondrial dysfunction. These mitochondrial defects boost ROS production and ultimately lead to DNA damage and p53 dependent activation of cellular senescence. Senescent cells have an increased lipid anabolic activity related to its increased metabolism activity.

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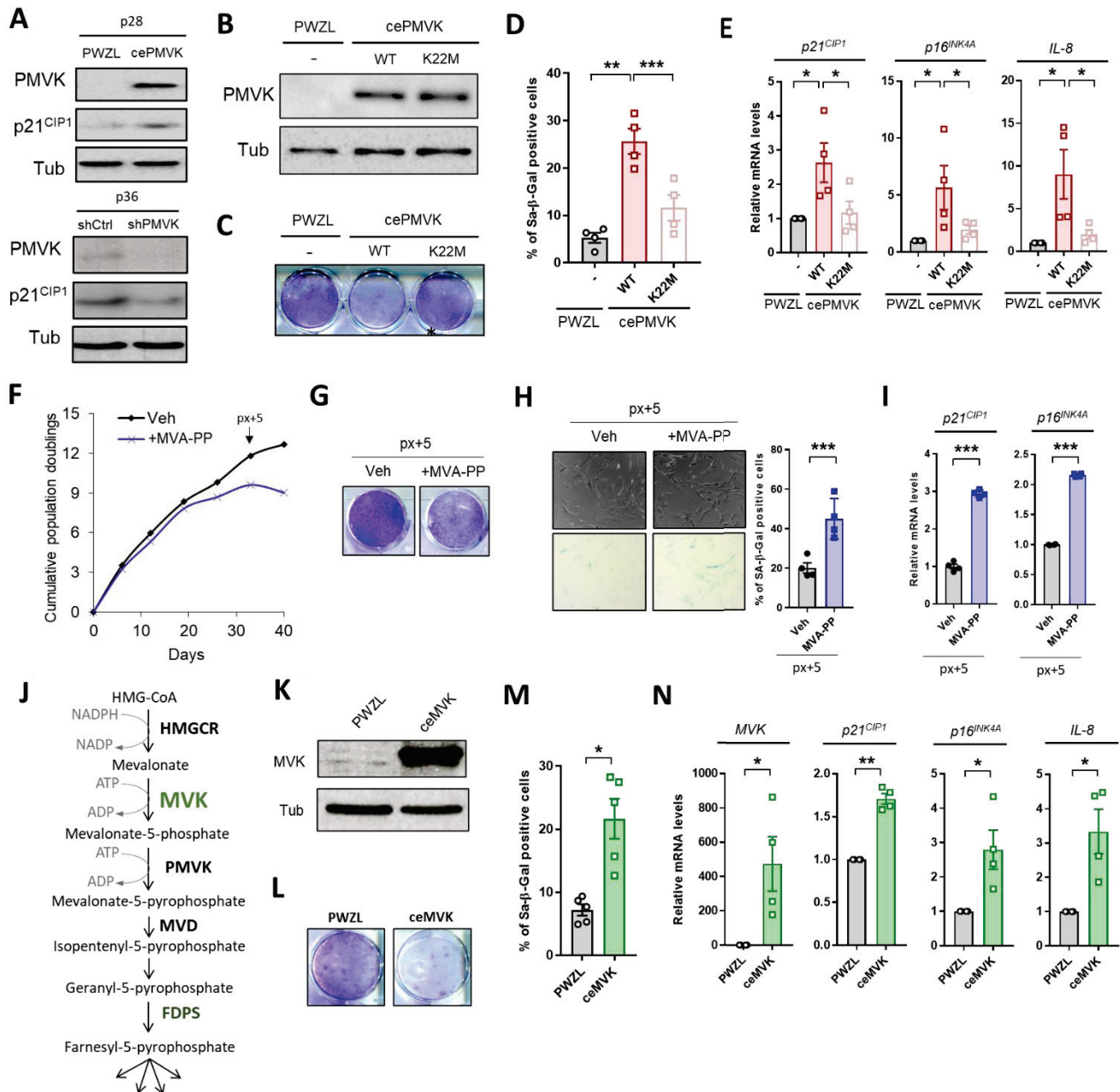
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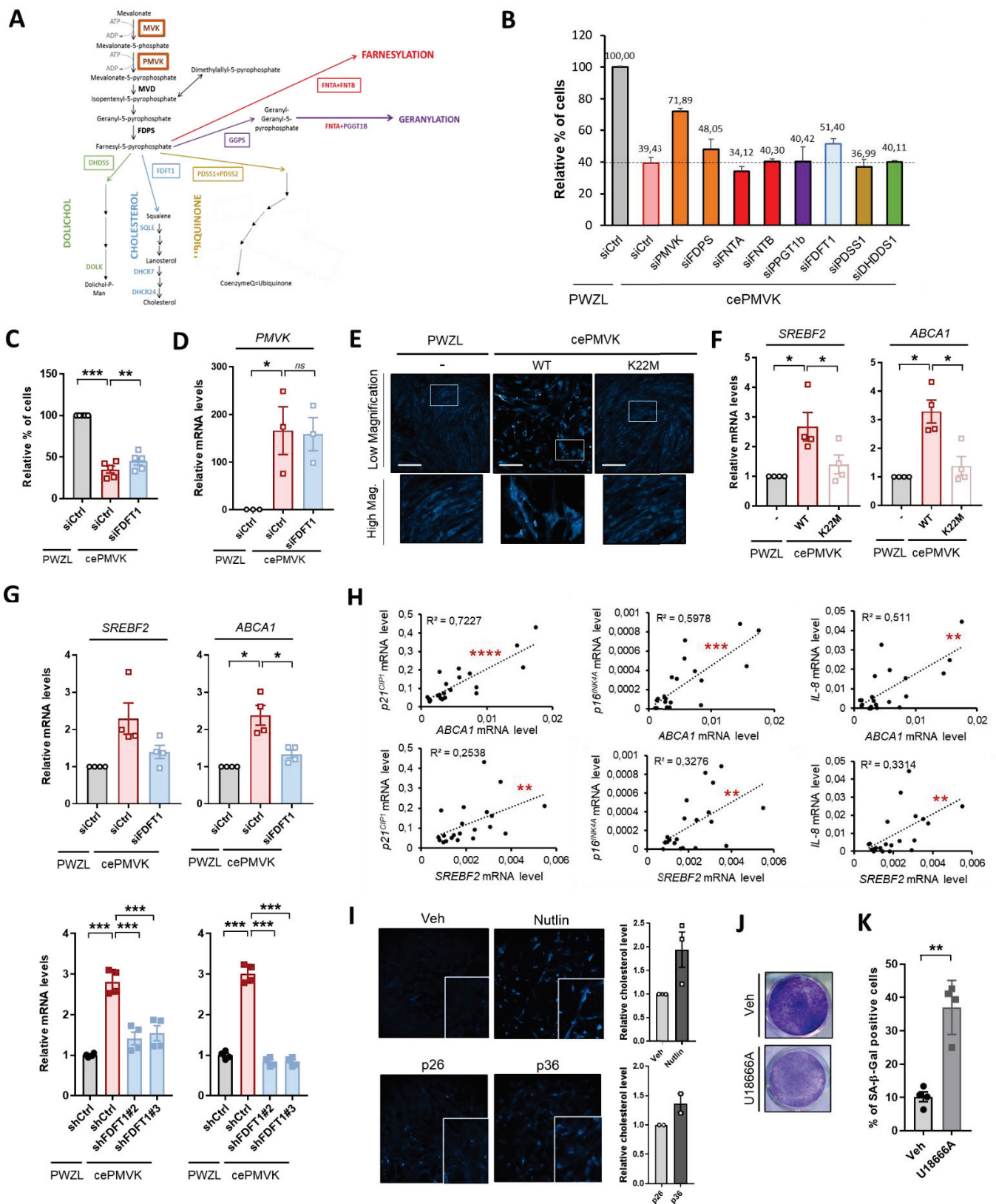
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**Supplemental Materials**



### Supplemental Figure 1.

**A.** Western blot on PMVK, p21<sup>CIP1</sup> and Tubulin in PMVK-overexpressing cells at early passage (p28) and in shPMVK-expressing cells at late passage (p36). Representative experiment (n=2). **B.** Western blot on PMVK and Tubulin in wild type PMVK-overexpressing cells (WT) and Kinase-Dead (KD)-PMVK-expressing cells (K22M). Representative experiment (n=3). **C.** Crystal violet staining 10 days after overexpressing PMVK or KD-PMVK. Representative experiment (n=5). **D.** Quantification of SA-β-gal positive cells in PMVK- and KD-PMVK-expressing cells. Mean +/- SEM of n=4 biological replicates. **E.** RT-qPCR of *PMVK*, *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>*, *IL-8* genes in PMVK- and KD-PMVK-overexpressing cells. Mean +/- SEM of n=5 biological replicates. **F.** Growth curves of MRC5 fibroblasts in medium supplemented or not with MVA-PP (5μM) every two days. Representative experiment (n=2). **G.** Crystal violet staining passage px+6 after starting the exogenous addition of MVA-PP. Representative experiment (n=2). **H.** Micrographs and quantification of SA-β-gal positive cells at passage px+6 after starting the exogenous addition of MVA-PP. Mean +/- SD of one representative experiment (n=2). **I.** RT-qPCR of *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* genes at px+6 after starting the exogenous

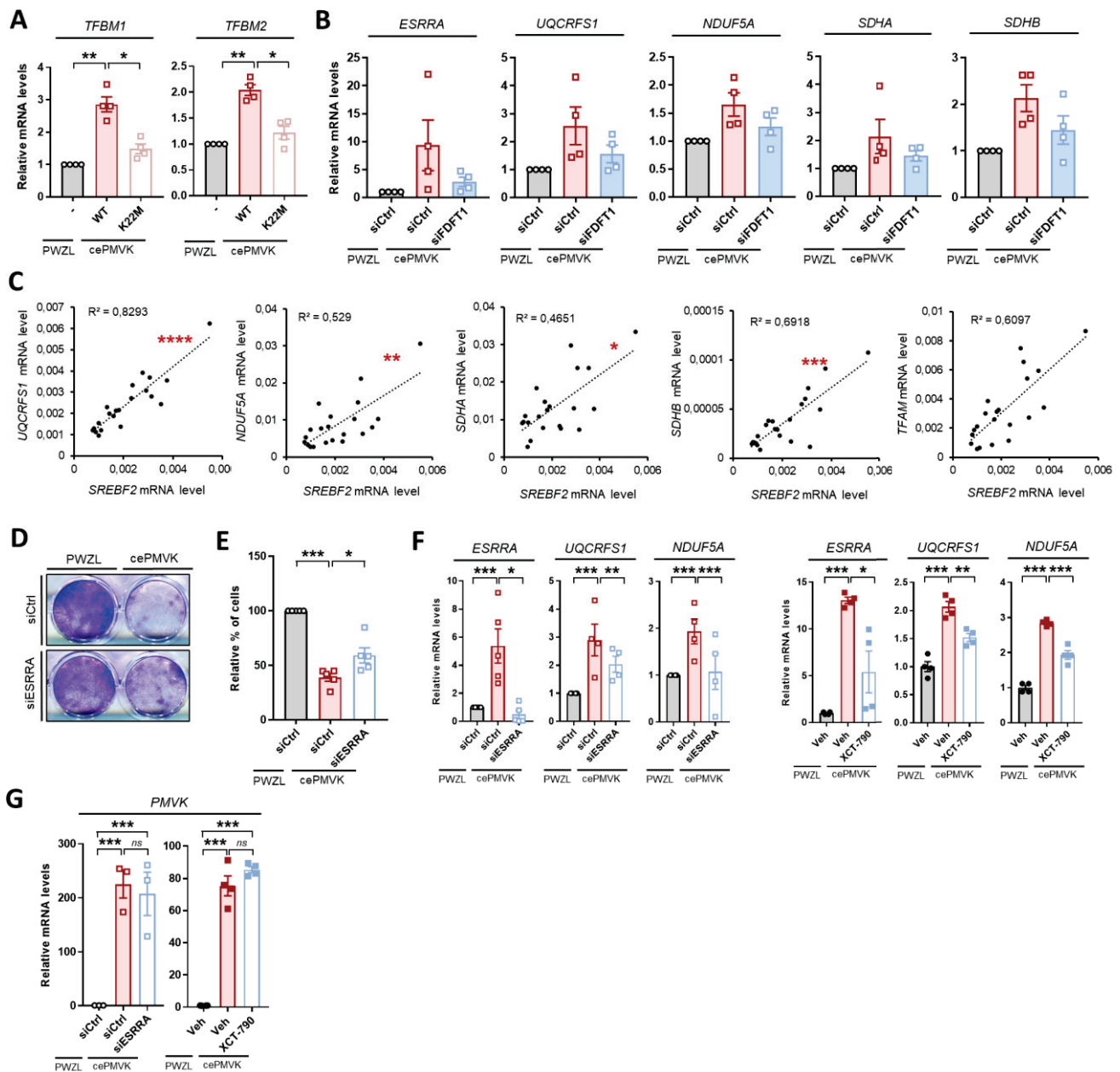
addition of MVA-PP. Mean +/- SD of one representative experiment (n=2). **J.** Schematic representation of the MVA pathway. **K.** Western blot on MVK and Tubulin in MVK-expressing cells. **L.** Crystal violet staining 8 days after expressing MVK. Representative experiment (n=4). **M.** Quantification of SA- $\beta$ -gal positive cells in MVK-overexpressing cells. Mean +/- SD of one representative experiment (n=4). **N.** RT-qPCR of *MVK*, *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* and *IL-8* genes in MVK-overexpressing cells. Mean +/- SD of one representative experiment (n=4).



**Supplemental Figure 2.**

**A.** Schematic representation of the MVA pathway and subbranches stemming from Farnesyl-5-Pyrophosphate, including farnesylation (red), geranylation (purple), ubiquinone synthesis (gold), cholesterol synthesis (blue) and dolichol biosynthesis (green). Squares enclose first specific enzyme of each subbranch. **B.** Quantification of number of cells after transfection cells

siRNA against PMVK, FDPS and each first specific enzyme of downstream subbranches of the MVA pathway in PMVK-overexpressing cells. Mean +/- SD of n=3 independent experiments. **C.** Quantification of number of cells after transfection with siRNA against FDFT1 in PMVK-expressing cells. Mean +/- SEM of n=4 independent experiments. **D.** RT-qPCR of *PMVK* gene in siFDFT1-transfected and PMVK-overexpressing cells. **E.** Micrographs of cholesterol assay in PMVK- and KD-PMVK overexpressing cells. Scale bar: 15µm. **F-G.** RT-qPCR of *SREBF2* and *ABCA1* genes in PMVK- and KD-PMVK overexpressing cells or in siFDFT1-transfected and PMVK-overexpressing cells. **H.** Linear regression analyses between *ABCA1* or *SREBF2* mRNA levels and *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* and *IL-8* mRNA levels. Spearman test. **I.** Micrographs and quantification of relative cholesterol level after cholesterol assay in Nutlin-treated cells (upper panel) and replicative senescent cells (lower panel). Mean +/- SEM of n=2-3 biological replicates. Scale bar: 10µm. **J.** Crystal violet staining in U18666A-treated MRC5 cells (2,5µM) 6 days after treatment. **K.** Quantification of SA-β-gal positive cells in U18666A-treated cells 72 hours after treatment. **L.** RT-qPCR of *p21<sup>CIP1</sup>* gene in U18666A-treated MRC5 cells, 72 hours after treatment.

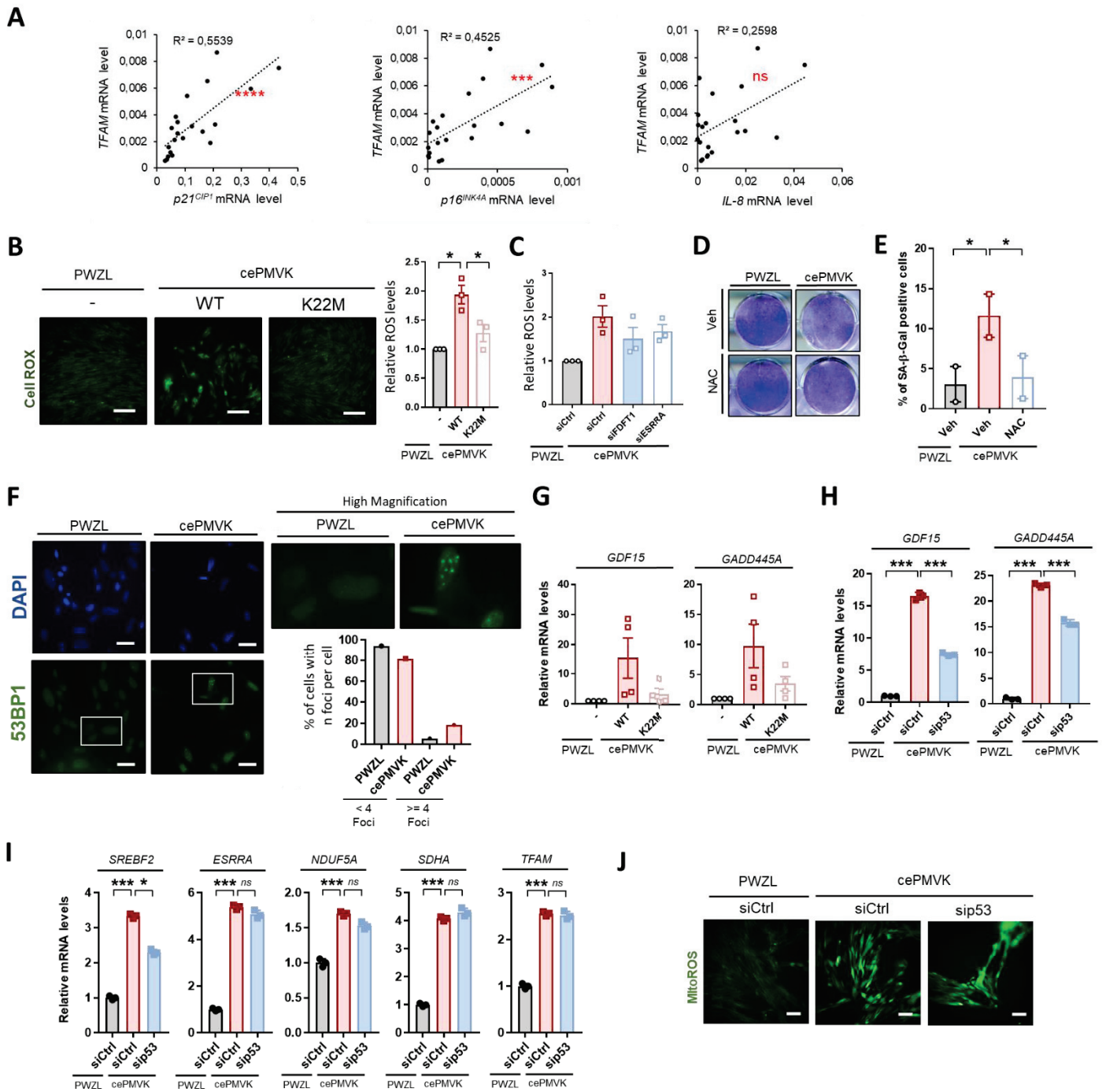


### Supplemental Figure 3.

**A.** RT-qPCR of *TFBM1* and *TFBM2* genes in WT- and KD-PMVK-overexpressing cells. **B.** RT-qPCR of *ESRRR* and  $ERR\alpha$ -target genes (including *UQCRCF1*, *NDUF5A*, *SDHA*, *SDHB*) in PMVK-overexpressing cells, previously transfected by siFDFT1. **C.** Linear regression analyses between *SREBF2* mRNA levels and *ESRRR* or  $ERR\alpha$ -target genes (*UQCRCF1*, *NDUF5A*, *SDHA*, *SDHB*) mRNA levels. Spearman test. **D.** Crystal violet staining in PMVK-overexpressing cells previously transfected by siFDFT1. Day 10. Representative experiment (n=4). **E.** Quantification of number in PMVK-overexpressing cells previously transfected by siFDFT1. Day 5. Mean +/- SEM of n=4 independent experiments. **F.** RT-qPCR of *ESRRR* and  $ERR\alpha$ -target genes (*UQCRCF1*, *NDUF5A*, *SDHA*, *SDHB*) in PMVK-overexpressing cells previously transfected with siESRRR (left panel) or treated for every two days during 21 days with XCT-790 (right panel). Mean +/- SEM of n=4 independent experiments for siESRRR and mean +/- SD of one representative experiment for XCT-790 experiment (n=3). **G.** RT-qPCR of PMVK mRNA levels during in PMVK-overexpressing cells previously transfected with siESRRR or treated for every two days during 21 days with XCT-790. Mean +/- SEM of n=4

independent experiments for siESRRA and mean  $\pm$  SD of one representative experiment for XCT-790 experiment (n=3).





### Supplemental Figure 4.

**A.** Linear regression analyses between *TFAM* mRNA levels and *p21<sup>CIP1</sup>*, *p16<sup>INK4A</sup>* or *IL*-mRNA levels. Spearman test. **B.** Total ROS quantification in WT- and KD-PMVK-overexpressing cells. Mean +/- SEM of n=3 independent experiments. **C.** ROS quantification in PMVK-overexpressing cells and previously transfected with, siCtrl, siFDFT1 or siESRRA. Mean +/- SEM of n=3 independent experiments. **D.** Crystal violet of PMVK-overexpressing cells upon NAC treatment during 12 days. **E.** Quantification of SA-β-gal positive cells positive cells in PMVK-expressing cells upon NAC treatment during 6 days. **F.** Immunofluorescence micrographs of cells PMVK-overexpressing cells, stained for 53BP1 protein (left panel) and quantification of the percentage of cells with less or more than 4 foci per nuclei. **G.** RT-qPCR of p53 target-genes *GADD445A* and *GDF15* in WT- or KD-PMVK-overexpressing cells. Mean

+/- SEM of n=4 independent experiments. **H.** RT-qPCR of p53 target-genes *GADD445A* and *GDF15* in PMVK-over expressing cells, previously transfected with siCtrl or sip53. Mean +/- SD representative of n=3 independent experiments. **I.** RT-qPCR of *SREBF2*, *ESRRA*, *ERRα*-target genes (*NDUF5A*, *SDHA*), and TFAM in PMVK-overexpressing cells previously transfected with siCtrl or sip53. Mean +/- SEM of n=4 independent experiments. **j.** Micrographs of mitochondrial ROS in PMVK-overexpressing cells, previously transfected with siCtrl or sip53. Mean +/- SD representative of n=2 independent experiments.

## Supplemental Table 1

GENE	SEQUENCES AND UPL PROBES	
Human - UPL	Sequence	UPL
ACTB-Forward	ATTGGCAATGAGCGGTC	#11
ACTB-Reverse	GGATGCCACAGGACTCCAT	
p21-Forward	TCACTGCTTGTACCCTTGTGC	#32
p21-Reverse	GGCGTTGGAGTGGTAGAAAT	
IL8-Forward	AGACAGCAGAGCACACAAGC	#72
IL8-Reverse	ATGGTTCCTCCGGTGGT	
FDFT1-Forward	AGTTTCGACGCTGTTATCCAG	#42
FDFT1-Reverse	GATAAAATATGCACACTGCGTTG	
ESRRA-Forward	GGGGCAGAAAGTACAAGC	#3
ESRRA-Reverse	ATCACTGGGGCTGCTGT	
TP53-Forward	AGGCCTTGGAACTCAAGGAT	#12
TP53-Reverse	CCCTTTTGGACTTCAGGTG	
SREBF2-Forward	GCTTTCTGCAACACAGCAAC	#2
SREBF2-Reverse	GACAGCAGTGCAGACTTA	
ABCA1-Forward	CCACAAAACATTGCTGCAT	#17
ABCA1-Reverse	GTCACCTCAGCTTCTCATGCT	
UQCERS1-Forward	AGCCTGTGTTGGACCTGAAG	#30
UQCERS1-Reverse	TGGGAATAACAACAGAAAGCAG	
NDUF5A-Forward	GGTGTGCTGAAGAAGACCACT	#76
NDUF5A-Reverse	TTGTGTACAATATTCTAGCCTCTCG	
SDHA-Forward	TCCACTACATGACGGAGCAG	#70
SDHA-Reverse	CCATCTCAGTTCTGCTAAAG	
SDHB-Forward	GGGGCCTGCAGTTCTTATG	#42
SDHB-Reverse	AGGCGCTCCTCTGTGAAGT	
TFAM-Forward	GAACAACCTACCCATATTTAAAGTCA	#61
TFAM-Reverse	GAATCAGGAAGTCCCTCCA	
TFBM1-Forward	TTGTTATGGCAGAACTCAGATG	#53
TFBM1-Reverse	CTTCTGTATTGGCTGCAAGT	
TFBM2-Forward	AATTTGGGAATAGAAGCAGTCC	#56
TFBM2-Reverse	ACTTGGGAACATTCCAACACTTT	
<b>Human - SYBR</b>		
ACTB-Forward	CATGTACGTTGCTATCCAGGC	
ACTB-Reverse	CTCCTTAATGTCACGCAGAT	
p16 <sup>INK4A</sup> -Forward	CGGTCGGAGGCCGATCCAG	
p16 <sup>INK4A</sup> -Reverse	GCGCCGTGGAGCAGCAGCAGCT	

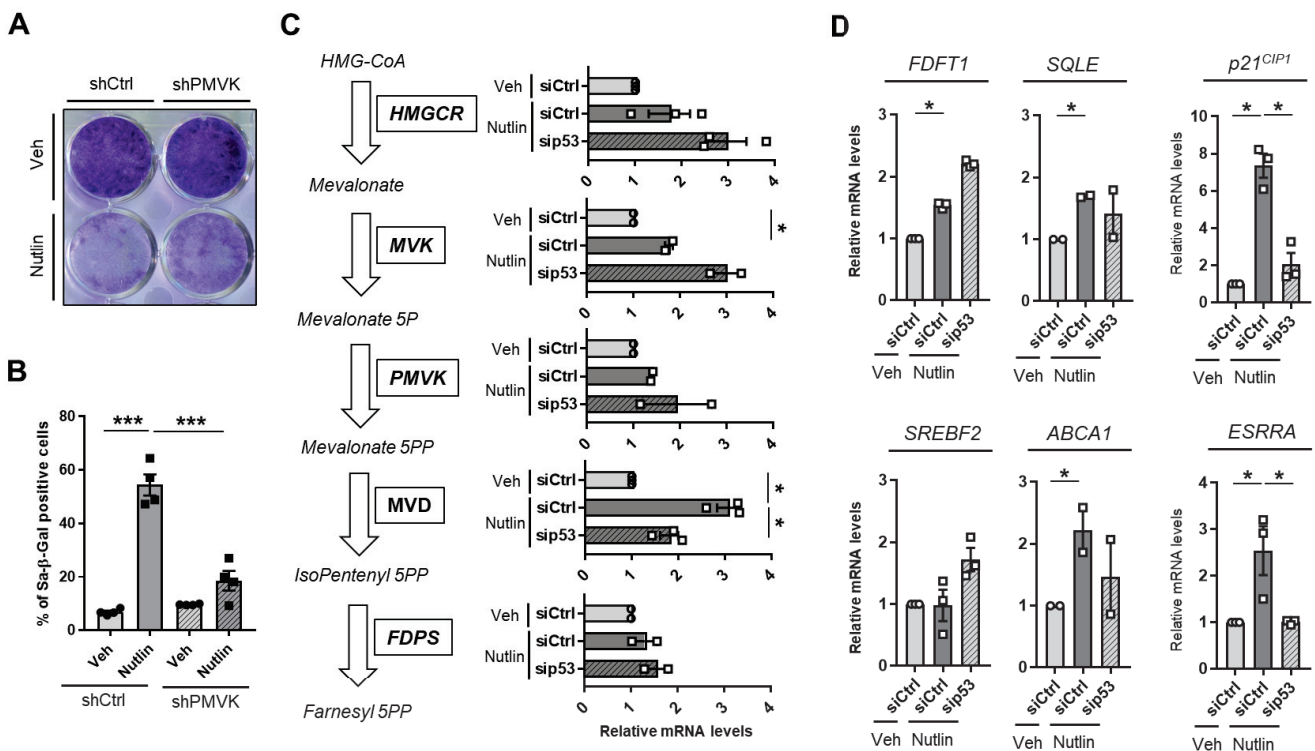
## Supplemental Table 2

Protein	Reference	Use	Dilution
p21 <sup>CIP1</sup>	P1484 (Sigma-Aldrich)	WB	1/500
MVK	sc-27585 (SantaCruz)	WB	1/500
PMVK	sc-390775 (SantaCruz)	WB	1/500
Tub	T6199 (Sigma-Aldrich)	WB	1/5000
GammaH2AX	2577S (Cell signaling)	IF	1/300
53BP1	4937 (Cell signaling)	IF	1/300

## 2.3. Additional Results

### MVA pathway, cholesterol biosynthesis and ER $\alpha$ regulation by p53

As seen in the results of the manuscript, replicative senescence (RS) is delayed by reduced MVA pathway. RS is mediated by telomere shortening, persistent DNA damage response, and p53 activation. A p53-dependent senescence-like state is mimicked by the addition of Nutlin-3A (Efeyan et al., 2007). Nutlin-induced senescence accumulate intracellular cholesterol (see previous manuscript). Moreover, reducing MVA pathway rescued not only RS but also Nutlin-induced senescence, as evidenced by a reversion of cell proliferation arrest (**Figure 26.A**) and increased SA- $\beta$ -gal activity (**Figure 26.B**). Taken together, these data suggest a role of MVA pathway in inducing p53-mediated cell cycle arrest. To test this hypothesis, we evaluated mRNA levels of MVA pathway genes upon Nutlin treatment. Nutlin increased the mRNA levels of different MVA pathway genes, including *MVK* and *MVD* (**Figure 26.A**), suggesting a role of p53 in regulating MVA pathway expression. Nevertheless, siRNA against p53 only reverted elevated *MVD* mRNA levels, highlighting a role for p53 in the sole transcription of *MVD* gene (**Figure 26.C**).



**Figure 26: Role of p53 in the regulation of expression of MVA pathway and cholesterol biosynthesis genes.** **A.** Crystal violet staining 12 days after Nutlin (10 $\mu$ M) treatment in shCtrl and shPMVK-expressing cells. Representative experiment (n=2). **B.** Quantification of SA- $\beta$ -gal positive cells 12 days after Nutlin (10 $\mu$ M) treatment in shCtrl and shPMVK-expressing cells. Mean  $\pm$  SD of one representative experiment (n=2). **C.** Relative mRNA levels of the enzymes of MVA pathway, including *HMGCR*, *MVK*, *PMVK*, *MVD* and *FDPS*, 4 days after Nutlin (10 $\mu$ M) treatment in cells previously transfected by siCtrl or sip53. Mean  $\pm$  SEM of n=2-3 independent biological replicates. **D.** Relative

mRNA levels of the enzymes of cholesterol biosynthesis pathway (upper left panel), namely *FDFT1* and *SQLE*, two cholesterol-sensing genes *SREBF2*, *ABCA1* (lower left panel), associated with *p21<sup>CIP1</sup>* and *ESRRA* (right panel). in cells previously transfected by siCtrl or sip53. Mean +/- SEM of n=2-3 independent biological replicates. For all experiments, one-way ANOVA Tukey's test.

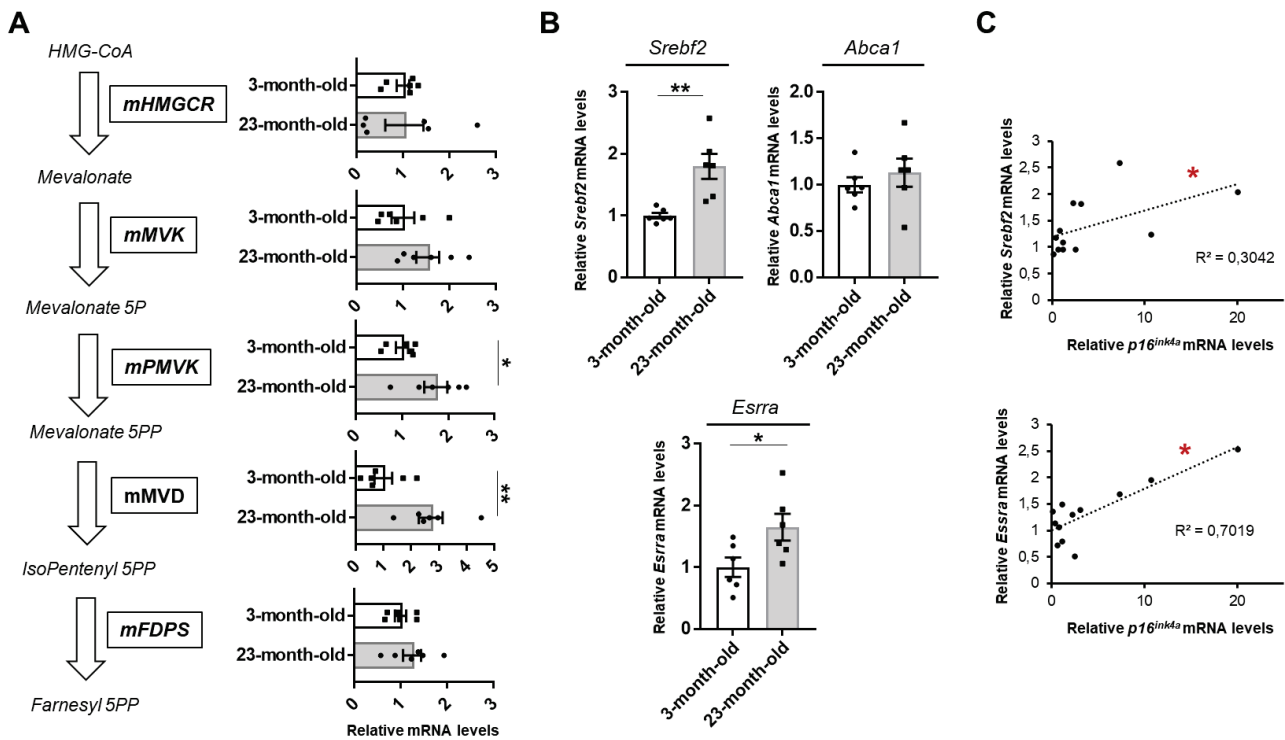
Moreover, Nutlin promoted increased mRNA levels of cholesterol synthesis genes, namely *FDFT1* and *SQLE* (**Figure 26.D**), and the cholesterol-sensing gene *ABCA1* and *ESRRA* (**Figure 26.D**), these two later being upregulated during MeViS. Noteworthy, *SREBF2* mRNA levels were not affected by Nutlin treatment. More importantly, siRNA against p53 only reverted transcriptional upregulation of *ESRRA* gene, and also a well described p53 target gene: *p21<sup>CIP1</sup>* (**Figure 26.D**).

Taken together, these results do not demonstrate a role of p53 in regulating all the MVA pathway or cholesterol biosynthesis genes. One unique gene of the MVA pathway, *MVD*, might be transcriptionally regulated by p53. Interestingly, *ESRRA* mRNA expression seems to be also regulated by p53.

### **Regulation of the expression of MVA pathway genes during aging**

The description of the mechanisms involved in the induction of MeViS raises immediately the question of the biological relevance of this senescence. Because MVA pathway is particularly activated in hepatocytes (Goldstein and Brown, 1990), we evaluated whether MVA pathway was regulated during aging, monitoring the mRNA levels of MVA pathway genes in liver of 3-month-old mice and 23-month-old mice. As previously observed, mRNA levels of *p16<sup>ink4a</sup>* and *ccl3* were increased in liver during aging (**Figure 25**).

Interestingly, the expression of some enzymes of the MVA pathway, including *Pmvk* and *Mvd*, were significantly upregulated during aging, while most of them (*Hmgcr*, *Mvk* and *Fdps*) remained unchanged (**Figure 27.A**). Moreover, the expression of two genes, *Sreb2* and *Abca1*, associated to cholesterol sensing and MeViS, was evaluated. Though mRNA levels of *Abca1* remained unchanged during aging, aged liver displayed an increase of *Sreb2* mRNA levels (**Figure 27.B**). Moreover, mRNA levels of *Esrra* were also upregulated during aging (**Figure 27.B**). Of note, mRNA levels of *Sreb2* and *Esrra* were also positively correlated with mRNA levels of *p16<sup>ink4a</sup>* (**Figure 27.C**), suggesting a potential interplay between these genes.



**Figure 27: Regulation of the expression of MVA pathway genes during aging.** **A.** Relative mRNA levels of the enzymes of MVA pathway, including *Hmgcr*, *Mvk*, *Pmvk*, *Mvd* and *Fdps*, in liver of 3-month-old and 23-month-old mice. Mean +/- SEM of n=6 mice (3 males and 3 females). **B.** Relative mRNA levels of *Srebf2*, *Abca1*, and *Esrra* genes in liver of 3-month-old and 23-month-old mice. Mean +/- SEM of n=6 mice (3 males and 3 females). For previous experiment, unpaired normal t-test. **C.** Linear regression analyses between relative *Srebf2* and *Esrra* mRNA levels and relative *p16<sup>ink4a</sup>* mRNA levels. Spearman test.

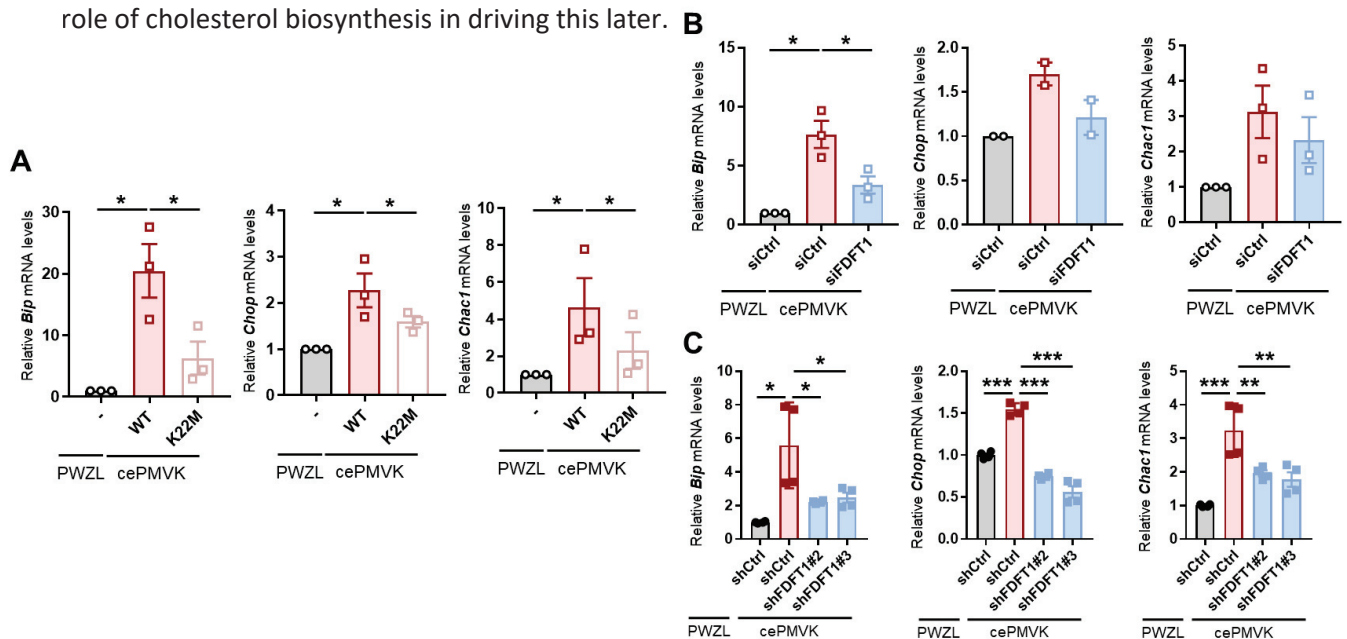
Taken together, these results display a potential age-dependent upregulation at mRNA levels of *Pmvk* and *Mvd* genes. This upregulation is associated with concomitant transcriptional upregulation of cholesterol-sensing gene *Srebf2* and *Esrra*, that could contribute to senescence-related phenotypes.

Involved in the modulation of metabolites fluxes, the post-transcriptional regulation of MVA should also be investigated to confirm this tendency. Unfortunately, our mass spectrometry approach failed to monitor the relative content of metabolites (MVA intermediates and cholesterol) in liver during aging but is needed to evaluate the potential age-related modulation of metabolites flux through MVA pathway.

### Cholesterol biosynthesis branch upregulates ER stress-associated genes in MeViS

Cholesterol is terminally synthesized in the ER lumen. Cholesterol sensing by the ER is also a major way to regulate cholesterol biosynthesis within the cell, as seen with the SREBP regulation by INSIG and SCAP1 ER-resident proteins (See INTRODUCTION - Chapter III - Section 3.2.2). Nevertheless,

cholesterol accumulation in the ER can cause ER stress (Feng et al., 2003). To evaluate ER stress in MeViS, we monitored the expression at mRNA levels of three genes involved in ER stress: *BiP*, *CHOP* and *CHAC1* (Hetz, 2012). The expression of these three genes were upregulated in PMVK-expressing cells, compared to control and KD-PMVK-expressing cells (Figure 28.A). Interestingly and more importantly, the knockdown of the first enzyme of cholesterol pathway FDFT1 by complementary si- and sh-RNA approaches reverted partially this upregulation (Figure 28.B-C), suggesting a functional role of cholesterol biosynthesis in driving this later.



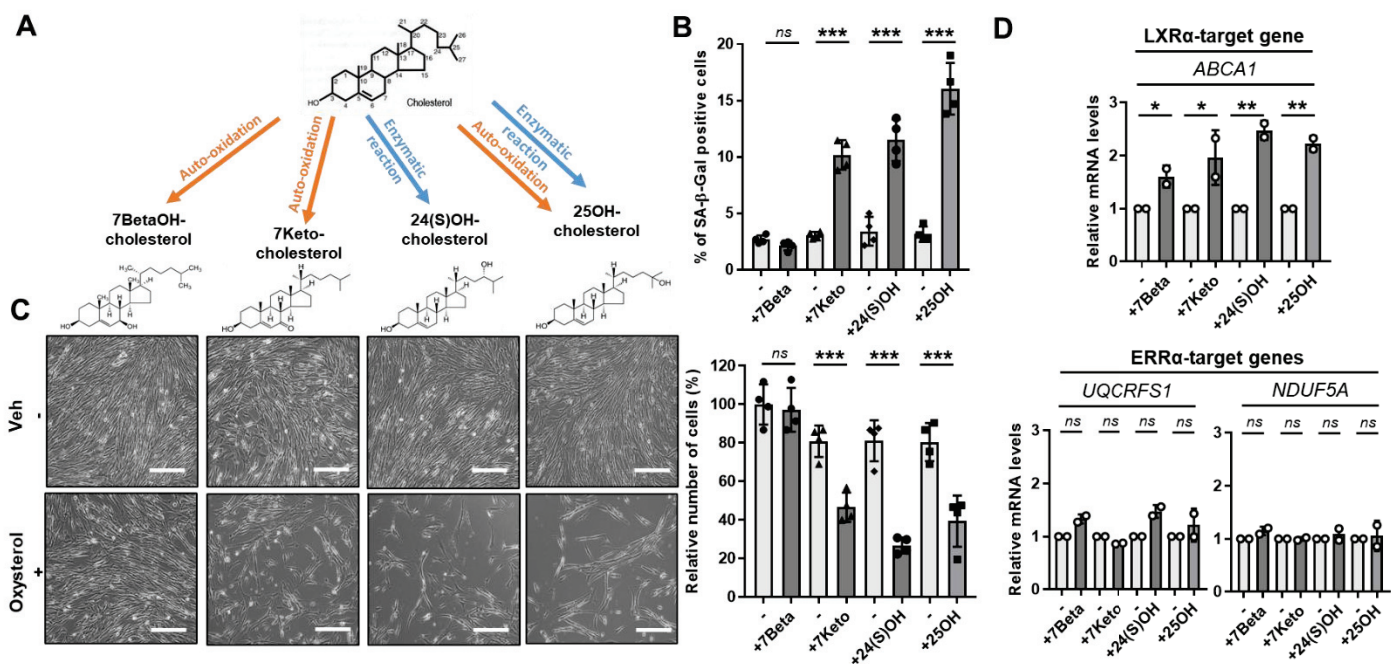
**Figure 28: Cholesterol biosynthesis subbranch upregulates ER-stress genes in MeViS.** **A.** Relative mRNA levels of *BiP*, *CHOP* and *CHAC1* genes in PMVK-expressing (WT) and KD-PMVK-expressing (K22M) cells. Mean +/- SEM of n=3 independent biological replicates. **B-C.** Relative mRNA levels of *BiP*, *CHOP* and *CHAC1* genes in PMVK-expressing cells, previously transfected by siRNA against FDFT1 or in double shFDFT1-PMVK-expressing cells. For siRNA, mean +/- SEM of n=2-3 independent biological replicates. For shRNA, mean +/- SD of one representative experiment (n=3). For all experiments, one-way ANOVA Tukey's test.

### Some oxysterols, cholesterol derivatives, induce a premature senescence

MeViS is associated by an increase of intracellular cholesterol. Excess of cholesterol can be resolved by cells by decreasing import/synthesis but also by promoting export or degradation. Indeed, excess of intracellular cholesterol is deleterious for the cells and can be either exported (*via* ABCA1 or ABCG1 transporters) or degraded by cytochrome P450 enzymes family into other active metabolites, that includes oxysterols, steroids hormones or bile acids. Oxysterols are thus cholesterol derivatives that can be produced by autoxidation, enzymatic reaction or both (Figure 29.A).

We monitored the effect of an exogenous addition of some of these cholesterol derivatives and observed an increased SA- $\beta$ -gal activity upon treatment with 7Keto-cholesterol, 24(S)OH-cholesterol or 25OH-cholesterol (**Figure 29.B**). Concomitantly, treatment with these oxysterols decreased cell density after a few days of treatment (**Figure 29.C**). Of note, increased SA- $\beta$ -gal activity and reduced cell density were specific of only some of the tested oxysterols, as 7BetaOH-cholesterol for example did not affect these parameters (**Figure 29.B-C**).

Because cholesterol is a ligand of ERR $\alpha$  (Wei et al., 2016) and MeViS is partly driven by ERR $\alpha$  transcriptional program, we assessed whether these cholesterol-derivatives could affect also this transcriptional ERR $\alpha$  program. As previously described (Janowski et al., 1996) and as a control of internalization of exogenous oxysterols, all oxysterol treatments activated the transcription of LXR $\alpha$ -target gene *ABCA1* (**Figure 29.D**). Nevertheless, none of oxysterols (pro-senescent or not) increased the expression of the two ERR $\alpha$ -target genes *UQCFS1* and *NDUF5A* (**Figure 29.D**).



**Figure 29: Some oxysterols, cholesterol derivatives, induce a premature senescence.** **A.** Metabolism of cholesterol molecule through auto-oxidation (orange), enzymatic reaction (blue) or mix to form oxysterol derivatives. **B.** Quantification of SA- $\beta$ -gal positive cells in cells upon treatment for 5 days with 7BetaOH-cholesterol (+7Beta: 20 $\mu$ M), 7Keto-cholesterol (+7Keto: 20 $\mu$ M), 24(S)OH-cholesterol (+24(S)OH: 20  $\mu$ M), or 25OH-cholesterol (+25OH: 5 $\mu$ M). Mean +/- SD of one representative experiment (n=3). Unpaired t-test. **C.** Micrographs of cells and quantification of cell number upon treatment for 5 days with 7BetaOH-cholesterol (+7Beta: 20 $\mu$ M), 7Keto-cholesterol (+7Keto: 20 $\mu$ M), 24(S)OH-cholesterol (+24(S)OH: 20  $\mu$ M), or 25OH-cholesterol (+25OH: 5 $\mu$ M). Scale bar: 10  $\mu$ m. Veh: Vehicle. Mean +/- SD of one representative experiment (n=3). Unpaired t-test. **D.** Relative mRNA levels of LXR $\alpha$ -target gene *ABCA1* (upper panel), and two ERR $\alpha$ -target genes *UQCFS1* and *NDUF5A* (lower panel) upon treatment for 5 days with 7BetaOH-cholesterol (+7Beta: 20 $\mu$ M), 7Keto-cholesterol (+7Keto: 20 $\mu$ M), 24(S)OH-cholesterol (+24(S)OH: 20 $\mu$ M), or 25OH-cholesterol (+25OH: 5 $\mu$ M). Mean +/- SD of one representative experiment (n=3). Unpaired t-test.



20 $\mu$ M), 24(S)OH-cholesterol (+24(S)OH: 20  $\mu$ M), or 25OH-cholesterol (+25OH: 5 $\mu$ M). Mean +/- SEM of n=2 independent biological replicates. For all experiments, one-way ANOVA Tukey's test.

Taken together, these results demonstrate that cholesterol oxidative are sufficient to induce a premature senescence response, and suggest that they could contribute in the induction of MeViS. More interestingly, this senescence is independent of the activation of ERR $\alpha$ . Taken together, these data suggest that MeViS could be regulated at least by two complementary mechanisms, namely (i) a cholesterol-mediated induction of ERR $\alpha$  and enhanced mitochondrial oxidative stress, coupled with (ii) an oxysterol-mediated and ERR $\alpha$ -independent induction of senescence.



**- DISCUSSION -**  
**and**  
**- PERSPECTIVES -**



The first two chapters focus on the role of ITPR2/MERCs calcium fluxes (Chapter I) or and on the mevalonate pathway/cholesterol (Chapter II), dealing either with (A) senescence mechanistic or (B) physiological relevance. Beyond these two independent studies, the three last chapters extend these results and open to discussion on the role of mitochondrial functions, cholesterol transfer in MERCs and more widely metabolism of senescent cells.



## **- CHAPTER I -**

### **1. - ITPRs, MERCs, ER-mitochondrial exchanges and cellular senescence -**

Our lab previously identified ITPR2 as a promoter of cellular senescence *in vitro*. This promotion is mediated by a calcium efflux from the ER through ITPR2, and a subsequent accumulation of calcium into the mitochondria through mitochondrial calcium uniporter (MCU) channel. As a consequence, mitochondrial membrane is depolarized, generating ROS and ultimately inducing senescence (Wiel et al., 2014; Ma et al., 2018). The study of *Itpr2* *-/-* mice proposes a functional investigation of the role of ITPR2 in the regulation of senescence *in vivo* and associated physio-pathological conditions, such as aging.

My work demonstrated that the loss of *Itpr2* increased lifespan, reduced immunosenescence and liver senescence, delaying age-related hepatic alterations. In addition to being a regulator of calcium fluxes, my project revealed that ITPR2 is able to promote mitochondria-ER contacts (MERCs) *in vivo*, reinforcing its role in mediating ER-mitochondrial fluxes. Finally, the use of an artificial linker gave the proof of concept that MERCs functionally promote the onset of premature senescence. Overall, my results paved the way to dissect the role of this direct apposition of endo-membranes in the context of cellular senescence and associated-pathophysiological conditions.

#### **1.1. Mechanistics of ITPR2-mediated senescence**

##### **1.1.1. ITPR2 mediates ER-mitochondrial calcium fluxes regulating senescence**

My PhD study explored the role of ITPR2 in regulating ER-mitochondria calcium fluxes. Notably, at basal level, *Itpr2* KO MEFs displayed increased ER calcium content coupled with decreased mitochondrial calcium content, without affecting cytosolic concentration. These results suggest a crucial role of ITPR2 in transferring calcium from ER to the mitochondria in MEFs. Other ITPRs, notably ITPR3, have been shown to promote calcium fluxes from ER to mitochondria (Hayashi and Su, 2007; Marchi et al., 2012; Kuchay et al., 2017; Wu et al., 2017; Filadi et al., 2018). The consequent

accumulation of mitochondrial calcium frequently induces apoptosis (Rizzuto et al., 2012) and MERCs structure coordinates this enhanced mitochondrial calcium uptake to initiate cellular suicide (Hayashi and Su, 2007; Li et al., 2009a; Iwasawa et al., 2011; Doghman-Bouguerra et al., 2016; Marchi et al., 2018). At sublethal concentrations, increased mitochondrial calcium content induces senescence (Wiel et al., 2014; Ma et al., 2018) through mitochondrial membrane (MM) depolarization, and ROS generation (Wiel et al., 2014; Ma et al., 2018). Namely, we observed that *Itpr2* loss reduces MM depolarization and mitochondrial ROS accumulation in MEFs, delaying cellular senescence, reinforcing the previously proposed mechanistic model.

### **1.1.2. What does promote ITPR2 expression or activity?**

ITPR2, MERCs and enhanced ER-mitochondrial fluxes favor the implementation of cellular senescence. Upstream, our lab demonstrated that oncogenic stress or telomere shortening, known inducers of cellular senescence, respectively in hMEC or fibroblasts, promote ER-mitochondrial calcium fluxes (Wiel et al., 2014) (**Figure 30.A**). Other senescence stresses may participate in the upregulation of ITPR2. Indeed, ITPR2 is targeted by post-translational oxidations that modulate its activity (Khan et al., 2013), and oxidative stress generated by superoxide  $O_2^{\cdot-}$  treatment promotes *Itpr2* expression (Bánsághi et al., 2014). Finally, the systemic metabolic stress observed in *ob/ob* mice enhances *Itpr2* expression in liver (Arruda et al., 2014). Overall, these last results suggest that multiple physiological and senescence-associated stresses regulate ITPR2 expression and activity (**Figure 30.A**). In addition to these physiological senescence stresses, transcriptional upregulation of *Itpr2* through knockdown of the transcription repressor RXRA triggers mitochondrial calcium accumulation and cellular senescence as well (Ma et al., 2018).

### **1.1.3. Systemic loss of *Itpr2* and role of other ITPRs**

My results demonstrated the role of ITPR2 in regulating senescence and more widely aging, *in vivo*, particularly in the liver, an ITPR2-rich organ (based on mRNA levels) (Vervloessem et al., 2015) (**Figure 12**). Moreover, no obvious mechanism of compensation of other ITPRs was observed in *Itpr2* KO mice or MEFs, as suggested by unchanged mRNA and protein levels of ITPR1 or ITPR3. Interestingly, an ITPR2-low organ (based on mRNA levels), the lung, displayed also reduction of senescence markers and could be associated also to the enhanced lifespan of *Itpr2* KO mice. Nevertheless, ITPR2 has been shown to be mainly regulated by post-transcriptional mechanisms (*See INTRODUCTION – Chapter II. Section 2.2.5.b*). This consideration of ITPR2-rich and ITPR2-low organs based on mRNA levels (Vervloessem et al., 2015) faces thus obvious limitations and should be extended at least to protein levels.

In other monitored organs (*e.g.* bone or kidney), *Itpr2* loss had no impact on senescence or on age-related features. As ITPR1 or ITPR3 have been associated with MERCs structure and functions (Marchi et al., 2014), their roles in these other organs in senescence-associated pathophysiological contexts, such as aging, remain an open question and could be further investigated. Concerning other ITPRs, no senescence-related studies have been so far conducted in *Itpr3* KO mice and could be eventually considered in the future, since systemic *Itpr1* KO faces a massive embryonic lethality (Matsumoto et al., 1996). Indeed, other data from our lab suggest that ITPR1 or ITPR3 participate also in cellular senescence upon MEK or MYC induction (Wiel et al., 2014) (data not shown) (**Figure 30.A**). In order to decipher the specific role of ITPR1 during aging, a conditional and organ-specific deletion of *Itpr1* could be considered as performed in recent studies (Perry et al., 2020; Yang et al., 2020).

ITPRs are part of membrane contact sites (MCS), called MERCs. These subcellular structures allow also efficient calcium exchanges between ER and mitochondria, but their role in senescence has been never investigated.

#### **1.1.4. MERCs: a new signaling platform regulating senescence**

##### **a. ITPR2: not only a calcium channel but also a regulator of MERCs establishment**

My PhD work demonstrated that the lack of *Itpr2* in liver and in MEFs was able to deplete MERCs in cells, indicating a functional role of ITPR2 in MERC establishment (**Figure 30**). In addition to being a regulator of calcium fluxes, ITPR2 is thus necessary for the implementation of MERCs, reinforcing its role in mediating ER-mitochondrial fluxes.

ITPRs are part of a super-complex linking ER membrane to outer mitochondrial membrane (OMM). Namely, glucose-related protein 75 (GRP75) interacts with ITPRs at the ER side, while it binds to VDAC at the mitochondrial side (Szabadkai et al., 2006 ) (**Figure 30.B**). A direct structural role of ITPRs in the establishment of MERCs has been only recently demonstrated. Last year, the group of G. Hajnóczky elegantly deciphered the specific role of each ITPR in MERCs establishment and calcium fluxes (Bartok et al., 2019), by using DT40 triple ITPRs KO cells and reintroducing each ITPR isoform. Interestingly, DT40 triple KO reduces drastically the number of MERCs (Bartok et al., 2019). In ITPR2-enriched organs, such as liver in our study, the knockout of ITPR2 is sufficient to disrupt MERCs. Moreover, among ITPRs channels, ITPR2 is the most efficient for calcium delivery between the ER and the mitochondria (Bartok et al., 2019), reinforcing our study suggesting a key role of ITPR2 in mitochondrial calcium uptake. In relation to my results, this later study confirms the role of ITPRs in regulating the establishment of MERCs and especially an essential role of ITPR2 (determinant in regards to the other ITPRs) in modulating mitochondrial calcium content.

### **b. Like ITPR2, MERCs regulate cellular senescence**

In my study, the potent role of MERCs in regulating senescence has been pointed out. Firstly, loss of *Itpr2* reduced concomitantly MERCs number and senescence *in vivo*. Secondly, MERCs number was positively associated with senescence markers, in liver and MEFs, suggesting a role of MERCs in regulating senescence. To confirm this hypothesis, the use of an artificial linker, developed by G. Hajnóczky (Csordás et al., 2006), allowed us to demonstrate for the first time the functional involvement of MERCs in promoting cellular senescence *in vitro*.

MERCs are known signaling platforms involved in the regulation of ER and mitochondrial biology through numerous exchanges (*e.g.* calcium and phospholipids) between these two organelles (Csordás et al., 2018). Variations of MERCs dynamics and subsequent ER and mitochondrial functions regulate cellular processes such as bioenergetics, inflammation, autophagy and apoptosis (Marchi et al., 2014). Our study reveals a new role of MERCs in promoting cellular senescence.

#### **i. MERCs favor ER-mitochondrial calcium fluxes**

An artificial linker forcing MERCs establishment induces a premature senescence. Regarding intracellular calcium content, these MERCs both enhanced mitochondrial calcium uptake and reduced ER calcium store triggering potent calcium homeostasis alterations in these organelles. To be noted, cytosolic calcium remained unchanged.

On the mitochondrial side, the MERCs-induced accumulation of mitochondrial calcium promoted mitochondrial membrane (MM) depolarization, mitochondrial ROS and subsequent p53-dependent senescence (**Figure 30.B**). Similar results were obtained in our lab in the context of oncogenic stress in human mammary epithelial cells (hMEC) (Wiel et al., 2014) or as a consequence of ITPR2 upregulation (Ma et al., 2018), reinforcing this mechanistic model. Noteworthy and as a mirror effect of MERCs enhancement, MERCs depletion observed in *Itpr2* KO MEFs reduced mitochondrial calcium accumulation MM depolarization in MEFs. According to other studies, the disruption of MERCs is known to limit mitochondrial calcium uptake (Doghman-Bouguerra et al., 2016). The particular role of mitochondria in senescence will be largely discussed in detail in Chapter III. Most of the literature about MERCs focus on the mitochondrial side of the MERCs, the ER side is rather less investigated.

On the ER side, MERCs induction decreased calcium content. ER calcium is determinant for chaperones involved in protein folding, such as Calreticulin or BiP/GRP78, and variations of ER calcium content can subsequently lead to an unfolded protein response (UPR) and ER stress (Krebs et al., 2015; Carreras-Sureda et al., 2018). ER stress is an integrated cellular response composed by three different branches that include three independent main sensors: IRE1, ATF6 and PERK (Hetz, 2012). ER stress is not considered as a classical mark of senescent cells (Hernandez-Segura et al., 2018a). Nevertheless,



Thapsigargin, a classical SERCA inhibitor leading to ER calcium depletion and subsequent ER stress, induces senescence in proximal tubular epithelial cells (Liu et al., 2014). Different branches of ER stress, such as ATF6 branch, may promote senescence depending on context (Cormenier et al., 2018; Druelle et al., 2016; Kim et al., 2019) and ER stress has been reported in different senescence models (Blazanin et al., 2017; Tezze et al., 2017; Wang et al., 2016b; Yuan et al., 2017). Overall, an essential question still remains: is the reduction of ER calcium observed in MERCs-induced senescent cells sufficient to induce an ER stress? (**Figure 30.B**). If so, it would be of interest to test whether reducing ER stress through chemical chaperones for example, such as 4-phenylbutyric acid (4-PBA), would impact senescence induced by MERCs. Furthermore, because the use of antioxidants rescued MERC-induced senescence and ER stress can generate ROS (Lee et al., 2007a; Kiviluoto et al., 2013; Carreras-Sureda et al., 2018), the MERC-induced oxidative stress, which is necessary to induce senescence, can also stem from ER stress.

#### ii. Potent roles of non-calcium fluxes in senescence?

Besides calcium, MERC is an exchange site for numerous other molecules, including phospholipids, cholesterol or even ROS (Csordás et al., 2018). My preliminary results suggest that the partial knockdown of ITPRs individually or in combination, is not able to rescue the strong senescence phenotype we observed after forcing MERCs (data not shown). This result could suggest that transfers of other metabolites or signaling actors of the MERCs are involved in the regulation of this type of senescence. Oxysterol-Binding Protein-Related Protein 5 (ORP5) and 8 (ORP8) are ER-anchored proteins involved in phospholipids exchanges at MERCs. Importantly, ORP5 administers phospholipid and calcium transfers, while ORP8 function is exclusively limited to phospholipid transfers. ORP5 may boost cell proliferation when it is constitutively expressed (Du et al., 2018) and inversely promote senescence when it is downregulated by siRNA (Rochin et al., 2019). More importantly, sole downregulation of the exclusive phospholipid-exchanger ORP8 does not affect senescence (Rochin et al., 2019). Taken together, these results suggest that at MERCs calcium transfers could be predominant on phospholipid transfers in the regulation of senescence and may reinforce our working model (**Figure 30**). Nevertheless, further studies need to be conducted to confirm these observations.

#### iii. MERCs modulation and regulation of cellular senescence

Beyond the role of lipids and calcium fluxes between ER and mitochondria, various other key MERC-resident proteins are able to modulate senescence (**Table 3**). Firstly, MERCs plasticity is tuned by their number, regulated by multiple scaffold and tethering factors that include among others MFN1 and MFN2 (Csordás et al., 2018). *Mfn1* and *Mfn2* mRNA and protein levels increase during TIS in

melanoma, contributing to heightened mitochondrial mass and subsequent enhanced oxidative phosphorylation (Martínez et al., 2019). According to functional studies, MFN1 and MFN2 have been linked to senescence. Indeed, *Mfn2* gene has been initially called Hyperplasia Suppressive Gene (HSG) at its discovery (Chen et al., 2004), suggesting a tumor suppressor role. Indeed, its depletion boosts cellular proliferation of both B-cell lymphoma cell line and MEFs (Chen et al., 2014). On the contrary, MFN2 triggers hyperfused mitochondria and is involved in the regulation of mesenchymal stem cells senescence induced by deletion of fibroblast growth factor (Son et al., 2017). To be noted, two recent studies reported an increased MERCs number coupled to an increased mitochondrial calcium uptake in RS of hippocampal neurons (Calvo-Rodríguez et al., 2016) and endothelial cells (Madreiter-Sokolowski et al., 2018). Taken together and in light of my results, these data reinforce the hypothesis of a role of MERCs in modulating senescence.

Function of proteins in MERCs	Name of proteins	Effect on proteins (-/+)	Effects on MERCs number	Effects on ER and/or MT	Senescence-Associated Phenotype	References
<b>TETHERING</b>	<b>MFN1</b>	+ (Stabilization)	N/A	Increases MT mass	Increases senescence	(Park et al., 2010)
	<b>MFN2</b>	- (Fragmentation)	N/A	Increases ER-MT Ca <sup>2+</sup> fluxes + enhanced OXPHOS	N/A	(Pulli et al., 2019)
	<b>Grp75</b>	+ (Overexpression)	N/A	N/A	Delays replicative senescence	(Kaul et al., 2003)
	<b>FIS1</b>	- (shRNA) + (Overexpression)	N/A N/A	Hyperfused MT Rescues hyperfused MT	Increases senescence Rescue DFO-induced senescence	(Lee et al., 2007b) (Yoon et al., 2006)
<b>CALCIUM TRANSFER</b>	<b>ITPR1</b>	N/A - (shRNA)	N/A N/A	N/A N/A	N/A Reduces OiS	(Arruda et al., 2014) (Wiel et al., 2014)
	<b>ITPR2</b>	- (shRNA) - (siRNA) - (KO)	N/A N/A Decreased	Limits ER-MT Ca <sup>2+</sup> fluxes / ROS / MMD Limits ER-MT Ca <sup>2+</sup> fluxes / MT ROS Limits ER-MT Ca <sup>2+</sup> fluxes MT ROS / MMD	Reduces OiS and RS Reduces senescence Reduces senescence (liver/MEFs)	(Wiel et al., 2014) (Ma et al., 2018) This manuscript
	<b>ITPR3</b>	- (shRNA)	N/A	N/A	Reduces OiS	(Wiel et al., 2014)
	<b>ITPR1/2/3</b>	- (Triple KO)	Decreased	Limits ER-MT Ca <sup>2+</sup> fluxes	N/A	(Bartok et al., 2019)
	<b>MCU</b>	- (shRNA)	N/A	Limits ER-MT Ca <sup>2+</sup> fluxes	Reduces OiS	(Wiel et al., 2014)
<b>OTHERS</b>	<b>PACS-2</b>	- (KO) - (KO)	N/A N/A	N/A N/A	Resistance to p53-dependent CCA and NF-KB program	(Atkins et al., 2014; Barroso-González et al., 2016)
	<b>p66Shc</b>	- (KO)	N/A	N/A	Delays RS	(Migliaccio et al., 1999)
	<b>HK2</b>	+ (Overexpression)	N/A	Reduces MT Ca <sup>2+</sup> uptake / MT ROS	N/A	(Pan et al., 2018)

**Table 3: MERC-key proteins involved in the regulation of senescence-associated features.** Summary of the studies reporting a potential role for MERCs proteins involved in tethering, calcium transfer or other functions, in the regulation of features of cellular senescence. In some studies, the role of modification of MERCs protein in the variations of MERCs number is indicated. N/A: not investigated. ER: Endoplasmic Reticulum; MT: Mitochondria; MMD: Mitochondrial Membrane Depolarization. OiS: Oncogene-induced Senescence. RS: Replicative Senescence. CCA: Cell Cycle Arrest.

The next step would be to decipher main actors involved in MERC-induced senescence. The “1<sup>st</sup> generation” of artificial linker we used (Csordás et al., 2006) faces some limitations because of its constitutive high expression, inducing a strong senescence phenotype, without excluding some

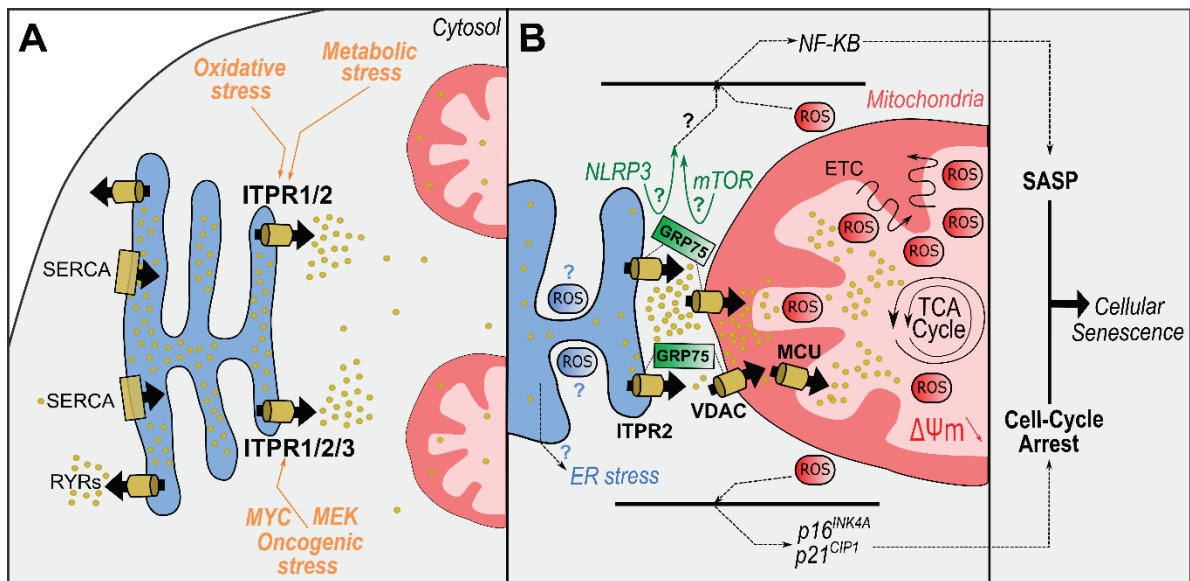
punctual cell death events. This strong phenotype limited our study of individual MERCs actor involved in MERC-induced senescence. Indeed, only modulating downstream effectors (such as ROS with antioxidants or p53 with siRNA) was able to revert this senescence. In order to overcome this technical issue, the “2<sup>nd</sup> generation” of inducible linker (Csordás et al., 2010; Booth et al., 2016) could be a more appropriate tool for an high-throughput siRNA screening based on MERCs key resident proteins.

Of note and beyond their quantity, the quality of MERCs has been proposed to depend on the exact distance between ER and mitochondria (<50nm but heterogeneous) (Giacomello and Pellegrini, 2016). Namely this “thickness” is defined as the width of the cleft separating OMM from ER, and subdivides MERCs in loose (~10nm) and tight (~25-40nm) structures (Cieri et al., 2018), with sometimes functional opposite roles. For example, while loose MERCs could promote ER-mitochondrial calcium transfers, very tight MERCs could limit them (Giacomello and Pellegrini, 2016). Altogether, the emergence of subcategories of MERCs adds another layer of complexity that should be acknowledged and taken into consideration for a further study of the role of MERCs in senescence.

#### **iv. A role of MERCs in SASP regulation**

Our study demonstrated that at least some SASP factors secreted by cells in MERC-induced senescence are NF- $\kappa$ B dependent. Interestingly, *Itpr2* KO liver and MEFs displayed a dampened inflammatory response whereas inducing MERCs enhances SASP and an inflammatory response, as evidenced by increased *Il-8* and *Ccl3* mRNA levels. A recent review described the link existing between MERCs and inflammation (Missiroli et al., 2018b). Whether MERCs can promote inflammation through SASP remains an open question to assess. SASP is known to be regulated by different upstream pathways, including NLRP3 and mTOR (*See INTRODUCTION - Chapter I. – Section 1.3.2.*). Interestingly, these two regulators are found in MERCs fraction (**Figure 30.B**). While the activity of mTOR pathway has not been investigated in our study, the investigation of NLRP3 in our fibroblast model is limited due to a low level of endogenous proteins. Nevertheless, these actors being both involved in MERCs structure and SASP open new perspectives and should be further considered (**Figure 30.B**).

The inflammatory part of SASP can promote paracrine senescence (Acosta et al., 2013; Chen et al., 2015; Bird et al., 2018) and acts as a positive feedback loop to reinforce the senescence phenotype (Acosta et al., 2008a; Kuilman et al., 2008). We can thus speculate that ITPR2, through a higher amount of functional and closer MERCs, could contribute to cellular senescence by two complementary and synergistic mechanisms, namely i) the increased mitochondrial calcium level and the subsequent ROS production which can induce cell cycle arrest and SASP and ii) the pro-inflammatory SASP production that is known to mediate autocrine and paracrine senescence. To test this last hypothesis, the effect of MERC-induced SASP on normal cells remains to be evaluated.



**Figure 30: Working model for the role of ITPR2 and MERCs in mediating cellular senescence.** **A.** In normal non-senescing cells, signals (orange) promote expression or activity of ITPRs. **B.** These signals could promote MERCs establishment for example through an upregulation of ITPR2, and an increased number of complex ITPR2-GRP75-VDAC1. Elevated expression or activity of ITPRs could enhance MERCs formation and subsequent ER-mitochondrial calcium exchanges. On the ER side, subsequent decreased in calcium content could generate ER stress, participating in elevated oxidative stress. On the mitochondrial side, calcium uptake by VDAC and MCU increases calcium concentration in the matrix and enhances ROS generation. These ROS participate in driving a NF-κB-dependent SASP and induction of cell-cycle arrest ultimately leading to cellular senescence. Present in MERCs fraction, NLRP3 and mTOR could participate to the regulation of SASP.

## 1.2. Physiological relevance of ITPRs-mediated senescence

### 1.2.1. ITPRs, MERCs and aging

Our study in mice demonstrated that *Itpr2* loss delayed aging. Indeed, enhanced global lifespan in females by this loss was associated with a decreased immuno-senescence and a reduction of age-related defects in liver, including fibrosis and steatosis. As a consequence, the metabolic stress triggered by hyperglycemia was better tolerated in old (26-month-old) *Itpr2* KO than WT control littermates. Except for this metabolic stress test, the differences between *Itpr2* KO mice and WT control littermates were observed post-mortem in 23-month-old mice. Whether these differences could have been there (even attenuated) earlier or could have been higher later remains unknown. Besides, other additional data reveal a reduction of some other senescence markers, not only in liver, but also in lung (**Figure 24**). Indeed, p19<sup>ARF</sup>, that contributes to aging-associated decline in lung function (Hashimoto et al., 2016b), was reduced in *Itpr2* KO mice (data not shown), suggesting a broader role of ITPR2 in regulating senescence and not restricted to the liver. Noteworthy, *Itpr2* mRNA transcripts were slightly upregulated around 1.5 in liver of 23-month-old mice compared to 3-month-old mice

**(Figure 25).** Nevertheless, ITPR2 protein levels or activity are unknown and could reflect other changes than these observed for mRNA levels. Notably, ITPR2 protein level could be further assessed during aging, while measuring ITPR2 activity remains challenging, due to limitations in measuring calcium fluxes *in vivo*. To go further in the characterization of age-related changes associated to ITPR2 and in line with its role in MERCs establishment, the ITPR2 localization in MERCs fraction during aging could also give us additional clues.

My results demonstrated also a positive association between intrahepatic MERCs number, senescence markers and some age-related parameters, such as intrahepatic fibrosis. Interestingly, ITPR1, which can also regulate senescence (Wiel et al., 2014), and the linker, which induces MERCs, have also been shown to promote steatosis and to alter glucose homeostasis in obese mice (Arruda et al., 2014), similarly to our results in *Itpr2* KO mice during aging. Obesity promotes senescence, which mediates organismal dysfunctions including steatosis and glucose homeostasis alterations (Ogrodnik et al., 2017; Palmer et al., 2015, 2019). Even though cellular senescence was not investigated in Arruda study (Arruda et al., 2014), we can speculate that ITPR1 and MERCs effects are mediated, at least in part, by cellular senescence. ITPR1 and ITPR2 may thus promote senescence and act as key drivers of age-related defects in the liver, including steatosis and alterations of glucose homeostasis. However, it is important to remind that MERCs contacts are crucial in the physiological role of liver. More particularly, reduced MERCs may also contribute to insulin resistance in liver and muscle (Tubbs et al., 2014b, 2018), raising the importance of a balanced number of MERCs. According to the physiological role of MERCs in liver, deficient MERC-mediated lipid transfer and phospholipid synthesis lead to Non-Alcoholic Steato-Hepatitis (NASH) phenotype (Hernández-Alvarez et al., 2019). Overall, MERCs homeostasis is crucial in liver, and an unbalanced number of MERCs is linked to age-related pathological conditions.

Beyond the role of ITPR in regulating liver phenotypes and associated metabolic syndromes, some recent reviews have nicely depicted the role of MERCs in global physiological aging (Janikiewicz et al., 2018; Moltedo et al., 2019). Indeed, MERCs are associated with numerous age-related pathologies, including neurodegenerative diseases (Area-Gomez et al., 2012; Manfredi and Kawamata, 2016; Lee et al., 2018), age-related muscular defects (Sebastián et al., 2016; Wang et al., 2018) or atherosclerosis (Moulis et al., 2019) (**Table 4**). To be noted, the majority of these pathologies are functionally linked to cellular senescence (**Table 4**). Our study thus suggests that senescence could partly mediate some effects of MERCs during aging (**Figure 30**).

Age-related pathologies		MERCs involvement (+/-)	MERCs involvement (REF)	Senescence involvement (+/-)	Senescence involvement (REF)
Neurodegenerative diseases	AD	(+)	(Hedskog et al., 2013; Area-Gomez et al., 2018)	(+)	(Bussian et al., 2018; Zhang et al., 2019)
	PD	(+)	(Rodriguez-Arribas et al., 2017; Lee et al., 2018)	(+)	(Chinta et al., 2018; Zhu et al., 2019)
	ALS	(+)	(Manfredi and Kawamata, 2016)	(+)	(Trias et al., 2019)
	Glaucoma	(+)	(Nivison et al., 2017)	(+)	(Skowronska-Krawczyk et al., 2015)
Metabolic syndroms	T2D	(-)	(Tubbs et al., 2014b, 2018)	(-)	(Helman et al., 2016)
		(+)	(Arruda et al., 2014; Thoudam et al., 2019)	(+)	(Ogrodnik et al., 2017; Aguayo-Mazzucato et al., 2019; Khosla et al., 2020)
	NAFLD	N/A	<N/A	(+)	(Papatheodoridi et al., 2020)
Age-related muscular defects	Sarcopenia	(+)	(Sebastián et al., 2016; Huang et al., 2018; Wang et al., 2018)	(+)	(García-Prat et al., 2016; Habiballa et al., 2019; Sugihara et al., 2018)
Atherosclerosis	-	(+)	(Moulis et al., 2019)	(+)	(Uryga and Bennett, 2016)
Osteoarthritis	-	N/A	N/A	(+)	(McCulloch et al., 2017)
Lung Chronic Dis.	-	N/A	N/A	(+)	(Schafer et al., 2017; Barnes et al., 2019)
Immunosenescence	-	N/A	N/A	(+)	(Ventura et al., 2017)

**Table 4: MERCs roles in senescence-associated age-related pathologies.** Summary of the studies reporting a (direct or indirect) role for MERCs in senescence-associated age-related pathologies. N/A: not investigated. AD: Alzheimer Disease. PD: Parkinson Disease. ALS: Amyotrophic Lateral Sclerosis. T2D: Type 2 Diabetes. NAFLD: Non-Alcoholic Fatty Liver Disease.

### 1.2.2. ITPR2, MERCs and other senescence-associated pathophysiological contexts

Beyond aging, cellular senescence participates in other physio-pathological conditions, such as cancer, wound healing or development. As *Itpr2* KO favors escape from OIS (Wiel et al., 2014), *Itpr2* KO mice should be more prone to develop cancerous lesions. Prior to my PhD, data generated by the lab using a skin papilloma induction protocol (Abel et al., 2009) displayed that *Itpr2* loss accelerated tumor initiation (data not shown). Noteworthy, the number of spontaneous lesions was unchanged at sacrifice between *Itpr2* KO mice and their control littermates. Taken together, these data indicate that ITPR2 could protect against some damage-induced cancer lesions, such as DMBA/TPA-induced papilloma. Our lab previously demonstrated that *ITPR2* expression is increased in benign tumors such as oncocytoma and prostatic intraepithelial neoplasia (Wiel et al., 2014) benign tissues being known to accumulate senescent cells (Collado et al., 2005). In line with these results, and according to OncoPrint database, *ITPR2* is mostly downregulated in cancer, as seen in cervical, esophageal, head and neck, melanoma, or pancreatic cancer. Besides, an association was found between clear renal cell

carcinoma and germline genetic variations of *ITPR2* (Zhang et al., 2017). Finally, while ITPR triple KO in T-lymphocytes precursors leads to a T-cell acute lymphoblastic leukemia (Ouyang et al., 2014), the high expression of ITPR2 is a marker of worse prognosis in cytogenetically normal acute myeloid leukemia (CN-AML) (Shi et al., 2015). Altogether, these results suggest that ITPR2 may mediate some tumor-suppressor effect of cellular senescence *in vivo*, while the last study in CN-AML may link anti- and pro-tumoral effects of cellular senescence. Moreover, other MERCs actors are regulated by oncogenic or tumor suppressor mechanisms. Through modification of calcium fluxes, MERCs promote cell death, mitochondrial bioenergetics, autophagy, and inflammation, all these four processes being involved in regulation of tumorigenesis (van Vliet et al., 2014; Sassano et al., 2017). To conclude, the precise role of ITPR2 and MERC establishment in control of tumorigenesis could be further assessed crossing *Itpr2* KO mice and some established KRAS-driven cancer mouse models for example. In the context of our study, a model of KRAS-driven HCC could be of high interest (Ye et al., 2014).

Cellular senescence is also involved in tissue regeneration and development (*See INTRODUCTION – Chapter I. – Section 1.4.2./3.*). In wound healing, the role of ITPR2 has not been strictly established, but a few studies suggest a role for ITPR2 in migration of fibroblasts and secretion of collagen (Belkacemi et al., 2018), these two processes being crucial in tissue regeneration (Enyedi and Niethammer, 2015). Finally, in development, the role of ITPRs seems to be dominated by ITPR1 as evidenced by massive embryonic lethality in *Itpr1* KO mice (Matsumoto et al., 1996). Beyond this detrimental cell autonomous effect of ITPR1 during embryonic development, a conditional ITPR1/ITPR2 double KO model (DKO) in cardiovascular cell lineages underlined the non-cell autonomous role of ITPR1 and ITPR2 in embryonic development through fetal-maternal exchanges (Yang et al., 2020). More broadly, the two mitofusins MFN1 and MFN2, accounting for determinant MERCs tethers, are required for proper embryogenesis (Chen et al., 2003), giving some clues of an involvement of MERCs structure in embryonic development. Nevertheless, no links with cellular senescence have been established so far between ITPRs or MERCs and wound healing or embryogenesis.

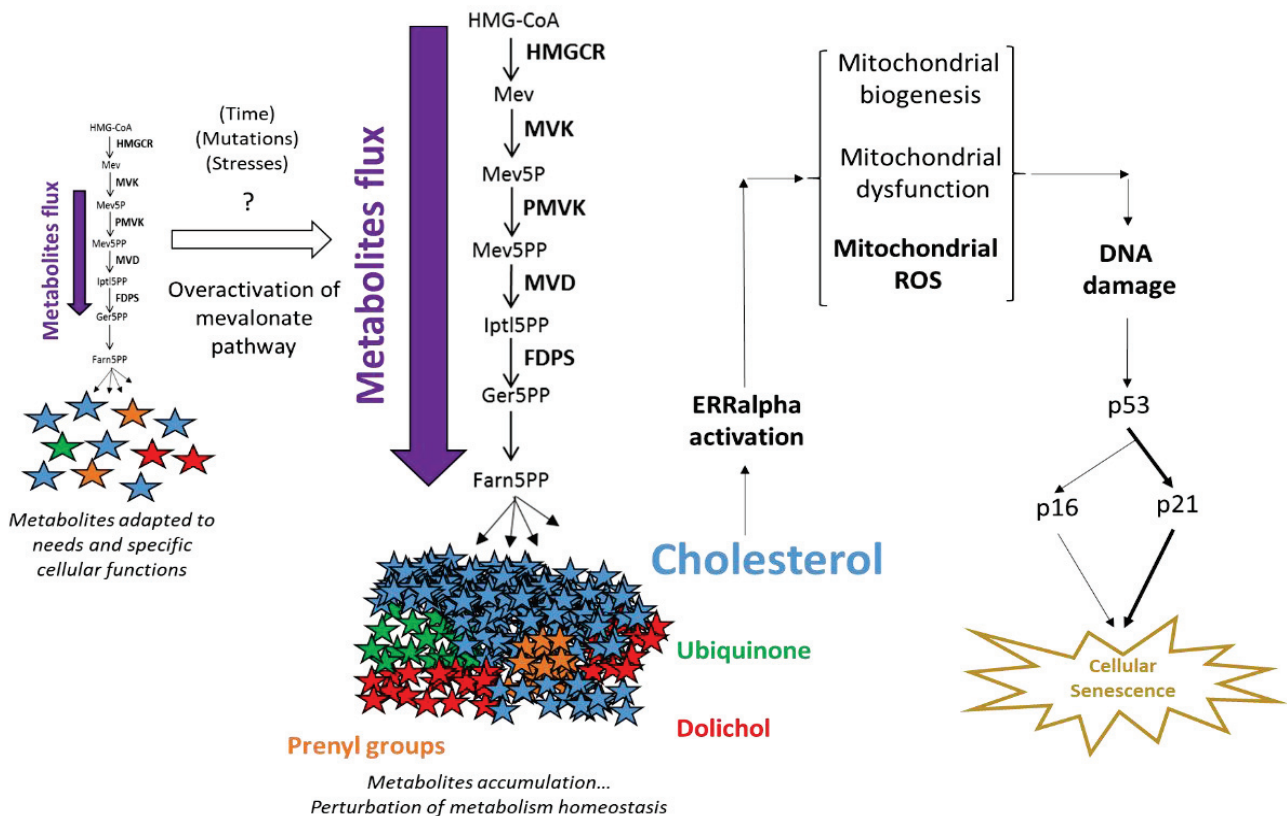
Overall, our results suggest that ITPRs and enhanced ER-mitochondrial calcium fluxes regulate cellular senescence and may be involved in pathophysiological contexts, such as aging. MERCs, which act as a signaling platform modifying both ER and mitochondrial functions and which could be reinforced by ITPR presence, may thus promote these ER-mitochondrial fluxes and subsequent senescence.



## - CHAPTER II -

### 2. - Mevalonate pathway, cholesterol and cellular senescence -

During my PhD study, I unraveled also a new subtype of metabolic stress-induced senescence arising from enhanced mevalonate (MVA) pathway activity. This MVA-induced senescence (MeViS) is mediated, at least in part, by the intracellular accumulation of cholesterol. This cholesterol is a ligand of  $ERR\alpha$  (Wei et al., 2016) and upregulates its program, triggering both mitochondrial biogenesis and membrane depolarization, generating ROS and leading ultimately to senescence (**Figure 31**).



**Figure 31:** Mechanistic model for the role of MVA pathway in regulating cellular senescence.

MVA pathway is regulated by nutrient supplies or cell fate, and the metabolite flux coming from this pathway is adapted to the cellular needs. With aging and stress, heightened activity of the MVA pathway enhances this metabolic flux leading to increased end-products, including cholesterol. Cholesterol accumulation triggers activation of  $ERR\alpha$  transcriptional program and leads to an enhanced mitochondrial biogenesis and subsequent dysfunction. These mitochondrial defects boost ROS production and ultimately lead to DNA damage and p53 dependent activation of cellular senescence.



## 2.1. Mechanistics of MeViS

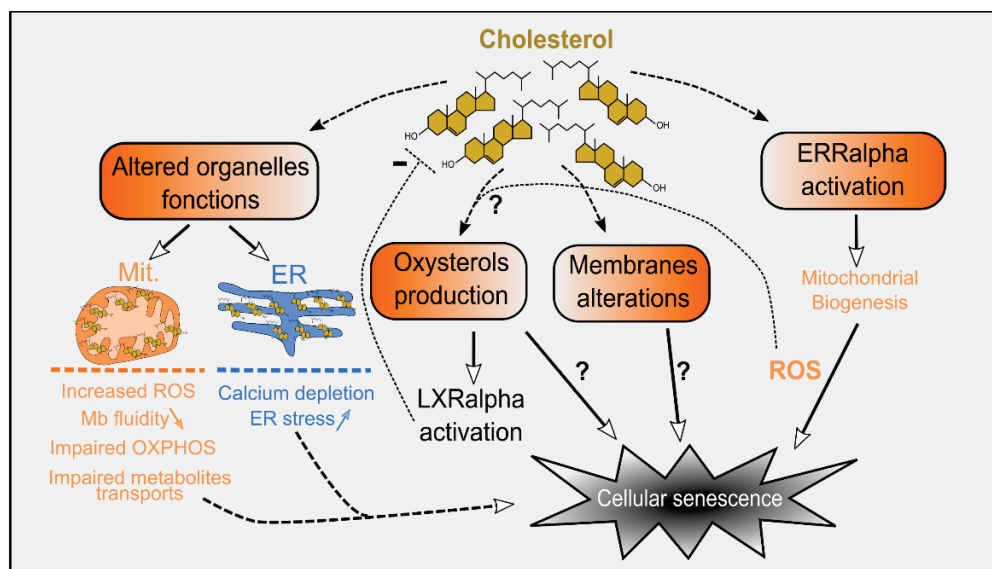
### 2.1.1. Consequences of abnormal cholesterol accumulation

As mentioned in my introduction, cholesterol homeostasis within the cells is maintained by 4 different mechanisms: synthesis/import opposed to degradation/export. Our results support that MVA-mediated synthesis of cholesterol mediates, at least partly, the senescence phenotype of MeViS (**Figure 31**). In line with these results, my data indicate that reducing MVA activity or the synthesis of cholesterol has a mirror effect and delays also replicative senescence. Early clues indicated that exogenous lipids like ceramide may induce senescence response (Venable et al., 1995) and further evidence confirmed that lipid-derivatives, such as 4-HNE, mediate similar pro-senescent signals (Riahi et al., 2015; Flor et al., 2016). Nevertheless, the particular role of endogenous bio-synthesized lipids was so far unknown. Taken together, my results shed light on the role of cholesterol accumulation as a functional inducer of senescence, through the activation of an ERR $\alpha$  program (**Figure 32**).

My results suggest that additionally to ERR $\alpha$  program, MVA-induced senescence activates transcription of ER stress genes, including *BiP* and *CHOP* (**Figure 28**). Moreover, knocking down the first enzyme of cholesterol biosynthesis reverted this increase (**Figure 28**). As cholesterol is terminally synthesized in the ER (Soccio and Breslow, 2004), we can speculate that it accumulates in the ER. ER cholesterol accumulation is known to promote ER calcium depletion and cytotoxicity *via* ER stress activation in macrophages (Feng et al., 2003). ER stress could thus participate in the induction of MeViS. This hypothesis should be further considered using for example chemical chaperones, such as 4-PBA, to reduce ER stress and evaluate impact on MeViS.

Cholesterol accumulation may be induced by different other ways than increase synthesis through the MVA pathway. ABCA1 and ABCG1 constitute two ABC transporters involved in the extracellular efflux of cholesterol. ABCA1/ABCG1 deletion is another genetic way to promote cholesterol accumulation and is at the origin of tissue alterations, as suggested by decreased insulin secretion of pancreatic B islet, and enhanced associated inflammation markers, including SASP marker IL-1 $\beta$  (Kruit et al., 2012). Moreover, an age-dependent decrease of ABCA1 level and subsequent accumulation of cholesterol can be observed in macrophages and result in age-related macular degeneration (AMD) (Sene et al., 2013). Nevertheless, in our model, siRNA strategy against ABCA1 and ABCG1 individually or in combination failed to induce intracellular accumulation of cholesterol in fibroblasts (data not shown), probably due to other compensatory export/degradation mechanisms. Nonetheless, the use of a chemical probe inducing build-up of intracellular cholesterol triggers premature senescence, thus reinforcing this functional role of cholesterol in promoting senescence.

Cholesterol derivatives, such as oxysterols, are increased as cholesterol level is elevated. Oxysterols may be generated either *via* enzymatic reactions (through the cytochrome p450 enzymes family) or by non-enzymatic reactions involving an auto-oxidation of cholesterol (Jusakul et al., 2011) and are known to be synthesized in fibroblasts (Lange et al., 2009). Oxysterols are more water-soluble than cholesterol and exhibit higher reactivity. Aside from their role as mutagens at high concentrations, they are involved in physio-pathological conditions, regulating for example Liver X Receptor alpha (LXR $\alpha$ ) program (Janowski et al., 1996) (**Figure 32**). Interestingly, upon MeViS induction, my results display an increased mRNA level of *ABCA1* and *ABCG1* genes, two LXR $\alpha$  transcriptional targets (Zhao and Dahlman-Wright, 2010). LXR $\alpha$  program is thought to confer a protective role against the toxic effect of cholesterol build-up through upregulation of these cholesterol efflux transporters and cholesterol carrier Apolipoprotein E (ApoE) in parallel to the degradation of enzymes of cholesterol synthesis (Zhao and Dahlman-Wright, 2010). Besides the activation of ERR $\alpha$ , my results thus suggest an upregulation of a LXR $\alpha$  program. As observed for *ABCA1* (Sene et al., 2013), LXR $\alpha$  has been recently found to be downregulated during aging, and especially in macrophages promoting age-related macular degeneration (AMD) (Choudhary et al., 2020). Once again, these data suggest a protective role of LXR $\alpha$  against cholesterol accumulation and senescence. Echoing this hypothesis and based on senescence knowledge, other studies report LXR $\alpha$  activation as an inhibitor of both endothelial senescence (Hayashi et al., 2014) neuro-inflammation in an AD model (Zelcer et al., 2007), or determinant for human lifespan (Mooijaart et al., 2007).



**Figure 32: Consequences of cholesterol accumulation in MeViS.** Cholesterol balance is necessary and its accumulation is deleterious for cells. Cholesterol accumulation in the cells leads to altered organelles functions. In mitochondria (Mit.), it enhances oxidative stress, decreases membrane fluidity, impairs oxidative phosphorylation (OXPHOS) and metabolites transports. In ER, it triggers calcium depletion and ER stress. Moreover, oxysterols are generated from excessive cholesterol content, leading to LXR $\alpha$  activation. This later promotes cholesterol efflux and acts as a negative feedback to

normalize cholesterol content. Membranes alterations can be found in cholesterol-overloaded cells and could participate in regulating cellular senescence. Finally, my result demonstrate that abnormal  $ERR\alpha$  activation promotes ROS-dependent cellular senescence.

Furthermore, my study functionally demonstrated that some oxysterols, such as 7Keto-cholesterol or 25OH-cholesterol, induce premature senescence in fibroblasts (**Figure 32**). Other studies reported the role of some oxysterols, such as 5,6-secoesterol or 27-hydroxycholesterol, in promoting also premature senescence respectively in hepatocytes or lung resident cells (Anticoli et al., 2010; Hashimoto et al., 2016c). To be noted, our model of oxysterol-mediated senescence was accompanied by  $LXR\alpha$  activation, but was independent of  $ERR\alpha$  activation (**Figure 32**). As cholesterol and ROS both increase in senescent cells, we can speculate an enhanced autoxidation of cholesterol and also the generation of oxysterols during MeViS (**Figure 32**). Overall, we propose thus, in addition to cholesterol- $ERR\alpha$  mechanism, a secondary mechanism driven by excessive generation of oxysterols that may participate in the  $ERR\alpha$ -independent promotion of MeViS. This additional molecular mechanism would give also some explanations for the partial rescue of MeViS with siESRRA (*See RESULTS – Chapter II. – Section 2.2.*). Unfortunately, at this stage our mass spectrometry approaches did not allow to address the exact quantification of 25-hydroxycholesterol or 7-Keto-cholesterol in MeViS or other senescence subtypes but should be further investigated.

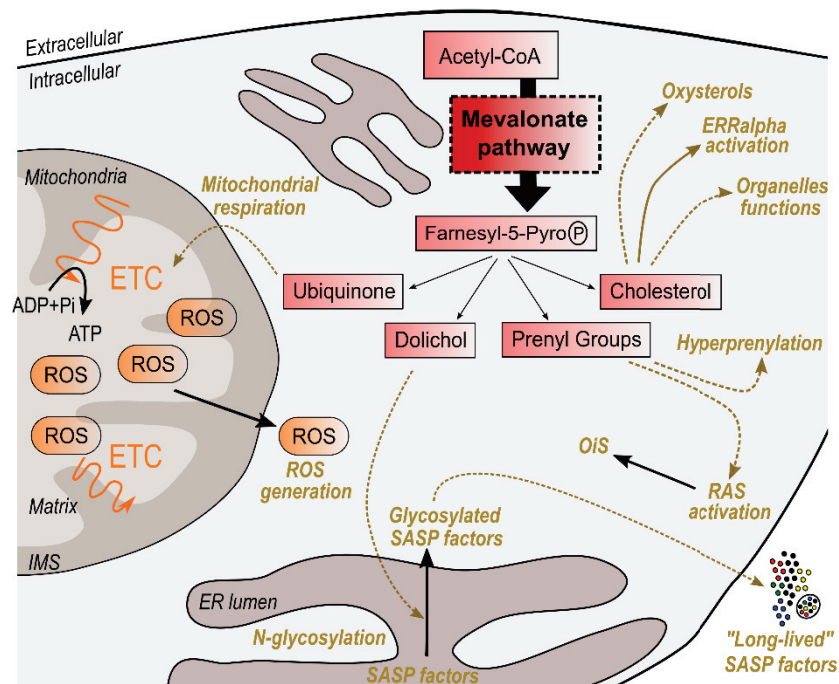
### **2.1.2. Involvement of non-steroid branches of the MVA pathway**

Our siRNA screen targeting the first enzyme of each sub-pathway identified cholesterol biosynthesis pathway as main driver of MeViS. Notably, except for *Fdft1* knock down which partially rescued cell proliferation arrest, no other individual knock down of the upstream enzymes controlling the others branches was able to rescue, even partially, the decreased cell number observed in MeViS. Based on this screen, we only measured cholesterol level without taking a look at the levels of other end-products, such as dolichol, ubiquinone or geranyl groups. In the same way as cholesterol accumulation, the build-up of other end-products in MeViS can be thus speculated, though some measurements (by mass spectrometry for example) should further confirm this hypothesis. These end-products in MeViS could contribute to the establishment or maintenance of MeViS.

Our results indicated that MeViS is accompanied by a robust SASP, that includes IL-8 and IL-6 expression. Most of the SASP proteins (soluble or receptors), including these two latter cytokines, are glycosylated and require a processing by Dolichol, one of the end-products of MVA pathway (Chojnacki and Dallner, 1988). This essential post-translational modification increases the stability but not the activity of proteins (Dewald et al., 2016; Waetzig et al., 2010). In tissues, SASP promotes multiple autocrine (Acosta et al., 2008a; Kuilman et al., 2008; Wajapeyee et al., 2008) and/or paracrine (Acosta

et al., 2013; Bird et al., 2018; Chen et al., 2015; Hoare and Narita, 2013) effects either to reinforce and spread cellular senescence (da Silva et al., 2019), or to enhance heterotypic interactions either with immune cells or surrounding normal or cancer cells (Coppé et al., 2006; Eggert et al., 2016; He and Sharpless, 2017; Krtolica et al., 2001; Laberge et al., 2012; Muñoz-Espín and Serrano, 2014b; Sagiv et al., 2016). These multiple signaling roles suggest that SASP scope depends on its stability, which can participate in the efficient delivery of signaling in neighboring and distant cells. Enhanced MVA pathway could thus contribute to sustain the supplies for glycosylation of the highly secretory senescent cells. Altogether, MVA pathway may fuel dolichol, geranyl and cholesterol molecules to sustain SASP activation/stability and subsequently reinforce the communications between senescent and surrounding (normal, immune and cancer) cells.

Moreover, accumulation of prenyl groups can overactivate -CAAX proteins, such as RAS, to trigger an OIS response (See INTRODUCTION – Chapter I. – Section 1.1.3). In the same time, the pathological-associated accumulation of farnesylated proteins, notably Lamin A, causes premature senescence, in HGPS (See INTRODUCTION – Chapter I. – Section 1.2.2.b). Finally, generation of ubiquinone can enhance mitochondrial ETC activity and promote the generation of ROS, which is known to participate in various subtypes of senescence (See INTRODUCTION – Chapter I. – Section 1.2.5).



**Figure 33: Potential contributions of downstream subbranches of MVA pathway to senescence.** End-products of the MVA pathway (red) could contribute to senescence in numerous ways. **A.** Cholesterol *via* the generation of oxysterols and ERR $\alpha$  program, but also alterations in endomembranes and organelles functions. **B.** Prenyl groups *via* hyperprenylation of some proteins, such as Lamin A, or hyperactivation of RAS which leads to Oncogene-induced senescence (OIS). **C.** Dolichol *via* hyper N-glycosylation of SASP factors and subsequent increased stability. **D.** Ubiquinone *via* an enhanced activity of ETC, and generation of excessive ROS.

### **2.1.3. MVA pathway regulation during senescence**

#### **a. Cellular senescence: a link with MVA pathway and cholesterol accumulation**

***MVA pathway in oncogene-induced senescence*** - Widely studied in the context of cancers, MVA pathway is upregulated by multiple oncogenic signals (Ras, PI3K/AKT, Myc) (Mullen et al., 2016). These oncogenic signals are known to be strong senescence inducers and drive OIS (Mullen et al., 2016) (Serrano et al., 1997; Zhu et al., 1998) (Braig et al., 2005; Chen et al., 2005; Denchi et al., 2005; Michaloglou et al., 2005; Roberson et al., 2005) (*See INTRODUCTION – Chapter I – Section 1.1.3.*). My preliminary results suggest that Raf-induced senescence upregulates some of the MVA pathway genes, including *HMGCR*, *MVD* and *FDPS*. This up-regulation has been observed in another model of OIS (Ras-induced senescence) (Pantazi et al., 2019). Moreover, this study demonstrates a functional role of cholesterol pathway in the senescence phenotype as suggested by a partial rescue of cell-cycle arrest with siMVD, siSQLE et siDHCR7 in OIS (Pantazi et al., 2019).

***MVA pathway in replicative senescence*** - As shown with a delayed RS in shPMVK expressing cells, our results shed light on the role of MVA pathway in regulating RS. Interestingly, mRNA levels were not particularly found elevated in RS, suggesting other layers of regulation. Even if not studied in the context of RS, transcripts of MVA pathway have been shown to be for example alternatively spliced (Medina et al., 2011) or even targeted for degradation by miRNA such as miR-33 and miR-122 in liver (Fernández-Hernando Carlos et al., 2013). Moreover, some enzymes of the MVA pathway are post-translationally regulated (Beg et al., 1978; Clarke and Hardie, 1990; Sapir et al., 2014) (*See INTRODUCTION – Chapter III. – Section 3.2.3.*). In line with senescence-associated context, an age-dependent post-translational modification (sumoylation) of HMGCS1 takes place in *C.elegans* (Sapir et al., 2014). For all these reasons, the post-transcriptional regulation of MVA pathway during RS should be further considered.

***Cholesterol accumulation and cellular senescence*** - Based on its role in MeViS, we monitored the intracellular cholesterol accumulation in known senescence models, such as replicative senescence or Nutlin-induced senescence. This accumulation was seen in both senescence contexts. Numerous other studies displayed an increased accumulation of lipid droplets in hepatocytes (Inoue et al., 2017; Ogrodnik et al., 2017), fibroblasts (Flor et al., 2016), or even periventricular glia (Ogrodnik et al., 2019), associated with some pathological issue such as age-dependent hepatic steatosis (Ogrodnik et al., 2017). This accumulation of intracellular lipids has been linked to increase of free fatty acids (Song et al., 2017) and free cholesterol (Cadenas et al., 2012; Maeda et al., 2009). Altered phospholipid synthesis or fatty acid processing changes (Cadenas et al., 2012; Gey and Seeger, 2013) recapitulate these changes in lipid metabolism of senescent cells. Moreover, my data indicated that artificial

accumulation of cholesterol was sufficient to initiate cellular senescence in normal cells, showing that intracellular cholesterol excess is a driver of senescence.

#### **b. MVA pathway, cholesterol and senescence: a role of p53?**

On one hand, our results demonstrated that MeViS is mediated by p53. On the other hand, additional data indicated that p53 stabilization by Nutlin-3a upregulates some genes of the MVA pathway, activates an ERR $\alpha$  program, and triggers intracellular cholesterol accumulation. These results recapitulate the observed phenotype, functionally involved in the promotion of MeViS. In normal cells, cholesterol accumulation in Nutlin-treated cells has been observed, but not in a senescence context, in three different cell lines of fibroblasts (BJ, HF, 67LR) (Saison-Ridinger et al., 2017). This p53-induced lipid droplet accumulation is associated with a slight increase of expression of MVA-pathway genes, but more particularly upregulation of downstream specific enzymes of cholesterol biosynthesis, such as Tm7sf2 (Saison-Ridinger et al., 2017). Stabilization of p53 could occur during replicative senescence (RS), and our data suggest it could generate a positive feedback loop to boost cholesterol production, thus reinforcing cellular senescence. Nevertheless, a recent study suggests an opposite role for p53 in Mouse Embryonic Fibroblasts (MEFs), demonstrating that Nutlin downregulates most of genes of the MVA pathway in a p53-dependent manner (Moon et al., 2019). Beyond this result, the role of p53 in regulating MVA pathway, cholesterol and their subsequent involvement in inducing senescence in our models (RS and Nutlin-induced senescence) should be thus further critically tested.

#### **c. SASP, inflammation and MVA pathway**

SASP has numerous roles in pathophysiological conditions including, among others, immune system recruitment, local inflammation, paracrine senescence or tumorigenesis (He and Sharpless, 2017) (*See INTRODUCTION – Chapter I. – Section 1.2.7.*). Our results suggested that MeViS induces SASP, while reducing MVA activity reduces IL-8 expression. MVA pathway inhibitors, such as statins or aminobiphosphonates (ABPs), have been shown to blunt SASP (Griveau et al., 2018; Wang et al., 2020) and some of its effects, including pro-tumorigenic activity (Liu et al., 2015b). Dampening SASP through statins could explain their numerous anti-inflammatory clinical outcomes *in vivo* (Liou et al., 2014; Montecucco and Mach, 2009). Indeed, simvastatin treatment reduces *in vitro* and *in vivo* the expression of main SASP pro-inflammatory cytokines including IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1 (Rezaie-Majd et al., 2002; Sakoda et al., 2016).

## 2.2. Physiological relevance of MeViS

Based on *in vitro* works, our study deciphered the molecular mechanism linking the enhanced MVA pathway activity and senescence program. This *in vitro* work was extended to RS context. All these data raise the question of physiological relevance of enhanced MVA pathway activity *in vivo*. Remarkably, numerous reports on the role of MVA pathway stem from the use of pharmacological inhibitors (statins and ABPs) or the study of genetic inherited disorders related to MVA pathway.

### 2.2.1. A cancer point of view

Some of my preliminary results suggest that, in a model of Raf-induced senescence, some of the MVA pathway genes (*Hmgcr*, *Mvd* and *Fdps*) and cholesterol synthesis genes (*Fdft1* and *Sqle*) are upregulated (data not shown), confirming other results obtained in Ras-induced senescence (Pantazi et al., 2019). To sustain high metabolic profile of cancer cells, MVA pathway is upregulated by known oncogenes, including RAS, PI3K/AKT or Myc, that are known to elicit OIS (Serrano et al., 1997; Miyauchi et al., 2004; Wu et al., 2007; Nogueira et al., 2008) and could participate in the transformation process (Mullen et al., 2016; Clendening et al., 2010; Hofmann et al., 2015; Wang et al., 2017b). In cancer cells, the interplay of MVA pathway with oncogene signaling is reinforced by the fact that MVA pathway fuels back many of these signaling proteins, notably through the generation of prenyl groups (Kusama et al., 2002; Cao et al., 2011). In pre-transformed cells, some clues indicate that the ectopic expression of HMGCR, not alone but in cooperation with RAS, promotes transformation (Clendening et al., 2010). For all these reasons, MVA pathway is necessary for the survival of many cancer subtypes (Efimova et al., 2018; Guterres et al., 2013; Hashimoto et al., 2016a; Kaymak et al., 2020; Laezza et al., 2015; Liang et al., 2018; Moon et al., 2019), which makes the use of MVA pathway inhibitors an interesting therapeutic approach broadly explored in cancer treatments (Buhaescu and Izzedine, 2007b; Goulitquer et al., 2018; Wang et al., 2020). Concerning cholesterol, not only its synthesis through MVA pathway, but also its import and export are often reprogrammed in cancer cells (Ding et al., 2019) and most of cancer cells accumulate intracellular cholesterol. Finally, while increased serum cholesterol levels are positively correlated with a higher risk of developing cancers, that include colon, rectal, prostatic and testicular cancer (Ding et al., 2019), it remains unknown whether increased intracellular cholesterol could favor the first steps of transformation.

Taken together and in light of my results, we can propose a model in which senescing cells in preneoplastic lesions display upregulation of MVA pathway. The bypass of senescence in these lesions could preserve this “advantageous” metabolic profile. MVA pathway activation during transformation in cancer cells could thus be considered as imprints of the past of senescing cells.

### **2.2.2. An aging point of view**

Our results suggested that reducing MVA pathway activity and subsequent cholesterol production may have a beneficial impact on aging, through the reduction of cellular senescence. At first sight, reducing an anabolic pathway to escape from a proliferation arrest and premature senescence seems to be counter intuitive. Nonetheless, it is important to remind that the only robust and universal non-pharmacological intervention to delay aging so far known is caloric restriction (CR) (Colman et al., 2009; Anderson and Weindruch, 2010). CR *in vivo* delays aging and age-pathologies in multiple organisms, but remains challenging to be studied in an *in vitro* context. We can speculate that a reduction of MVA pathway could mimic part of CR *in vitro* and *in vivo*. Not surprisingly, CR and low food intake limits both fecal excretion (Bennion and Grundy, 1975) and total serum levels of cholesterol delaying atherosclerosis in humans (Fontana et al., 2004). However, no *in vivo* study reporting MVA pathway activity or intracellular cholesterol levels in tissues upon CR has been so far conducted.

In the context of physiological aging, some final products of MVA pathway and sub-branches, including dolichol or cholesterol, have been shown either to accumulate *in vivo* with aging in mouse and other mammals (Parentini et al., 2005) or to be linked to age-related pathologies (Wood et al., 2002) whereas other, such as ubiquinone, are reduced in aged-tissues (Bentinger et al., 2010). Moreover, a haploinsufficient mouse model of *Myc* +/- displays increased lifespan, that is correlated with a decreased expression of various genes of the MVA pathway including *Hmgcs1*, *Hmgcr*, *Mvd* and *Srebf2*. More interestingly and beyond these observations, functional studies through the pharmacological inhibition of MVA pathway during physio-pathological aging process were performed in worms, mouse and even in human (Jacobs et al., 2013; Jahn et al., 2020; Varela et al., 2008). The first study showed that complementary use of statins and ABPs alleviates premature age-related features and enhances lifespan of a murine model of premature aging (Varela et al., 2008). The second study has been conducted on more than 70 years old patients, and demonstrated a decreased mortality in statin-treated cohorts (Jacobs et al., 2013). Finally, the third one showed that this statin-mediated lifespan extension is observed as well in *C. elegans* (Jahn et al., 2020). Of note, these three studies demonstrate a pro-longevity role of blunting MVA pathway. Concerning cholesterol and ERR $\alpha$  program, some functional data indicate beneficial effects in senescence-associated pathologies of dampening them. Firstly, age-dependent decrease of the cholesterol efflux channel ABCA1 is associated with an overload of intracellular cholesterol in macrophages and a subsequent age-related macular degeneration (AMD) (Sene et al., 2013). Secondly, ERR $\alpha$  may mediate age-related osteoporosis, as *ERR $\alpha$*  -/- female mice display reduced osteoporosis (Delhon et al., 2009; Teysier et



al., 2009). While no study on osteocytes has been performed in our hands in the context of senescence, we can speculate a reduction of senescent cells in bones of *ERRα* *-/-* mice during aging. Interestingly, this anti-osteoporosis effect can be mimicked with inhibitors of the MVA pathway, as demonstrated by the use of ABPs in the management of osteoporosis in postmenopausal women (Kanis et al., 2008). Moreover, in a context of Idiopathic Pulmonary Fibrosis (IPF) where senescence has been involved (Schafer et al., 2017a), a recent study demonstrated that IPF cells display an enhanced MVA pathway activity, suggesting a causative role of increased metabolites to promote fibrosis in lung (Larson-Casey et al., 2019b).

Overall and in view of my results, the dysregulation of MVA pathway with aging and the consequent accumulation of final products (cholesterol among others) could be determinant to drive cellular senescence, aging and age-related pathologies *in vivo*. Multiple beneficial effects of MVA pathway inhibitors, cholesterol reduction and *ERRα* ablation in reducing senescence-associated age-related disorders, reinforce this hypothesis.

### **2.2.3. MVA-pathway related metabolic diseases**

My study strongly suggests that cholesterol functionally promotes cellular senescence. Others report indirectly features of senescence-associated phenotypes in using tools to induce cholesterol accumulation. Namely, chemical-induction of cholesterol build-up is known to inhibit the release of cytochrome c, thus promoting resistance to cell death (Montero et al., 2008; Lucken-Ardjomande et al., 2008; Appelqvist et al., 2011), a mechanism described in senescence phenotype (*See INTRODUCTION – Chapter I. – Section 1.2.3.*).

As cellular senescence is involved in numerous pathophysiological conditions, it is of interest to check these senescence-associated contexts with regard to metabolic diseases. Hypercholesterolemic patients present a reduced lifespan due to an increased risk of cardiovascular diseases (Mabuchi H et al., 1989; Civeira, 2004). In line with this, age-related atherosclerosis is partly linked to an increased cholesterol content and is for this reason managed by the use of statins (Shepherd et al., 1995; Civeira, 2004). Interestingly, senescent vascular smooth cells and lipid-loaded (“foam cells”) macrophages accumulate during atherosclerosis stage and senolytics treatment have been shown to limit their progression in mice (Roos et al., 2016; Uryga and Bennett, 2016; Childs et al., 2016). Based on these results, and though not investigated in these contexts, we can thus speculate a role played by MVA pathway in the accumulation of cholesterol in vascular smooth cells or macrophages contributing to progression of age-related atherosclerosis. To assess this question, an

interesting approach would be first to constitutively activate the MVA pathway in liver, overexpressing PMVK for example, and decipher the consequences on liver physio-pathology, including histological (steatosis, fibrosis) and functional (inflammation, metabolism tolerance test) studies. Secondly, the potential detrimental effect could be confronted by the action of senolytics.

Mutations in MVA pathway concern some inherited disorders, including MVK deficiency (MKD) or porokeratosis (PK). MKD is a recessively inherited autoinflammatory disorder (Favier and Schulert, 2016), subdivided in two different well-defined clinical phenotypes, namely hyperimmunoglobulinemia D coupled to periodic fever syndrome and mevalonic aciduria. MKD is a rare disease concerning 300 cases worldwide, as described in the medical literature (Favier and Schulert, 2016) However, clinical studies report neither a delayed or premature aging phenotype, nor changes in the risk of developing cancers, nor wound healing disruption. Besides, mutations in some MVA pathway enzymes and loss of function of PMVK are found in some sporadic and familial PK (Zhang et al., 2015; Wang et al., 2016a; Leng et al., 2018). PK is a clonal disorder affecting the keratinization process of the skin (Otsuka et al., 1991). Of note, it has been known for several decades that PK is associated with a premalignant context (Otsuka et al., 1991), a well-defined tissue context where accumulation of senescent cells is observed (Collado et al., 2005). Moreover, one study has reported an increase of p16<sup>INK4A</sup> expression in keratinocytes of PK patients epidermis (Uryu and Furue, 2017), though no other study has so far been conducted to evaluate other senescence markers in this context. Taken together, MKD and PK underline and remind us the importance of MVA pathway in physiological contexts, suggesting that a systemic chronic reduction of this pathway could be also detrimental.

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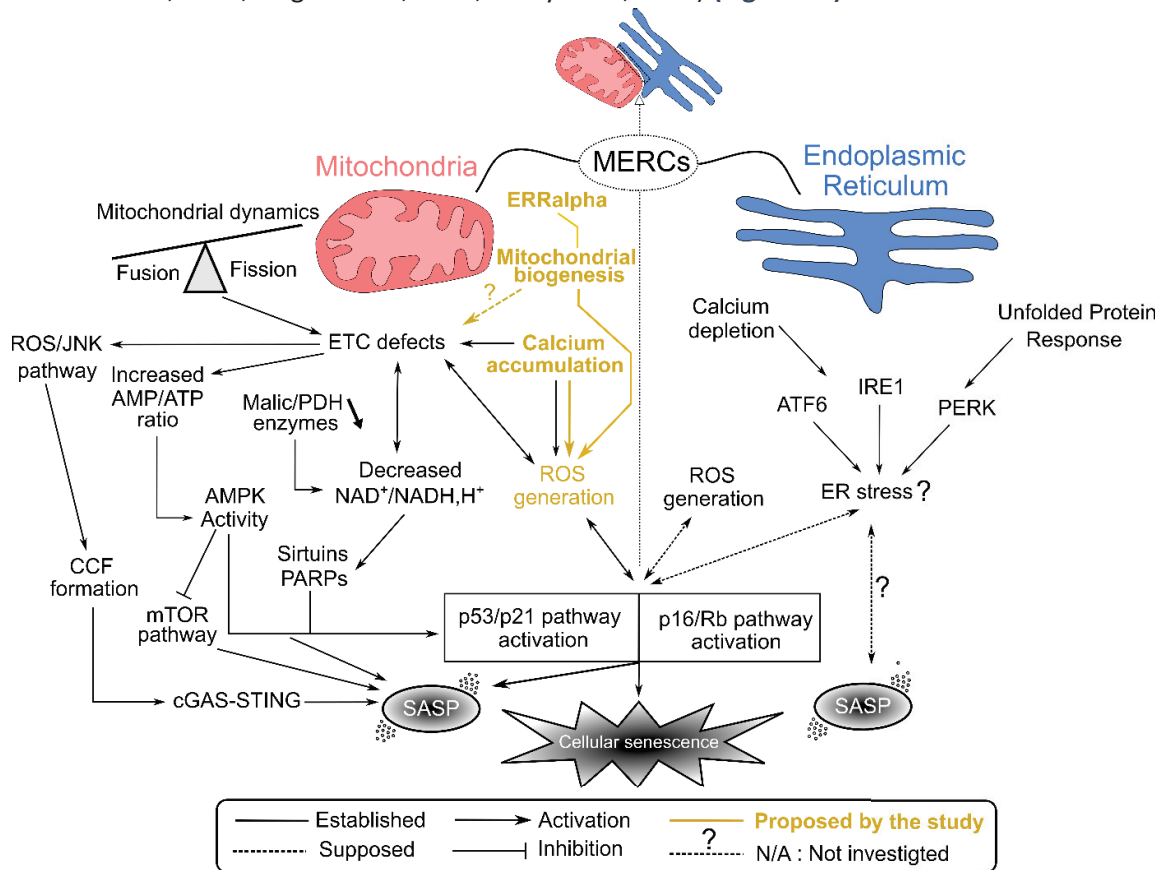


## - CHAPTER III -

### 3. - Mitochondria: signaling organelles in the regulation of senescence -

My two apparently independent PhD projects converge functionally to the study of the mitochondrial functions and draw attention to the role of this organelle in senescence regulation. Mitochondrial dysfunction has been widely reported during cellular senescence, though the intrinsic mechanisms leading to this dysfunction are not fully understood.

ETC dysfunction, alterations of mitochondrial matrix enzymes, such as ME or PDH, have been found to promote senescence (Stöckl et al., 2007; Jiang et al., 2013; Kaplon et al., 2013; Ziegler et al., 2015). Mitochondria-associated senescence was then reported to be mediated notably by ROS-p38 pathway and/or correlative decreased ATP generation, triggering AMPK-dependent senescence (Moiseeva et al., 2009; Ziegler et al., 2015; Wiley et al., 2016) (Figure 34).



**Figure 34: Regulation of cellular senescence by mitochondria and ER.** Summary of the mitochondrial (on the left) and ER (on the right) rearrangements that modulate cellular senescence. Our model (in gold) proposes that mitochondrial dysfunction could rise either from excessive mitochondrial calcium accumulation or abnormal mitochondrial biogenesis, this later being mediated by cholesterol-ERR $\alpha$  program. Mitochondrial functions can be linked to ER ones, and interplay between these organelles

exist notably *via* MERCs connections. This unbalanced scheme shed light on the under-investigated role of ER in modulating senescence, and should be further considered, in link or not with MERCs.

My study proposes two distinct (but not exclusive) paths to explain how changes in mitochondrial functions can induce a senescence phenotype: calcium or biogenesis-related changes (Figure 34).

### 3.1. Calcium and mitochondrial functions

My data demonstrated that ITPR2 and MERCs favor calcium fluxes from the ER to the mitochondria to promote mitochondrial membrane (MM) depolarization, ROS generation, and a subsequent senescence. Coupled to previous results obtained in the lab in different models including hMEC and fibroblasts (Ma et al., 2018; Wiel et al., 2014), our model suggests that enhanced ITPR2- or MERC-induced ER-mitochondria calcium transfers are sufficient to initiate a senescence response.

#### 3.1.1. Mitochondrial calcium and metabolism

In normal conditions, mitochondria constitute a temporary reservoir of calcium and various physiological changes of calcium content in these mitochondria have been reported (Rizzuto et al., 2012). Originally, calcium accumulation was observed in energized mitochondria, unravelling a crucial role of calcium in modulating mitochondrial bioenergetics and aerobic metabolism (Rizzuto et al., 2012). Indeed, at least three mitochondrial enzymes are regulated by calcium. Notably, calcium directly regulates mitochondrial  $\alpha$ -ketoglutarate- and isocitrate-dehydrogenases both displaying EF-hand calcium-binding motif, and indirectly pyruvate dehydrogenase, which is modulated by a calcium-dependent phosphatase (Denton, 2009). Not restricted to dehydrogenases, some IMM metabolite transporters, such as the two mammalian aspartate-glutamate carriers, include also ER-hand calcium-binding site (Contreras et al., 2007). Overall, increased mitochondrial calcium either in intermembrane space or matrix promotes respectively metabolites transport and metabolites processing in TCA cycle through these calcium-sensitive proteins (Satrústegui et al., 2007; Denton, 2009).

Interestingly, MERCs promote mitochondrial metabolism (Bravo et al., 2011; Rizzuto et al., 2012; Carreras-Sureda et al., 2019), a high calcium concentration reflecting a high oxidative metabolism in the mitochondria. In this context, reduced MERCs contacts and ER-mitochondria calcium fluxes through Cyclophilin KO (Rieusset et al., 2016; Tubbs et al., 2014b) may lead to dysregulation of TCA cycle and fatty acid  $\beta$ -oxidation (Tavecchio et al., 2015). Mitochondrial ETC accounts for the major intracellular sources of ROS, that include also ER through oxidative folding, peroxisomes through fatty acids  $\beta$ -oxidation, or lysosome membranes through NAPDH oxidases

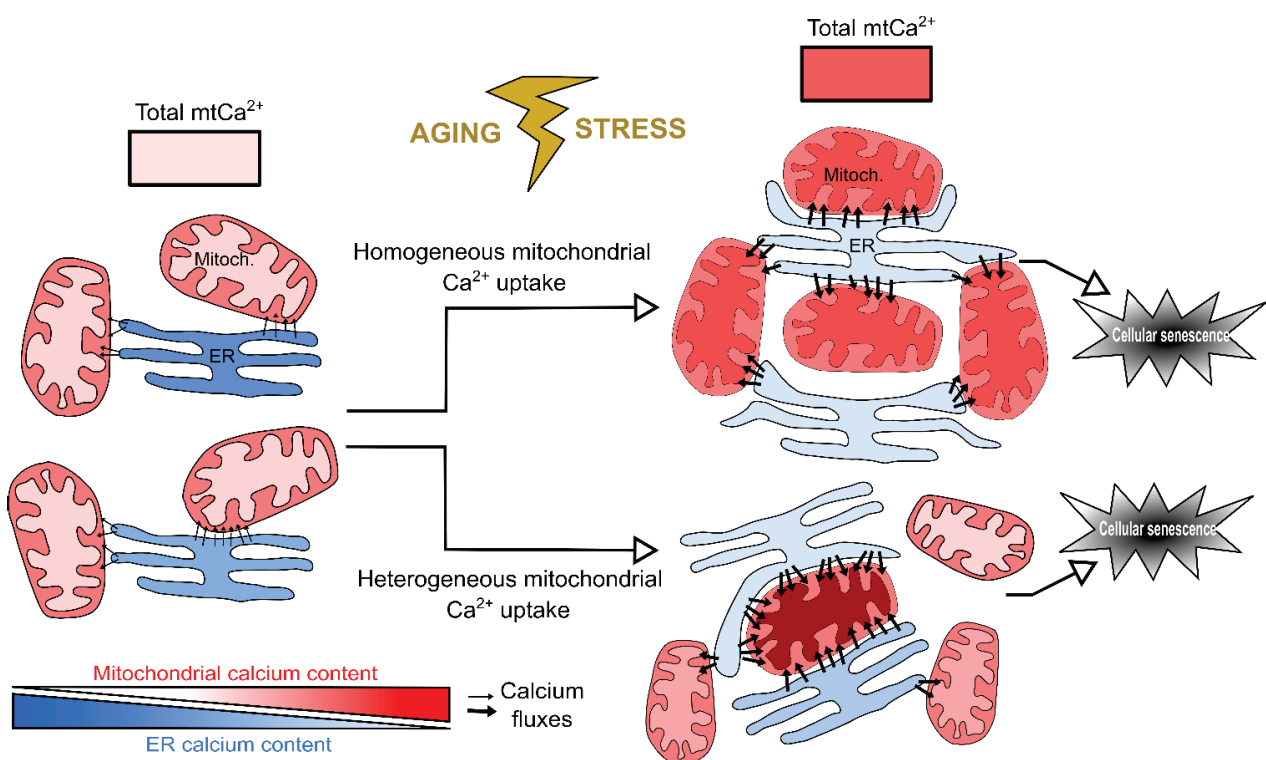
(Gorrini et al., 2013). The use of antioxidants rescues MERC-induced senescence, indicating that mitochondrial ROS mediate the senescence phenotype. Our model thus proposes that an enhanced calcium flux to the mitochondria leads to mitochondrial dysfunction, as suggested by the partial uncoupling of the MM. Nevertheless, the exact mechanisms of mitochondrial dysfunction remain puzzling. Beyond the ultimate production of ROS, decreased ETC activity, increased AMP/ATP ratio and reduced  $\text{NAD}^+/\text{NADH,H}^+$  ratios are known to mediate senescence in context of dysfunctional mitochondria. The functional study of mitochondria, through a global Seahorse analysis, coupled to measurements of AMP/ATP and  $\text{NAD}^+/\text{NADH,H}^+$  is an interesting approach to further decipher mitochondrial dysfunction in this model.

In parallel, senescent cells display modulations of mitochondrial metabolism. Functional studies displayed that disrupting ETC chain or malic enzymes may promote senescence (Jiang et al., 2013; Moiseeva et al., 2009; Ziegler et al., 2015; Wiley et al., 2016). Observational studies also show that changes in mitochondrial metabolism accompanies cellular senescence, with a reduced ETC activity, an excessive generation of ROS, an increased AMP/ATP ratio coupled with activation of AMPK, and finally a reduced  $\text{NAD}^+$  synthesis (Moiseeva et al., 2009; Ziegler et al., 2015; Wiley et al., 2016). Indeed, increased ROS generation sometimes associated with partial MM depolarization/uncoupling were reported during OIS in epithelial cells (Wiel et al., 2014) and during RS in various cell models including fibroblasts (Hutter et al., 2004), hippocampal neurons (Calvo-Rodríguez et al., 2016), mesenchymal stem cells (Stab et al., 2016) or endothelial cells (Madreiter-Sokolowski et al., 2018). Mitochondrial calcium overload was also observed in endothelial and hippocampal neurons (Calvo-Rodríguez et al., 2016; Madreiter-Sokolowski et al., 2018). Interestingly, FCCP and partial mitochondrial uncoupling is able to promote senescence in fibroblasts and hMEC (Stöckl et al., 2007; Wiel et al., 2014). According to my results and in line with these studies, it is not excluded that mitochondrial calcium overload could participate in these mitochondrial metabolic alterations of senescing cells, that could be easily monitored through the use of a calcium ratiometric genetic or chemical reporter.

### **3.1.2. Mitochondrial calcium and cell survival**

Aside from its role in metabolism, mitochondrial calcium participates in cell survival and particularly necrosis and apoptosis (Rizzuto et al., 2012). The mitochondrial permeability transition pore (mPTP) locates at the IMM and is essential for the release of apoptosome components (i.e. cytochrome c), thus constituting the initial step of apoptosis (Rasola and Bernardi, 2014). Of note, calcium waves in mitochondria favor its opening and a concomitant collapse of the membrane potential, ultimately triggering MOMP and release of apoptosome components (Rizzuto et al., 2012;

Szalai et al., 1999). My results suggest that enhanced ITPR2 and MERC-mediated sub-lethal loadings of mitochondrial calcium promote senescence. In order to reconcile mitochondrial calcium and three different cells fates, namely survival, senescence and apoptosis, two models can be proposed. A first “calcium homogeneity theory” postulates a global homogenous uptake of mitochondrial calcium in all mitochondria (**Figure 35**). An alternative theory is based on mitochondria heterogeneity (Kuznetsov and Margreiter, 2009) and recent discovery of minority MOMP, a non-lethal mechanism involving MOMP only in a few subfraction of mitochondria (Ichim et al., 2015) (**Figure 35**). “Calcium homogeneity” and “minority MOMP” theories could be addressed with high-resolution calcium imaging in live cells upon MERC induction, and further open new perspectives in understanding mitochondrial heterogeneity and sub-lethal stresses in link with senescence.



**Figure 35: Linking enhanced mitochondrial calcium uptake and induction of senescence.** One of my working models proposes that aging and various stress could induce an increase of mitochondrial calcium content ( $\text{Total mtCa}^{2+}$ ) concomitantly to a decrease of ER calcium content, these intracellular variations triggering ultimately cellular senescence. MERCs favor these ER-mitochondrial calcium exchanges and participate in senescence. Without altering the terminal induction of senescence, this increased mitochondrial calcium uptake can be either (i) homogeneous (upper right panel) or (ii) heterogeneous (lower right panel).

Whether calcium accumulates highly in some punctual mitochondria or increase gradually in all mitochondria should be critically tested. Not evaluating these both theories but linking together calcium, senescence and apoptosis, a recent study displayed an increased MERCs number and

mitochondrial calcium levels in senescing endothelial cells (Madreiter-Sokolowski et al., 2018). This higher steady-state mitochondrial calcium level observed in these senescing cells exceeds a threshold upon reestratol exposure and subsequently switch from senescence to apoptosis (Madreiter-Sokolowski et al., 2018). This mitochondrial calcium threshold is not reached in non-senescent cells (Madreiter-Sokolowski et al., 2018). If confirmed, this hypothesis could constitute an important axis of research for discovering new senolytics specifically modulating mitochondrial calcium levels.

### **3.2. Mitochondrial biogenesis and mitochondrial functions**

My results emphasized that MeViS is driven partly by the cholesterol-mediated activation of an ERR $\alpha$  transcriptional program. ERR $\alpha$  participates to the regulation of mitochondrial biogenesis, with PPARGC $\alpha$  (PGC1 $\alpha$ ), NRF-1 or NRF-2 (Scarpulla, 2008). Interestingly, some preliminary results suggest that siRNA against PGC1 $\alpha$  may overcome MeViS with lower effect than ERR $\alpha$  (data not shown), reinforcing the functional role of mitochondrial biogenesis to drive MeViS. According to my data, ERR $\alpha$  was necessary to induce mitochondrial ROS production and subsequently senescence program. Whether MeViS displays increased mitochondrial mass, mtDNA and/or abnormal dynamics is still an open question that should be further addressed.

Paradoxically to a global senescence-associated decline in ETC chain activity (Ziegler et al., 2015), an increased mitochondrial mass is seen in various senescence models including RS, OiS and oxidative-induced senescence (Lee et al., 2002; Maneiro et al., 2003; Moiseeva et al., 2009; Stab et al., 2016; Ziegler et al., 2015). More particularly, in fibroblasts, oncogene activation increases mitochondrial mass and mtDNA prior to the senescence cell-cycle arrest (Moiseeva et al., 2009). Moreover, oxidative stress-induced senescence could be mediated by an enhanced translational program, as suggested by its reversion with cycloheximide (Lee et al., 2002). More recently and in line with our results, mitochondrial biogenesis has been recently proposed to be functionally involved in the induction of senescence of lung epithelial cells (Summer et al., 2019) or fibroblasts (Takenaka et al., 2020).

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## **- CHAPTER IV -**

### **4. - MERCs and Cholesterol: beyond ITPR2 and MVA pathway -**

Taken together, my results unraveled, beyond mitochondria, two new actors in regulating cellular senescence: MERCs and cholesterol. Remarkably, MERCs are crucial in cholesterol transport from ER to mitochondria and could contribute in regulating cellular senescence. Indeed, some studies reported a role of cholesterol mitochondrial import in the regulation of cellular processes notably in regulating mitochondrial functions and cell survival.

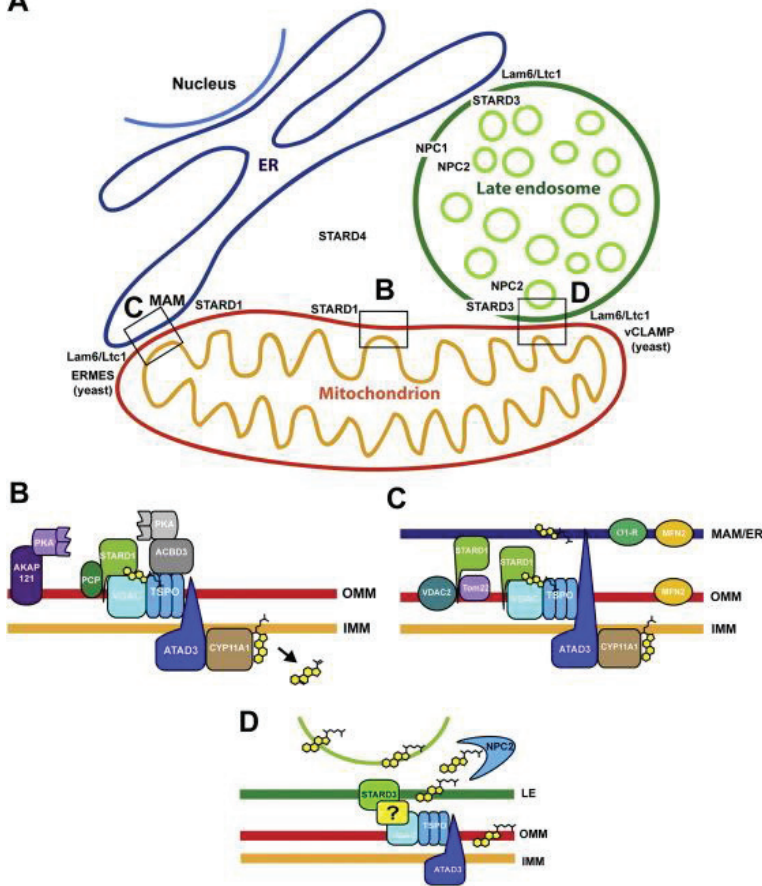
#### **4.1. Localization of cholesterol accumulation in MeViS**

Our results indicated that MeViS accumulates intracellular cholesterol. Nonetheless, where exactly this cholesterol is accumulated remains unknown. This accumulation seems to be punctuated and suggests a localized build-up. Among potential sites of cholesterol accumulation, ER lumen, where cholesterol is terminally synthesized (Soccio and Breslow, 2004), can be suspected. Cholesterol can be as well stored in lipid droplets in esterified forms, as reported by others studies of senescing hepatocytes (Inoue et al., 2017; Ogrodnik et al., 2017) or fibroblasts (Flor et al., 2016).

Finally, mitochondria can import cholesterol through facilitated diffusion bound to lipid transfer proteins (LTP), including for example steroidogenic acute regulatory protein (StAR), renamed STARD1 (**Figure 36**) (Elustondo et al., 2017). Indeed, excess of intracellular cholesterol is deleterious for the cells and can be either exported (*via* ABCA1 or ABCG1 transporters) or degraded by cytochrome P450 enzymes family into other active metabolites, that include oxysterols, steroids hormones or bile acids. The first steps of this degradation take place in mitochondria and depends on the cell type. Steroidogenic cells require the CYP11A1 to transform cholesterol into pregnenolone, which is the precursor of all steroids (including testosterone or estrogen) (Elustondo et al., 2017) (**Figure 36**) Hepatic cells require CYP7A1 to transform cholesterol into 7-Hydroxycholesterol, a precursor of bile acid (Zhao and Dahlman-Wright, 2010). Non-steroidogenic/hepatic cells present some enzymes involved in the catabolism of cholesterol notably producing oxysterols, as observed in fibroblasts (Lange et al., 2009). Within the cell, cholesterol is not transported by vesicular trafficking, but by facilitated or simple diffusion at membrane contact sites (MCS), that include MERCs (Elustondo et al., 2017).



A



**Figure 36: Mitochondrial import of cholesterol import.** A. The mitochondria import cholesterol from different locations within the cell, including cytosol (B) or membrane contact sites (MCS) linked either to ER (C) or endosome (D). STARD1 is located at the OMM and is crucial to mediate cholesterol import through the OMM. ATAD3 is necessary to maintain a reduced distance between OMM and IMM, allowing efficient diffusion until mitochondrial matrix. (Elustondo et al., 2017)

#### 4.2. Cholesterol accumulation in ER or mitochondria: a senescence point of view

**Cholesterol accumulation in ER** - Cholesterol accumulation in the ER promotes calcium depletion and cytotoxicity *via* ER stress activation in macrophages (Feng et al., 2003) and may thus participate in the promotion of cellular senescence through this mechanism. Less investigated than mitochondrial cholesterol, no study has so far reported an increased ER cholesterol content in ER during cellular senescence, opening new perspectives in linking cholesterol, ER stress and senescence.

**Cholesterol accumulation in mitochondria** – As double-membrane organelle, mitochondria require cholesterol for regulating OMM and IMM fluidity. Nevertheless, mitochondria are considered as low-cholesterol organelles (in contrary to ER for example), concentrating between 0,5 and 3% of the total cholesterol of plasma membranes, and making them sensing organelles of variations in intracellular cholesterol content (Maxfield and Tabas, 2005). Indeed, unphysiological levels of mitochondrial cholesterol were largely reported in solid tumors (Maxfield and Tabas, 2005; Elustondo et al., 2017).

Importantly, mitochondria are the site of the first crucial steps of the catabolism of cholesterol, degrading the later into oxysterols, steroid hormones or bile acids, depending on the cell type (Elustondo et al., 2017). Like calcium variations, increased mitochondrial cholesterol content is known

to disrupt mitochondrial functions (Elustondo et al., 2017). Reducing mitochondrial membrane fluidity (Baggetto et al., 1992; Montero et al., 2008), the increased mitochondrial cholesterol impairs also the function of metabolites exchangers, such as pyruvate and 2-oxoglutarate transporters (Paradies et al., 1992; Coll et al., 2003). Furthermore, the accumulation of mitochondrial cholesterol triggers ETC defects and subsequent deficient oxidative phosphorylation (Baggetto et al., 1992; Yu et al., 2005; Kennedy et al., 2014) and results frequently in the excessive generation of ROS (Baggetto et al., 1992; Yu et al., 2005; Elustondo et al., 2017). Of note, all these defects participate in the promotion of cellular senescence (*See INTRODUCTION – Chapter I. – Section 1.3.4.*) (**Figure 34**), though no study clearly reported a functional link between, increased mitochondrial cholesterol content, mitochondrial dysfunction, and cellular senescence. Nevertheless, some clues indicated a senescence phenotype, as suggested by the resistance to cell death in mitochondrial cholesterol-overloaded cells in multiple reports (Montero et al., 2008; Lucken-Ardjomande et al., 2008; Appelqvist et al., 2011), one of the main features of the senescence phenotype (*See INTRODUCTION – Chapter I. – Section 1.2.3.*). A more precise characterization of the senescence phenotype in these contexts could be addressed in future perspectives.

To assess these questions, the precise localization of cholesterol within the cell, for example coupling co-IF with organelle markers and biochemical characterization *via* gradual differential centrifugations, should be firstly addressed. Then, and subject to the condition that STARD1 is expressed in our fibroblast model, the subsequent use of siRNA against this key import protein could give additional indications of the functional involvement of cholesterol transport in the regulation of cellular senescence.

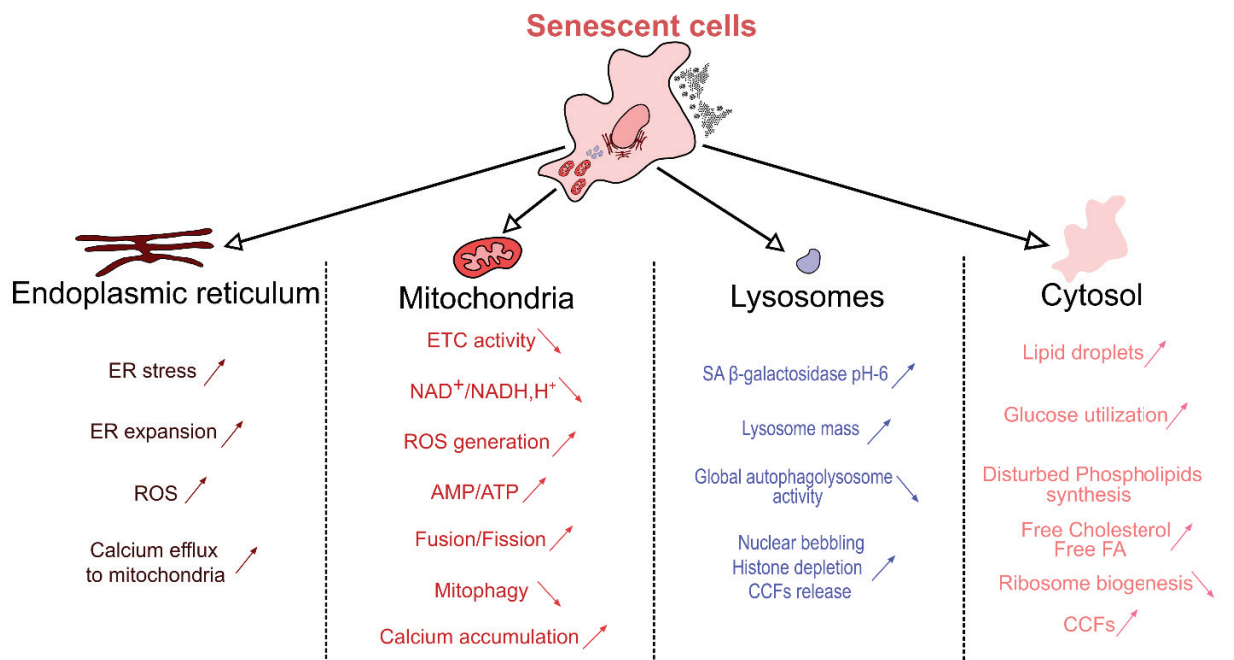
**- CONCLUSION -**



## CONCLUDING REMARKS - CHALLENGING THE METABOLISM OF SENESCENT CELLS

### Metabolism of senescent cells – Not a “low” metabolism

Long regarded as a “low” and inactive metabolism, similar to the one observed in quiescent cells, the metabolism of senescent cells has revealed its “active” nature. Indeed, senescent cells display metabolic rearrangements that do not freeze the metabolism, but deeply modify it compared to normal cells. Though they remain disparate (**Figure 37**), some of these metabolic rearrangements, including ER stress or mitochondrial dysfunction, can also drive cellular senescence, highlighting their functional importance in regulating senescence.



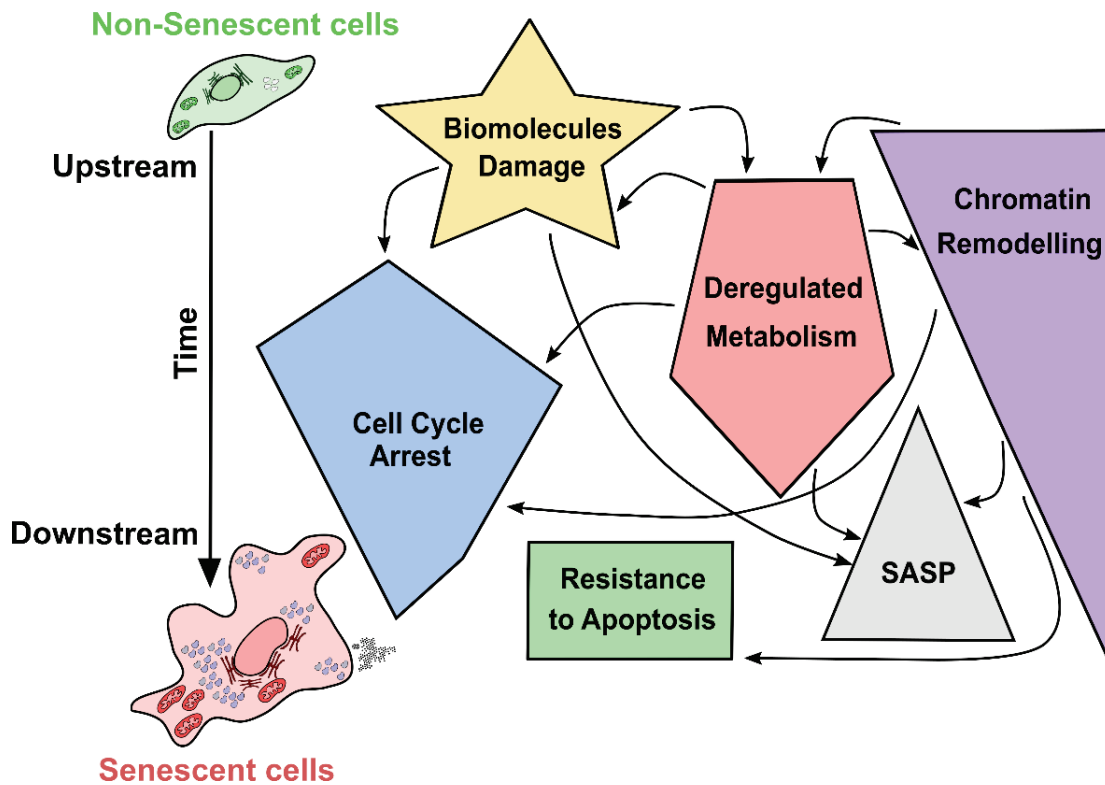
**Figure 37: Overview of the senescence-associated metabolism rearrangements.** Summary of the main metabolic rearrangements observed during cellular senescence. These changes are subdivided depending if they are related to biology of endoplasmic reticulum, mitochondria, lysosomes or cytosol. Non-exhaustive list.

The apparent differences between quiescent and senescent metabolism could be partly explained by the Senescence-Associated Secretory Phenotype (SASP). Indeed, while quiescent cells stop proliferating to enter in a state of dormancy, senescent cells do not only undergo cell cycle arrest but also start secreting myriad of SASP factors. Secretion being an important energy- and substrate-consuming process for cells, it is thus not surprising to observe metabolic rearrangements within senescing cells. Senescent cells could adapt their metabolism in order to provide both energy and bio-substrates, ultimately covering the important needs for secretion.

Metabolic homeostasis of senescent cells differs thus from normal or cancer cells, but still remains optimized to ensure a proper survival.

### Metabolism reorganization is central in the senescence program

As seen in the introduction of this work, the main features of cellular senescence includes (1) permanent cell cycle arrest, (2) cellular and subcellular morphological changes, (3) resistance to apoptosis, (4) senescence-associated- $\beta$ -galactosidase activity, (5) macromolecular damage, (6) specific epigenetic landscape and (7) secretory phenotype (SASP) (**Figure 5**). Interestingly, some of these features are directly linked with cell metabolism, and metabolic regulators account for crucial mediators of senescence program (*See INTRODUCTION – Chapter I. – 1.3.4.*). In line with our results, we propose a central role for deregulated metabolism in the induction of senescence features (**Figure 38**). Metabolism of senescent cells can thus in this model initiate, integrate and regulate pro-senescent signals in order to coordinate the senescence response.



**Figure 38: An integrative view of cellular senescence: the central role of metabolism.** The establishment of senescence is a dynamic process. From upstream signal to terminal outcomes, that includes cell cycle arrest, resistance to apoptosis or SASP, multiple signals are integrated by cells to coordinate senescence program. We propose a central role for metabolism in initiating, integrating and regulating pro- and anti- senescent signals.

### **Organelles dysfunction, metabolic reorganization and cell survival during senescence**

A growing number of studies report an increase in organelles content, including ER expansion and elevated mitochondrial and/or lysosomes masses during cellular senescence (**Figure 37**). Usually observed by an enlarged morphology, this global increased organelle mass is paradoxically coupled with numerous organelles dysfunctions, such as for example decreased ETC or reduced autophagolysosome activity. Organelle biogenesis could be seen as an adaptation for cells to overcome the gradual decline in organelle functions. Acting as a vicious circle, organelle biogenesis could also reinforce organelle dysfunction without eliminating defective organelles, as seen with the decreased mitophagy for example. This paradoxal link between organelles dysfunctions and biogenesis/dynamics is one way to elucidate in the future some secrets of the metabolism of senescent cells.

My PhD work shed light on the effect of enhanced lipid anabolism and heightened calcium fluxes in regulating senescence. Whether they can participate in a global boosted or blunted metabolism in senescent cells remains to be further elucidated. Nevertheless, we propose that they may contribute to increase both available bio-substrates (MVA pathway) and energy (mitochondrial calcium and ERR $\alpha$ -mediated mitochondrial biogenesis) to sustain needs for optimal survival and secretion of senescent cells. These results paved the way to investigate further whether organelles dysfunction only constitute a consequence of senescence or actively participate in the establishment of the phenotype, and decipher how metabolism links survival and senescence.

Indeed, while exposed to numerous organelle dysfunctions, it remains surprising to observe that senescent cells survive to these dysfunctions, and even elicit pro-survival signals, acquiring this ability to resist to cell death mechanisms. Remarkably, this adaptation reveals many unanswered questions. For metabolism of senescent cells, the term “dysfunction” could be thus replaced by the term “re-adaptation”. Because of the interplay between survival and metabolism, the link between survival mechanisms and metabolism reorganization in senescent cells should be addressed in the future.

« Moins on a de connaissances, plus on a de convictions »  
Boris Cyrulnik - XXème siècle



# - ANNEXES -



Aside from the two projects presented in this manuscript, I participated in the study of other senescence regulators we are interested in, including phospholipase A2 receptor 1 (PLA2R1). PLA2R1 encodes a transmembrane protein, that interacts with extracellular matrix (ECM) proteins such as secreted phospholipase A2 (sPLA2), and some collagen and integrin isoforms. Our lab showed that in primary human cells, PLA2R1 overexpression induces premature senescence and its knock-down both delays telomere-dependent senescence and favors escape from senescence induced by oxidative and oncogenic stress. (Vindrieux et al., 2013; Bernard and Vindrieux, 2014). Mechanistically, this senescence is mediated at least in part by JAK/STAT signaling (Vindrieux et al., 2013). Beyond these studies, an interesting question is to know whether PLA2R1 may contribute to premature aging. Premature aging can be mimicked by disrupting the production of lamin to generate a truncated form of the later, called progerin, that results in misshapen nuclei, genomic instability and ultimately triggers either cellular senescence *in vitro* or premature aging *in vivo*.

The two published papers in which we addressed this question are presented here. The first study that I was involved in, displays that PLA2R1 mediates in part progerin-induced senescence *in vitro* and some features of the premature aging phenotype of progeria mouse models (notably in bones). Moreover, progerin-induced senescence upregulates FDPS, the terminal enzyme of the MVA pathway, this later being necessary for establishing senescence. The second study in which I participated, demonstrates the beneficial effects of inhibiting JAK/STAT pathway that mediates PLA2R1-induced senescence, in a mouse model of progeria. JAK/STAT inhibitor alleviates premature aging phenotype and reduces bone fractures, increases bone mineral content, and improves grip strength.



# Targeting the phospholipase A2 receptor ameliorates premature aging phenotypes

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## Abstract

Hutchinson–Gilford progeria syndrome (HGPS) is a lethal premature aging that recapitulates many normal aging characteristics. This disorder is caused by mutation in the LMNA gene leading to the production of progerin which induces misshapen nuclei, cellular senescence, and aging. We previously showed that the phospholipase A2 receptor (PLA2R1) promotes senescence induced by replicative, oxidative, and oncogenic stress but its role during progerin-induced senescence and in progeria is currently unknown. Here, we show that knockdown of PLA2R1 prevented senescence induced by progerin expression in human fibroblasts and markedly delayed senescence of HGPS patient-derived fibroblasts. Whole-body knockout of *Pla2r1* in a mouse model of progeria decreased some premature aging phenotypes, such as rib fracture and decreased bone content, together with decreased senescence marker. Progerin-expressing human fibroblasts exhibited a high frequency of misshapen nuclei and increased farnesyl diphosphate synthase (FDPS) expression compared to controls; knockdown of PLA2R1 reduced the frequency of misshapen nuclei and normalized FDPS expression. Pamidronate, a FDPS inhibitor, also reduced senescence and misshapen nuclei. Downstream of PLA2R1, we found that p53 mediated the progerin-induced increase in FDPS expression and in misshapen nuclei. These results suggest that PLA2R1 mediates key premature aging phenotypes through a p53/FDPS pathway and might be a new therapeutic target.

## KEYWORDS

cellular senescence, p53, progeroid diseases, signaling

## 1 | INTRODUCTION

The LMNA gene encodes lamin A and C proteins, which are located in the nuclear lamina where they contribute to rigidity and shape of the nuclear envelope and regulate chromatin organization and gene

expression. Hutchinson–Gilford progeria syndrome (HGPS), a premature aging disease, is caused by a mutation in the LMNA gene, which leads to the activation of a cryptic splice donor site in exon 11 (Eriksson et al., 2003). The mutant prelamin A mRNA is then translated into progerin, an internally truncated protein that fails to undergo processing to mature lamin A and induces premature senescence (Goldman et al., 2004). ZMPSTE24 deficiency also leads to

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failure of maturing lamin A, to premature senescence and to progeria syndrome (Bergo et al., 2002).

The concept that cellular senescence contributes to pathologies linked to aging has been demonstrated over past few years. Indeed, eliminating senescent cells in mice with a progeroid syndrome delays some age-associated disorders, while in wild-type mice it reduces aging-related diseases and extends lifespan (Baker et al., 2016, 2011). The list of age-related diseases improved by delaying senescence or eliminating senescent cells is increasing and includes osteoporosis, type 2 diabetes, and atherosclerosis (Childs et al., 2016, 2017; Farr et al., 2017; Minamino et al., 2009). However, the role of cellular senescence in premature aging remains largely unclear.

Cellular senescence can be induced by replicative exhaustion, reactive oxygen species (ROS), genotoxic drugs, and ionizing radiation and results in stable proliferation arrest and the acquisition of a specific senescence-associated secretory phenotype (SASP). In the context of aging and age-related diseases, proliferation arrest is thought to limit organ renewal and the SASP is thought to alter the organization and function of tissues (Ovadya, & Krizhanovsky, 2014). We previously observed in primary human cells that constitutive expression of the phospholipase A2 receptor 1 (PLA2R1) induces premature senescence and that its knockdown delays telomere-dependent senescence and stimulates escape from senescence induced by oxidative and oncogenic stress. PLA2R1 encodes a transmembrane protein that can bind to secreted phospholipase A2 (sPLA2) and some collagen and integrin isoforms; and may regulate cellular senescence through the activation of JAK/STAT signaling and the  $ERR\alpha$  transcription factor (Augert et al., 2009, 2013; Bernard, & Vindrieux, 2014; Griveau et al., 2016; Vindrieux et al., 2013; Vindrieux et al., 2013).

These results raise the interesting question of whether PLA2R1 may contribute to premature aging. In this study, we used progerin-expressing fibroblasts, HGPS patient-derived fibroblasts, and a mouse model of progeria to address those questions.

## 2 | RESULTS

### 2.1 | Inhibiting PLA2R1 expression overcomes progerin-induced premature senescence

Hutchinson–Gilford progeria syndrome is caused by the expression of progerin, a truncated form of lamin A (Goldman et al., 2004). To study the role of PLA2R1 in progerin-induced senescence, we used normal human fibroblasts overexpressing progerin; control cells expressed lamin A. As expected, the proteins were localized in the nucleus and progerin was functionally active as it altered nuclear shape and we observed that PLA2R1 increased upon progerin expression (Supporting Information Figure S1a,b and Figure 1a). Next, we knocked down PLA2R1 expression using two different shRNA sequences (Figure 1a and Supporting Information Figure S1b). Constitutive expression of progerin resulted in proliferation arrest as judged by reduced number of cells observed using crystal violet staining (Figure 1b) and growth curves (Figure 1c) and reduced

expression of the proliferation marker Ki67 (Figure 1d), and increased frequency of SA- $\beta$ -Gal-positive cells (Figure 1e and Supporting Information Figure S1c) and increased expression of p21 (CDKN1A) and the SASP component IL-8 (Figure 1f,g). Knockdown of PLA2R1 with two independent shRNAs abolished all these hallmarks of cellular senescence (Figure 1b–g and Supporting Information Figure S1c). We conclude that PLA2R1 mediates premature senescence induced by the constitutive expression of progerin.

### 2.2 | Loss of PLA2R1 extends replicative potential of HGPS patient-derived cells

Primary dermal fibroblasts derived from patients with HGPS have a limited replicative potential compared to fibroblasts from healthy people (Eriksson et al., 2003). We introduced control or two different shRNA directed against PLA2R1 and verified PLA2R1 knockdown in two independent HGPS-derived cells (Figure 2a). PLA2R1 knockdown extended the ability of these cells to proliferate, reduced the percentage of SA- $\beta$ -Gal-positive cells, and reduced p21 and IL-8 expression (Figure 2b–g). In the same time frame of this experiment, we used control dermal fibroblasts without mutation in *LMNA* gene. These cells did not display premature senescence, and they were not impacted by the knockdown of PLA2R1 (Supporting Information Figure S2). Thus, PLA2R1 contributes to the reduced replicative potential of cells derived from patients with HGPS.

### 2.3 | Loss of PLA2R1 reverts accumulation of misshapen nuclei

Hutchinson–Gilford progeria syndrome patient-derived cells exhibit a high frequency of misshapen nuclei which may contribute to disease pathogenesis (Goldman et al., 2004). Therefore, we next determined the impact of inhibiting PLA2R1 expression on nuclear shape in normal human fibroblasts constitutively expressing progerin and in HGPS cells stained with antibodies to lamin A/C. As expected, progerin expression led to an increase in the frequency of severely misshapen nuclei, and this increase was largely reverted upon PLA2R1 knockdown (Figure 3a,b). Similar results were obtained in HGPS cells (Figure 3c). These results demonstrate that the absence of PLA2R1 sustains normal nuclear shape in the presence of progerin.

### 2.4 | PLA2R1 loss reduces defects in a mouse model of progeria

We next determined whether PLA2R1 contributes to progeria-like phenotypes *in vivo*. For this, we used *Zmpste24*-deficient mice which display accumulation of prelamin A, misshapen nuclei, and some hallmarks of progeria, including prominent bone alterations manifested as multiple rib fractures and reduced bone mineral content (Bergo et al., 2002; Figure 4a,b). Although knockout of *Pla2r1* did not change lifespan (Supporting Information Figure S3a), it reduced the number of rib fractures and increased bone mineral content (Figure 4a,b) and it improved grip strength (Supporting Information

**FIGURE 1** Phospholipase A2 receptor (PLA2R1) contributes to progerin-induced senescence. MRC5 cells were infected with retroviral vectors encoding the indicated genes and shRNAs and selected. (a) Fifteen days after selection, RNA was isolated, reverse-transcribed, and PLA2R1 transcripts were quantified by qPCR and normalized against ACTB levels. (b) Similar amounts of selected cells were seeded after the end of selection; cells were fixed 8 days later and stained with crystal violet. (c) At each passage, cells were counted and the number of population doublings was calculated, and the same number of cells was reseeded. (d) RNA was isolated 15 days after the end of the selection, reverse-transcribed, and Ki67 transcript levels were assessed by quantitative PCR and normalized against ACTB levels. (e) Twelve days after the end of the selection, cells were stained for SA- $\beta$ -Gal activity, and the percentage of SA- $\beta$ -Gal-positive cells was calculated. (f, g) RNA was prepared 15 days after the end of the selection, and RT-qPCR were performed against p21 (CDKN1A) or IL-8. Results were normalized against ACTB. All results in this figure are representative of at least three independent experiments. Error bars indicate SDs of triplicate measurement. Statistical analysis was performed with Student's *t* test (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.005).

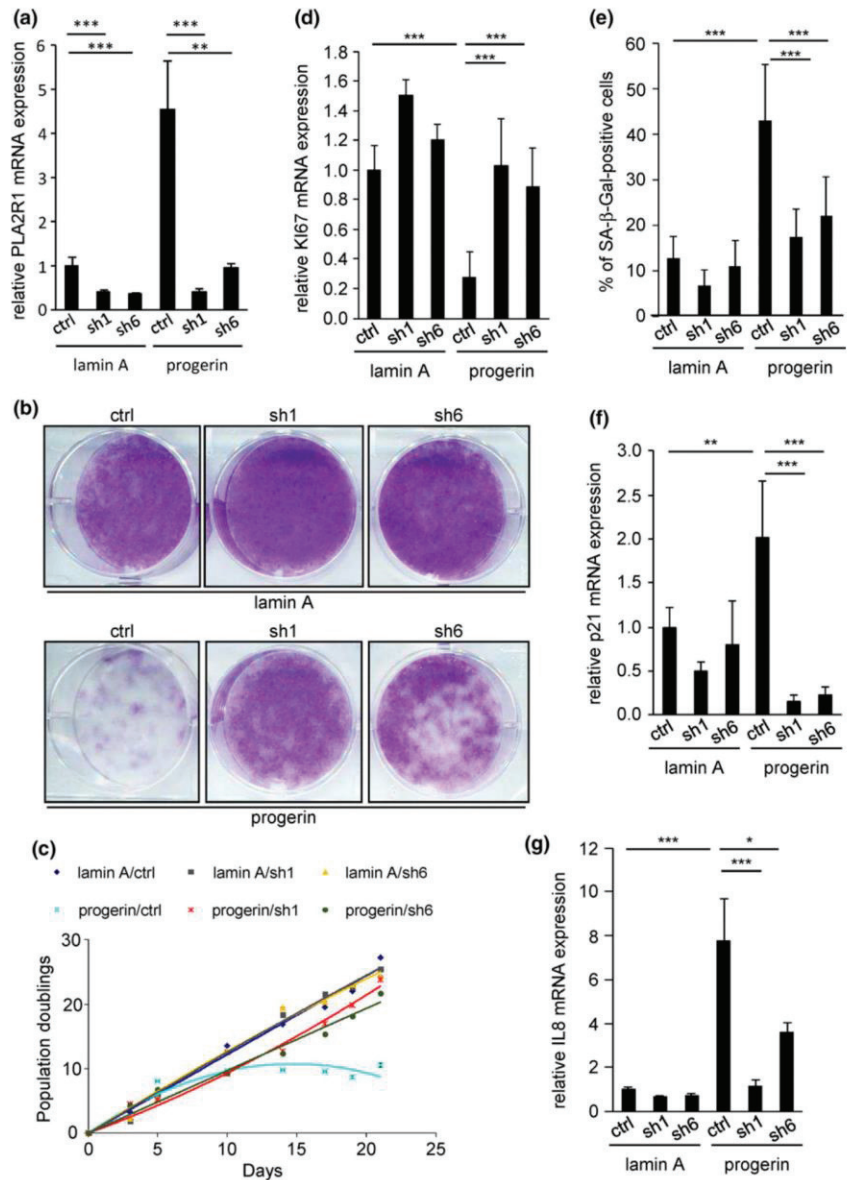
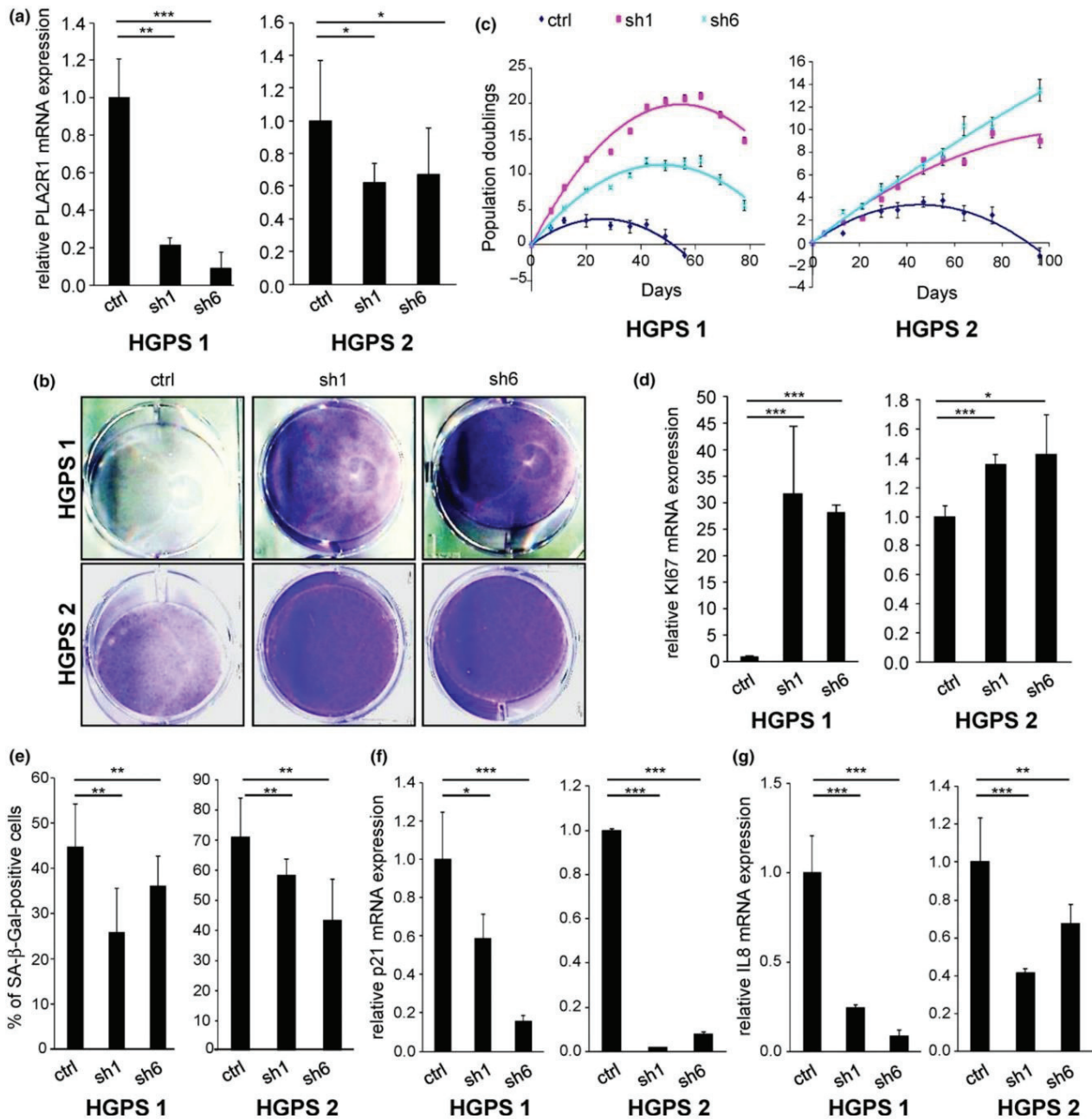


Figure S3b). Moreover, knockout of *Pla2r1* reduced trabecular separation in vertebral bone of *Zmpste24*-deficient mice, as judged by histomorphometric analyses (Figure 4c). Interestingly, we found higher levels of the p53 target and senescence marker p21 as well as of IL-8 senescence marker mRNA in bone of *Zmpste24*-deficient mice than in wild-type and that knockout of *Pla2r1* normalized those levels (Figure 4d,e). We conclude that loss of *Pla2r1* in a mouse model of progeria reduces bone alterations and signs of cellular senescence.

## 2.5 | Loss of PLA2R1 reduces farnesyl diphosphate synthase expression and thereby improves nuclear shape abnormalities and promotes senescence escape

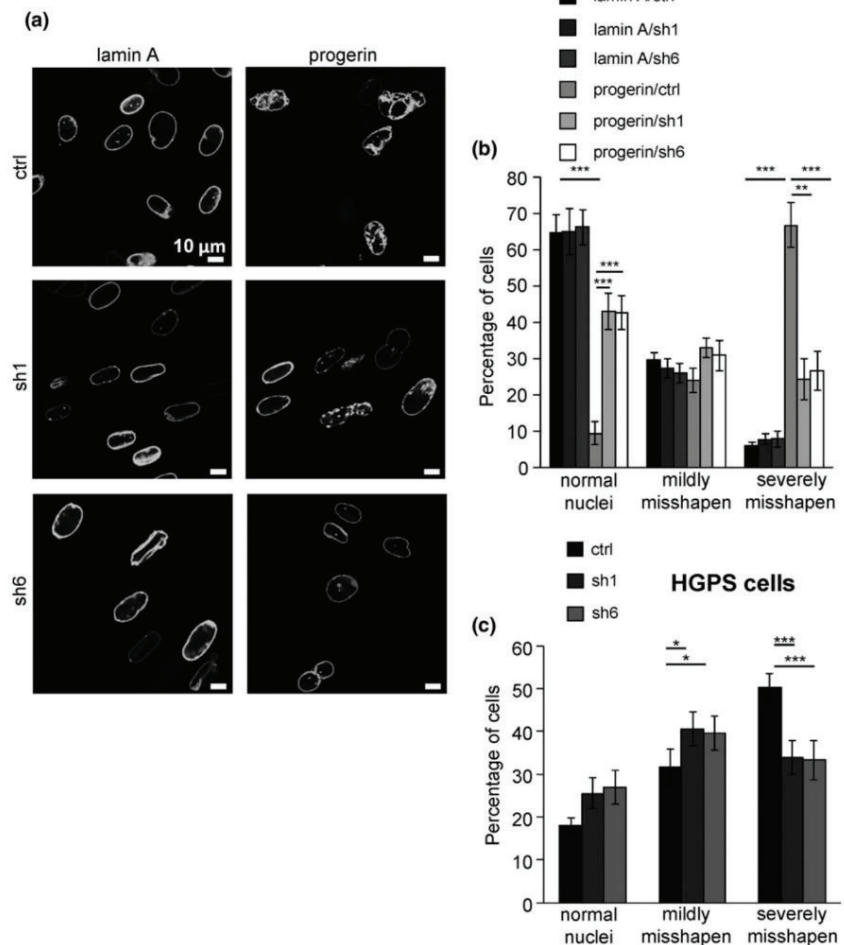
Farnesyl diphosphate synthase (FDPS) may promote the formation of misshapen nuclei upon progerin expression, and it is also a target

of bisphosphonate drugs which are used for clinical trials against HGPS (Gordon et al., 2016; Varela et al., 2008). We found increased FDPS expression in progerin-expressing cells, in human HGPS cells, and in mouse model (Figure 5a–c). The loss of PLA2R1 normalized FDPS expression in all three conditions (Figure 5a–c). To determine whether the reduced FDPS expression contributed to senescence escape and improved nuclear shape in PLA2R1-targeted cells, we used pamidronate, a bisphosphonate drug that inhibits FDPS activity. Indeed, incubating progerin-expressing cells with pamidronate reduced senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -Gal) activity; increased cell proliferation and Ki67 expression; and reduced p21 and IL-8 expression to levels observed in control cells (Figure 5d–i). Moreover, pamidronate reduced the frequency of misshapen nuclei (Figure 5j,k). These results suggest that reduced FDPS activity mediates the effects of PLA2R1 knockdown on progerin-induced senescence and misshapen nuclei.



**FIGURE 2** Loss of phospholipase A2 receptor (PLA2R1) extends the lifespan of Hutchinson–Gilford progeria syndrome (HGPS)-derived cells. HGPS-derived fibroblasts (HGPS 1 and 2) were infected with retroviral vectors encoding a scrambled or two different shRNAs directed against PLA2R1 and selected. (a) Twelve days after infection, RNAs were extracted reverse-transcribed, and qPCR was performed against PLA2R1 and ACTB for normalization. (b) Five days after infection, the same amounts of cells were seeded. Twelve days later, cells were fixed and stained using crystal violet. (c) Population doubling was calculated at each passage, and the same number of cells was reseeded. (d) The levels of the proliferation marker Ki67 were quantified by qPCR and normalized against ACTB levels 12 days after infection. (e) Cells were stained 8 days after infection for SA-β-Gal activity, and the number of SA-β-Gal-positive cells was counted for each condition. (f, g) Twelve days after infection, RNAs were prepared, and p21 or IL-8 mRNA levels were measured by RT-qPCR and normalized against ACTB mRNA. All results in this figure are representative of at least two independent experiments. Error bars indicate SDs of triplicate measurement. Statistical analysis was performed with Student's *t* test (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.005).

**FIGURE 3** Phospholipase A2 receptor (PLA2R1) promotes progerin-induced misshapen nuclei. (a, b) MRC5 cells were infected with retroviral vectors encoding lamin A-GFP or progerin-GFP together with control or PLA2R1 shRNA sequences. Ten days after selection, cells were analyzed by confocal microscope. Representative pictures are shown in panel a, and counting of normal, mildly misshapen, and severely misshapen nuclei was performed and is displayed in panel b. (c) Ten days after the end of the selection, immunofluorescence using a lamin A/C antibody was performed in HGPS-derived fibroblasts encoding either control or shRNA directed against PLA2R1. The same classification as in b was used. Around 200 nuclei were counted for each condition (mean  $\pm$  SEMs). The experiments shown are representative of at least three biological repeats. Statistical analysis was performed with Student's *t* test (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.005).



## 2.6 | Dominant-negative p53 reduces FDPS expression and improves nuclear shape

We showed earlier that PLA2R1 knockdown causes senescence escape by reducing DNA damage and p53 pathway activation (Augert et al., 2009). p53 also contributes to progerin-induced senescence, and it may regulate FDPS expression in cancer cells (Kudlow, Stanfel, Burtner, Johnston, & Kennedy, 2008; Laezza et al., 2015). However, whether p53 affects nuclear shape and whether it regulates FDPS expression in noncancer cells is not clear. As expected, progerin increased P-ATM and  $\gamma$ H2AX DNA damage marks (Figure 6a), p53 phosphorylation (Figure 6a), the p53 transcriptional target p21 (Figures 1f, 5h, and 6a), and FDPS (Figures 5a and 6a), and these inductions were abolished upon PLA2R1 knockdown (Figure 5a). Expressing a dominant-negative p53 (p53DN) overcame progerin-induced senescence: p53DN increased proliferation of progerin-expressing cells and reduced p21 and IL-8 expression (Supporting Information Figure S4). Similar to PLA2R1 inactivation, p53DN also reduced the frequency of misshapen nuclei and FDPS expression in progerin-expressing cells (Figure 6b–e). Furthermore, p53 activation by nutlin-3, a known activator of p53 (Vassilev et al.,

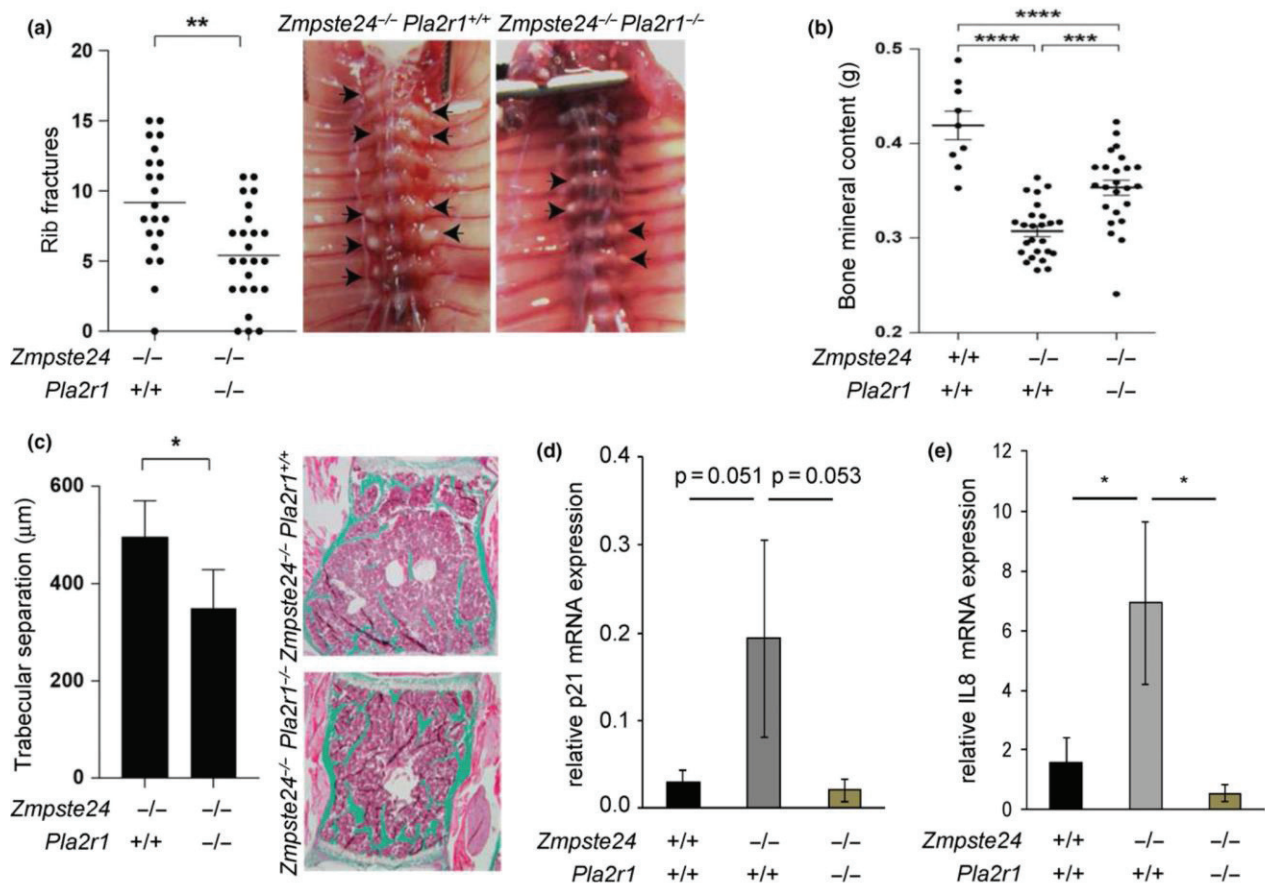
2004), led to increased FDPS mRNA similar to the observed p21 increase and led to binding of p53 on the p53 binding site previously described on FDPS promoter in cancer cells (Laezza et al., 2015; Figure 6f–g). The results suggest that p53 contributes to progerin/PLA2R1-induced misshapen nuclei and cellular senescence by upregulating FDPS expression.

## 3 | DISCUSSION

In this study, we found that PLA2R1 mediates effects of progerin expression on cellular senescence and some premature aging hallmarks. The knockdown of PLA2R1 overcame the proliferation arrest and other characteristics of senescent cells (SA- $\beta$ -Gal activity and increased IL-8) induced by progerin, either ectopically or endogenously expressed in mutated cells. Because PLA2R1 participates in senescence induced by short telomeres, oncogene, and oxidative stress, the current results reinforce the concept that PLA2R1 is a master regulator of cellular senescence (Augert et al., 2009; Bernard, & Vindrieux, 2014; Vindrieux et al., 2013).

It has previously been suggested that FDPS could promote the formation of misshapen nuclei upon progerin expression, and it is



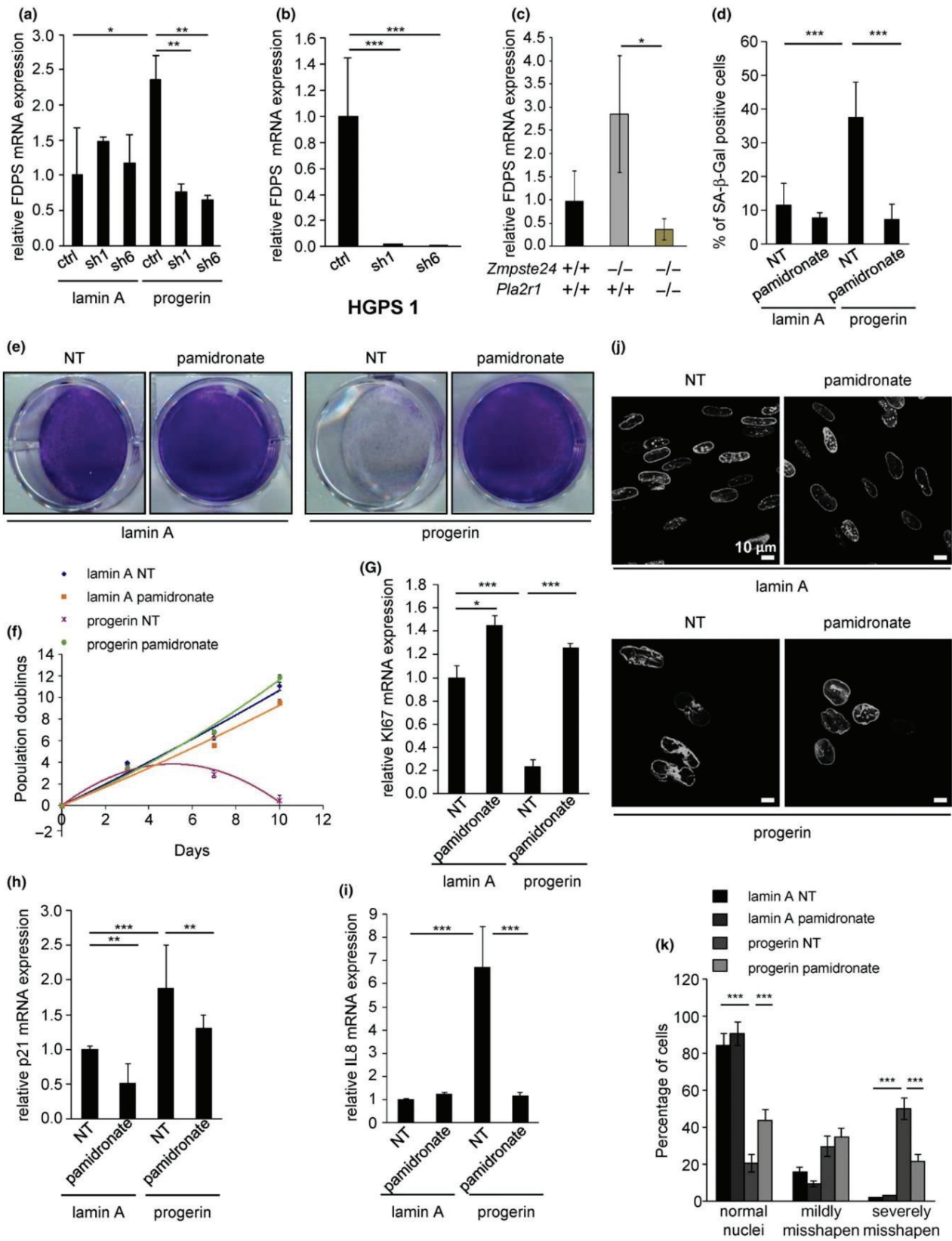


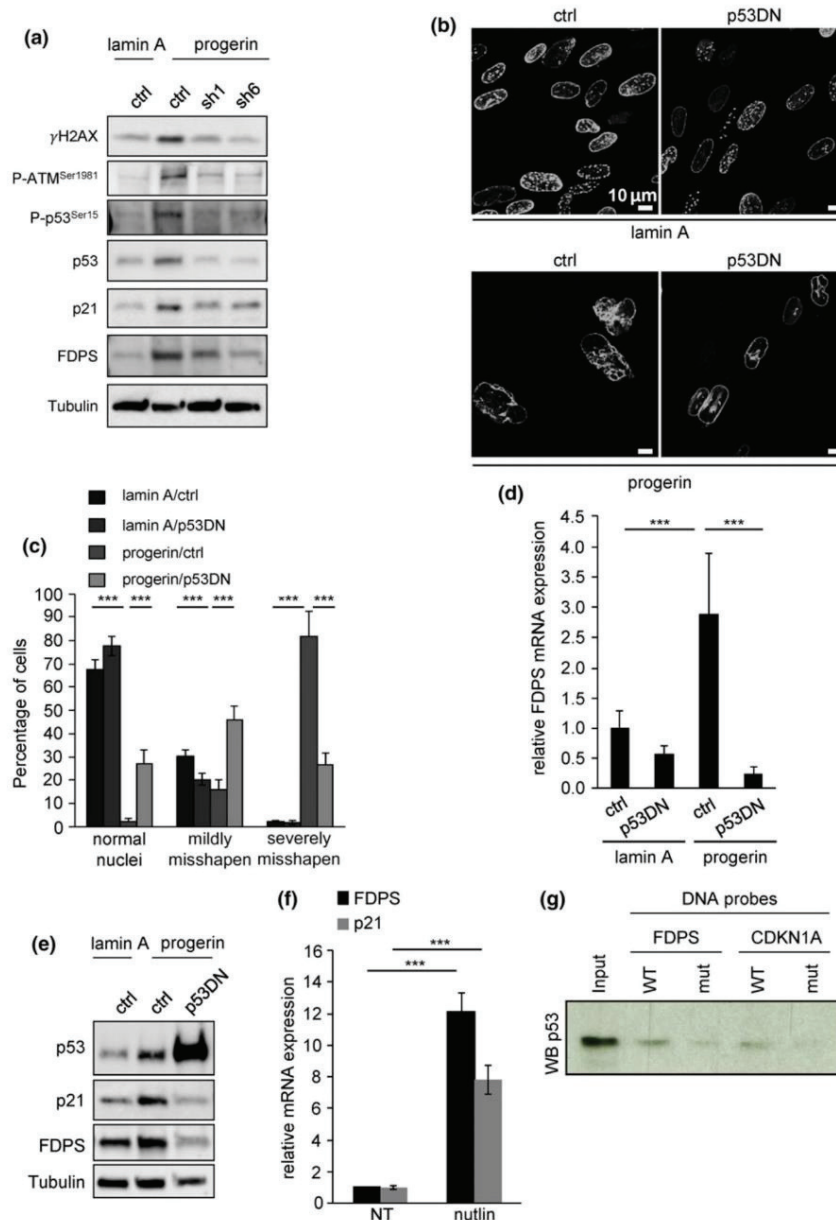
**FIGURE 4** Loss of *Pla2r1* partially rescues defects in a murine HGPS model. (a) Number of rib fractures in *Zmpste24*<sup>-/-</sup>*Pla2r1*<sup>+/+</sup> (n = 21) and *Zmpste24*<sup>-/-</sup>*Pla2r1*<sup>-/-</sup> (n = 24) mice. Ventral view of spinal columns from *Zmpste24*<sup>-/-</sup>*Pla2r1*<sup>+/+</sup> and *Zmpste24*<sup>-/-</sup>*Pla2r1*<sup>-/-</sup> mice. Arrowheads indicate rib cage calluses, and signs of rib fractures at costovertebral junctions. (b) DEXA analyses of bone from 12- to 15-week-old mice. (c) Trabecular separation of L4 vertebrae (n = 5 per genotype, mean ± SDs) and representative Masson–Goldner trichrome-stained sections of L4 vertebrae. (d, e) Bone mRNA levels of p21 and IL-8 (n ≥ 3 per genotype) after normalization against ACTB levels (mean ± SEMs)

also a target of bisphosphonate drugs which are used for clinical trials against HGPS (Gordon et al., 2016; Varela et al., 2008). Bisphosphonates target FDPS, and according to our results, FDPS levels increased upon progerin and prelamin A expression (i.e., in HGPS-derived cells and *Zmpste24*-deficient bone, respectively) and targeting FDPS with pamidronate overcame progerin-induced senescence

and misshapen nuclei. These results suggest that under normal conditions PLA2R1 contributes to progerin-induced phenotypes by stimulating FDPS expression. Progerin was found to increase PLA2R1 levels. Even if we do not know how, induction of PLA2R1 in the context of replicative senescence has already been described (Augert et al., 2009), suggesting that PLA2R1 is induced and it participates

**FIGURE 5** A progerin/PLA2R1 pathway increases FDPS expression and thereby induces senescence and misshapen nuclei. (a, b) Fifteen days after selection, RNAs were extracted from MRC5 or HGPS-derived cells, and mRNA levels of FDPS were quantified by RT–qPCR for each indicated condition. Results were normalized against ACTB levels. (c) RNA was extracted from the bone of mice of the indicated genotypes before RT–qPCR experiments to measure FDPS levels. FDPS levels were normalized against ACTB levels. The number of mice per group ranged from 3 to 5. (d–k) MRC5 cells were infected with retroviral vectors encoding lamin A-GFP or progerin-GFP. After selection and seeding the same quantity of cells, cells were treated every 3 days with 5 μM pamidronate (a bisphosphonate FDPS inhibitor). (d) After 15 days of treatment, cells were stained for SA-β-Gal activity, and the percentage of SA-β-Gal-positive cells was calculated. (e) Eight days after selection, the same amounts of cells were seeded. Ten days later, cells were fixed and stained with crystal violet. (f) At each passage, cells were counted and the number of population doublings was calculated, and the same number of cells was reseeded. (g–i) Fifteen days after selection, RNA was prepared, reverse-transcribed, and indicated transcript levels were quantified by qPCR and normalized against ACTB levels. (j, k) Fourteen days after selection, cells were analyzed by confocal microscopy. Representative pictures are shown, and counting of normal, mildly misshapen, and severely misshapen nuclei was performed. The experiments shown are representative of at least three biological repeats. Statistical analysis was performed with Student’s t test (\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.005).





**FIGURE 6** p53 promotes FDPS expression and misshapen nuclei in progerin-expressing cells. (a) MRC5 cells infected with retroviral vectors encoding lamin A or progerin together with or without a retroviral vector encoding shRNA directed against PLA2R1. Fifteen days after selection, protein extracts were prepared and analyzed using antibodies targeting the indicated proteins. (b–e) MRC5 cells were infected with retroviral vectors encoding lamin A or progerin together with or without a retroviral vector encoding a dominant-negative form of p53 (p53DN). (b) Twelve days after selection, photographs of nuclei and (c) quantification of normal, mildly misshapen, and severely misshapen nuclei were performed. (d) Ten days after infection and selection, RNAs were prepared, reverse-transcribed, and FDPS transcripts were quantified by qPCR. Results were normalized against ACTB mRNA levels. (e) Twelve days after selection, protein samples were prepared. Samples were analyzed by immunoblot using antibodies targeting the indicated proteins. (f) MRC5 cells were treated with nutlin-3, and 5 days later, RT-qPCR against FDPS and p21 was performed. Results were normalized against ACTB mRNA levels. (g) DNA pull-down assay showing p53 binding to a DNA motif in the FDPS promoter. A lysate of MRC5 primary human fibroblasts treated with nutlin-3 was incubated with FDPS probe or CDKN1A probe, harboring either a wild-type (WT) p53-binding motif or a mutant one with two base substitutions (mut). p53 binding was assessed by western blot. The experiments shown are representative of at least two biological repeats. Statistical analysis was performed with Student's *t* test (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.005$ ).

to different types of senescence. PLA2R1 regulates senescence through induction of a mitochondrial program leading to ROS production, DNA damage, and the p53 pathway (Augert et al., 2009;

Griveau et al., 2016). Progerin is able to induce DNA damage accumulation, eventually through ROS generation (Musich, & Zou, 2011; Richards, Muter, Ritchie, Lattanzi, & Hutchison, 2011; Wheaton

et al., 2017). Our data support a role of PLA2R1 in progerin-induced DNA damage as its knockdown reverts accumulation of DNA damage marks such as  $\gamma$ H2AX and P-ATM. DNA damage signaling leads to phosphorylation and activation of p53 transcriptional activity, both being observed in progerin-expressing cells and reversed in PLA2R1 knockdown cells. p53 has been reported to impact progerin-induced senescence (Kudlow et al., 2008) and once to bind to the FDPS promoter and to regulate FDPS expression in cancer cells (Laezza et al., 2015). Thus, it is conceivable that p53 downstream of progerin and PLA2R1 could mediate the increased FDPS expression. Accordingly, we found that p53 loss of function abolishes progerin-induced senescence, misshapen nuclei, and FDPS increase, whereas its activation by nutlin-3 induces FDPS expression suggesting the presence of a progerin/PLA2R1/p53/FDPS pro-senescent axis in HGPS cells.

Beyond *in vitro* results, little was known on the potential role of PLA2R1 in regulating age-related diseases, aging, or premature aging *in vivo*. Some HGPS and aging hallmarks are replicated in *Zmpste24*-deficient mice due to loss of lamin A maturation (Bergo et al., 2002; Burtner, & Kennedy, 2010; Gordon, Rothman, Lopez-Otin, & Misteli, 2014; Ibrahim et al., 2013). Our results using *Pla2r1* and *Zmpste24* double-knockout mice support a functional role for PLA2R1 in controlling cellular senescence and some aging hallmarks. Indeed, the loss of *Pla2r1* partially rescues the decreased bone content, the increased rib fracture, and the increased senescence markers observed in the bone of the *Zmpste24* knockout mice. Our results thus reveal an inverse correlation between senescence markers and bone content and fragility, suggesting that the correlation might be functionally linked. We can thus speculate that loss of PLA2R1, by reducing senescence, could improve this mark of aging. Supporting this hypothesis, a causal role for senescent cells has recently been described in bone loss during aging as eliminating senescent cells or inhibiting JAK kinases, in old mice, delayed bone loss (Farr et al., 2017). We have previously reported that PLA2R1 exerts its pro-senescent effects through JAK kinases reinforcing a potential link between PLA2R1, the mechanisms it controlled, and bone defects (Bernard & Vindrieux, 2014; Vindrieux et al., 2013). We can speculate that JAK kinase inhibitors could also be candidate drugs to fight premature aging, at least for the bone defects part.

The bisphosphonate family of drugs is used to fight bone loss during aging (Russell, Watts, Ebetino, & Rogers, 2008). Independently of this known effect, bisphosphonates also decrease misshapen nuclei in HGPS cells, which are proposed to participate in the disease, and trials using this drug have been set up for this reason (Gordon et al., 2016; Varela et al., 2008). Our results point out that bisphosphonate strongly impacts senescent phenotype reinforcing a potential functional link between bone fragility during aging and increase cellular senescence. It has also been reported that bisphosphonates can impact bone-independent marks of aging like emphysema (Ueno et al., 2015), emphysema being thought to be regulated by cellular senescence (Tarasviciene-Stewart, & Voelkel, 2008), further suggesting that FDPS/bisphosphonates can regulate cellular senescence and aging beyond their well-known bone effects.

In conclusion, our results demonstrate that PLA2R1 could be a new factor that contributes to hallmark phenotypes of premature aging and suggest that it could constitute a novel target to impact some aging parameters.

## 4 | MATERIALS AND METHODS

### 4.1 | Cell culture and reagents

MRC5 normal human fibroblasts (ATCC, Manassas, VA, USA) and virus-producing GP293 cells (Clontech, Mountain View, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, USA) containing GlutaMAX and supplemented with 10% FBS (Sigma-Aldrich, Saint Louis, USA) and 1% penicillin/streptomycin (Life Technologies). Dermal fibroblasts from patients with HGPS carrying the 1824 C > T mutation were obtained from the Progeria Research Foundation (HGADFN003; Peabody, MA) and the NIA Aging Cell Culture Repository (AG03199; New Jersey, USA); dermal fibroblasts from control patient without LMNA mutation were obtained from the NIA Aging Cell Culture Repository (AG03258; New Jersey, USA) and were cultured in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin. Information of primary fibroblasts and their usage in the figures are displayed in Supporting Information Table S1. Cells were maintained at 37°C under a 5% CO<sub>2</sub> atmosphere. Pamidronate (506600, Merck Millipore, Billerica, USA), a FDPS inhibitor, was used at 5  $\mu$ M. Nutlin-3 (Sigma-Aldrich), a p53 inducer, was used at 5  $\mu$ M.

### 4.2 | Vectors, transfection, and infection

The following vectors were supplied by Addgene: pBABE-puro-GFP-wt-lamin A (#17662) and pBABE-puro-GFP-progerin (#17663). PLA2R1-shRNA-encoding retroviral vectors have been previously described (Augert et al., 2009).

Virus-producing GP293 cells were transfected with vectors using the GeneJuice reagent according to the manufacturer's recommendations (Merck Millipore). Cells were transfected with the VSVg (1  $\mu$ g) and the retroviral vector of interest (5  $\mu$ g). Two days after transfection, the viral supernatant was mixed with fresh medium (1/2) and hexadimethrine bromide (8  $\mu$ g/ml; Sigma-Aldrich), and was then used to infect target cells for 6 hr. One day postinfection, selection was started with neomycin (100 mg/ml), puromycin (500 ng/ml), or both.

### 4.3 | RNA extraction, reverse transcription, and real-time quantitative PCR

RNA was extracted with phenol-chloroform using Upzol (Dutscher, Brumath, France). The Maxima First cDNA Synthesis Kit (Life Technologies) was used to synthesize cDNA from 1  $\mu$ g of total RNA. The reverse transcription (RT) reaction mixture was diluted 1/20 and used as cDNA template for quantitative PCR (qPCR) analysis. TaqMan qPCR analyses were carried out on a FX96 Thermocycler (Bio-

Rad, Hercules, USA). The PCR mixture contained TaqMan mix (Roche, Boulogne-Billancourt, France), 200 nM of primers, the Universal Probe Library probe (100  $\mu$ M) for the gene of interest (TaqMan Gene Expression Assays [Primers/probe]; Life technologies), and 1.67  $\mu$ l cDNA template. Reactions were performed in triplicate. The relative amount of mRNA was calculated using the comparative Ct ( $\Delta\Delta$ CT) method, following data normalization against ACTB for housekeeping genes. The PCR primers used for the qPCR are listed in Supporting Information Table S2.

#### 4.4 | Senescence-associated $\beta$ -galactosidase analysis, crystal violet staining, and growth curves

To perform SA- $\beta$ -Gal assays, cells were washed twice with PBS, fixed for 5 min in 2% formaldehyde/0.2% glutaraldehyde, rinsed twice in PBS, and incubated at 37°C overnight in SA- $\beta$ -Gal solutions as described (Augert et al., 2009). For crystal violet staining, cells were seeded on 6- or 12-well plates; staining was performed 8–10 days later as described (Augert et al., 2009). For growth curves, selected cells were seeded at the same density, split twice a week and counted; the population doubling was calculated at each passage.

#### 4.5 | Quantification of misshapen nuclei

Cells expressing lamin A-GFP or progerin-GFP vectors and HGPS patient-derived and control cells were seeded in 8-well chamber-slide plates (Dutscher); 2 days later, immunofluorescence was performed using a lamin A/C antibody (sc-6215; Santa Cruz Biotechnology). The cells were analyzed with a Zeiss LSM 780 NLO confocal microscope using a 63 $\times$  oil-immersion objective. Images were captured and processed using the Zen software (cropping, addition of scale bars). Nuclei were classified into three categories according to their shape: normal, mildly misshapen, or severely misshapen. Around 200 nuclei were counted for each condition.

#### 4.6 | Immunoblot

Twelve to fifteen days after retroviral infection with the indicated vectors, cells were directly lysed into Laemmli buffer supplemented with 10%  $\beta$ -mercapto-ethanol. The lysates were resolved on 12% or 4%–20% Mini-PROTEAN gels (Bio-Rad) and transferred to nitrocellulose membranes (0.22  $\mu$ m; Bio-Rad). The membranes were blocked with TBST–milk 5% for 1 hr and incubated with primary antibodies overnight at 4°C. Secondary antibodies were incubated for 1 hr at room temperature. Antibodies and dilutions used were as follows: p21 (C-19, sc-397, Santa Cruz Biotechnology, 1:500), p53 (DO-1, sc-126, Santa Cruz Biotechnology, 1:1,000), phospho-p53Ser15 (9284, Cell Signaling, 1:500),  $\alpha$ -tubulin (T6199, Sigma-Aldrich, 1:5,000), FDPS (HPA028200, Atlas Antibodies, 1:200), phospho-ATM S1981 (ab81292, Abcam, 1:1,000), PLA2R1 (AMAB90772, Atlas Antibodies, 1:500), and phospho-histone H2AX (2577S, Cell Signaling, 1/1,000). HRP-conjugated secondary antibodies were anti-mouse (115-035-003) and anti-rabbit (111-035-003) from Jackson Laboratories.

#### 4.7 | Mice

Mice were maintained on a mixed background (C57BL/6 and SV129). *Pla2r1*<sup>-/-</sup> mice (Hanasaki, Yokota, Ishizaki, Itoh, & Arita, 1997) were bred with *Zmpste24*<sup>-/-</sup> mice (Bergo et al., 2002) to produce *Zmpste24*<sup>-/-</sup>*Pla2r1*<sup>-/-</sup> mice; littermate *Zmpste24*<sup>-/-</sup>*Pla2r1*<sup>+/+</sup> mice were used as controls. The ability of mice to hang for more than 30 s on to an upside-down grid was assessed every week. Mice were considered having an impaired grip when they were unable to hang 2 weeks in a row. Mice were monitored daily. Mouse experiments were approved by the Research Animal Ethics Committee in Gothenburg.

#### 4.8 | Bone analyses

Whole-body bone mineral content was determined by dual-energy X-ray absorptiometry (DXA) using a Lunar PIXImus densitometer (Wipro, GE Healthcare). Bone histomorphometry was used to analyze trabecular bone in an undecalcified lumbar vertebra (L4) stored in 70% EtOH (Erben, & Glosmann, 2012). Each sample was dehydrated in increasing concentrations of EtOH, defatted in xylene, and embedded in methyl methacrylate. The embedded sample was sectioned longitudinally in a coronal plane using an automated rotary microtome (Leica RM2265; Leica Microsystems, Wetzlar, Germany) and a tungsten-carbide knife. Plastic 4- $\mu$ m sections were obtained from a standardized site of the vertebral body cavity. The sections were stained with Masson–Goldner trichrome, and trabecular separation was determined. In each section, the analysis of trabecular bone using OsteoMeasure 7 histomorphometry system (OsteoMetrics, Atlanta, GA, USA) was performed using a region of interest with the total area of 1.5 mm<sup>2</sup>.

#### 4.9 | DNA pulldown assay

MRC5 primary human fibroblasts were treated with 5  $\mu$ M nutlin-3. Five days later, cell lysate was prepared in HKMG buffer containing 10 mM Hepes pH 7.9, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 10% glycerol, 0.5% NP-40, 1 mM DTT, and protease inhibitors (Complete EDTA-free, Roche) and was precleared with preequilibrated streptavidin-coupled Dynabeads (Invitrogen). Annealed pairs of complementary oligonucleotides with the sense oligonucleotide biotinylated at the 5' end (Sigma) were used for DNA pulldown. Oligonucleotide sequences are listed in Supporting Information Table S3. These DNA probes were incubated overnight at 4°C with the cell lysate. DNA-bound proteins were then collected by incubation for 1 hr at 4°C with streptavidin-coupled Dynabeads (Invitrogen), washed four times in HKMG buffer, and separated by SDS-PAGE. p53 was detected by western blot (p53 antibody, DO-1, sc-126; Santa Cruz).

#### 4.10 | Statistical analysis

Values are mean  $\pm$  SD or SEM as indicated in the figure legend. Statistical analyses were performed using Student's *t* test or one-way

ANOVA with Bonferroni's post hoc test when comparing three or more groups (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.005$ ).

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## CONFLICT OF INTEREST

None declared.

## AUTHOR CONTRIBUTIONS

A.G. and C.W. designed and performed the experiments, analyzed the results, and wrote the manuscript. D.B. and M.O.B. supervised, conceived, and designed the experiments; analyzed the results; and wrote the manuscript. B.L.C., D.V.Z., S.D., M.W., N.M., J.M., and D.V. performed the experiments and/or analyzed the results. All authors reviewed or edited the manuscript and approved its final version.

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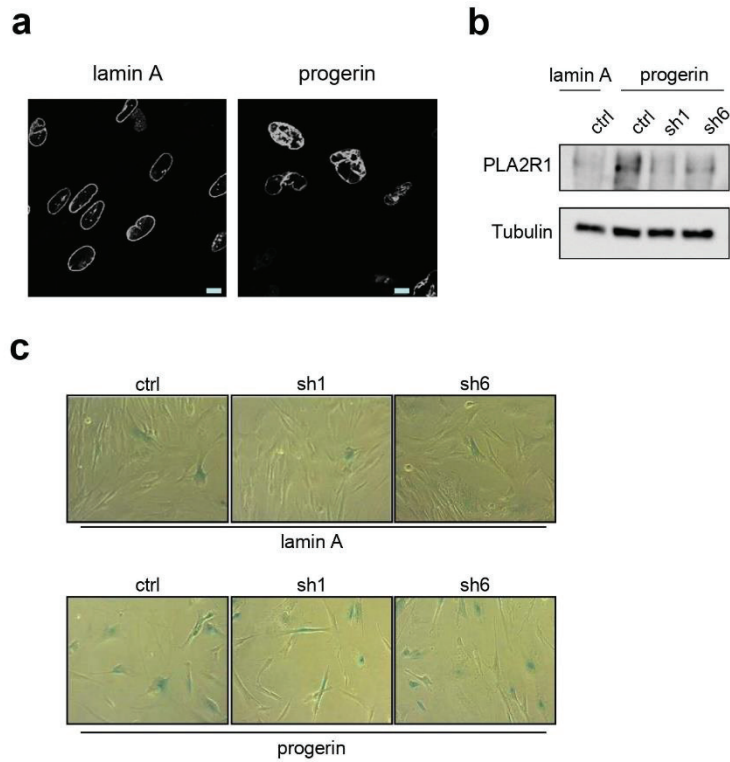
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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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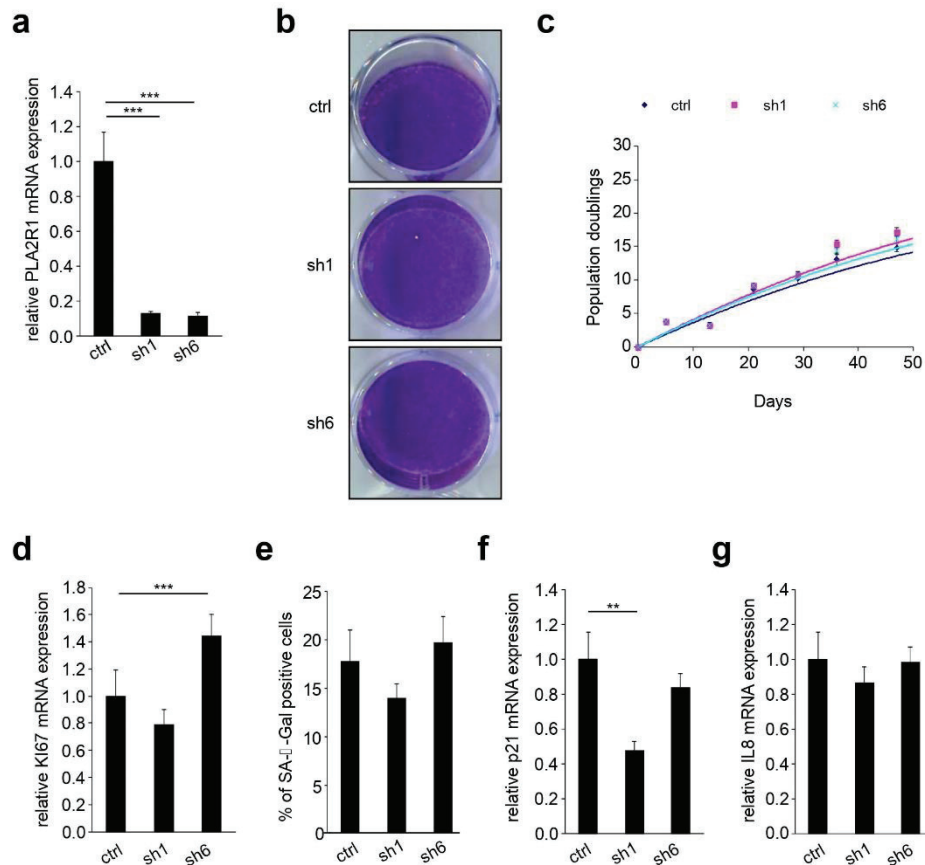
## Sup Figure 1



**Supplementary Figure 1** (a) Progerin-induced misshapen nuclei in normal human MRC5 fibroblasts. MRC5 cells were infected with lamin A-GFP or progerin-GFP encoding retroviral vectors. Confocal experiment was performed ten days after selection and representative images are shown. (b) MRC5 cells infected with retroviral vectors encoding lamin A or progerin together with or without a retroviral vector encoding shRNA directed against PLA2R1. Fifteen days after selection, protein extracts were prepared and analyzed using PLA2R1 and tubulin antibodies. (c) Representative photos of the SA-b-Gal staining used for the quantification displayed Figure 1e.

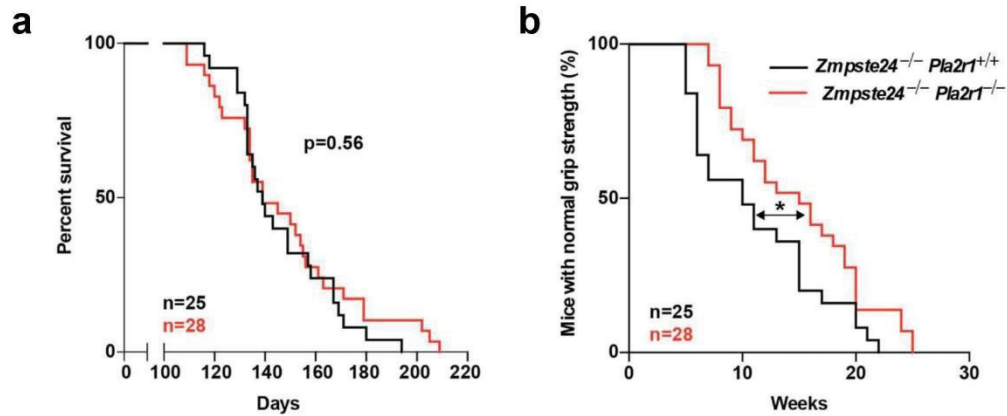


## Sup Figure 2



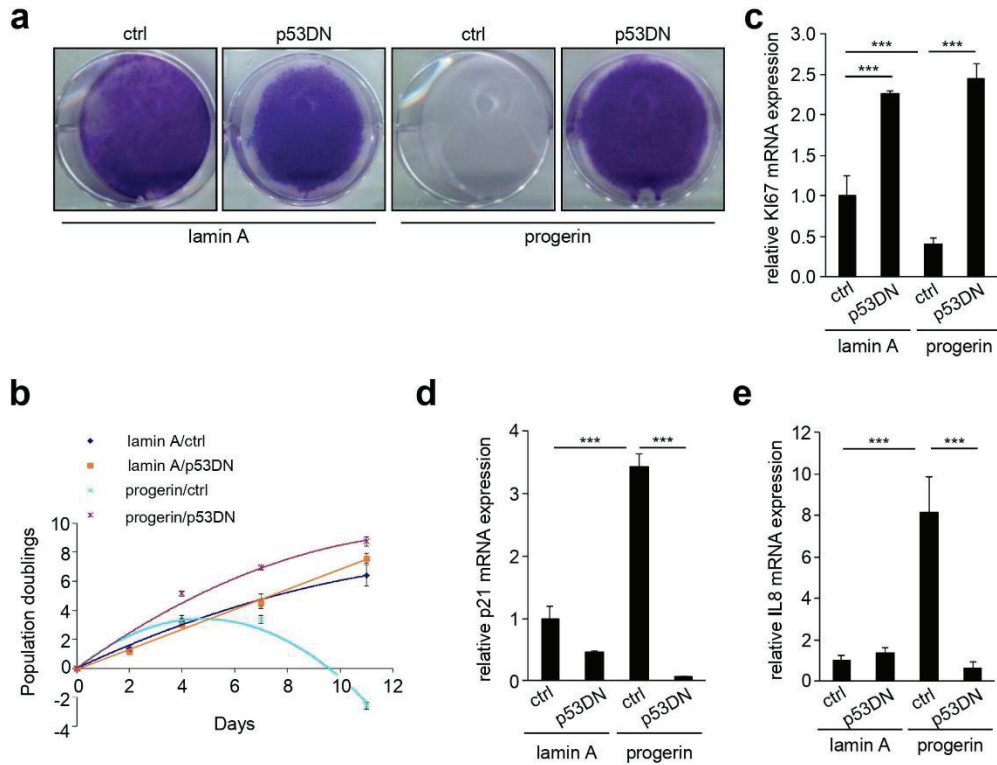
**Supplementary Figure 2** Behavior of control dermal fibroblasts without LMNA gene mutation. Cells were infected with retroviral vectors encoding control or two different shRNAs directed against PLA2R1. (a) Twelve days after infection, RNA was prepared. PLA2R1 knockdown was verified by RTqPCR. PLA2R1 transcript levels were normalized against ACTB levels. (b) Twelve days after seeding the same amount of cells, cells were fixed and stained using crystal violet. (c) At each passage, cells were counted and the number of population doublings was calculated at each passage and the same number of cells was re-seeded. (d) RNA was prepared 12 days after infection. Ki67 proliferation marker levels were quantified using RTqPCR after normalization to ACTB levels. (e) SA-β-Gal assays were performed 8 days after infection and the number of SA-β-Gal positive cells counted. (f-g) RNA was prepared 12 days after infection. p21 (CDKN1A) or IL8 mRNA levels were measured by RTqPCR and their levels normalized to the level of ACTB mRNA. Error bars indicate SDs of triplicate measurement. Statistical analysis was performed with the Student's t-test (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.005$ ).

## Sup Figure 3



**Supplementary Figure 3** Loss of PLA2R1 improves grip strength but not survival in *Zmpste24*<sup>-/-</sup> mice. (a) Kaplan-Meier plot showing survival of *Zmpste24*<sup>-/-</sup> *Pla2r1*<sup>+/+</sup> (n=25) and *Zmpste24*<sup>-/-</sup> *Pla2r1*<sup>-/-</sup> (n=28) mice. (b) Kaplan-Meier plot showing the percentage of *Zmpste24*<sup>-/-</sup> *Pla2r1*<sup>+/+</sup> (n=25) and *Zmpste24*<sup>-/-</sup> *Pla2r1*<sup>-/-</sup> (n=28) mice with normal grip strength. (\*P < 0.05).

## Sup Figure 4

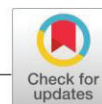


**Supplementary Figure 4** Loss of p53 function bypasses progerin-induced senescence. MRC5 cells were infected with retroviral vectors encoding the indicated products and selected. (a) Similar amount of cells were seeded. Eight days later, cells were fixed and stained using crystal violet. (b) At each passage, cells were counted and the number of population doublings calculated and the same number of cells was re-seeded. (c-e) RNAs were prepared, reverse transcribed, and indicated transcript levels were quantified by qPCR and normalized against ACTB levels. The experiments shown are representative of at least 3 biological repeats. Error bars indicate SDs of triplicate measurement. Statistical analysis was performed with the Student's t-test (\*\*\*)  $p < 0.005$ .

## Sup Table 1

Name	Cell type (origin)	Source	LMNA mutation	Name in the Figures	Figure using these cells
MRC5	Human fibroblasts (lung)	ATCC	WT	MRC5	Figure 1a-g; Figure 3a-b; Figure 5a, 5d-k; Figure 6a-h
HGADFN003	Human fibroblasts from HGPS patient (skin)	Progeria Research Foundation	1824 C > T	HGPS 1	Figure 2a-g; Figure 3c; Figure 4b
AG03199	Human fibroblasts from HGPS patient (skin)	NIA Aging Cell Culture Repository	1824 C > T	HGPS 2	Figure 2a-g
AG03258	Human fibroblasts from control patient (skin)	NIA Aging Cell Culture Repository	WT	control	Supplementary Figure 2

Table 1. Information of primary fibroblasts used and their usage.



## The JAK1/2 inhibitor ruxolitinib delays premature aging phenotypes

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### Abstract

Hutchinson–Gilford progeria syndrome (HGPS) is caused by an *LMNA* mutation that results in the production of the abnormal progerin protein. Children with HGPS display phenotypes of premature aging and have an average lifespan of 13 years. We found earlier that the targeting of the transmembrane protein PLA2R1 overcomes senescence and improves phenotypes in a mouse model of progeria. PLA2R1 is regulating the JAK/STAT signaling, but we do not yet know whether targeting this pathway directly would influence cellular and in vivo progeria phenotypes. Here, we show that JAK1/2 inhibition with ruxolitinib rescues progerin-induced cell cycle arrest, cellular senescence, and misshapen nuclei in human normal fibroblasts expressing progerin. Moreover, ruxolitinib administration reduces several premature aging phenotypes: bone fractures, bone mineral content, grip strength, and a trend to increase survival in a mouse model of progeria. Thus, we propose that ruxolitinib, an FDA-approved drug, should be further evaluated as a drug candidate in HGPS therapy.

### KEYWORDS

cellular senescence, JAK/STAT pathway, progeria, ruxolitinib

Hutchinson–Gilford progeria syndrome (HGPS) is a lethal premature aging disorder. HGPS is caused by a mutation in the *LMNA* gene, leading to the production of progerin, a truncated prelamin A form, which induces misshapen nuclei, cellular senescence, and aging (Goldman et al., 2004). The functional involvement of cellular senescence in promoting aging and age-related pathologies has been confirmed during the last few years (Childs et al., 2017).

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway regulates cellular senescence and some age-associated alterations, as evidenced by the use of the JAK1/2 inhibitor ruxolitinib, an FDA-approved drug used in the management

of myeloproliferative disorders, which decreased bone loss and adipose tissue inflammation and improved physical condition during aging in mice (Farr et al., 2017; Xu et al., 2015; Xu, Tchkonja, & Kirkland, 2016). These effects of ruxolitinib have been attributed to the inhibition of the senescence-associated secretory phenotype (SASP) (Farr et al., 2017; Xu et al., 2015, 2016).

The phospholipase A2 receptor (PLA2R1) is a transmembrane protein able to interact with some secreted PLA2 (sPLA2), collagens, and integrins. However, little is known about the role of this protein and of these interactions. PLA2R1 promotes cellular senescence in different contexts, and JAK/STAT signaling mediates

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the pro-senescent effects of PLA2R1 (Augert et al., 2009; Bernard & Vindrieux, 2014; Griveau et al., 2018; Vindrieux et al., 2013). Consistently, the JAK/STAT pathway drives the cell cycle arrest associated with PLA2R1-induced senescence, thus affecting senescence beyond the sole inhibition of the SASP as described by others (Farr et al., 2017; Xu et al., 2015, 2016) or/and because the SASP can be also a critical mediator of the full senescence (Acosta et al., 2008). Moreover, we recently observed that PLA2R1 contributes to progeria phenotypes *in vitro* in progerin-expressing cells and *in vivo* in the *Zmpste24*<sup>-/-</sup> murine model of progeria (Griveau et al., 2018).

These results raise the interesting possibility that targeting JAK/STAT signaling directly might overcome senescence and reduce disease phenotypes in progeria. In this study, we defined the impact of ruxolitinib, an FDA-approved JAK1/2-competitive inhibitor that binds to and inhibit their kinase activities (Mascarenhas & Hoffman, 2012), on the development of senescence and premature aging phenotypes induced by mutant prelamin A.

To define the ability of ruxolitinib to influence progerin-induced senescence, we used normal human fibroblasts (MRC5) constitutively expressing progerin or control cells expressing lamin A (Griveau et al., 2018). The expression of progerin strongly inhibited cell proliferation when compared to lamin A-expressing cells (Figure 1a,b). The addition of ruxolitinib prevented the progerin-associated growth inhibitory effect (Figure 1a,b). As expected, the constitutive expression of progerin induced premature senescence, as evidenced by an increase in senescence markers, such as senescence-associated  $\beta$ -galactosidase staining (SA- $\beta$ -Gal), increased expression of the cyclin-dependent kinase inhibitors (CKI) CDKN1A and CDKN2A, and increased expression of the IL-8 SASP component (Figure 1c-f). Strikingly, all of these senescent characteristics were abolished upon ruxolitinib treatment (Figure 1c-f). Moreover, similar results were obtained using HGPS-derived fibroblasts in which ruxolitinib increased the replicative potential and decreased several senescence markers (Figure S1). Hence, the JAK1/2 inhibitor ruxolitinib inhibits progerin-induced senescence in normal human fibroblasts.

Earlier studies revealed that PLA2R1 contributes to progerin-induced senescence by stimulating farnesyl diphosphate synthase (FDPS) expression and misshapen nuclei formation (Augert et al., 2009; Griveau et al., 2018; Vindrieux et al., 2013). FDPS controls the production of a farnesyl lipid, which is attached to the carboxyl terminus of progerin and which is required for its ability to cause disease and induce the characteristic nuclear shape abnormalities observed in HGPS cells (Varela et al., 2008). We argued that if JAK/

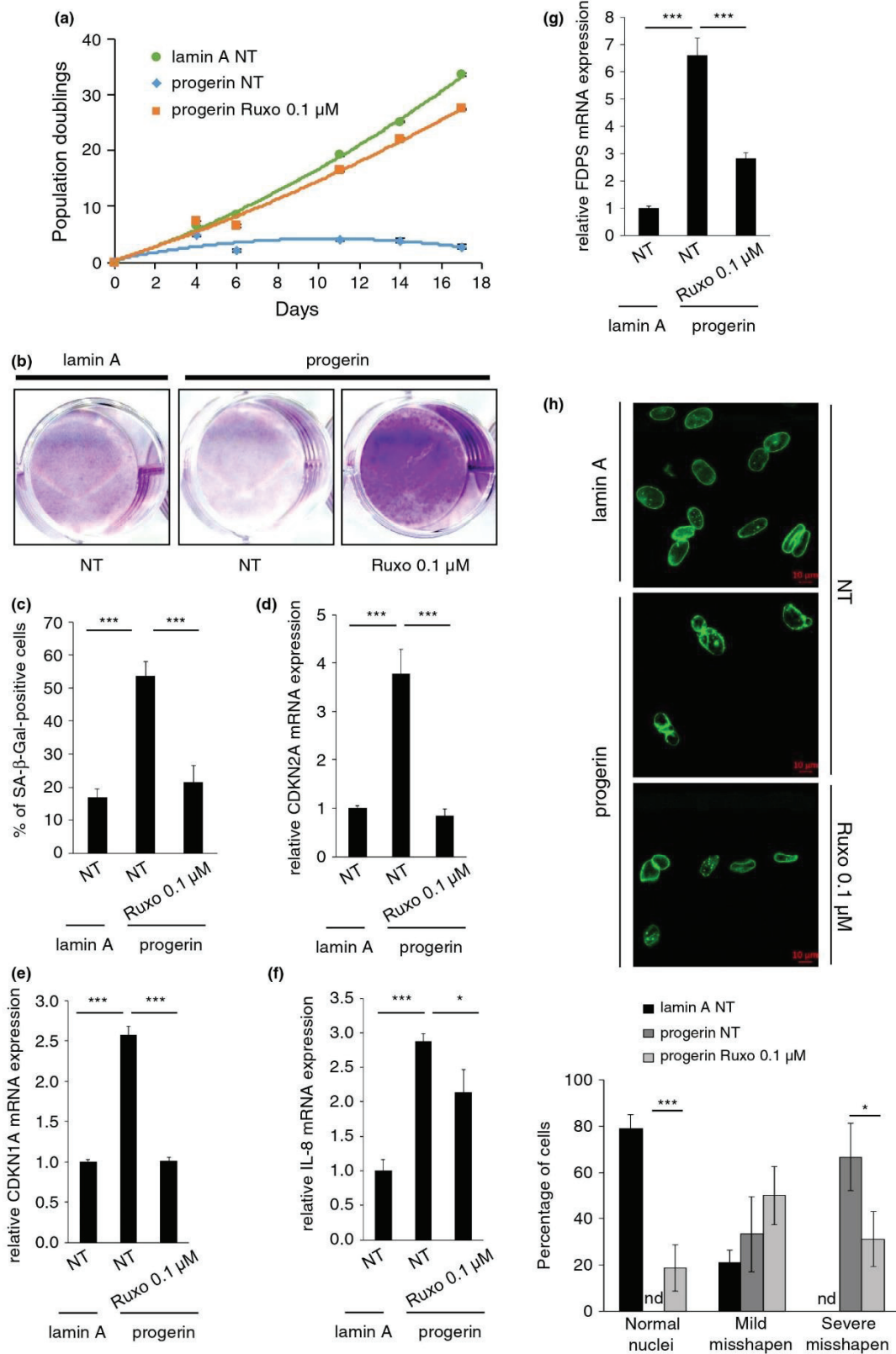
STAT signaling mediates PLA2R1-induced senescence in progerin-expressing cells, ruxolitinib should prevent those phenotypes. Indeed, that was the case: Ruxolitinib reduced the high level of FDPS expression and misshapen nuclei in progerin-expressing cells (Figure 1g,h), without changing GFP-progerin protein level (Figure 1h and Figure S2). Overall, these results confirm that JAK1/2 inhibition by ruxolitinib impacts not only the secretome of senescent cells, as already suggested by others (Farr et al., 2017; Xu et al., 2015, 2016), but also the cell cycle arrest, which can itself be induced by the SASP (Acosta et al., 2008).

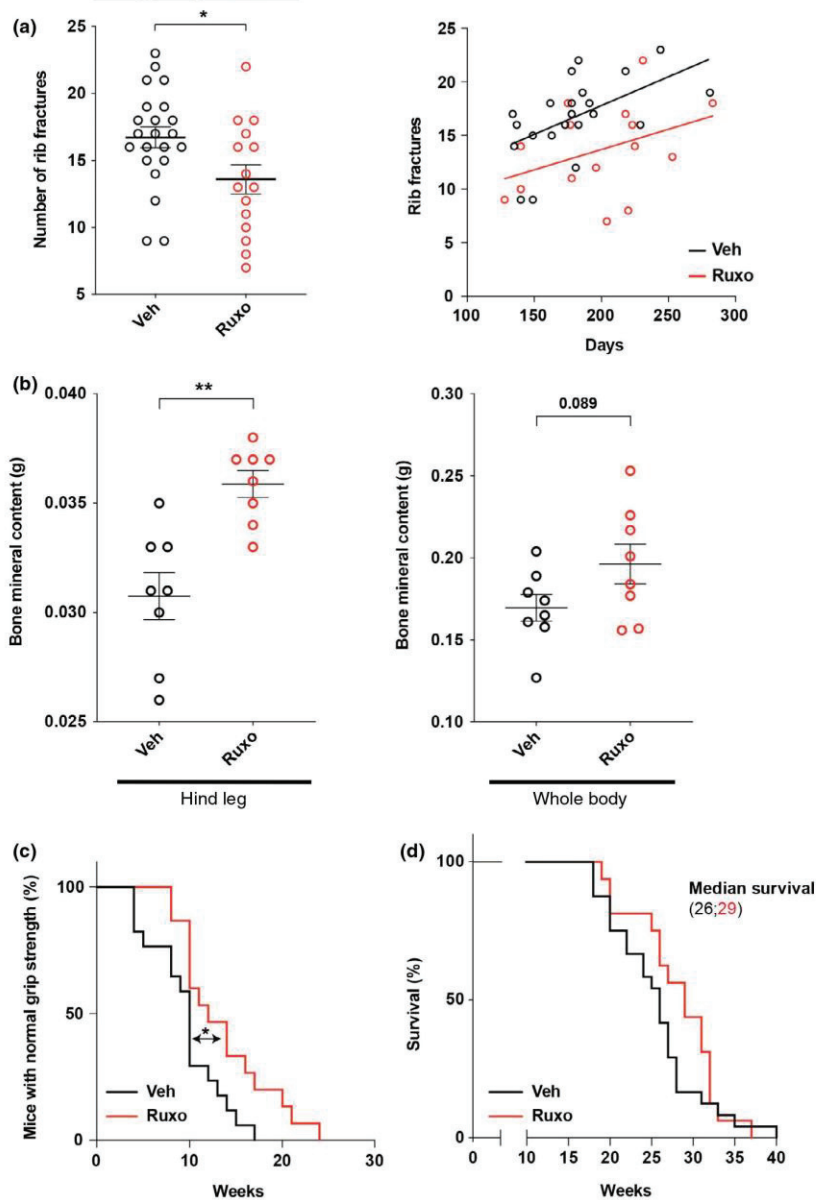
Next, we determined whether ruxolitinib treatment could affect progeria-like phenotypes *in vivo*. Full-length prelamin A accumulates in cells of *Zmpste24*-deficient mice, which triggers nuclear shape abnormalities and several hallmarks of progeria, including bone abnormalities, rib fractures, muscle weakness, low body weight, and premature death (Bergo et al., 2002). Ruxolitinib administration reduced the number of rib fractures and increased grip strength, bone mineral content (BMC), and survival (Figure 2a-d). These results represent the first proof of concept in cells and mice for the use of ruxolitinib in the treatment of premature aging syndromes.

The effect of ruxolitinib on progeria phenotypes is a phenocopy of that observed in *Pla2r1* knockout mice, that is, reduced numbers of rib fractures, increased BMC, and improved grip strength (Griveau et al., 2018). Moreover, similar to *Pla2r1*<sup>-/-</sup> mice, ruxolitinib did not affect body weight or bone mineral density (BMD) (Figure S3a,b). These results support the idea that JAK1/2 mediates PLA2R1-induced cellular senescence corroborating our previous findings (Griveau et al., 2016; Vindrieux et al., 2013). Even if unusual, increased BMC without increased BMD has already been reported in human and mouse studies (Andreassen & Oxlund, 2001; Khamoui et al., 2017; Van Loan, Johnson, & Barbieri, 1998; Windahl, Vidal, Andersson, Gustafsson, & Ohlsson, 1999), and this can eventually occur in the context of weight loss (Khamoui et al., 2017; Van Loan et al., 1998), the murine model of progeria used in our study losing weight. This increase in BMC without increase in BMD was explained by increased bone area (Figure S3c).

We administered ruxolitinib in subcutaneously implanted slow-release pellets. Advantages of this system are that it delivers drug continuously over a long period of time and it reduces the daily stress associated with traditional drug delivery approaches. A potential disadvantage is that drug doses will likely be lower than with traditional methods. Hence, it is possible that the effects of ruxolitinib observed in the current study are underestimated. This argument

**FIGURE 1** Ruxolitinib prevents progerin-induced senescence. MRC5 cells were infected with retroviral vectors encoding lamin A or progerin, and subsequently, puromycin was selected. Cells were then treated every 2 days with ruxolitinib at 0.1  $\mu$ M. (a) Growth curves were performed by counting and seeding the same number of cells at each passage. Cell population doubling was calculated. (b-c) Twelve days after the beginning of the treatment, the same number of cells was seeded, (b) fixed and stained by crystal violet, or (c) assessed for SA- $\beta$ -Gal activity. (d-g) RNAs were isolated 15 days after the beginning of the treatment and reverse-transcribed, and RT-qPCR was performed for CDKN2A, CDKN1A, IL-8, or FDPS. Results were normalized against ACTB. (h) Fifteen days after the beginning of the treatment, cells were analyzed by confocal microscopy. Representative pictures are shown, and counting of normal, mildly misshapen, and severely misshapen nuclei was performed. Nd means not detected. Results presented in this figure are representative of at least two independent experiments. Error bars indicate SD of measurements taken in triplicate. Statistical analysis was performed with Student's t test (\* $p$  < .05; \*\* $p$  < .01; \*\*\* $p$  < .005)





**FIGURE 2** Ruxolitinib delays premature aging phenotypes. (a) Left, number of rib fractures in *Zmpste24*<sup>-/-</sup> mice treated with vehicle ( $n = 22$ ) or ruxolitinib ( $n = 15$ ) at time of death. Right, plot showing the number of rib fractures (y-axis) and survival (x-axis) and the linear regressions obtained for each condition. (b) Dual-energy X-ray absorptiometry (DEXA) analyses of the bone mineral content (BMC) from mice at time of death are shown. ( $n = 8$ , five females and three males; per group, average age of mice analyzed is 26.75 weeks and 27.875 weeks for vehicle- or ruxolitinib-treated groups, respectively). (c) Kaplan–Meier plot showing the percentage of *Zmpste24*<sup>-/-</sup> vehicle-treated ( $n = 24$ ) and *Zmpste24*<sup>-/-</sup> ruxolitinib-treated ( $n = 16$ ) mice with normal grip strength. (d) Kaplan–Meier curves showing survival of vehicle-treated and ruxolitinib-treated mice ( $n = 24$  and  $n = 16$ , respectively). Errors bars indicate SEM. Statistical analyses were performed using the two-sided Student's *t* test for (a) and (b) and the log-rank test

could conceivably explain the robust effects of ruxolitinib on cultured cells and the relatively modest effects *in vivo*. Regardless, some improvements in disease phenotypes *in vivo* were statistically significant and reproducible. Overall, we believe our study should encourage the design of comprehensive preclinical trials to adjust dosage and routes of administration in progerin-knock-in mice, such as the *Lmna*<sup>G609G/G609G</sup> mouse model, which more faithfully recapitulates phenotypes of HGPS, alone or in combination with compounds such as farnesyl transferase inhibitor (FTI) (Gordon et al., 2016, 2012). Indeed, our results support that ruxolitinib, by inhibiting FDPS expression that is upstream of FT, impacts the same pathway than FTI. They may then cooperate to revert progerin effects.

Ruxolitinib is currently used as an antitumor agent in the context of myeloproliferative disorders and is proposed to decreased

inflammation (Bose & Verstovsek, 2017; Ghoreschi & Gadina, 2014; Lussana & Rambaldi, 2017), in agreement with previous observations that ruxolitinib represses pro-inflammatory SASP production (Farr et al., 2017; Xu et al., 2015, 2016). Although the use of ruxolitinib is relatively safe, there are reports of reduced blood cell counts and increased risk of infection and nonmelanoma skin cancer, which would be important to consider before testing in HGPS patients (Fabiano et al., 2015; Saeed, McLornan, & Harrison, 2017). Beyond ruxolitinib and JAK inhibition, another key mediator of the pro-inflammatory SASP, namely the NF- $\kappa$ B transcription factor, is also an important mediator of HGPS (Osorio et al., 2012). Taking into account the lack of current therapeutic opportunities for the devastating HGPS disease, ruxolitinib, possibly in combination with other validated drugs, could thus constitute a



new strategy (Gordon et al., 2016, 2012) to improve the health of children with HGPS.

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#### CONFLICT OF INTEREST

None declared.

#### AUTHOR CONTRIBUTIONS

AG, CW, and DVZ performed experiments. AG, CW, and DVZ analyzed experiments. MOB and DB designed the study. CW and DB wrote the manuscript with the contribution of all coauthors. MOB and DB supervised the study.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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## **Online Supporting Information**

### **Experimental procedures**

#### **Cell culture and reagents**

MRC5 normal human fibroblasts (ATCC, Manassas, VA, USA), dermal fibroblasts from patient with HGPS carrying the 1824 C > T mutation were obtained from the NIA Aging Cell Culture Repository (AG03199; New Jersey, USA) and virus producing GP293 cells (Clontech, Mountain View, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, USA) containing GlutaMax and supplemented with 10% FBS (Sigma-Aldrich, Saint-Louis, USA) and 1% penicillin/streptomycin (Life Technologies). Cells were maintained at 37°C under a 5% CO<sub>2</sub> atmosphere. Ruxolitinib (HY-50856, Clinisciences, Nanterre, France) was used at 0.1 μM.

#### **Vectors, transfection and infection**

The following vectors were supplied by Addgene: pBABE-puro-GFP-wt-lamin A (#17662), pBABE-puro-GFP-progerin (#17663). Virus-producing GP293 cells were transfected with vectors using the GeneJuice reagent according to the manufacturer's recommendations (Merck Millipore). Five million cells seeded in 10-cm dishes were transfected with the VSVg (1 μg) and the retroviral vector of interest (5 μg). Two days post-transfection, the viral supernatants were mixed with fresh medium (1/2) and hexadimethrine bromide (8 μg/ml; Sigma-Aldrich), and was then used to infect MRC5 cells for 6 h. One day post-infection, puromycin selection was initiated (500 ng/ml).

### **Senescence-associated $\beta$ -Galactosidase (SA- $\beta$ -Gal), crystal violet staining, and growth curves**

For senescence-associated- $\beta$ -Galactosidase assays, cells were washed twice with PBS, fixed for 5 min in 2% formaldehyde/0.2% glutaraldehyde, rinsed twice in PBS, and incubated at 37°C overnight in SA- $\beta$ -Gal solutions as described (Augert et al. 2009). For crystal violet staining, cells were seeded onto 6-well plates, prior to being stained 12 days later as previously described (Augert et al. 2009). For growth curves, selected cells were seeded at the same density, split as indicated and counted. The population doubling was calculated at each passage.

### **RNA extraction, reverse transcription, and real-time quantitative PCR**

RNA was extracted with phenol-chloroform using Upzol (Dutscher, Brumath, France). The Maxima First cDNA Synthesis Kit (Life Technologies) was used to synthesize cDNA from 1  $\mu$ g of total RNA. The reverse transcription (RT) reaction mixture was diluted 1/20 and used as cDNA template for quantitative PCR (qPCR) analysis. TaqMan qPCR analyses were carried out on a FX96 Thermocycler (Biorad, Hercules, USA). The PCR mixture contained TaqMan mix (Roche, Boulogne-Billancourt, France), 200 nM of primers, the Universal Probe Library probe (100  $\mu$ M) for the gene of interest (TaqMan Gene Expression Assays (Primers/probe); Life technologies) and 1.67  $\mu$ l cDNA template. Reactions were performed in triplicate. The relative amount of mRNA was calculated using the Comparative Ct ( $\Delta\Delta$ CT) method, following data normalization against actin for housekeeping genes. Sequence of the primers used were described previously (Griveau et al. 2018). Sequences of the primer used are: CDKN1A, fwd 5'-TCACTGTCTTGTACCCTTGTGC-3', rev 5'-GGCGTTTGGAGTGGTAGAAAT-3', UPL probe 32; CDKN2A, fwd 5'-GTGGACCTGGCTGAGGAG-3', rev 5'-CTTTCAATCGGGGATGTCTG-3', UPL probe

34; IL8, fwd 5'-AGACAGCAGAGCACACAAGC-3', rev 5'-ATGGTTCCTTCCGGTGGT-3', UPL probe 72; FDPS, fwd 5'-GAGTACCCGCCAACAAGC-3', rev 5'-ATCTAACCAGCGGGACA-3', UPL probe 15; GAPDH, fwd 5'-AGCCACATCGCTCAGACAC-3', rev 5'-GCCCAATACGACCAAATCC-3', UPL probe 60; ACTB, fwd 5'-ATTGGCAATGAGCGGTTC-3', rev 5'-GGATGCCACAGGACTCCAT-3', UPL probe 11.

### **Quantification of misshapen nuclei**

MRC5 expressing lamin A-GFP or progerin-GFP vectors treated or not with Ruxolitinib were seeded in 8-well chamber slide plates (Dutscher). The cells were analyzed with a Zeiss LSM 780 NLO confocal microscope using a 63× oil-immersion objective. Images were captured using the Zen software. Nuclei were classified according to their shape: normal, mildly misshapen or severely misshapen. At least 200 nuclei were counted for each condition.

### **Mice and Ruxolitinib treatment**

Mice knockout for *Zmpste24* (Bergo et al. 2002) were maintained on a pure C57BL/6N background and monitored daily and weighed weekly. As described previously, these mice appear normal during the first weeks of age, with a 5-10% lower body weight compared to wild-type animals. At 5-15 weeks old, mice show some signs of muscle weakness, and have a lower body weight than wild-type mice. Starting at 15 weeks of age, mice gradually lose weight, start to move more slowly, and appear more hunchbacked. Above 20 weeks, mice are less active, are wobbling or limping and keep losing weight. Mice have a lifespan of 20-35 weeks.

Mice, males and females, were randomly selected to be treated with either a vehicle or with Ruxolitinib. Ruxolitinib was administered through sub-cutaneous implantation of slow-release pellets (Innovative Research of America, Sarasota, FL, USA) of placebo or 30 mg/kg/day of Ruxolitinib, starting at 6 weeks of age. Two doses of pellets (18 mg or 30 mg of Ruxolitinib over 60 days) were designed to counterbalance the difference in weight between males and females. Mice were sacrificed when becoming moribund or when they had lost more than 20% of their weight over two consecutive weeks. Mouse experiments were approved by the Animal Research Ethics Committees in Gothenburg and Linköping, Sweden.

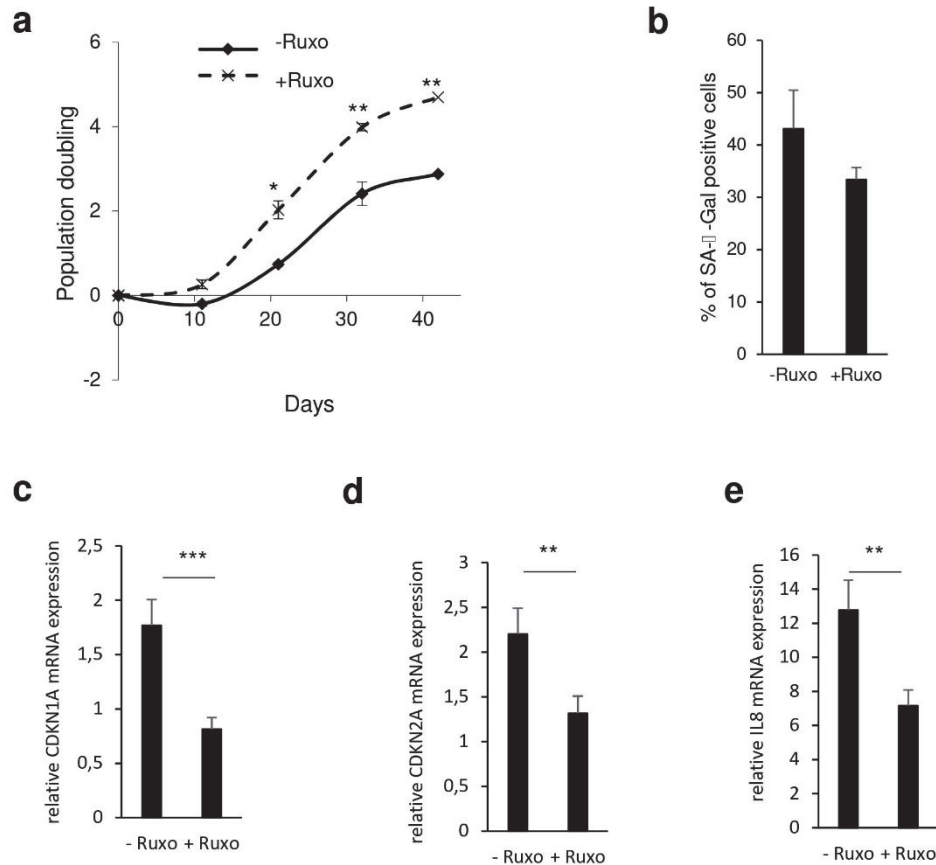
### **Phenotypical tests in mice**

The ability of mice to hang for more than 30s on to an upside-down grid was assessed every week (yes or no assessment). Mice were considered as having an impaired grip when they were unable (no) to hang 2 weeks in a row. Bone mineral content and Bone mineral density were determined on whole body (with ROI excluding head and tail) and on hind legs stored in 70% ethanol by dual-energy X-ray absorptiometry (DEXA) using a Lunar PIXImus densitometer (Wipro, GE Healthcare).

### **Statistical analyses**

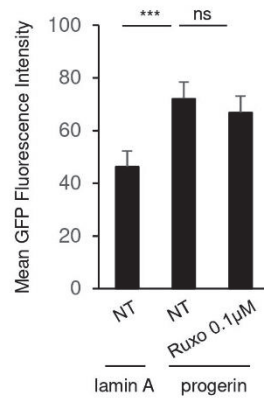
Statistical analyses were performed using Microsoft Excel or GraphPad v 8.2. Statistical significance for mouse survival and grip strength was assessed by a Log-rank (Mantel Cox) test. All other parameters were analyzed with unpaired two-sided Student t-test. (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.005$ ).

## Supplemental Figure 1



**Figure S1** Ruxolitinib delays replicative senescence in HGPS-derived fibroblasts. (a) Cells were treated or not every 2 days with 0.1  $\mu$ M of Ruxolitinib. Growth curves were performed and cumulative population doubling displayed. (b) SA- $\beta$ -Gal staining were performed after 30 days of treatment. (c-e) RNAs were prepared 30 days after the beginning of the treatment, reverse transcribed, and RT-qPCR were performed for CDKN1A, CDKN2A and IL8. Results were normalized using GAPDH. Error bars indicate SD of measurements taken in triplicate. Statistical analysis was performed with the Student's t test (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.005$ ).

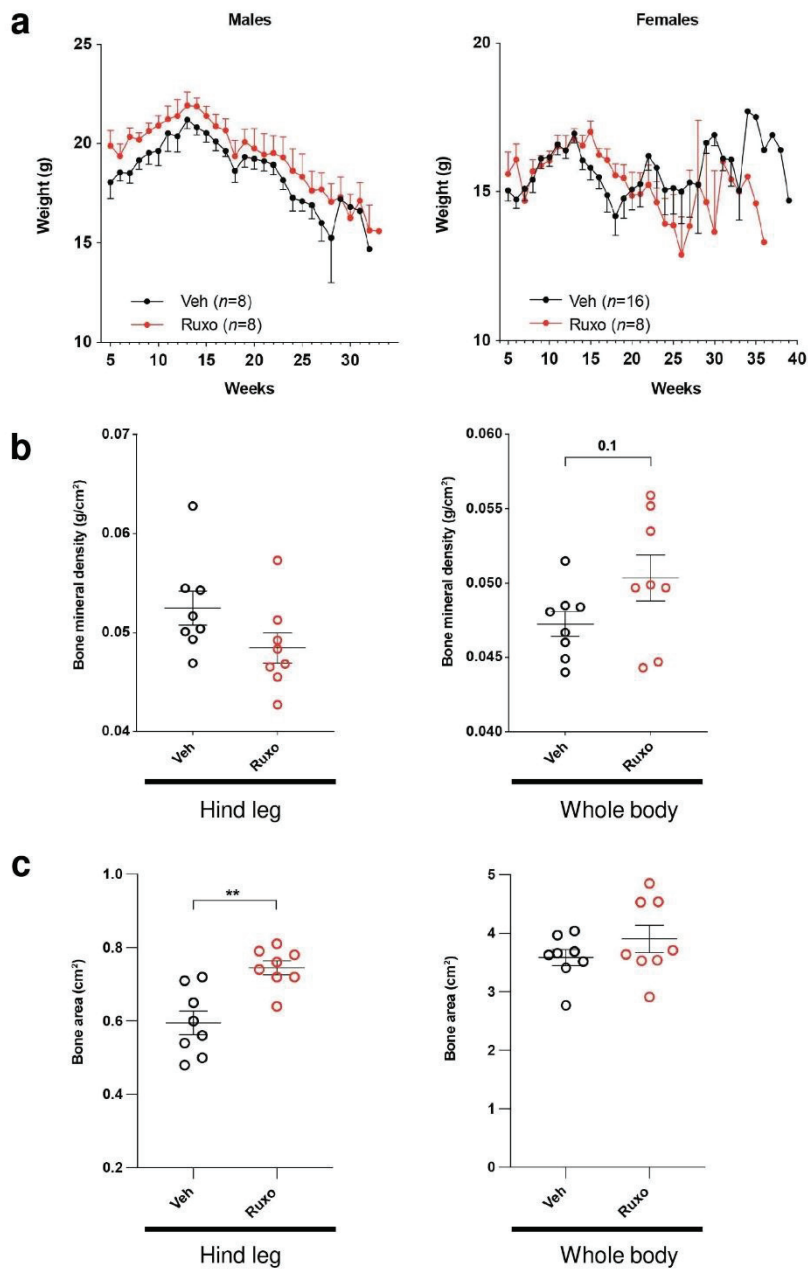
## Supplemental Figure 2



**Figure S2** Ruxolitinib does not affect levels of progerin. MRC5 cells were infected with retroviral vectors encoding lamin A-GFP or progerin-GFP, selected and then treated every 2 days with Ruxolitinib at 0.1  $\mu$ M. Fifteen days after the beginning of the treatment, cells were analyzed by confocal microscopy and GFP fluorescence intensity of nuclei quantified with ImageJ Software. Data are presented as mean fluorescence of 10-15 fields  $\pm$  SEM. Statistical analysis was performed with the Student's t test (\*\*\*)  $P < 0.005$ ).



### Supplemental Figure 3



**Figure S3** Ruxolitinib does not impact weight and bone mineral density of *Zmpste24*<sup>-/-</sup> mice. (a) Weight curves of *Zmpste24*<sup>-/-</sup> treated with vehicle or with Ruxolitinib. Left, males; right, females. (b-c) Dual-energy X-ray absorptiometry (DEXA) analyses of the bone mineral density (BMD) and bone area from mice at time of death are shown. (n = 8; 5 females and 3 males; per group, average age of mice analyzed is 26.75 weeks and 27.875 for vehicle or Ruxolitinib treated groups, respectively). Left, data obtained from hind leg; Right, data from whole-body.



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