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# The antioxidant transcription factor NRF2 as a new regulator of the extracellular matrix in human skin fibroblasts

Mélanie Salamito

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**Mélanie SALAMITO**

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**Le facteur de transcription antioxydant NRF2 comme nouveau régulateur de la matrice extracellulaire des fibroblastes de peau humaine**

**The antioxidant transcription factor NRF2 as a new regulator of the extracellular matrix in human skin fibroblasts**

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Co-encadrante de thèse  
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To my parents and grandparents,



À mes parents et grands-parents,



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<i>In press</i>	

## Abstract

The extracellular matrix (ECM) provides structural support for cells and tissues but it also functions as signaling platforms that can influence almost every decision made by the cells through chemical and mechanical signals. Dysregulation or deficiency of the ECM components can lead to various acquired and inherited disorders. Structural changes in the skin ECM also represent one of the hallmarks of the aging process. In that context, my PhD aimed at identifying new regulators of the dermal ECM synthesis and organization.

Over the past years, *Caenorhabditis elegans* has emerged as a powerful genetic model organism with predictive values in mammals to study the aging process. Our attention was particularly drawn to two genes: *aak-2* and *skn-1* for their regulation of *C.elegans* longevity through upregulation of several collagen genes. Collagens are the major proteins of the ECM. We therefore investigated if AMPK and NRF2, which are the mammalian orthologs of AAK-2 and SKN-1 respectively, could also regulate ECM gene expression in human skin primary fibroblasts, which are the main producers of ECM in skin.

As with aging AMPK and NRF2 activities decline, I used siRNA and/or shRNA to silence AMPK and NRF2 to mimic dermal aging and to investigate potential changes in functional and mechanical properties of the secreted ECM. Since the first set of data obtained with NRF2 was the most promising, I further focused my PhD work on the transcription factor NRF2.

NRF2 is a master regulator of the oxidative stress response and regulates the transcription of numerous cytoprotective genes. RNA-seq analysis of siNrf2 fibroblasts revealed that, additionally to NRF2 conventional targets, ECM genes were the most represented differently expressed genes. Composition and topology of the ECM produced by shNrf2 dermal fibroblasts were analyzed using a combination of light and electron microscopies. We showed that NRF2 silencing in human skin fibroblasts reduces collagen I expression and impacts collagen fibril formation and organization. Our data suggest that ZNF469, a zinc finger protein identified in our study as a new target of NRF2, is involved in the collagen I transcriptional regulatory cascade triggered by NRF2. ZNF469 is indeed involved in connective tissue diseases and has been suggested as a regulator of collagen expression.

In conclusion, this PhD work identifies NRF2 as a positive regulator involved in ECM regulation and homeostasis in human skin fibroblasts and in that, represents a promising new target in anti-aging strategies.

**Keywords:** human dermal fibroblasts, extracellular matrix, NRF2, collagens, fibrillogenesis, RNA-seq, RNA silencing strategies

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## Abbreviations

$\alpha$ -SMA:  $\alpha$ -smooth muscle actin  
ACC: acetyl-coA carboxylase  
AD: atopic dermatitis  
ADAM: a disintegrin and metalloproteinase  
AFM: atomic force microscopy  
AMPK: AMP-protein activated kinase  
ARE: antioxidant response element  
BCS: brittle cornea syndrome  
BM: basement membrane  
BMP: bone morphogenetic protein  
CAF: cancer-associated fibroblast  
CCN1: cysteine-rich protein 61  
ChIP: chromatin immunoprecipitation  
COL: collagenous domain  
COMP: cartilage oligomeric protein  
DDR: discoid domain receptor  
DEJ: dermal-epidermal junction  
DMF: dimethylfumarate  
EBP: elastin binding protein  
ECM: extracellular matrix  
EDS: Ehler-Danlos syndrome  
EGF: epidermal growth factor  
EMILIN: elastin microfibril interface located protein  
ERK: extracellular signal-regulated kinase  
FACIT: fibril associated with interrupted triple helix  
FAF: fibrosis-associated fibroblast  
FGF: fibroblasts growth factor  
FSP1: fibroblast-specific-protein 1  
GAG: glycosaminoglycan  
GSH: glutathione  
GO: gene ontology  
HA: hyaluronic acid  
IGF1: insulin growth factor 1  
JNK: c-Jun N-terminal kinase

KEAP1: Kelch-like ECH-associating protein 1  
LAP: latency-associated-peptide  
LLC: large latent complex  
LTBPs: latent TGF- $\beta$  binding proteins  
LOX: lysyl oxidase  
MACIT: membrane associated collagens with interrupted triple helices  
MAGPs: microfibril associated glycoproteins  
MAPK: mitogen activated protein kinase  
mi-RNA: micro-RNA  
MMP: matrix metalloproteinase  
MOI : multiplicity of infection  
NC : non-collagenous domain  
NRF2: nuclear factor erythroid 2-related factor  
P4H: prolyl-4-hydroxylase  
PAH: polycyclic aromatic hydrocarbons  
PDGF: placental derived growth factor  
PDI: protein disulfide isomerase  
PG: proteoglycan  
PM: particulate matter  
RISC complex: RNA-Induced Silencing complex  
ROS: reactive oxygen species  
SCC: squamous cell carcinoma  
SHG: Second Harmonic Generation  
SLRPs: small leucine rich repeat proteoglycans  
SME: smad binding element  
SPARC: secreted protein aspartic and rich in cystein/osteinectine  
SSc: systemic sclerosis  
TEM: transmission electron microscopy  
TGF- $\beta$ : transforming growth factor  $\beta$   
TGM: transglutaminase  
TIMP: tissue inhibitor metalloproteinase  
TNF- $\alpha$ : tumor necrosis factor  $\alpha$   
TSS: transcription start site  
XRE: xenobiotic response element

## Preamble: Can *C. elegans* longevity factors be transposed to human skin?

I started my PhD in September 2016 in the laboratory of Dr. Hugo Aguilaniu at the IGFL in Lyon, working on biological aging pathways in *C.elegans*. For unforeseen circumstances and moving of Hugo Aguilaniu to Brazil, I joined Florence Ruggiero's team in March 2017. This PhD project has thus been mainly conducted in both the laboratory of Florence Ruggiero at the IGFL in Lyon, who is a world-renowned expert in extracellular matrix proteins and especially in collagens, and the Advanced Research Department of L'Oréal in Aulnay-sous-Bois, which has a strong expertise in skin biology and in the transcription factor NRF2.

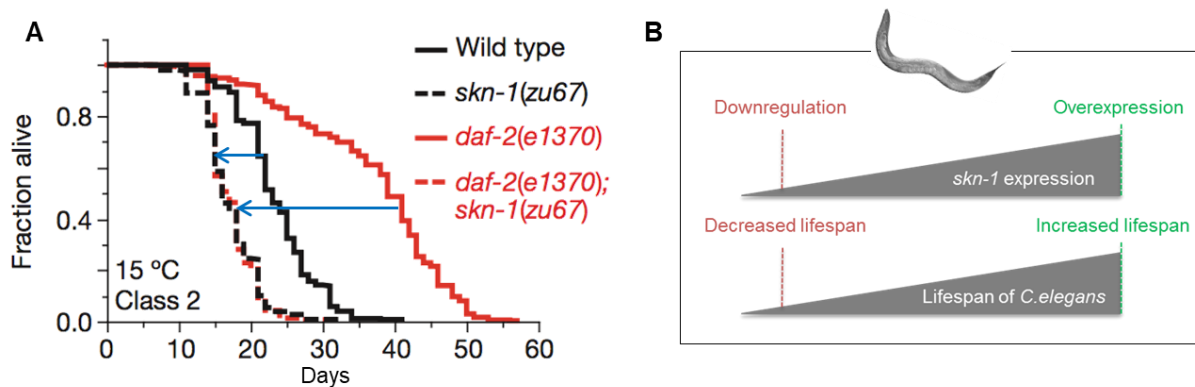
Over the past thirty years, *C.elegans* has been proven to be an excellent model organism for genetic analyses, with powerful predictive values in mammals (Riddle et al., 1997). Remarkably, intensive research using *C. elegans* has established most, if not all, fundamental signaling pathways implicated in the biological aging process (Kaletta and Hengartner, 2006). One key pathway of interest is the insulin/IGF-1/FOXO signaling axis, which is highly conserved from worm to humans (Kenyon, 2010). Further research has shown that this signaling axis is modulated in response to a number of physiological perturbations that have been associated with enhanced lifespan, such as caloric restriction, hormonal perturbation *via* germline signaling or down regulation of mitochondrial metabolism (Kenyon, 2010).

Our attention was particularly drawn to the *C. elegans* longevity factors AAK-2 and SKN-1. The *aak-2* gene is encoding the catalytic  $\alpha$  subunit of the AMPK activated protein kinase. Worms mutated for *aak-2* are short-lived compared to wild-type animals, while overexpression of *aak-2* prolongs worm lifespan (Apfeld et al., 2004). Research in mammals has identified AMPK as a key factor of the general aging process (Salminen and Kaarniranta, 2012) and, since then, AMPK has emerged as a potential target to slow down aging in humans and has captured the attention of many scientists (Longo et al., 2015). Interestingly, it was shown that in AAK-2 overexpressing worms 31 out of 549 differentially expressed genes were encoding collagens, which are the most abundant extracellular matrix (ECM) proteins (Ewald et al., 2015). This therefore suggests a link between AMPK activity and ECM gene expression. I will expand on this subject in the "additional results" section.

The second factor, SKN-1, is the worm ortholog of the mammalian transcription factor NRF2, for nuclear factor erythroid-2-related factor 2 (also known as nuclear factor erythroid-derived 2-like 2) which is encoded by *NFE2L2* in human. *C.elegans* studies have evidenced



this transcription factor as a key regulator of longevity. Worms mutated for *skn-1* have a shortened lifespan (An and Blackwell, 2003) and moderate overexpression of *skn-1* extends the worm lifespan (Figure 1) (Tullet et al., 2008) whereas high levels of SKN-1 in worms has been shown to be harmful. Additionally, the expression of *skn-1* in *C.elegans* was found to be up-regulated in major interventions that are known to increase longevity, such as reduced insulin/IGF-1 signaling, dietary restriction or inhibition of TOR (Onken and Driscoll, 2010; Robida-Stubbs et al., 2012; Tullet et al., 2008). These three signaling pathways have also been shown to increase the longevity in mice with a concomitant up-regulation of NRF2 (Bartke et al., 2013; Johnson et al., 2013; Longo et al., 2015). Despite some divergences in the DNA binding sequences of the transcription factor and in its regulation between *C.elegans* and mammals, SKN-1/NRF2 function seems to be remarkably conserved across evolution, including its major role in antioxidant and detoxification processes (An and Blackwell, 2003). The recent study from Ewald and collaborators that links SKN-1 to the expression of extracellular matrix genes (Ewald et al., 2015) prompted us to investigate the role of NRF2 in human skin. As we obtained very interesting preliminary data, my PhD work mainly focused on NRF2 and its action on ECM gene expression.



**Figure 1. SKN-1 is a master regulator of longevity.**

(A) Longevity analyses of *daf-2* mutants (*daf-2(e1370)*), *skn-1* mutants (*skn-1(zu67)*), and the double *daf-2*, *skn-1* mutants (*daf-2(e1370);skn-1(zu67)*). *skn-1* mutants have decreased lifespans. Mammalian homologues of *daf-2* and *skn-1* are respectively IGF-1 and NFE2L2. From Ewald et al., 2015. (B) Schema illustrating the master regulator role of *skn-1* in *C.elegans* longevity.

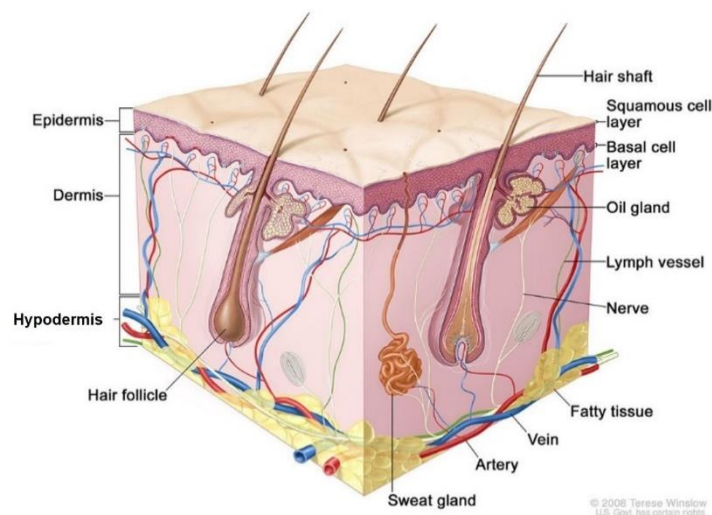
# **I. Introduction and objectives**

# 1. Introduction to human skin

## 1.1. Properties and functions of the human skin

Skin is the largest organ of the human body with a surface of two square meters and representing 15% of an adult's body weight (Kanitakis, 2002). Its thickness depends on the anatomic sites, and varies from 0.3 mm on the lips and around the eyes to 4 mm on the palms and soles.

Being the outermost layer of the human body, skin ensures multiple vital functions by ensuring an efficient barrier against external stresses like UV, mechanical forces, chemical, microbiological factors but also by regulating water loss. Skin is also an important endocrine organ responsible for the synthesis and secretion of hormones and essential vitamins, notably vitamin D, but also an essential sensory organ with its ability to sense heat, pressure, touch, and pain, thanks to its different sensory receptors. It can therefore be considered as a real 'brain on the outside' (Tobin, 2017). Besides, skin plays a fundamental role in the homeostasis of the body by regulating its temperature and immune response (Matejuk, 2018). Last but not least, the skin has an important psychological and social function by affecting our self-esteem and integration in the society (McLafferty et al., 2012). The skin is therefore a highly dynamic organ with multiple complex functions, which allows us to adapt to and interact with the environment throughout life.



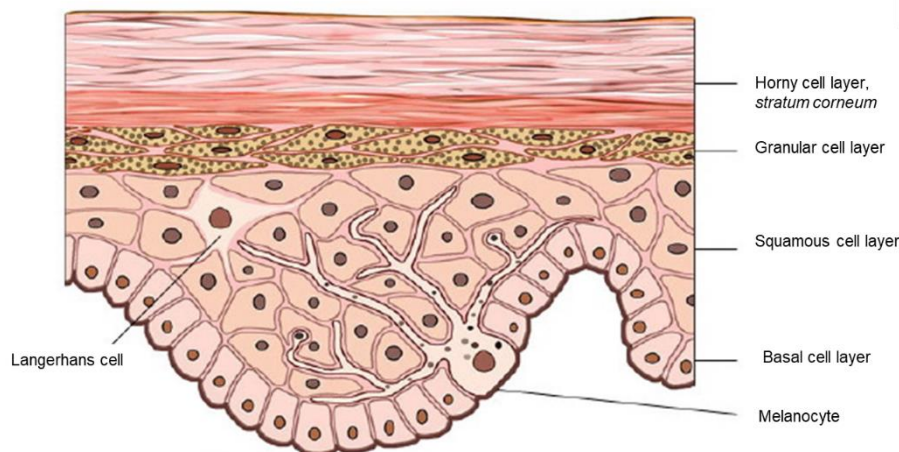
**Figure 2. Structure and organization of the human skin.**

Skin is divided in three main layers: epidermis, dermis and hypodermis, and includes various appendages as notably hair follicle, sweat glands and nerves. Modified from Terese Winslow LLC, Medical And Scientific Illustration.

Skin is composed of three main layers that differ in their structure and organization. The most external layer is the epidermis and the connective layer below is called the dermis, which contains the skin appendages. These two layers are separated by the dermal-epidermal junction, and supported by an adipose subcutaneous tissue, the hypodermis (Figure 2).

## 1.2. The epidermis

The epidermis is a stratified squamous epithelium, which continuously undergoes renewal. Epidermis is primarily composed of keratinocytes (95%), which go through different differentiation steps from the deepest layer to the external layer. Based on the different stages of keratinocyte differentiation, the epidermis is subdivided into four layers: the basal layer, the spinous layer, the granular layer and the stratum corneum (Figure 3). The differentiation process is tightly regulated to ensure epidermal homeostasis. Keratinocytes produce keratin, which is a fibrous and highly insoluble protein that confers protection to the epidermis (Fuchs and Green, 1980).



**Figure 3. Representation of keratinocyte differentiation throughout epidermis, with the associated four layers.**

From DermNetNZ.org.

The basal layer is composed of cubic proliferative keratinocytes with a perpendicular orientation to the underlying dermis. These keratinocytes are anchored to the basement membrane through specialized junctional complexes, the hemidesmosomes, and synthesize keratins 5 and 14. In the spinous layer, keratinocytes have a lower proliferation rate, begin to flatten and become polygonal cells, which are connected to each other by desmosomes.

Spinous keratinocytes express keratins 1 and 10, whereas the expression of keratins 5 and 14 is decreased (Eckert et al., 2005). The upper granular layer is characterized by flattened non-proliferative keratinocytes which produce keratohyalin granules. These latter are filled with profilaggrin and intermediary filament of keratins. At this stage, keratinocyte express late differentiation markers, such as loricrin and involucrin. Stratum corneum contains the last differentiation stage of keratinocytes called corneocytes. They are enucleated dead cells but biochemically highly active. The protein-rich corneocytes can be considered as 'bricks' embedded in a matrix of ceramides, cholesterol and fatty acids, which derived from the processed and degraded filaggrin and keratins (Waller and Maibach, 2005). Cohesion between corneocytes is ensured by corneodesmosomes, which are further degraded at the surface of the skin, thereby leading to the detachment of the corneocytes. This process is called desquamation and allows epidermal renewal (Wikramanayake et al., 2014).

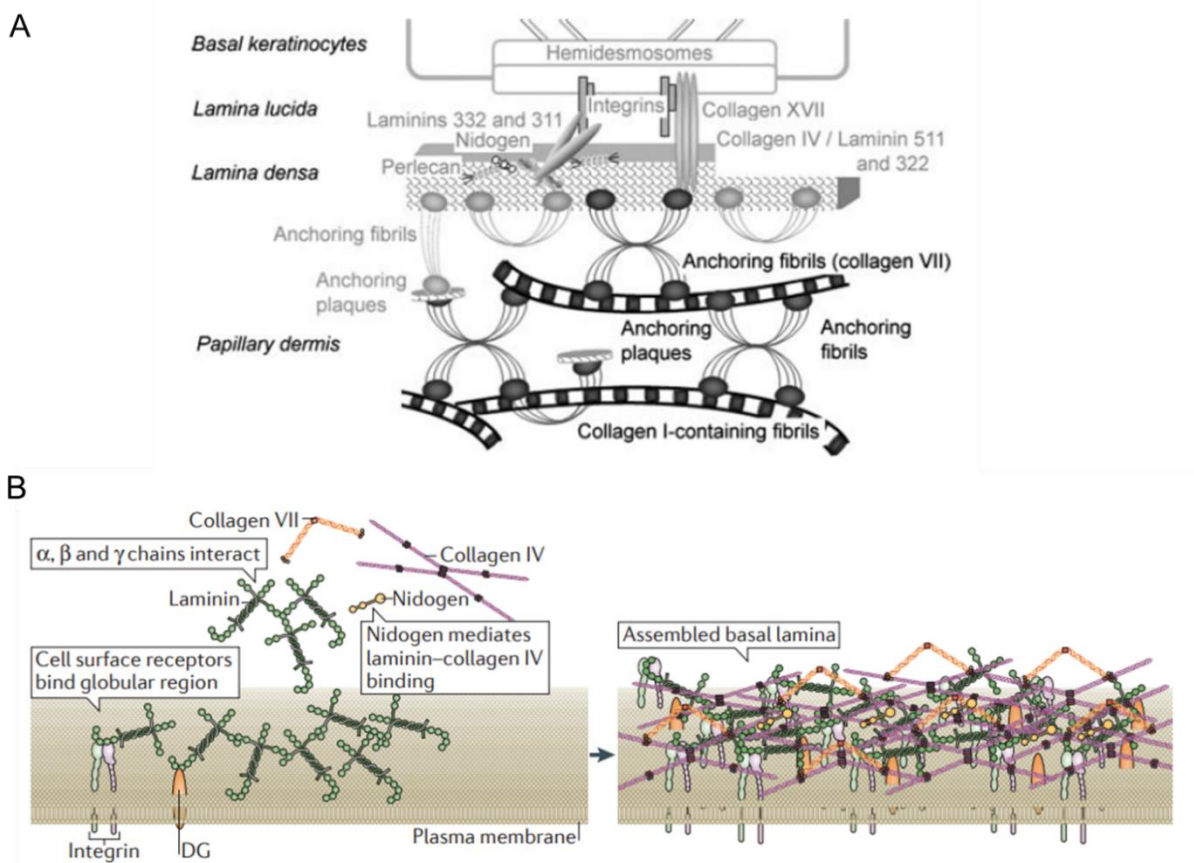
The epidermis is also composed of melanocytes (3%), which ensure skin pigmentation and UV radiation protection, Langerhans cells (2%), involved in skin immunological responses, and Merkel cells (0,5%), contributing to sensory reception (Tobin, 2017).

### 1.3. The dermal-epidermal junction

The dermal-epidermal junction (DEJ), also called the epidermal basement membrane (BM), is localized at the interface between the epidermis and the dermis and ensures their cohesion. The DEJ is a network composed of cell adhesion complexes and organized sheets of extracellular matrix proteins, originated from both epidermal keratinocytes and dermal fibroblasts. Electron microscopy allows distinguishing different layers within the DEJ: the keratinocytes plasma membrane that contains specialized adhesive junctions called hemidesmosomes, and the BM composed of an electron-dense lamina lucida and a thicker sublamina densa zone located in the upper part of the papillary dermis (Figure 4A) (Has and Nyström, 2015). Collagen IV and laminins form independent networks that are mainly connected by nidogens and the proteoglycan perlecan (Figure 4B). Collagen IV is able to associate into dimers and tetramers thereby generating an interlaced three-dimensional network. The major laminins found in the DEJ are laminin-332, laminin-311 and laminin-511 (Nyström and Bruckner-Tuderman, 2019). The epidermal-dermal network is strengthened by the presence of collagen VII, which forms anchoring fibrils reinforcing cohesiveness between the epidermal basement membrane and the dermis (Villone et al., 2008). Collagen VII has

indeed been shown to directly interact with the laminin 5 and collagen IV and with the collagen fibrils from the upper papillary dermis (Brittingham et al., 2006; Chen et al., 1997).

In addition to be a mechanical support for the surrounding cells, the DEJ also ensures major biological functions by filtrating and supplying nutrients and oxygen coming from the dermis to the epidermis. It also acts as a reservoir of growth factors that are bound to the proteoglycans, which enables an active communication between keratinocytes and fibroblasts (Iozzo et al., 2009). Hence, this exchange zone is directly involved in skin homeostasis, such as wound repair and epidermal differentiation (Has and Nyström, 2015; Iriyama et al., 2011; Marionnet et al., 2006).



**Figure 4. The dermal-epidermal junction organization.**

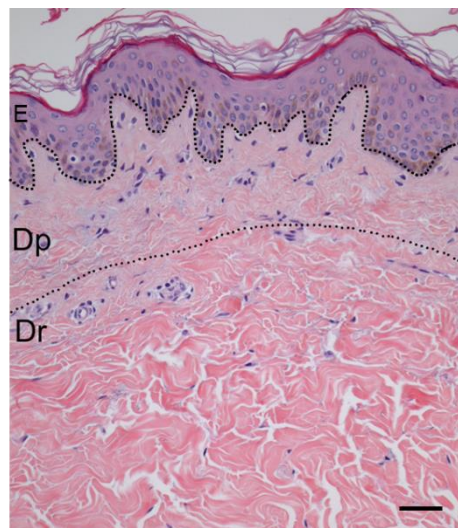
(A) Global overview of the dermal-epidermal junction in human skin. From Villone et al., 2008. (B) Basal lamina composition and assembly. Two major collagens participate in the assembly of the basal lamina: collagen IV and collagen VII. In addition, laminins form a sheet on the cell surface by recruiting soluble laminin. This sheet is reinforced by interactions among chains of different laminin molecules and by the binding between laminin globular regions and cell surface receptors (for example, integrins and dystroglycan). Collagen IV binds to laminins via nidogens and forms a scaffold on top of the laminins. This scaffold is further secured by collagen VII, which, in its unbent form (sometimes termed anchoring fibrils), can link the basal lamina to the reticular lamina to form the basement membrane. From Mouw et al., 2014.

## 1.4. The dermis

### 1.4.1. Structure and main components

In contrast to the epidermis, which is mainly constituted of keratinocyte layers, the dermis is a connective tissue containing, in adult, very few cells and large amounts of an acellular component, the dermal ECM. Fibroblasts, which constitute the main dermal cell population, are major ECM producers. The dermal ECM is a complex macromolecular network built of interlaced fibrils formed by collagens and elastin, in which proteoglycans and glycoproteins fill the space to form a hydrated structure (Frantz et al., 2010; Nyström and Bruckner-Tuderman, 2019). Hence, it forms a tightly organized structure that allows the dermis to withstand compressive forces. Composition and organization of ECM structures determine the biomechanical properties of the skin.

Histologically, the dermis is subdivided into the upper papillary dermis and the lower reticular dermis, which differ in cell-type and ECM microenvironment (Figure 5). The papillary dermis is characterized by fibroblasts with a higher proliferation rate and smaller size than the fibroblasts found in the reticular dermis. The ECM in the papillary compartment is characterized by thin collagen fibers wherein numerous papillary fibroblasts are found, whereas the ECM in reticular dermis is a dense, well-structured network with large collagen bundles and thick elastin fibers and contains sparse reticular fibroblasts (Haydont et al., 2019a; Nauroy et al., 2017). Dermal fibroblasts and ECM will be further detailed in section 2 of this introduction.

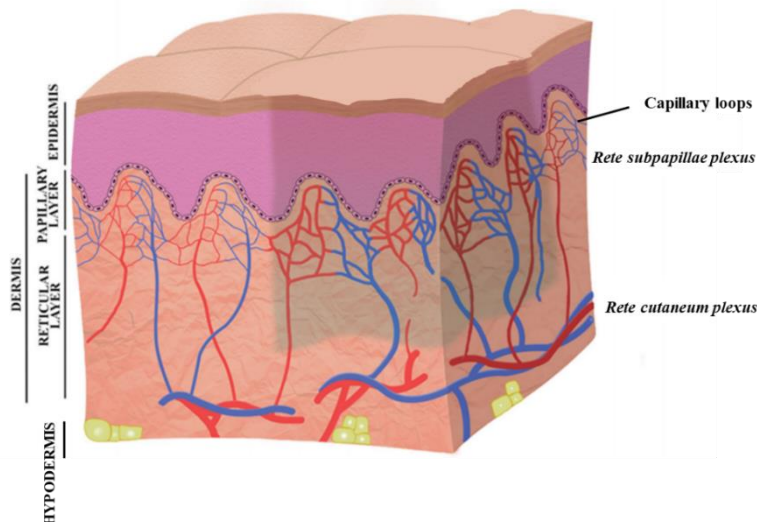


**Figure 5. Histological characteristics of the human skin.**

Histological section of a mammary skin biopsy obtained from a 19 year old donor and stained with hematoxylin, eosin, and saffron (HES). Scale bar=50  $\mu$ m. E, Epidermis; Dp, papillary dermis; Dr, reticular dermis. Modified from Mine et al., 2008.

### 1.4.2. Dermal vascular, lymphatic and nervous systems

The dermis also possesses vascular, lymphatic and nervous systems, which are associated to the skin appendages. The vascular and lymphatic systems include endothelial and immune cells additionally present in the human dermis. Endothelial cells are forming capillaries that ensure the cutaneous microcirculation (Figure 6). This micro-vascular system is organized as two horizontal plexuses: the first one is located just below the DEJ, called the *rete subpapillae plexus*, since it separates the papillary dermis from the reticular dermis; and the second one named the *rete cutaneum plexus* is located at the dermal-hypodermal junction (Braverman, 2000). The superficial vascular plexus forms capillary loops along the dermal papillae and therefore provides nutrients and oxygen to the upper non-vascularized epidermis. The two horizontal plexuses are interconnected through arterioles and venules and also supply the skin appendages, namely sweat glands and hair follicles, with nutrients (Deegan and Wang, 2019). Moreover, the vascular system also plays important thermoregulatory functions and ensures the skin barrier function by transporting immune cells and mediators upon inflammatory stimuli. Dermal immune cells are composed of various cell-types, which mainly include dendritic cells, macrophages and mast cells. They are ensuring skin immunity by protecting the skin and the whole body against pathogens and internal stressors, and are of major importance in the inflammation, wound healing or angiogenesis processes (Matejuk, 2018).



**Figure 6. Schematic cross-section of the skin illustrating the cutaneous microcirculation.**

The skin microvascular system is organized into two horizontal plexuses: the *rete subpapillae plexus* and the *rete cutaneum plexus* that respectively separate the papillary dermis from the reticular dermis and the reticular dermis from the hypodermis. Capillary loops at the dermal papillae are important structures for the oxygen and nutrient supply to the epidermis. Modified from van Raath et al., 2019.

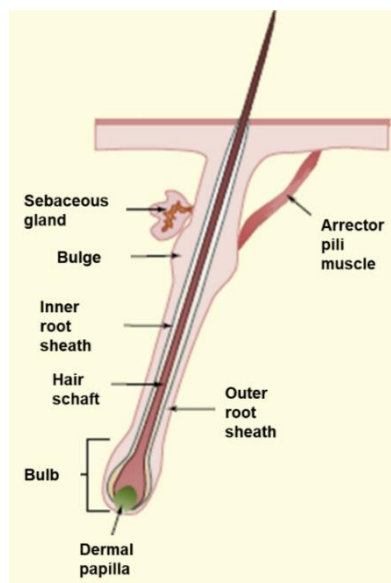


The dermis is also densely innervated and contributes to the high sensitivity of the skin. It is of note that the epidermis is also innervated although to a lesser extent compared to the dermis. The dermis contains various types of nerve endings, which allow discriminating different sensations such as pain or temperature (Laverdet et al., 2015). The nerve endings are also connected to the capillaries to regulate blood pressure and to sweat glands and to the arrector pili muscle to control the sudation and the hair elevation in response to temperature changes. In addition, neuromediators transmitted through the nerve endings are of major importance in the wound healing process (Ashrafi et al., 2016).

### 1.4.3. Skin appendages

Skin appendages are complex epithelial structures anchored into the dermis. They include eccrine and apocrine sweat glands and the pilosebaceous unit, which is itself composed of the hair follicle, sebaceous glands and the arrector pili muscle (Figure 7) (Kanitakis, 2002).

Sweat glands (Figure 7) are connected to the skin surfaces through sweat ducts. Their main function is to regulate the temperature of the body by releasing water and electrolytes upon heat or cold, which is sensed through the nerve endings. Sweat glands also secrete hydrating factors and antimicrobial peptides, which both contribute to the skin barrier function (Schitteck et al., 2001; Watabe et al., 2013).



**Figure 7. The pilosebaceous unit.**

The pilosebaceous unit is composed of the hair follicle (hair shaft, inner and outer root sheath and bulb), sebaceous gland and arrector pili muscle. Modified from Schneider et al., 2009.

The hair follicle (Figure 7) is a very complex organ resulting from an ectodermal-mesodermal interaction, and is therefore composed of several cells types including keratinocytes, melanocytes and specialized fibroblasts (Philpott, 2018). The hair is a fiber that results from the aggregation of terminally differentiated dead keratinocytes forming a structure called the hair shaft. The bulb is a structure located at the bottom of the hair shaft and contains highly proliferative epithelial cells, melanocytes, and dermal papilla fibroblasts. The dermal papilla can be considered as the signaling center of the hair follicle since it is composed of a very specialized fibroblast subpopulation that controls the hair shaft diameter and length, as well as the hair cycle (Schneider et al., 2009). The sebaceous gland is connected to the upper part of the hair follicle (Figure 7) and secretes sebum around the hair, which is a lipid-rich liquid that contributes to the skin impermeability (Zouboulis et al., 2016). The arrector pili muscle is a small muscle that connects the hair follicle to the dermis and further contributes to the skin isolation properties when contracted. Together, the pilosebaceous unit is an illustration of the complex interplay between the epidermis and the dermis, which plays a major role in skin homeostasis.

### 1.5. The hypodermis

The hypodermis is the deepest layer of the skin. It is an energy reserve, which acts as a thermal insulator and protects the body from shocks. Even though the hypodermis has a very different structure from the fibrous dermis, these two layers are well connected through the vascular network, nerve endings and the continuity of skin appendages. The main resident cells of the hypodermis are the adipocytes. With a mesenchymal origin, adipose cells originate from the same progenitor as reticular fibroblasts (Driskell and Watt, 2015).

## 2. The dermis: a highly dynamic and complex compartment

In this second part, I will specifically focus on the dermal compartment of the skin and detail the properties and functions of its two main constitutive interconnected components: the ECM and the fibroblasts.

### 2.1. The dermal extracellular matrix (ECM)

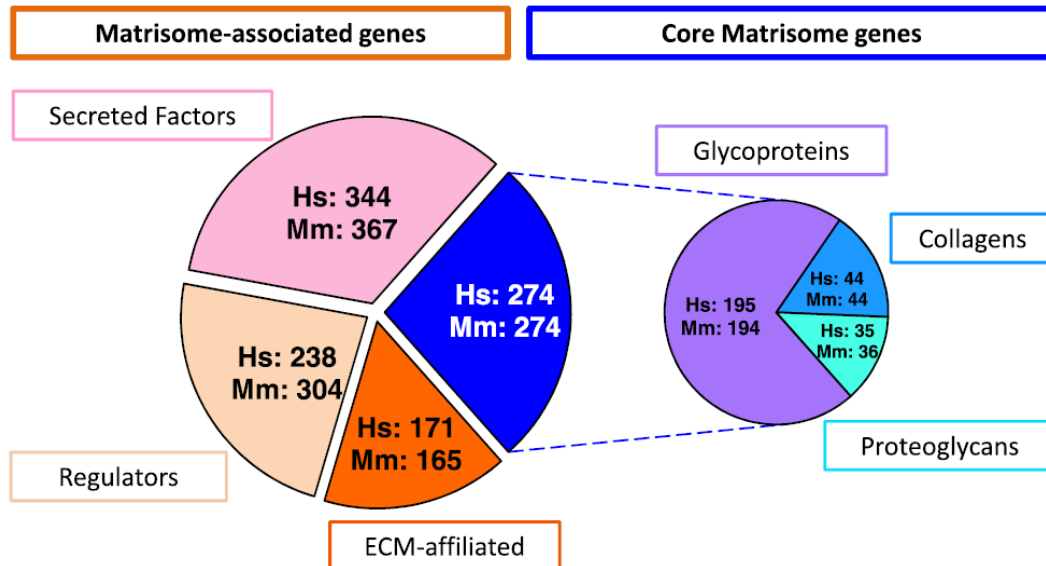
#### 2.1.1. An introduction to the extracellular matrix components: the matrisome

Cells are not the only components of tissues and organs. The extracellular space is indeed filled with several macromolecules that bind to each other and form a three-dimensional network called the extracellular matrix (ECM), which is found in almost all tissues and organs of multicellular organisms (Frantz et al., 2010). ECM was first believed to be an inert component only providing a structural scaffold to the surrounding cells. However, it is now clear that ECM is also absolutely vital for cell functions and tissue integrity by regulating many biological functions, including proliferation, survival, adhesion and differentiation. The crucial functions of ECM proteins are highlighted by the wide range of pathologies arising either from aging, acquired diseases and/or from genetic mutations in matrix genes, including cardiovascular, fibrotic and skeletal diseases, and cancers (Taha and Naba, 2019; Theocharis et al., 2019).

Although basically composed of proteins, polysaccharides and water, ECM displays unique tissue specific compositions that result from complex biochemical and biophysical interplays between the cells of each tissue and their respective microenvironment. In vertebrates, hundreds of genes encode ECM proteins (Hynes, 2009). The complex composition of ECMs and the development of high-throughput technologies termed “omics”, such as RNA-sequencing or proteomics, has raised the need for a complete list of secreted proteins found in the extracellular space, indispensable for the detailed characterization of tissue specific ECMs (Hynes and Naba, 2012). However, biochemistry of ECMs remains a scientific challenge since ECM proteins are large proteins forming highly insoluble cross-linked aggregates. In 2012, by combining bioinformatics and *in vivo* proteomics approaches, Naba and collaborators defined the ‘matrisome’, as the list of genes encoding ECM and ECM-

associated proteins, both in human and mouse (Figure 8) (Hynes and Naba, 2012; Naba et al., 2012). Using a novel *in silico* orthology-based approach and bioinformatics, the zebrafish matrisome was then characterized (Nauroy et al., 2018) followed by the drosophila and *C.elegans* ones (Davis et al., 2019; Teuscher et al., 2019).

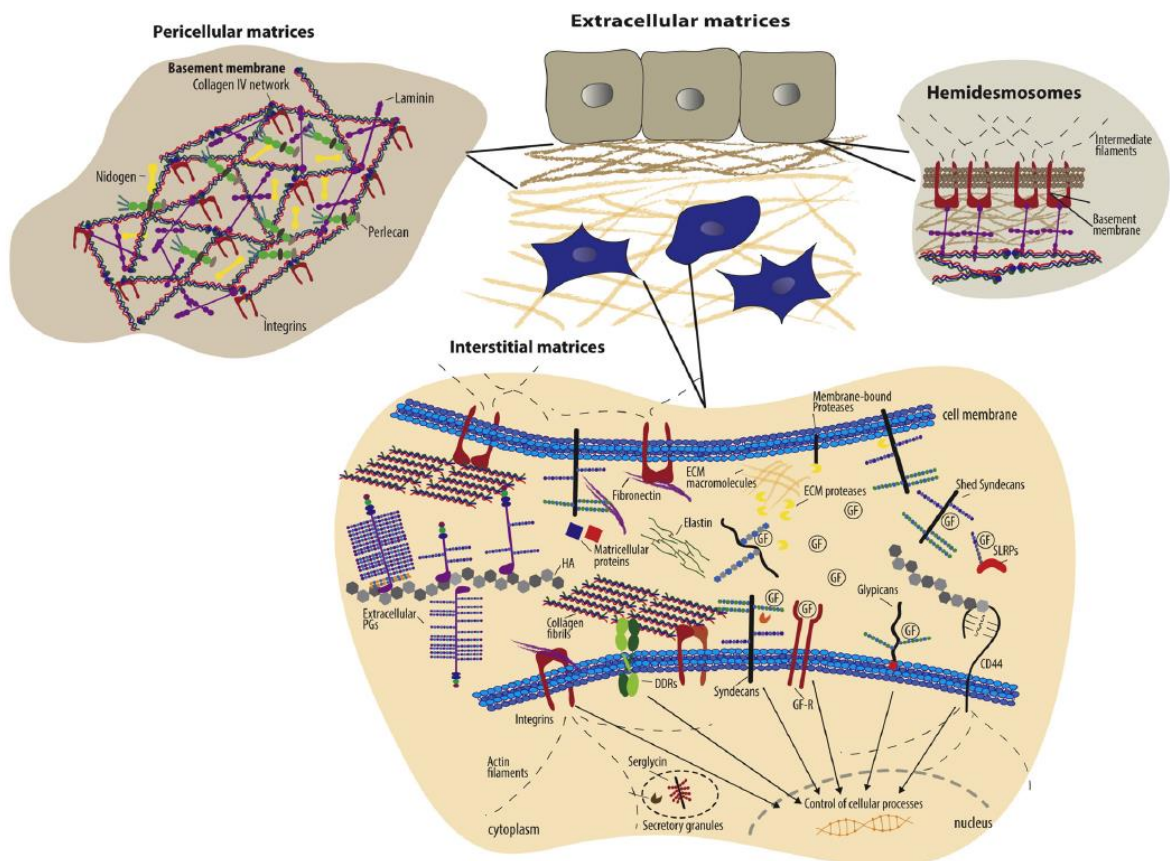
The mammalian matrisome includes 1027 genes in the human genome and 1110 genes in the mouse genome, accounting for about 4% of the whole genome in both species (Figure 8) (Naba et al., 2016). The matrisome is divided into two main categories: i) the core matrisome and ii) the matrisome-associated genes. The core matrisome comprises almost 300 genes in both humans and mice and contains structural and well-conserved proteins including the collagens, proteoglycans and glycoproteins. Matrisome-associated genes encode the proteins that are present in the extracellular space but not considered as “pure” matrix proteins. This category includes: secreted factors (e.g. growth factors, cytokines), ECM regulators (e.g. MMPs, ADAMs and cross-linking enzymes) and ECM affiliated proteins (e.g. semaphorins, syndecans, mucins, lectins). It is of note that this last subcategory comprises proteins that some scientists may or may not consider as ECM proteins, but that are regularly found in ECM-enriched preparation.



**Figure 8. Human and mouse matrisomes.**

Pie charts representing the number of human genes encoding core matrisome and matrisome-associated proteins. “Hs” indicates genes in the human genome and “Mm”, genes in the murine genome. From Naba et al., 2015.

Additionally, ECMs are classified into two main subsets: the pericellular and interstitial matrices, which both differ in their localization, topography and composition (Figure 9) (Theocharis et al., 2016). Pericellular matrices, which include basement membranes, are specialized ECMs that separate, for example, epithelial or endothelial cells from their surrounding connective tissue (Sekiguchi and Yamada, 2018). Interstitial ECMs are found in most connective tissues, they mainly contain fibrous extracellular network that surrounds cells and provides an architectural support to organize tissues.



**Figure 9. Overview of the major types of extracellular matrices.**

Basement membrane, a type of pericellular matrix, is found between epithelial cells and connective tissue. This layer is composed of a collagen IV network that associates with ECM components including nidogen, laminin, perlecan, and minor collagens like collagen XV and XVIII. Epithelial cells are anchored to basement membranes by hemidesmosomes formed via interactions of integrins with laminins. Interstitial matrices are composed of collagen fibrils, elastin, secreted PGs, and matricellular proteins. They interact with each other creating a dynamic and complex three-dimensional network. From Theocharis *et al.*, 2016.

In dermis, both of these ECMs coexist with the basement membrane at the dermal epidermal junction, which that has been previously introduced in the section 1, and the interstitial matrix of the dermis, on which I will focus in this section.

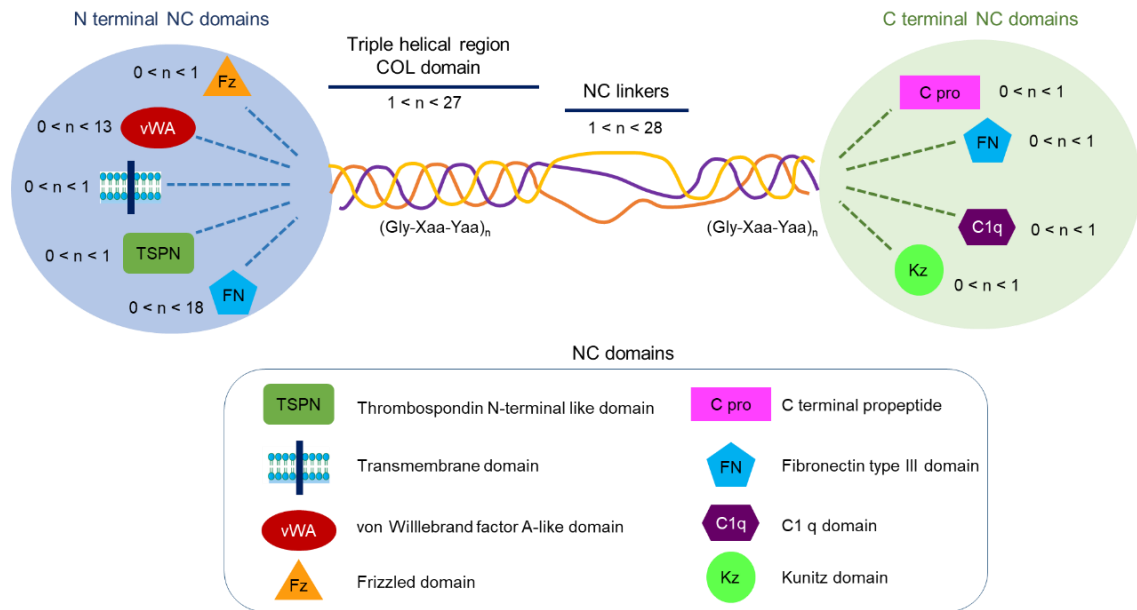
### 2.1.2. Composition and structure of the dermal interstitial matrix

The dermal interstitial matrix is mainly composed of collagen and elastic fibers, proteoglycans and water. Collagens, that aggregate into large fibers in the extracellular space, are the most abundant proteins of dermal ECM. Elastic fibers are made of elastin molecules and incorporate diverse other glycoproteins that are critical for the formation of the elastic fiber, such as fibrillins and fibulins. Together, collagen and elastic fibers form a three-dimensional network in which proteoglycans fill the space and interspace collagen fibers to finally stabilize and hydrate, through the binding to water, the dermal ECM structure. The composition and ratio of these different components are directly responsible for the topology, strength, and elasticity of the human skin.

- Collagen fibers network

#### Generalities and main characteristics of collagens

The name “collagen” was given in the XIX<sup>th</sup> century to the glue that was generated from boiling connective tissue. In Greek, “kolla” means glue and the suffix “gen”, giving birth to. Collagens are the most abundant proteins in mammals, accounting for 30% of the total protein mass, and are well conserved proteins across species (Bretaud et al., 2019). In humans, the collagen superfamily today comprises 28 members encoded by 44 genes. The 28 collagens have been named with Roman numerals (I to XXVIII) in the order of their discovery (Kadler et al., 2007; Ricard-Blum and Ruggiero, 2005).



**Figure 10. Structure and possible domains of collagen molecule.**

All collagens contain at least one and up to 27 triple helical regions (also named collagenous domain - COL) that are composed of  $(\text{Gly-Xaa-Yaa})_n$  repeats where Gly is a glycine, Xaa often a proline and Yaa often a hydroxyproline. Non-collagenous (NC) regions are intercalated between collagenous domains. Several other non-collagenous domains can be present at the C- or N-terminal part of the molecule and repeated up to 18 times, as it is the case for the fibronectin type III domain. From Salamito et al., in press.

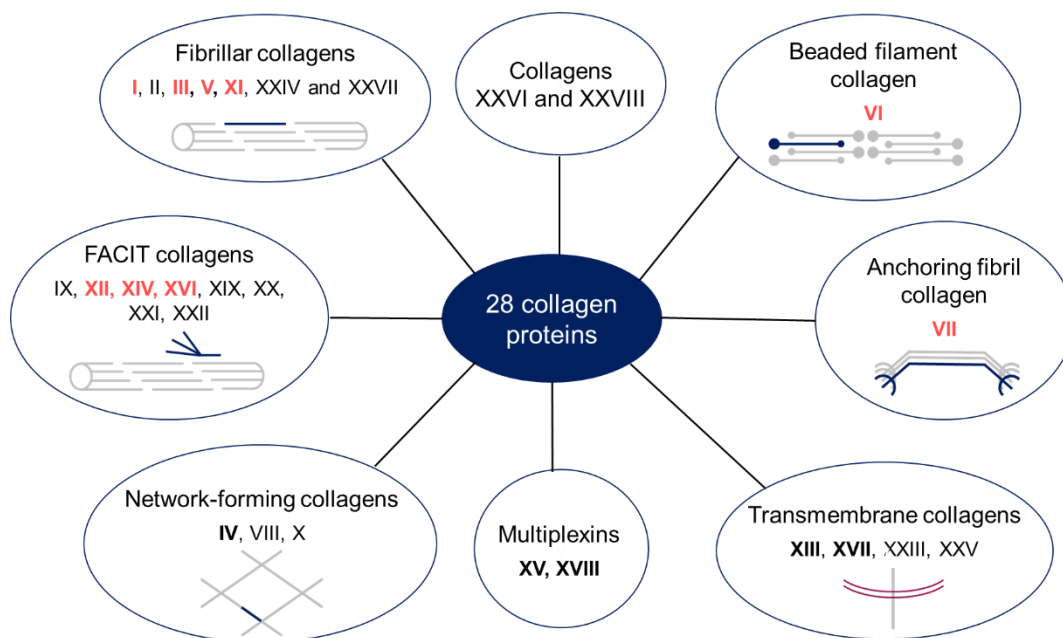
All collagens are trimeric molecules with at least one triple helical domain due to the presence of  $(\text{Gly-X-Y})_n$  triplet repeats, with X and Y respectively often being proline and hydroxyproline residues (Figure 10). They are composed of three associated polypeptide chains, called  $\alpha$ -chains, coiled into a type II polyproline left-handed helix. Either being identical or different, these three chains respectively form either a homotrimer or a heterotrimer. In most tissues, the prevalent form of collagen I is the heterotrimer composed of two  $\alpha 1$  chains and one  $\alpha 2$  chain  $[\alpha 1(I)]_2 \alpha 2(I)$ . The collagen  $\alpha 1(I)$  and  $\alpha 2(I)$  chains are respectively encoded by *COL1A1* and *COL1A2* genes. In addition to their triple helical domains, named collagenous domain (COL), collagens comprise non-collagenous domains (NC).

Collagens are able to form specific supra-molecular aggregates according to which (but not only) collagens have been classified into seven subfamilies (Figure 11). The fibrillar collagens, the Fibril Associated with Interrupted Triple Helix (FACIT), the network-forming collagens, the multiple triple helix domains and interruptions (multiplexins), the beaded filament collagens, the anchoring fibril collagens and transmembrane collagens (also called MACIT for Membrane Associated Collagens with Interrupted Triple Helices). Nevertheless, these collagen subfamilies include 26 out of the 28 collagens. Even though collagens XXVI and XXVIII are sometimes included within the beaded filament collagens, they cannot be strictly



assigned to any of these subsets and they remain “orphan” collagens (Byron et al., 2013; Ricard-Blum, 2011). This extraordinary diversity of supramolecular aggregates is linked to the various functions ensured by collagens, that range from the maintenance of structural and biochemical properties of a given tissue to important biological functions such as cell proliferation, cell adhesion or cell death to name but a few (Gelse et al., 2003; Ricard-Blum, 2011). A book chapter introducing the collagen superfamily can be found in annex of this manuscript (Salamito et al., in press).

Collagens from all seven subfamilies are present in human skin (Figure 11), in which the main components of the dermal interstitial ECM are by far the fibrillar collagens.

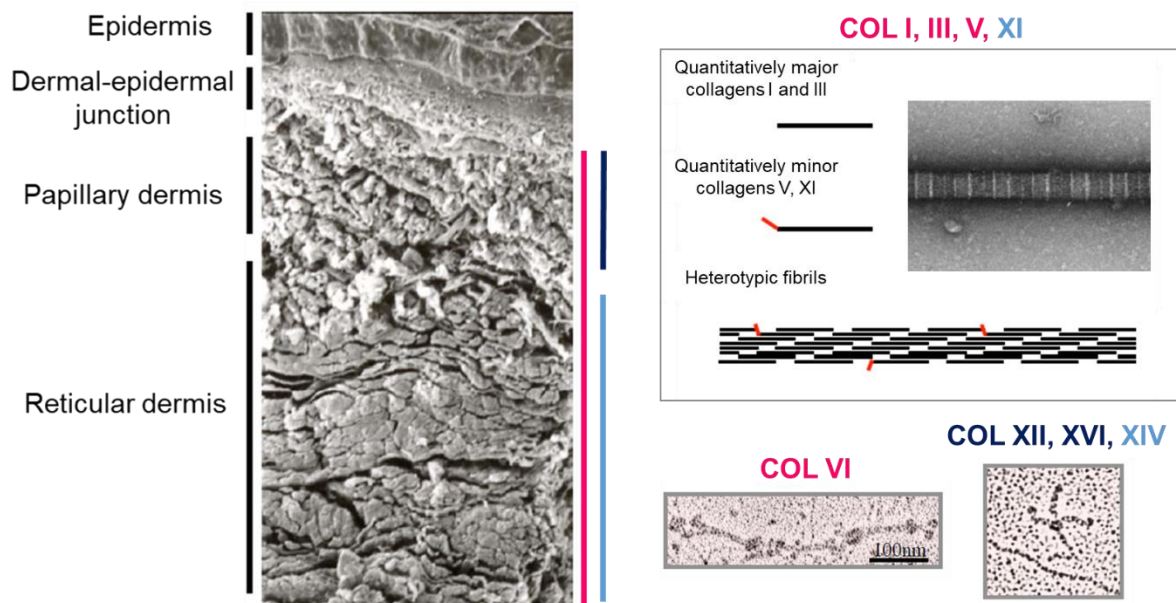


**Figure 11. The 28 human collagens classified into the different subfamilies according to their supramolecular organization.**

Collagens in bold are the ones expressed in human skin (epidermis, DEJ and dermis), and the ones in bold and red are the ones specifically express in human dermis. Modified from Salamito et al., in press.

## Dermal collagens are mainly fibrillar collagens

Collagens are representing 70 to 80% of the skin dry weight, therefore, they are the major constituents of the dermal ECM. Dermal collagens are mainly the fibrillar collagens I, III and V, respectively representing 80%, 15% and 5% of dermal collagens (Haydout et al., 2019a). Collagens I, III and V assemble to form highly organized heterotypic fibrils (Figure 12). While collagen I and III are the main structural components, the minor collagen V is one of the initiators and regulators of the collagen-fibril process, named fibrillogenesis, that will be further detailed in the coming section (Ruggiero et al., 2005). It is interesting to note that another collagen from the fibrillary subfamily, collagen XI, has been recently reported as expressed in the reticular dermis (Haydout et al., 2019c; Nauroy et al., 2017).



**Figure 12. Skin dermal collagens: localization and supramolecular organization visualized by Transmission Electron Microscopy(TEM).**

Fibrillar collagens (I, III, V and XI) are the main constituents of the dermis, they form fibrils with a characteristic banded pattern (alternately grey and light grey). Except for collagen XI $\alpha$ 1, they are all localized in both papillary and reticular dermis. Collagen VI is also expressed in the whole dermis and forms beaded filaments visualized by TEM. The possible incorporation of collagen XI into the collagen fibers was not shown in (Nauroy et al, 2017) and is solely based on its known function in cartilage. FACIT collagens have a “trident”-like structure in TEM. While collagens XII and XVI are mainly expressed in papillary dermis, collagen XIV is mainly found in reticular dermis. Modified from Ruggiero et al., 2005 and Kadler, 2017.

The remaining 5% of the dermal collagens comprises collagens VI, VII, XII, XIV, and XVI (Figure 12). The beaded filament forming collagen VI exerts both anchoring and bridging functions and is widely expressed throughout the whole dermis and in the DEJ of skin (Lamandé and Bateman, 2018; Ruggiero et al., 2005). Collagen VII is chiefly expressed by the

basal keratinocytes but several studies have demonstrated that it is also expressed in the upper papillary dermis. It is the main component of anchoring fibrils, which are adhesive suprastructures ensuring the connection of the epidermal basement membrane with the dermis (Ryynänen et al., 1992). Even though collagen VII is a minor component of skin, it is of crucial importance for maintaining skin integrity, since mutations in the *COL7A1* gene cause a congenital skin fragility condition associated with skin blistering and chronic wounds, named epidermolysis bullosa (Has et al., 2018). Finally, the FACIT collagens types XII, XIV and XVI are also localized in human dermis (Ruggiero et al., 2005; Wälchli et al., 1994). Collagen XII and XIV have been identified at the surface of collagen fibers respectively in the papillary and the reticular dermis while collagen XVI was described at the surface of fibrillin microfibrils. Their interaction with collagen fibers can be mediated by some proteins, for example by the small proteoglycans decorin and fibromodulin, or by the Cartilage Oligomeric Protein (COMP) (Agarwal et al., 2012). Collagen XVI can associate with the surface of microfibrils of fibrillin-1/2 in the papillary dermis close to the epidermal basement membrane (Kassner et al., 2003).

#### Biosynthesis of fibrillar collagens: from the formation of the triple helix to fibrillogenesis

The biosynthesis of collagens is a complex multistep process, going from transcriptional control in the nucleus to the aggregation of collagen trimeric molecules into striated fibrils in the extracellular compartment. So far, only the main steps of the biosynthesis of the fibrillar collagen subfamily have been elucidated and will be further described.

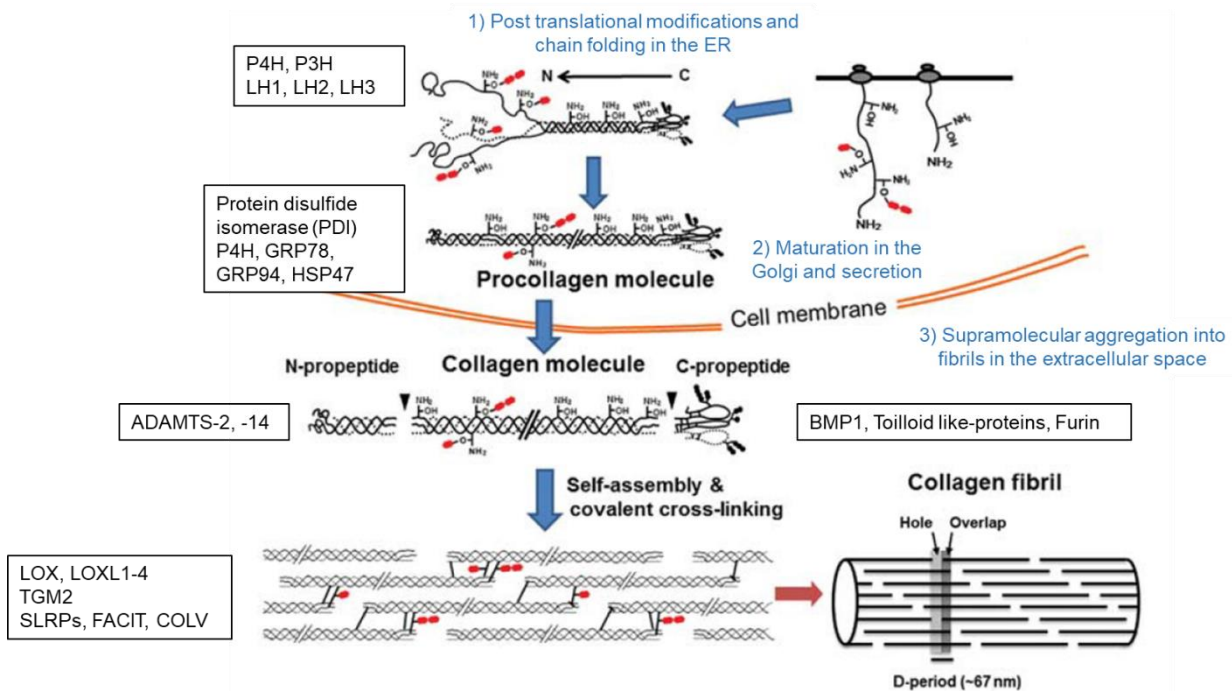
In dermal fibroblasts, collagen expression is largely controlled by the transforming growth factor  $\beta$  (TGF- $\beta$ ) family, but many other cytokines and growth factors are involved, i.e. placental derived growth factors (PDGF) and the fibroblasts growth factors (FGF) families (Gelse et al., 2003). The regulatory mechanisms of collagen biosynthesis are further described in section 2.2.2 of this chapter. As for all secreted proteins, after transcription, collagen pro $\alpha$ -chains mRNAs are translated by membrane bound ribosomes and translocated to the lumen of the rough endoplasmic reticulum. Then, collagen pro $\alpha$ -chains undergo 3 major post-translational events to become functional, which will be further described and illustrated in [Figure 13](#).

- 1) *Post translational modifications and formation of the procollagen molecule in the ER*

Collagen pro $\alpha$ -chains undergo numerous and complex post-translational modifications in the ER among which are: (1) cleavage of the signal peptides; (2) hydroxylation of most of the prolines at the Yaa position of the Gly-Xaa-Yaa triplets by Prolyl-4-hydroxylase (P4H). Prolyl-3-hydroxylase also hydroxylates selected proline residues in several collagens (Rappu et al., 2019); (3) partial hydroxylation of specific lysines and (4) glycosylation of hydroxylysines generated in step 3. Those post-translational modifications are followed by a critical step of collagen biosynthesis that is the specific and controlled assembly of the newly synthesized collagen pro $\alpha$ -chains into a triple helix. This occurs in a zipper-like manner, starting from the C-terminus and propagating towards the N-terminus. Collagen C-propeptides play a major role in the selection of the pro $\alpha$ -chains and procollagen folding. C-propeptide association depends on specific recognition sequences and on the activity of protein disulfide isomerase (PDI) to create disulfide bonds between three individual C-propeptides that facilitate the initiation of the triple helix formation (Bulleid et al., 1997; Fessler et al., 1985; Lees et al., 1997). *In vivo*, the presence of hydroxyprolines (about 10% of the total amino-acids in fibrillar collagens) enables binding of the HSP47 chaperon protein and the subsequent procollagen trimer stabilization (Makareeva and Leikin, 2007). HSP47 is required for the secretion of stable triple helix collagen into the extracellular space and monitors the integrity of the triple helix. As a molecular chaperon, it prevents local unfolding of the triple helix and/or pro $\alpha$ -chains aggregates. Other chaperones also contribute to the triple helix quality control such as the binding immunoglobulin protein BiP.

## 2) *Maturation in the Golgi apparatus and secretion of the collagen molecule*

Procollagen molecules are further translocated to the Golgi apparatus, which ensures accurate procollagen glycosylation and trafficking. Before their transport to the extracellular space, procollagens start to aggregate laterally within the Golgi to plasma membrane carriers. When procollagens reach the plasma membrane, carriers connect to the ECM through deep projections of the plasma membrane, called “fibripositors” (Canty and Kadler, 2005; Kadler, 2017). The precise nature of these vesicles is still a matter of considerable debate. Specifically, the question of how large rod-shape molecules of up to 700 nm in length can fit into secretory vesicles remains puzzling. Collagen trafficking is far from being completely understood and would need further investigations (Banos et al., 2008; Canty and Kadler, 2005; McCaughey and Stephens, 2019).



**Figure 13. Biosynthesis of fibrillar collagens.**

During the synthesis of pro  $\alpha$ -chains in the ER, specific peptidyl lysine residues are hydroxylated to form hydroxylysine (-OH-NH<sub>2</sub>) and, subsequently, specific glycosylated hydroxylysine residues (O-linked glycosylation). For the latter, either single galactose (a red hexagon) or a glucose-galactose (two red hexagons) is attached. After these and other modifications, two pro $\alpha$ 1 chains (solid line) and one pro $\alpha$ 2 chain (dotted line) associate with one another and fold into a triple helical molecule from the C- to the N-terminus to form a procollagen molecule in the ER. This procollagen molecule further undergoes modifications in the Golgi apparatus and is then packaged and secreted into the extracellular space. Then both N- and C-propeptides are cleaved to release a collagen molecule. Then, collagen molecules spontaneously assemble into a fibril that is stabilized by covalent intra- and inter-molecular covalent cross-linking. During fibrillogenesis, molecules are packed in parallel and are longitudinally staggered by an axial repeat distance, D period (~67 nm) creating two repeated regions, i.e. overlap and hole regions, in the fibril. Modified from Yamauchi and Sricholpech, 2012.

### 3) Supramolecular organization into striated fibrils in the extracellular space

Once secreted in the extracellular space, N- and C-propeptides of fibrillar procollagens are entirely (e.g. collagens I and III) or partially (e.g. collagens V and XI) removed by specific matrix enzymes including BMP-1, mTLD and TLL-1 for the C-propeptide and ADAMTS family members for the N-propeptide. Mature collagens spontaneously organize into fibrils that consist in end-overlap alignment of collagen molecules in a quarter-staggered packing pattern. Fibrils have a starting diameter of 12 nm and can further co-aggregate to form fibers with a diameter to over 500 nm, depending on the tissue and the developmental stage (Craig et al., 1989; Kadler, 2017). Interestingly, it has been shown that fibroblasts in culture are able to

synthesize collagens with the characteristic banded pattern, but these *in vitro* fibrils are not able to form any higher-order organization like the *in vivo* collagen fibers (Canty and Kadler, 2005). Fibril structures are strengthened by intra- and inter- molecular crosslinks catalyzed by lysyl oxidases between lysine and hydroxylysine residues (Myllyharju and Kivirikko, 2004; Vallet and Ricard-Blum, 2019; Yamauchi and Sricholpech, 2012)

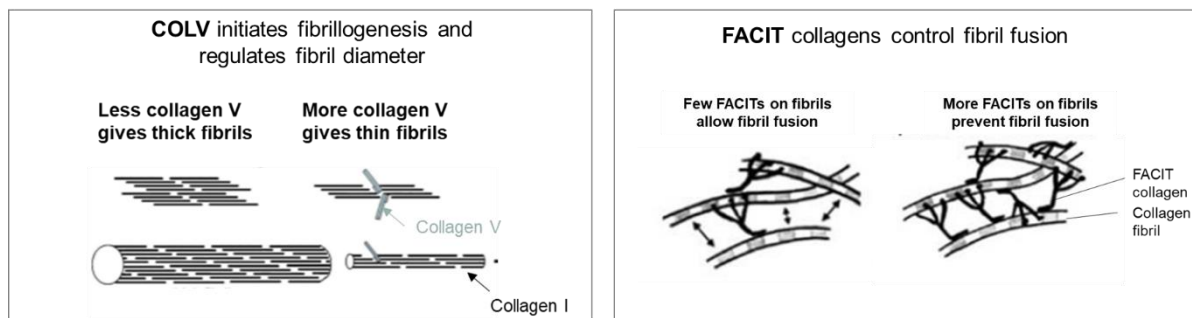
Transglutaminase-2 (TGM2) is another important cross-linker of collagen fibrils. TGM2 catalyzes isopeptide bonds between lysine and glutamate residues in collagens I, III, V and XI (Esposito and Caputo, 2005). These intermolecular bonds are prerequisites for the mechanical resilience ensured by collagen fibrils as well as for the stability of the collagen network (Gelse et al., 2003; Wang et al., 2014).

### Regulation of fibrillogenesis

Collagen fibril length, number and diameter vary between tissues and organs but also within a given tissue, as it is the case in dermis that is sub-divided into the papillary and reticular dermis as introduced above. Collagen fibrils form complex macromolecular alloys, composed of different collagen types, but also of various non-collagen ECM proteins (Bruckner, 2010). Indeed, collagen fibrils are able to interact with many binding partners that initiate, organize and regulate the fibrillar collagen network (Holmes et al., 2018). A snap-shot of the main collagen-fibrils organizers and regulators will be further described.

Even though they are minor components of collagen fibrils, collagens V and XI are both required to initiate the assembly of the predominant collagen I and collagen II into fibrils, respectively (Blaschke et al., 2000; Wenstrup et al., 2004a). They are therefore considered as fibril nucleators. Due to the partial cleavage of their N-propeptides, these two collagens also regulate the lateral growth and diameter of collagen fibrils (Marchant et al., 1996). While triple helical domains of collagens V and XI are buried within the fibrils, their retained large N-terminal domains cannot be incorporated and are found at the surface of the fibrils in the gap regions (Kadler et al., 2008). N-propeptides thus regulate fibril diameter by sterically hindering further addition of collagen I or II molecules (Figure 14) (Birk, 2001; Mienaltowski and Birk, 2014). Lack of the pro $\alpha$ 1(V) chain in *Col5a1<sup>-/-</sup>* mice leads to embryonic death caused by defective assembly of collagen I/V fibrils. Heterozygous *Col5a1<sup>+/-</sup>* mice are viable, but they display a 50% reduction in the number of dermal collagen fibers. In addition, they show a larger diameter than the ones observed in healthy skin and an irregular contour, called “cauliflower” fibrils (Wenstrup et al., 2004a). Mutations in *COL5A1* are the major molecular cause of a

severe connective tissue disease, called classic Ehlers-Danlos syndrome (EDS) (Wenstrup et al., 2004b). EDS is characterized by the formation of abnormal collagen fibrils, similarly to what has been described in *Col5a1<sup>+/-</sup>* mice. Thus, in addition to controlling the initiation and the diameter of fibrils, collagen V also regulates the number of collagen fibrils in tissues, making it one of the key components of fibrillogenesis, especially in skin.



**Figure 14. Regulation of fibrillogenesis by collagens.**

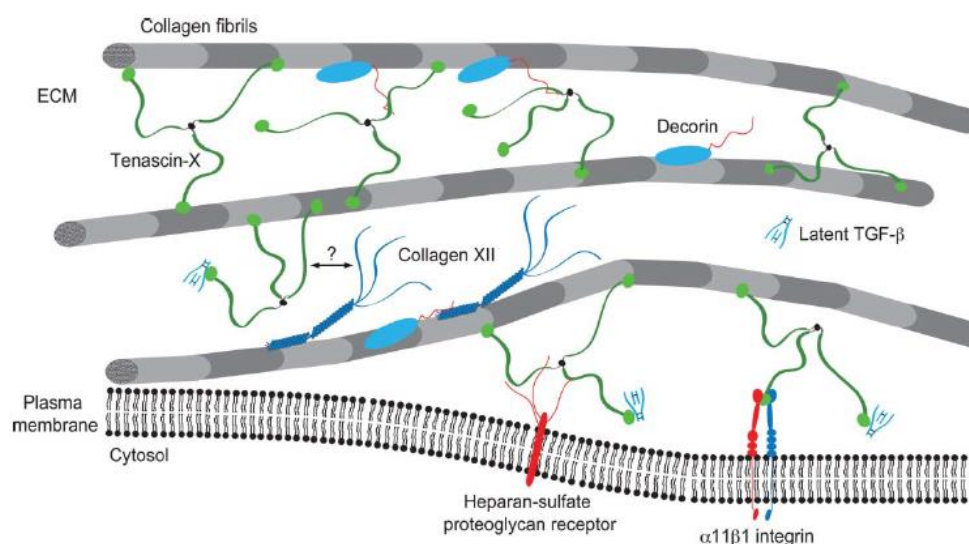
Collagen V and FACIT collagens are negative regulators of fibril diameter by initiating fibrillogenesis and interspacing collagen fibrils. Modified from Gordon and Hahn, 2010.

In addition to fibrillar collagens, the FACITs collagens, notably collagen IX in cartilage, and XII and XIV in several soft connective tissues including skin are able to regulate collagen fibrillogenesis through their interaction with collagen fibrils by either interspacing them or preventing fibril fusion (Figure 14) (Ansorge et al., 2009; Bella and Hulmes, 2017; Gordon and Hahn, 2010). They also have been suggested to bridge collagen I and III fibrils (Haydout et al., 2019a).

Members of the Small Leucine Rich Repeat Proteoglycans (SLRPs) family, which include decorin, lumican, fibromodulin, biglycan and asporin, are also able to interact with collagen fibrils and regulate collagen fibrillogenesis (Kalamajski and Oldberg, 2010). Decorin is the most described SLRP: mice deficient for this proteoglycan are viable, but have a very fragile skin with a reduced tensile strength and irregular collagen fibers (Figure 15) (Danielson et al., 1997). Lumican is a principal component of the cornea, the dermis, and muscle connective tissues. Lumican-null mice show skin laxity and fragility, which are typical features of classic EDS. Numerous abnormally thick collagen fibers are found in the skin of these mice, indicating a dysregulated fibrillogenesis process (Chakravarti et al., 1998). In contrast to decorin and lumican, asporin-null mice do not display any significant alterations in the formation or the diameter of collagen fibrils. However, the skin of *Aspn<sup>-/-</sup>* mice is characterized by an increased mechanical toughness, probably caused by changes in the

glycosaminoglycan chain composition and the increased amounts of decorin and biglycan detected in the skin of these mice (Maccarana et al., 2017).

Other matricellular proteins, such as the tenascin X, periostin, SPARC, COMP or thrombospondin-2, (Bornstein et al., 2000) regulate fibrillogenesis. Tenascin X determines the number of collagen fibrils and the interspace between the fibrils. This further impacts the three-dimensional organization of the fibrils (Figure 15) (Valcourt et al., 2015). Lack of tenascin X in *Tnx<sup>-/-</sup>* mice results in skin hyper-extensibility, similar to patients with classical-like EDS, which has been classified as an EDS subtype caused by mutations in *TNXB* gene (Mao et al., 2002). The presence of COMP, an ECM protein initially described in cartilage, accelerates the fibril formation process, and is able to interact directly with collagen I (Agarwal et al., 2012; Halász et al., 2007). Periostin deficiency results in the formation of aberrant collagen fibrils, as shown in human dermal fibroblasts and in mice (Egbert et al., 2014; Norris et al., 2007).



**Figure 15. Fibrillogenesis is controlled by various ECM components.**

Fibrillogenesis is regulated by nucleator collagens (V and XI), FACITs, SLRPs and matricellular proteins as tenascin X. Finally, integrin and fibronectin would organize this three-dimensional macromolecular network. From Valcourt et al., 2015.

Finally, fibrillogenesis is controlled by fibronectin and integrin, which have been suggested to act as molecular organizers (Kadler et al., 2008). They determine the site of fibril formation at the cell surface in the extracellular space. Several studies showed that the inhibition of either integrin and/or fibronectin results in a suppression of collagen fibril assembly,



as it was shown for the example by the inhibition of integrin  $\alpha 2\beta 1$  in vascular smooth muscle cells (Li et al., 2003; McDonald et al., 1982).

- Elastic fiber network and other glycoproteins

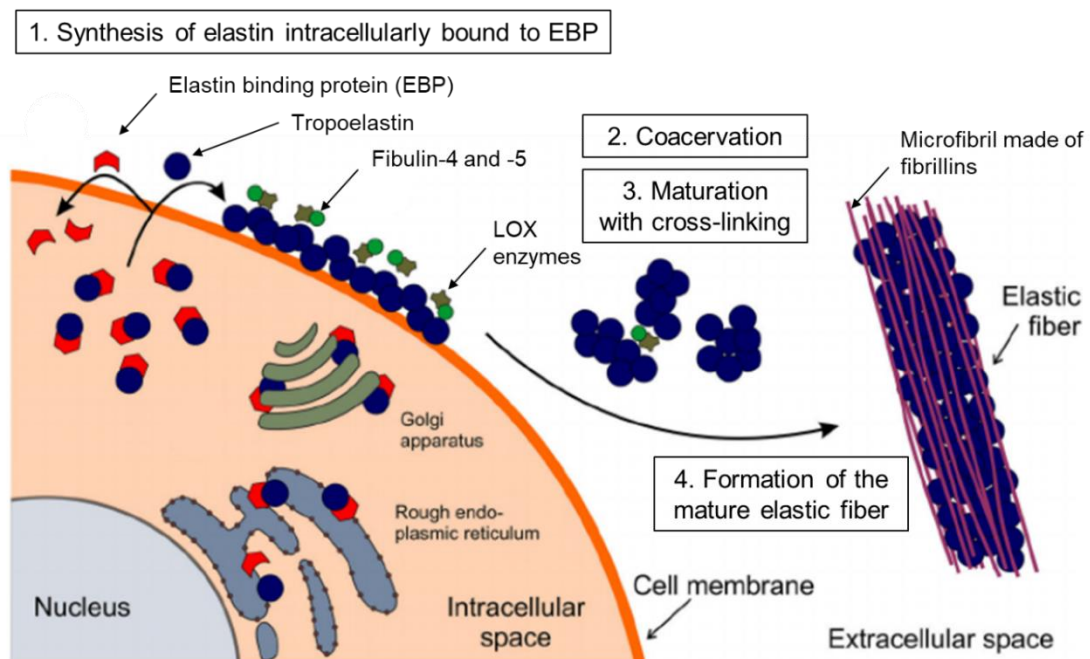
The second fibrous network of major importance in dermis is made of elastic fibers, which confers unique elasticity and resilience properties to human skin. These fibers constitute up to 5% of the dry weight of the skin (Wise and Weiss, 2009). They ensure complementary biomechanical properties to the tensile strength conferred by collagen fibers since they enable skin to undergo thousands cycles of resilience and recoil generated by repetitive mechanical stresses (Gosline et al., 2002). Being extremely stable and insoluble, elastic fibers are produced during fetal development and are then formed for the whole life. Elastic fibers are composed of an outer mantle of fibrillin-rich microfibrils and an inner core of amorphous cross-linked elastin. They are synthesized and organized by fibroblasts, through a complex and well-orchestrated process involving various ECM components. Conversely, these elastic fibers are able to interact with a vast array of matrix molecules, that will be further presented (Kielty et al., 2002; Muiznieks and Keeley, 2013).

#### Formation of elastic fibers, a process called elastogenesis

In humans, elastin is encoded by one single gene (Muiznieks and Keeley, 2013). The protein product of the elastin gene is the elastin precursor, named tropoelastin, which is further bound to a specific chaperone, the elastin binding protein (EBP) before being secreted. EBP stabilizes and prevents tropoelastin from premature aggregation before secretion into the extracellular space (Figure 16). Then, coacervation occurs, a process during which tropoelastin monomers self-assemble and form globular hydrophilic droplets bound to the cell membrane. Tropoelastin monomers then undergo maturation by being cross-linked by the lysyl oxidases LOX, LOXL1 and LOXL2. This enzymatic reticulation enables the formation of larger insoluble aggregates (Liu et al., 2004; Schmelzer et al., 2019). Tropoelastin aggregates then assemble with microfibrils of fibrillins that act as a protein scaffold for further formation of the mature elastic fibers (Figure 16).

Hence, fibrillins are essential actors and constituents of elastic fibers. In humans, there are three closely related fibrillin proteins: fibrillin-1, -2 and -3 encoded by *FBN1*, *FBN2* and

*FNB3* respectively and therefore able to form microfibrils. While the expression of fibrillin-2 and -3 are mostly restricted to embryogenesis, fibrillin-1 synthesis persists throughout adult life, thus making it the predominant isoform in dermis (van Loon et al., 2020). However, fibrillin-2 is also detected in the upper papillary dermis close to the epidermal dermal junction and its enhanced expression has been reported in wound healing and sclerosis contexts (Brinckmann et al., 2010). Studies on the presence of fibrillin-2 in post-natal microfibrils have revealed that this isoform could form the structural core of the microfibril and therefore be masked by an outer layer of fibrillin-1 (Charbonneau et al., 2010). These two fibrillins have been shown to strongly influence biomechanical properties of elastic fibers (Rock et al., 2004). Additionally, fibrillin microfibrils regulate the bioavailability of TGF- $\beta$  through its binding to latent TGF- $\beta$  binding proteins (LTBPs) (Krieg and Aumailley, 2011). TGF- $\beta$  is probably the most powerful cytokine that activates collagen and other ECM components synthesis in the skin, and will be further detailed in section 2.2.2.



**Figure 16. Different steps of the elastogenesis.**

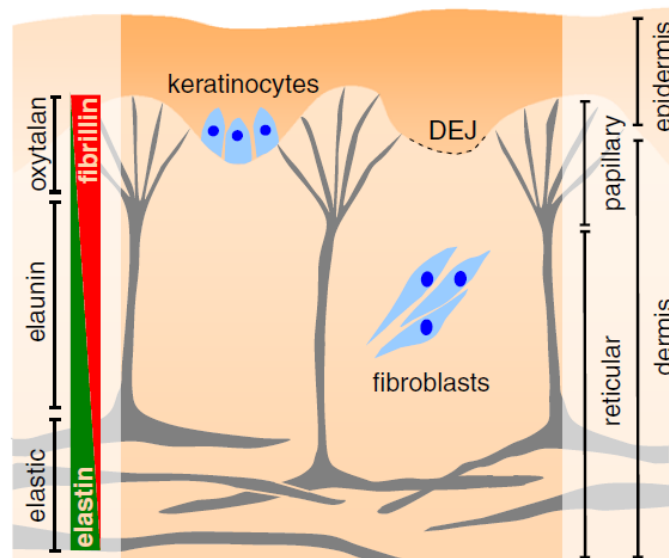
From the tropoelastin synthesis until the formation of the mature elastic fiber, many ECM components are required. Modified from Schmelzer et al., 2019.

Besides fibrillins, a wide range of ECM proteins also regulate elastogenesis including fibulin-4 and -5, the microfibril associated glycoproteins (MAGPs), the elastin microfibril interface located proteins (EMILINs) and proteoglycans such as decorin, biglycan, versican and perlecan (Shin and Yanagisawa, 2019). All of these regulators are thus integrated into the elastic fibers. Fibulins are incorporated into the elastic fibers and play an important role in

elastogenesis (Kielty et al., 2002). Knock-out of fibulin-4 (FBLN4) or fibulin-5 (FBLN5) in mice impairs the biosynthesis of elastic fibers in dermis. Notably, fibulin-4 plays an irreplaceable role in elastogenesis, since *Fbln4*<sup>-/-</sup> mice die just after birth due to severe vascular and lung defects and a complete absence of elastic fibrils in the dermis (McLaughlin et al., 2006). The *Fbln5*<sup>-/-</sup> mice display fewer, more fragmented, and structurally looser elastic fibers in their dermis compared to their wild-type counterparts (Nakamura et al., 2002; Yanagisawa et al., 2002). Mechanistically, fibulin-5 initiates the elastic fiber assembly, and stabilizes them by binding to integrins and tropoelastin molecules. Deposition of fibulin-5 on microfibrils also promotes coacervation and cross-linking of tropoelastin monomers (Hirai et al., 2007). Mutations of fibulin-4 or -5 are associated with *cutis laxa*, a genetic disease affecting connective tissues, and are, especially the dermis, characterized by hyper-elastic biomechanical properties of soft tissues due to abnormal elastic fibers (Berk et al., 2012). The microfibril-associated proteins MAGP-1 and -2 are both expressed in the dermis, interact with fibrillins and favor the formation of elastic fibers specifically in the papillary dermis (Jensen et al., 2001; Lemaire et al., 2007). EMILIN-1 is expressed at the interface between tropoelastin monomers and microfibrils, and interacts with fibrillins (Fitoussi et al., 2019; Zanetti et al., 2004). Decorin, biglycan and versican co-localize with elastic fibrils in the dermis. Finally, perlecan stabilizes elastic fibers through its interaction with fibrillins-1 and -2 and tropoelastin (Gheduzzi et al., 2005).

#### Organization of dermal elastic fibers throughout dermis

As for collagen fibers, the elastic fiber network structure differs in structure and organization between the papillary and reticular dermis (Sherratt, 2009). Mature and horizontal elastic fibers in the deepest part of the dermis (*i.e.* reticular dermis) are connected to perpendicularly arranged thinner elaunin fibers in the papillary dermis that further merge with oxytalan fibers, which intersperse into the DEJ (Figure 17). Closely related to the DEJ, oxytalan fibers are mainly, if not essentially, composed of fibrillin microfibrils, whereas mature collagen fibrils, located in the deep dermis, are mainly constituted of elastin, which accounts for 90% of the volume of the elastic fibers. In the elaunin fibers, the two proteins are present in equal quantities. This depicts an inverted ratio in the content of elastin and fibrillin in the elastic fibers between the upper and deeper dermis (Figure 17) (Haydont et al., 2019a; Sherratt, 2009).



**Figure 17. Elastic fiber network in dermis.**

The elastic fiber system in the skin is confined to the dermis where composite elastic fibers in the reticular dermis give way to arrays of fibrillin microfibril bundles at the DEJ. The fibrillin microfibrils of the elastic fiber system are synthesized by both keratinocytes and dermal fibroblasts. From Sherratt 2009.

### Matricellular proteins: other important glycoproteins of the dermal ECM

Matricellular proteins are secreted extracellular glycoproteins that do not self-assemble into ECM fibrils, but instead shape the ECM through multiple interactions between cell receptors, ECM components, cytokines and growth factors. Some of them have already been introduced as fibrillogenesis regulators. These proteins are also important mediators of cell-matrix interactions, and can thus impact cell migration and counter cell adhesion by regulating intracellular signaling and ECM-cell adhesions. These proteins are highly expressed during embryonic development, whereas their expression is limited to tissue repair or wound healing processes during adulthood (Adams, 2018; Bornstein, 2009). Matricellular proteins mainly include tenascins, thrombospondins, CCN proteins, Secreted Protein Aspartic and Rich in Cystein/osteinectine (SPARC) periostin and fibulins. In addition to fibulins, that have already been described, the major matricellular proteins in human dermis are thrombospondin-1 and -5 (TSPN-1 and TSPN-5), tenascin-C and -X and periostin (Nyström and Bruckner-Tuderman, 2019).

TSPN-1 plays a key role in collagen homeostasis, since this glycoprotein is able to bind to latent TGF- $\beta$  and to matrix metalloproteinases (MMP). Increased expression of TSPN-1 and TGF- $\beta$  are associated with some fibrotic skin diseases (Kim et al., 2015; Rosini et al., 2018).

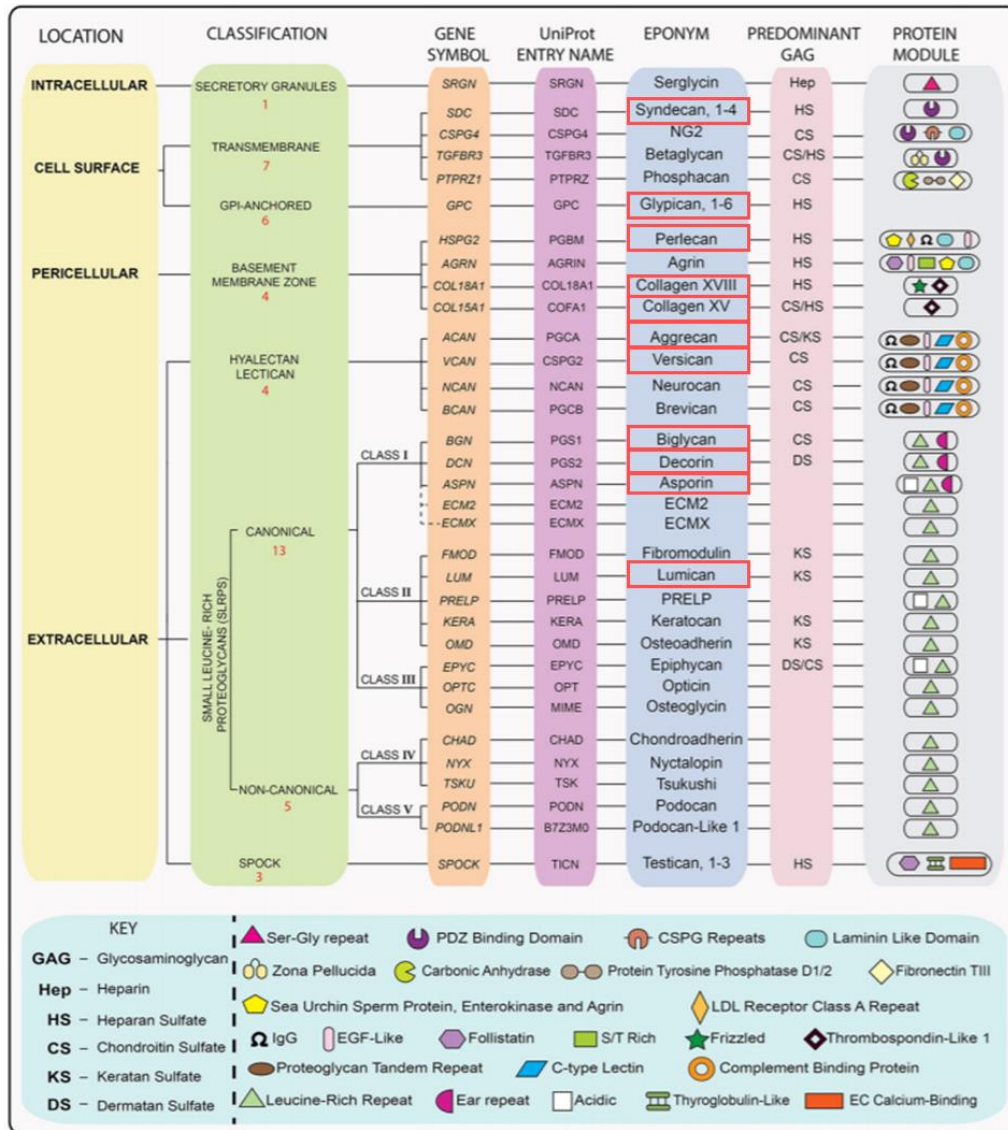
TSPN-5, also known as COMP, was shown to influence collagen fibril organization in papillary dermis by binding to collagen XII and XIV (Agarwal et al., 2012).

Tenascin-X and -C are molecular organizers of the dermal ECM. Tenascin-X has been localized to dermal elastic fibers and mutations in the *TNXB* gene lead to less numerous and immature elastic fibers in patients with classical-like EDS (De Paepe and Malfait, 2012; Egging et al., 2007; Zweers et al., 2004). Tenascin-C expression in skin is associated with wound healing and tumors (Latijnhouwers et al., 1997).

Unlike most matricellular proteins, periostin expression is not restricted to skin embryogenesis or pathogenesis situations. Periostin is indeed secreted by papillary dermal fibroblasts throughout the whole life. Periostin has been identified as an important regulator of fibrillogenesis in skin and its overexpression has been reported in fibrotic tissues (Egbert et al., 2014; Mitamura et al., 2018; Norris et al., 2007). In addition, the aberrant expression of this protein has been associated with various other skin diseases as atopic dermatitis, melanoma or scleroderma, that are triggered by immune responses (Kuwatsuka and Murota, 2019; Mitamura et al., 2018).

- Stabilization and hydration of the ECM through proteoglycans

Proteoglycans (PGs) are specific glycoproteins, in which one or more glycosaminoglycan (GAG) chains and/or O- and N-oligosaccharides are attached to a core protein (Iozzo and Schaefer, 2015). Iozzo and Schaefer have suggested a nomenclature taking into account the cellular and subcellular localization of the PGs, as well as their sequence homology and some common protein domains in the core protein (Figure 18) (Iozzo and Schaefer, 2015). These glycoproteins are of particular importance in the dermis since they are able to interact with a vast array of ECM proteins, but they also have the particularity to sequester water thanks to the highly negative charged GAG chains they carry. As such, in addition to link and stabilize the network of collagen and elastic fibers, PGs strongly contribute to skin hydration and thus confer to the skin a large osmotic swelling pressure that contributes to its viscoelastic biomechanical properties (Haydout et al., 2019a). In addition, PGs regulate the bioavailability of growth factors and modulate cell signaling through additional interactions with cell receptors (Smith and Melrose, 2015). Hence, PGs are essential to maintain skin integrity and functions.



**Figure 18. Classification of proteoglycans.**

The four families are based on their cellular and subcellular location, homology at the protein and genomic levels and the presence of unique protein modules which are often shared by members of a given class. The key for the various modules is provided in the bottom panel. PG in red boxes have been evidenced in skin (epidermis, DEJ and dermis). Modified from Iozzo and Schaefer, 2015.

The prevalent PGs in skin include the small leucine rich proteoglycans (SLRPs) decorin biglycan and versican (Lee et al., 2016; Nyström and Bruckner-Tuderman, 2019). They all are produced by dermal fibroblasts although these produce more decorin than biglycan and versican (Li et al., 2013). Skin also contains particularly high levels of hyaluronic acid (HA), a non-sulfated GAG that has unique capacities in retaining water in tissues.

Decorin and biglycan stabilize the elastin and collagen fiber networks of the human dermis through direct binding to both fibers. It is worthy to note that there are very few direct interactions between elastin and collagen fibers. Cohesion and interactions between these two

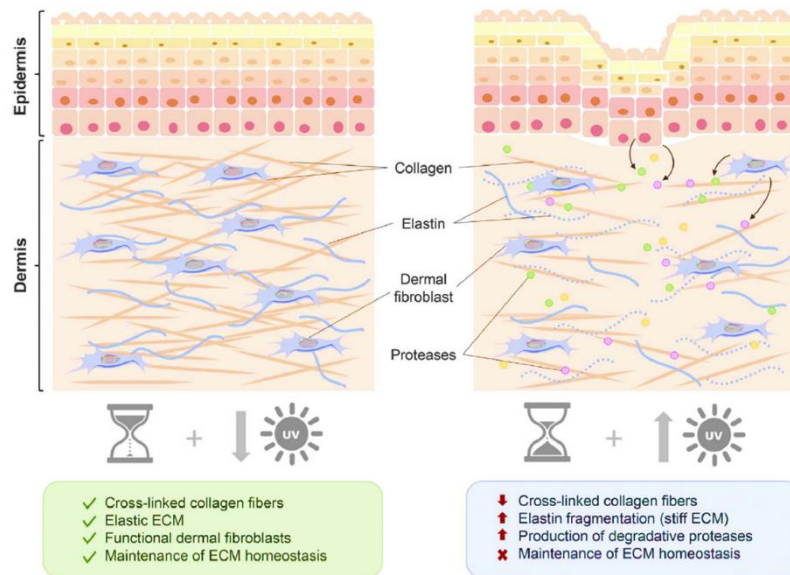
networks are instead ensured by PGs, but also by the collagen VI beaded filaments (Haydont et al., 2019a; Seidler and Dreier, 2008). Mice studies have demonstrated that decorin has a more important function in skin than biglycan. Mice invalidated for decorin exhibit thin and fragile skin with a reduced strength due to an improper fibrillogenesis, whereas a knock-out of biglycan alters the dermal thickness; however, the mice do not display any clinical skin phenotype (Corsi et al., 2002; Danielson et al., 1997).

As mentioned above, HA is only composed of non-sulfated, highly hydrophilic GAGs and does not contain any core protein. Of all the tissues in the body, skin is the richest organ in HA (Stern and Maibach, 2008) where it ensures vital functions as a major source of hydration (Papakonstantinou et al., 2012). In the human dermis, HA is highly expressed in the upper papillary dermis (Anderegg et al., 2014). HA is not only a space-filler with high binding capacities for water, it can also self-assemble or bind to other ECM proteins. In the dermis, its main binding partner is versican. Finally, HA plays an important role in the skin homeostasis by interacting with cell surface receptors, notably CD44.

### 2.1.3. Remodeling of the ECM

ECM is a highly dynamic structure that is synthesized during development and then undergoes continuous remodeling in response to external stimuli, such as applied forces, injuries or during wound healing in healthy and pathological conditions but also in physiological situations as diverse as during exercise or pregnancy. ECM remodeling is tightly controlled by diverse ECM degrading proteases, including the sheddases a disintegrin and metalloproteinases (ADAMs), ADAM with thrombospondin motifs (ADAMTS) and matrix metalloproteinases (MMPs). MMPs activity is counterbalanced by the tissue inhibitor metalloproteinases (TIMPs), but also by other enzymes, such as the lysyl oxidase (LOX) and transglutaminases (TGM), which cross-link ECM fibers and control the stiffness of the matrix (Frantz et al., 2010; Karamanos et al., 2019). In healthy conditions, the degraded ECM can be renewed and this remodeling is indeed crucial to maintain a proper tissue integrity and functionality.

Dysregulation of ECM remodeling appears to initiate and promote the progression of various diseases, including fibrosis and cancers as examples (Coentro et al., 2019; Kass et al., 2007) but it also represents an aging hallmark, especially in the skin (Figure 19) (Freitas-Rodríguez et al., 2017; Phillip et al., 2015).



**Figure 19. Intrinsic changes in skin ECM during photoaging.**

UV radiations and the accumulation of damage in intracellular components over time generate an imbalance between synthetic and degradation pathways involved in skin ECM remodeling by promoting protease production, leading to a loss of ECM structure and organization. From Freitas-Rodríguez et al, 2017.

## 2.2. Biology of the fibroblast: the major dermal cell population

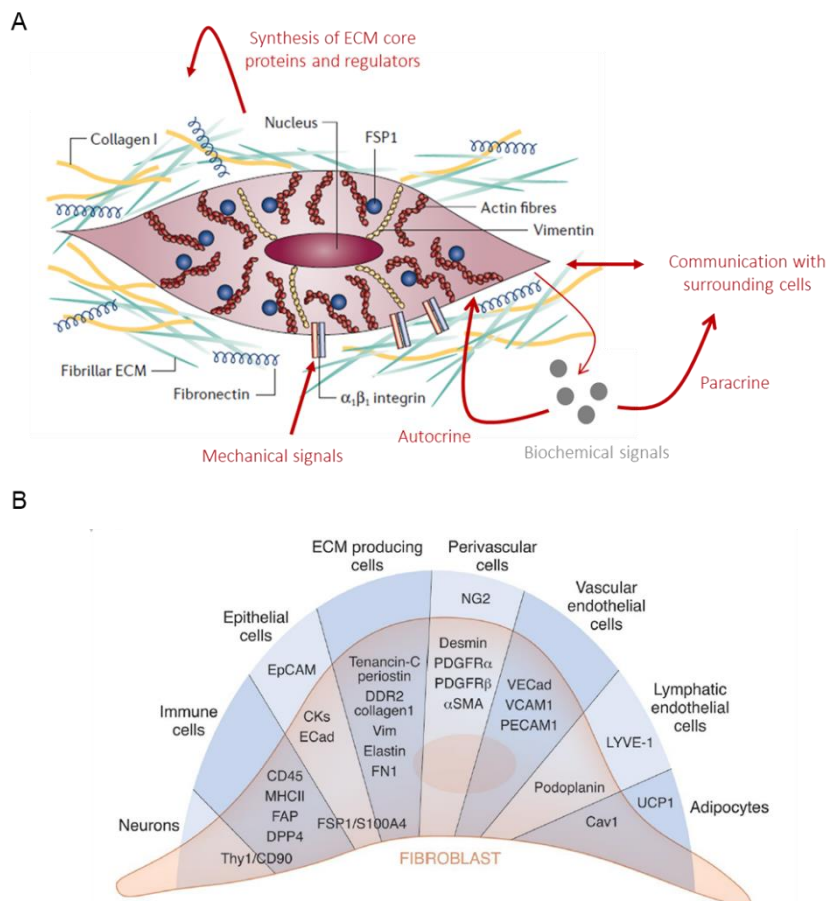
### 2.2.1. Fibroblasts are heterogeneous and plastic cells

Fibroblasts are abundant cells throughout the human body and among the most studied cell types as they are easily isolated and cultivated *in vitro*. Nevertheless, they still remain poorly defined and enigmatic cells. Fibroblasts are mesenchymal cells that have been firstly described as spindle-shaped cells synthesizing and organizing extracellular matrix proteins into the extracellular space (Figure 20A). Notably, fibroblasts are the major cells responsible for the production of collagen molecules. Therefore, they are responsible for the integrity and homeostasis of most connective tissues such as cartilage, tendons and dermis (Kalluri and Zeisberg, 2006). However, this definition does not take into account the remarkable heterogeneity and plasticity of the fibroblast population.

The heterogeneity of fibroblasts can be illustrated by the vast array of biomarkers, the expression of which can vary according to the tissue and even to the location within a given tissue (Figure 20B) (Chang et al., 2002). This is actually perfectly illustrated with the dermis,



which comprises different fibroblast subtypes that will be presented in section 2.2.3. Despite intensive investigations in the last decade, there is still no specific fibroblast biomarker identified and even less for each subpopulation of fibroblasts, since most of the biomarkers are also expressed in other cell types, such as perivascular, epithelial or immune cells (LeBleu and Neilson, 2020). This is a very limiting step that complicates fibroblast characterization. As an example, collagen I was first believed to be specific for fibroblasts, but it can also be synthesized by bone marrow-derived cells (Buchtler et al., 2018; Florin et al., 2004). So far, fibroblast-specific-protein 1 (FSP1) has been shown to provide the best specificity for fibroblast detection *in vivo* (Strutz et al., 1995). Therefore, isolation of fibroblasts from tissues still uses the combination of both inclusive and exclusive biomarkers (LeBleu and Neilson, 2020).



**Figure 20. Functions and biomarkers of a fibroblast.**

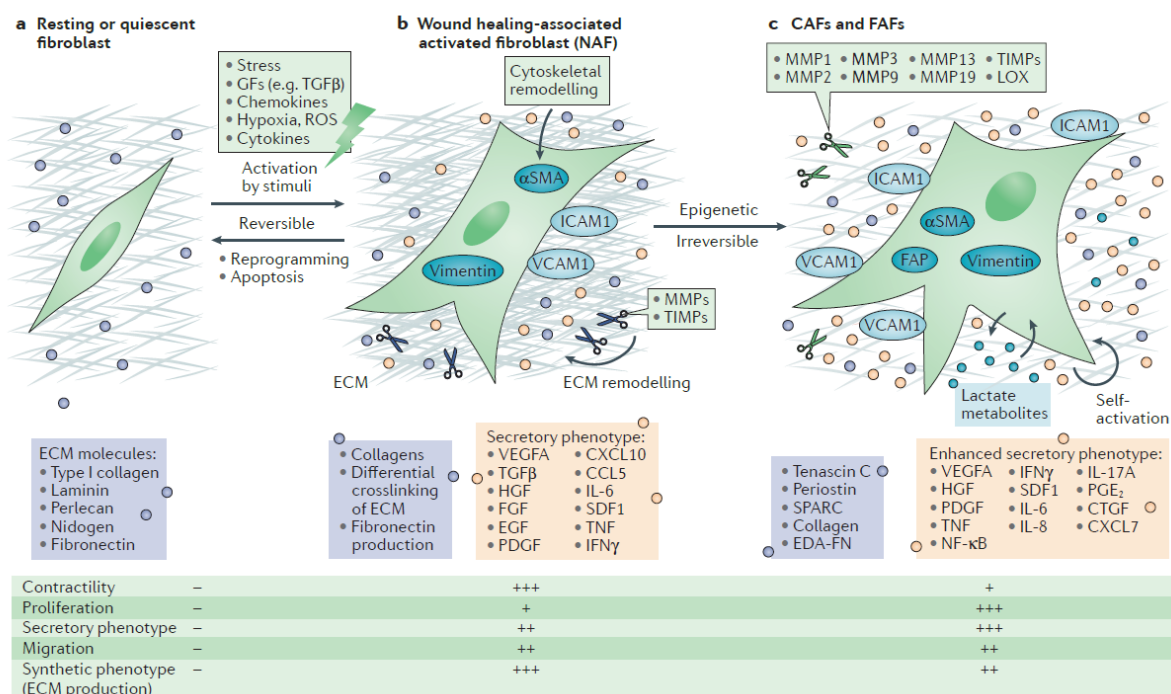
(A) Illustration of the main functions of a fibroblast: secretion of ECM core proteins (collagens, glycoproteins, proteoglycans) and ECM regulators (MMPs, TIMPs, LOX, TG); cellular communication through the secretion of biochemical signals that can act in an autocrine and paracrine manner, and also through mechanical signals perceived from its surrounding matrix which can modulate its activity. Modified from Kalluri and Zeisberg, 2006. (B) Biomarkers of a fibroblast, with relative inclusive (in red) and exclusive (in blue) biomarkers. From Lebleu and Neilson, 2020.

Additionally, fibroblasts exhibit a very specific plasticity in response to autocrine and paracrine signals such as cytokines and growth factors. Depending on the nature of the biochemical or mechanical signals perceived either from its matrix or from one of its neighboring cells, the fibroblast is able to adapt and to regulate its ECM production. Thus, the function of a fibroblast is going far beyond being an intensive ECM producer. It is also crucial for coordinating immune and angiogenic responses in order to remodel its cellular microenvironment (Figure 20A) (Saraswati et al., 2019; Van Linthout et al., 2014). It is the case for human dermis in which fibroblasts actively communicate with and respond to biochemical and mechanical signals from both the ECM, proximal endothelial cells and keratinocytes from the above epidermis as well as adipocytes from the hypodermis. This plasticity confers to the fibroblasts a particular important role in skin physiology, notably in wound healing. Dysregulation of fibroblast activity can therefore lead to skin alterations or pathologies, such as during aging, or in skin fibrosis or cancer (Jevtić et al., 2020; Russo et al., 2020; Werner et al., 2007).

## 2.2.2. Fibroblast activation and regulation of ECM deposition

- Cutaneous activation of fibroblasts

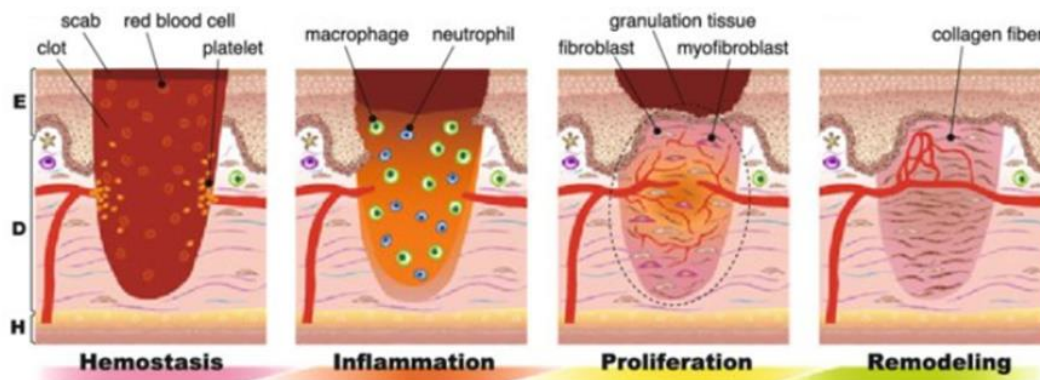
While fibroblasts are first highly proliferative in fetal dermis, they switch to a quiescent state in early post-natal life (Rognoni et al., 2018). Quiescent fibroblasts in healthy dermis have a resting phenotype with a reduced transcriptional activity, decreased proliferative and migration capacities, negligible metabolic activity, and thus low ECM protein synthesis (Figure 21A). However, quiescent fibroblasts can acquire an ‘activated’ phenotype upon different types of stimuli in physiological and pathological contexts to synthesize large amounts of ECM components (Kalluri and Zeisberg, 2006).



**Figure 21. Multi-step activation of fibroblasts.**

(A) Quiescent or resting fibroblasts are inert and are identified as spindle-shaped single cells embedded in physiological extracellular matrix (ECM). (B) In response to tissue injury and the associated stimuli, the quiescent fibroblasts are reversibly activated to facilitate repair and regeneration. The normal activated fibroblasts (NAFs) gain expression of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) and become stellate in shape. They gain contractile properties. The acquired synthetic functions are associated with secretory and migratory functions that amplify their activation, recruitment and proliferation. The reversibility of such activation may be mediated by reprogramming or apoptosis of the wound healing-associated activated fibroblasts when the repair process is complete. (C) Activated fibroblasts may gain further secretory phenotypes. This process is associated with persisting and unabated injurious stimuli, such as the development of cancer lesions. Epigenetic regulation may limit the regression of such activated states. Such fibrosis-associated fibroblasts (FAFs) and cancer-associated fibroblasts (CAFs) gain enhanced proliferative properties and are a functionally diverse population. From Kalluri, 2016.

The presence of activated fibroblast was first reported during wound healing (Gabbiani et al., 1971) and has been later more largely identified when tissue injury occurs (Figure 21B) (Zeisberg et al., 2000). The wound healing process is divided into four major steps. The first step is the formation of a fibrin clot to quickly seal the wound, followed by the inflammatory phase with the recruitment of macrophages and neutrophils, which secrete cytokines and growth factors that stimulate keratinocyte proliferation and angiogenesis, and attract reticular fibroblasts to the injury site, where they further differentiate into myofibroblasts. Then, the active proliferation phase is initiated and includes the re-epithelialization by keratinocytes and the production of granulation tissue, composed of an abundant and disorganized ECM, by the myofibroblasts. In the last phase the granulation tissue is remodeled, degraded, and replaced by a newly synthesized dermal ECM by papillary fibroblasts (Figure 22).



**Figure 22. The four phases of the wound healing process.**

Wound healing process first starts with a clot formation/hemostasis; followed by the inflammation phase with the macrophage and neutrophils that attract keratinocytes and fibroblasts for the coming proliferation phase with reepithelialization and formation of the granulation tissue. The last phase is the remodeling of the ECM at the wound site. From Kawasumi et al., 2013.

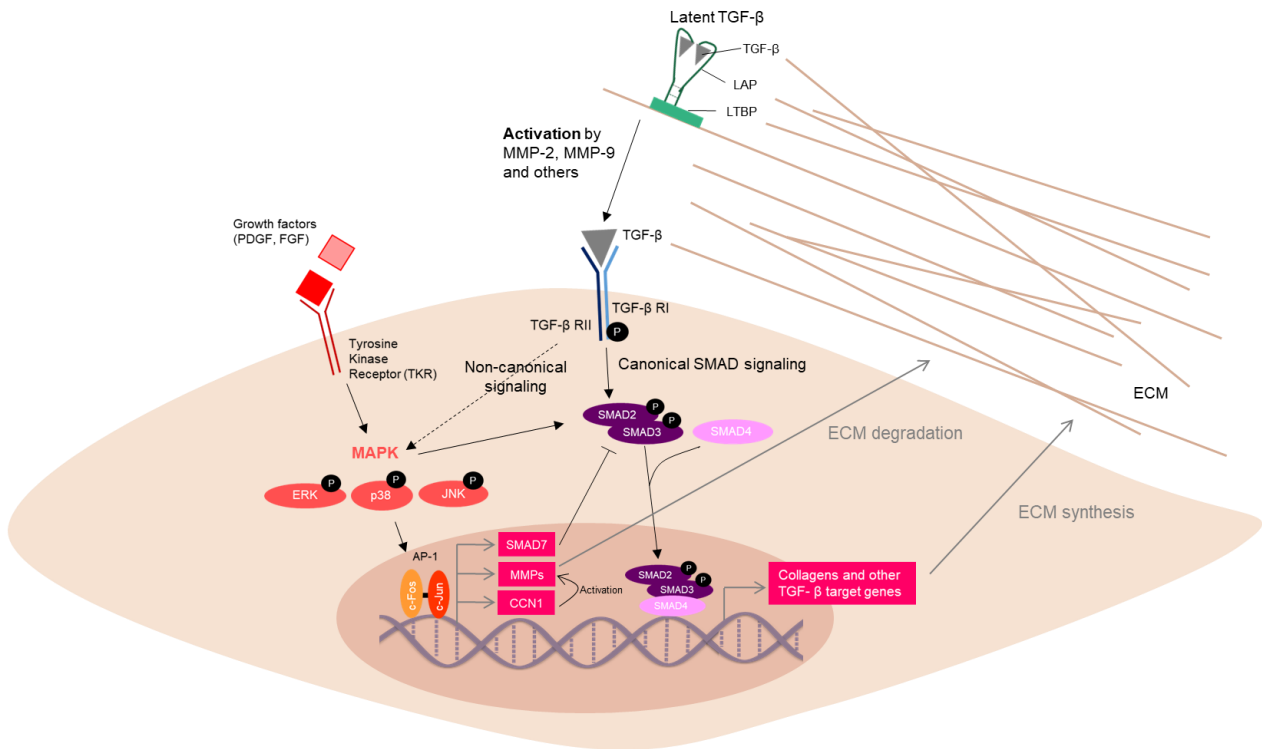
Fibroblasts are also able to undergo morphological changes when they differentiate into 'myofibroblast' in response to growth factors such as epidermal growth factor (EGF), TGF- $\beta$  or PDGF secreted by surrounding epithelial and inflammatory cells, in presence of elevated levels of reactive oxygen species (ROS), or if ECM rigidity is altered (Tomasek et al., 2002; Zeisberg et al., 2000). Myofibroblasts express  $\alpha$ -smooth muscle actin ( $\alpha$ SMA), encoded by the *ACTA2* gene), which is normally specific to the smooth muscle.  $\alpha$ SMA associates with cellular actin microfilaments to form stress fibers, conferring both contractile capacities and a stellate morphology. In addition, myofibroblasts secrete cytokines and growth factors that attract immune cells and they are also intense producers of ECM core components such as collagen I, but also of ECM degrading proteases such as MMPs which are involved in damaged ECM

repair (Hinz, 2016). In the context of wound healing, differentiation of fibroblasts into myofibroblasts has been suggested to be reversible as the number of myofibroblasts dropped to normal situation after wound repair. Currently there are two hypotheses, either they are going back to their resting phenotype or they are undergoing apoptosis while adjacent fibroblasts are repopulating the dermis (Desmoulière et al., 1995; Nedelec et al., 2001; Tomasek et al., 2002).

Chronic activation of fibroblasts is responsible for skin fibrosis and skin cancers, in which differentiated fibroblasts are respectively named Fibrosis-Associated Fibroblasts (FAFs) and Cancer-Associated Fibroblasts (CAFs) (Figure 21C). The activated state of these fibroblasts is considered as 'irreversible' contrary to myofibroblasts. Constitutive activation seems to be mediated through epigenetic modifications such as specific DNA methylations patterns or expression of miRNAs (LeBleu and Neilson, 2020). For instance, epigenetic alterations have been shown to activate aberrant differentiation of fibroblasts into FAFs in fibrotic disorder systemic sclerosis (SSc) (Bergmann and Distler, 2017). CAFs are hyper proliferative fibroblasts which promote tumor growth and progression by secreting growth factors and producing a stiffer ECM (Lu et al., 2012).

- Regulation of ECM deposition and degradation

During embryogenesis, dermal fibroblasts actively secrete ECM to build the dermal architecture that will contribute in epidermal differentiation and certainly in yet undiscovered other skin structures. On the contrary, in post-natal life, as previously described, dermal fibroblasts are quiescent and quite only secrete ECM proteins in response to activation by growth factors and cytokines, including the TGF-  $\beta$  family, placental derived growth factors (PDGF) and fibroblasts growth factors (FGF) family as the main actors (Figure 23).



**Figure 23. Signaling pathways regulating ECM deposition and degradation in dermal fibroblasts.** TGF- $\beta$  is the main inducer of ECM synthesis, and also inhibits ECM degradation through the induction of both canonical SMAD pathway and non-canonical pathway involving MAPK. MAPK can also be activated by other growth factors such as PGDF and FGF that further activate ECM deposition and degradation.

The TGF- $\beta$  family, which comprises TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3, Bone Morphogenic Proteins (BMP) and activins, are strong inducers of ECM secretion (Morikawa et al., 2016). TGF- $\beta$ 1 to 3 are involved in wound healing with each isoform exerting specific functions, that are context dependent. TGF- $\beta$ 3 promotes scarless healing in embryos and reduces scarring in adult skin, while TGF- $\beta$ 1 has been shown to be the major form in adult skin with key roles in wound healing and skin fibrosis that will be presented (K. K. Kim et al., 2018; Lichtman et al., 2016).

When secreted, TGF- $\beta$  molecules are sequestered in the ECM in a latent state. TGF- $\beta$ , the latency-associated-peptide (LAP) and latent TGF- $\beta$  binding proteins (LTBPs) altogether form the large latent complex (LLC) (Figure 23). While LAP maintains TGF- $\beta$  in a latent state, LTBPs directly sequester it through the interaction with ECM proteins such as fibrillin elastin or decorin, which regulate TGF- $\beta$  bioavailability (Thomson et al., 2019). TGF- $\beta$  activation occurs through cleavage by MMP-2 or 9 of the cross-links between TGF- $\beta$  and LAP/ LTBPs, through the presence of TSP-1 that competes for interaction with LAP and LTBPs, or by cell-surface integrins that can bind to the RGD motifs of the LAPs which prevents them from

sequestering TGF- $\beta$  (Atanasova et al., 2019; Margadant and Sonnenberg, 2010; Robertson and Rifkin, 2016).

Once released, TGF- $\beta$  activates its downstream signaling pathways through binding to its two transmembrane serine/threonine kinases receptors, namely TGF- $\beta$ RI and TGF- $\beta$ RII. Binding of TGF- $\beta$  to TGF- $\beta$ RII enables its dimerization with phosphorylated TGF- $\beta$ RI, which further activates phosphorylation of Smad2 and Smad3 and heterodimerize with a common partner Smad4. The activated Smad complex then translocates into the nucleus where it activates transcription of TGF- $\beta$  target genes, either alone or in combination with other factors, by binding the Smad binding element (SME) or CAGA box. In human skin fibroblasts, SMEs have been identified in the promoter regions of the collagen genes *COL1A2*, *COL3A1*, *COL6A1*, *COL6A3* and also in fibronectin, decorin and versican (Chen et al., 1999; Verrecchia et al., 2001). Additionally, TGF- $\beta$  increases collagen and elastin synthesis by stabilizing their mRNAs (Bowen et al., 2013; Davidson, 2002; Kähäri et al., 1992).

In addition to the canonical Smad signaling pathway, TGF- $\beta$  also activates non-canonical (non-Smad) pathways, which include three families of mitogen activated protein kinases (MAPKs): extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) and PI3K/Akt pathways (Cole et al., 2018; Ricard-Blum et al., 2018). MAPK further activates the transcription factor activator protein 1 (AP-1), composed of c-Fos and c-Jun. AP-1 transcription factor activates the expression of several target genes, including MMPs (MMP1 and MMP3), responsible for the remodeling of dermal ECM (J.-M. Kim et al., 2018) and Smad7 that in turn further inhibits phosphorylation of Smad2/Smad3 and therefore collagen synthesis (Quan et al., 2005), and cysteine-rich protein 61 (CCN1) that has been shown to upregulate MMP1 (Qin et al., 2013).

Through these different signaling cascades, TGF- $\beta$  induces ECM gene expression, and inhibits ECM degradation (Figure 23). Hence, it is considered as the main regulatory pathway for ECM homeostasis in dermal fibroblasts. TGF- $\beta$  upregulation causes abnormal accumulation of ECM protein in fibrosis, whereas its downregulation has a significant impact on skin aging (Quan and Fisher, 2015).

PDGF family includes four members PDGFA, PDGFB, PDGFC and PDGFD that can either form homo- or heterodimers. PDGFs further bind to tyrosine kinase receptors PDGFR $\alpha$  and PDGFR $\beta$  resulting in the activation of MAPK signaling and defects in ECM secretion and angiogenesis (Figure 23) (Barrientos et al., 2008; Lin et al., 2006). As examples, PDGFB and

C have been shown to stimulate TGF- $\beta$  secretion and fibroblast proliferation. PDGFC also controls ECM degradation by activating TIMP-1 expression and regulation of MMP1 activity (Jinnin et al., 2005).

FGFs increase fibroblast proliferation, ECM synthesis, and stimulate angiogenesis by enhancing endothelial cell proliferation. They are thus important factors in skin wound healing and skin aging. FGFs activate the MAPK signaling cascade through its binding to tyrosine kinase transmembrane receptors (Figure 23) (Barrientos et al., 2008; de Araújo et al., 2019). This family of growth factors comprises 23 members, among which the basic FGF (bFGF or FGF-2) is of major importance in dermal fibroblasts (de Araújo et al., 2019). FGF-2 stimulates fibroblast proliferation and ECM degradation by stimulating the expression of collagenases and elastases (Sasaki, 1992; Yang et al., 2018).

In addition to these three growth factor families, other cytokines and growth factors influence ECM deposition. For instance, the insulin growth factor 1 (IGF1) activates elastic fibers formation, whereas the epidermal growth factor (EGF) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) have opposite effects (Sproul and Argraves, 2013). TNF- $\alpha$  has also been shown to counterbalance TGF- $\beta$  activity through inhibition of collagen I expression (Verrecchia and Mauviel, 2004).

### **2.2.3. The dynamic interplay between dermal fibroblasts, ECM and surrounding cells**

- Communication between the fibroblasts and ECM: definition of the 'tissue skeleton'

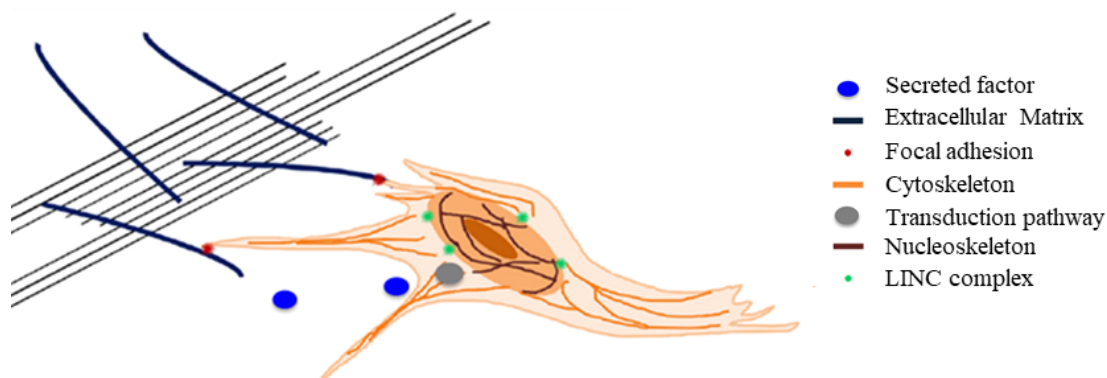
The relationship between fibroblasts and ECM has been shown to be essential for maintaining tissue integrity and homeostasis (Manou et al., 2019). As an example among many, it has been shown that the alteration of this dialogue between fibroblast and its microenvironment is one of the causes of skin aging and aberrant wound repair (Vedrenne et al., 2012). Fibroblasts actively communicate with the surrounding ECM components by connecting to their membrane receptors (Theocharis et al., 2016). Integrins, discoid domain receptors (DDR), the HA specific receptor CD44, or cell surface proteoglycans, such as syndecans and glypicans are the classical ECM receptors, with integrins being by far the most studied ones. Integrins are transmembrane proteins mediating the interaction between the cell and extracellular collagens, fibronectins and laminins (Hynes, 2002). They are dimeric proteins



constituted of two non-covalently linked subunits:  $\alpha$  and  $\beta$ . So far, 18  $\alpha$ -subunits and 8  $\beta$ -subunits were identified in vertebrates, resulting in the formation of 24 unique combinations (Kechagia et al., 2019).

Communication between fibroblasts and the ECM is also mediated by growth factors (GFs) and cytokines, including chemokines, interleukins and tumor necrosis factors, that are deposited within the ECM. The ECM constitutes a “reservoir” of these extracellular signaling molecules and regulates their bioavailability (Hynes, 2009). In addition fibroblasts also perceive and respond to mechanical signals (Humphrey et al., 2014). Change in ECM physical properties or even topology can elicit mechanical signals transmitted from the ECM to the cells *via* integrins and the subsequent formation of focal adhesion complexes. This further induces reorganization of the intracellular cytoskeleton, which is itself connected to the nucleoskeleton, the cellular transcriptional machinery and the genomic DNA. Mechanical solicitations can therefore impact gene transcription and eventually cell function and behavior (Cole et al., 2018).

This complex protein network that bridges the ECM to the nucleus has been recently defined as the “tissue skeleton” by Haydont and collaborators (Figure 24) (Haydont et al., 2019b). It has been shown that aging substantially modifies the tissue skeleton in human dermal fibroblasts (Haydont et al., 2019b).



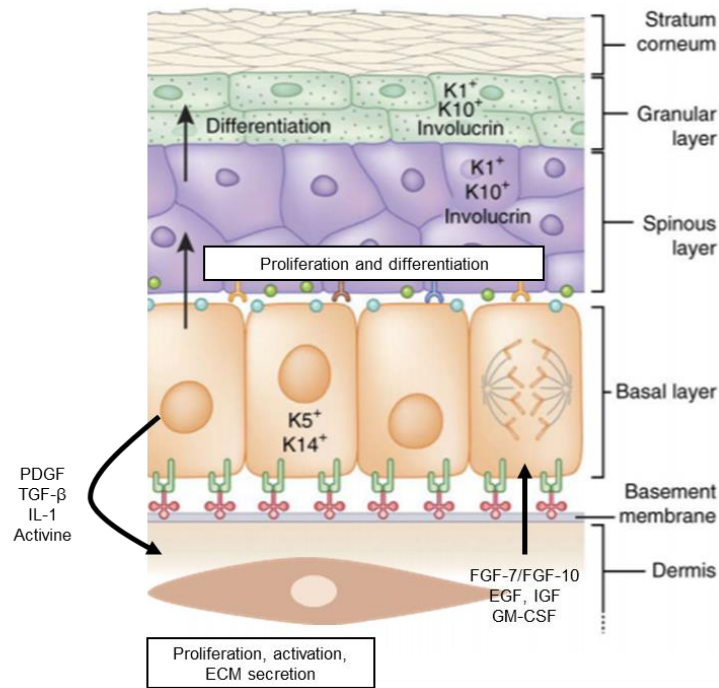
**Figure 24. Illustration of the ‘tissue skeleton’ network that connects the fibroblast to its microenvironment.**

This network connects ECM and genomic DNA via successive molecular relays: focal adhesion points, cytoskeleton, LINC complexes, and nucleoskeleton. The ‘tissue skeleton’ participates to the transmission of mechanical and biochemical stimulations to the nucleus and transformation into genetic and epigenetic responses (gene expression, DNA compaction, histone acetylation, spatial organization of DNA). It therefore illustrates the dialogue between fibroblast and its microenvironment. Modified from Haydont et al., 2019b.

- Dermal fibroblasts contribute to epidermal homeostasis

There is a dynamic interplay between the dermal fibroblasts and keratinocytes in skin. Dermal fibroblasts are able to regulate keratinocyte proliferation, migration and differentiation through paracrine signals and mechanical signals from the ECM (Jevtić et al., 2020). As previously introduced this communication is fundamental during wound healing and its disruption can lead to skin aging (Cole et al., 2018; Vedrenne et al., 2012; Werner et al., 2007). *In vivo* and *in vitro* studies have allowed elucidating some of these intercellular mechanisms.

In 1975, Rheinwald and Green established that 3T3 fibroblasts that have been irradiated to decrease their proliferation but not their synthetic activity, are required for keratinocyte culture since they stimulate their proliferation (Rheinwald and Green, 1975). This was later confirmed by using post-mitotic human dermal fibroblasts as a feeding layer for keratinocyte culture (Limat et al., 1989). Further studies led to identification of the various growth factors secreted by fibroblasts, such as EGF, IGF-1, GM-CSF and FGFs (FGF-1, FGF-7 and FGF-10) that control keratinocytes proliferation and migration through a paracrine loop between the epidermis and dermis (Figure 25) (Seeger and Paller, 2015). Among them, FGF-7, also known as Keratinocyte Growth Factor (KGF), is of particular importance for epidermal homeostasis. FGF-7 is expressed at low levels under normal conditions, but is highly produced during wound healing (Werner et al., 1992). Secretion of IL-1 from keratinocytes induces the synthesis of FGF-7 in fibroblasts, which in turn stimulates keratinocyte proliferation (Maas-Szabowski et al., 1999). By stimulating keratinocyte proliferation, FGF inhibits keratinocyte differentiation (Ogawa et al., 2008). In turn, keratinocytes also impact the fibroblast phenotype by expressing PDGF, IL-1- $\alpha$  and  $\beta$  and TGF- $\beta$ , which induce fibroblast differentiation into myofibroblasts, and regulate their proliferation and secretion of ECM (Figure 25) (Werner et al., 2007). Even if these soluble factors mediate communication between these two cell types, their physical interaction is required to activate keratinocyte proliferation since keratinocytes treated with conditioned media from fibroblasts do not exhibit the same proliferative capacities (Smola et al., 1993). Moreover different subtypes of fibroblasts differently impact epidermal differentiation through the release of specific growth factors and cytokines (Hausmann et al., 2019; Sorrell et al., 2004).

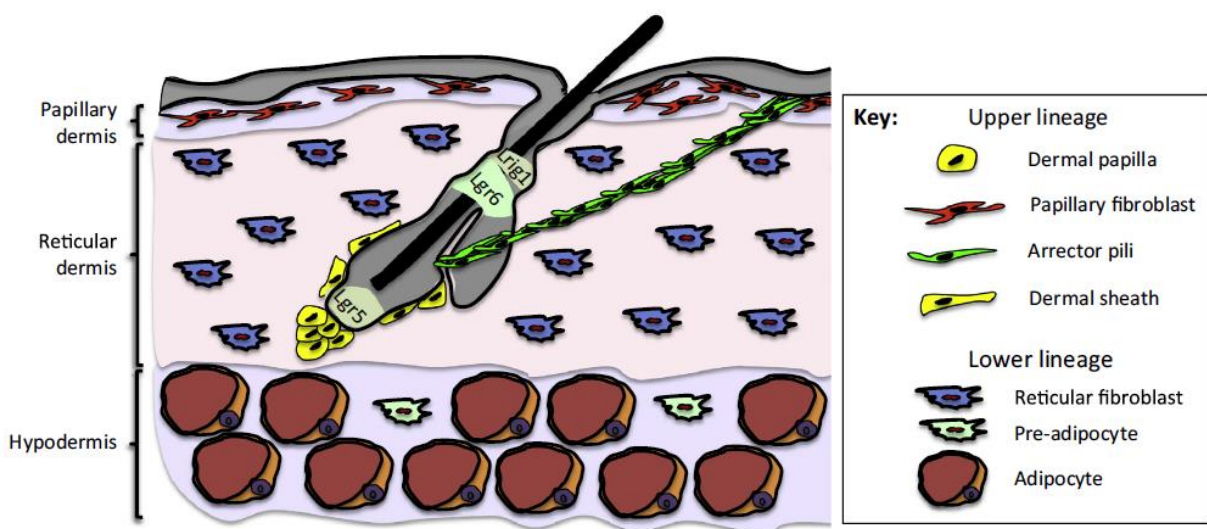


**Figure 25. Crosstalk between keratinocytes and dermal fibroblasts.**

Keratinocytes and fibroblasts are communicating both through secreted factors and physical interaction. Fibroblasts are regulating keratinocyte proliferation, migration and differentiation. In turn, keratinocytes are regulating fibroblasts proliferation, differentiation into myofibroblasts and secretion of ECM. Modified from Hsu et al., 2014.

## 2.2.4. Heterogeneity of dermal fibroblasts

Although all fibroblasts are secreting and organizing their surrounding matrix, there is an important heterogeneity between the fibroblasts residing in the different connective tissues of the human body (Chang et al., 2002; Rinn et al., 2006). When cultured *in vitro*, fibroblasts from different tissues indeed display differences in their morphology and behavior, which has been partly explained by the different patterns of HOX gene expression during embryonic development observed at different locations (Rinn et al., 2008). Additionally, fibroblasts exhibit remarkable functional diversity even within a same tissue, as it is the case for dermis in which different fibroblasts subtypes have been identified (Driskell and Watt, 2015).



**Figure 26. Heterogeneity of dermal fibroblasts in mouse skin.**

The locations of three epidermal stem cell populations (Lgr1+, Lgr6+, and Lgr5+) in the hair follicle are shown. The three dermal layers are also indicated: the papillary dermis, reticular dermis and hypodermis/white adipose layer. The fibroblasts of the dermal papilla (DP) and arrector pili muscle (AP) are derived from the same lineage as the papillary dermis, while reticular fibroblasts, pre-adipocytes and adipocytes share a common origin. From Driskell and Watt, 2015.

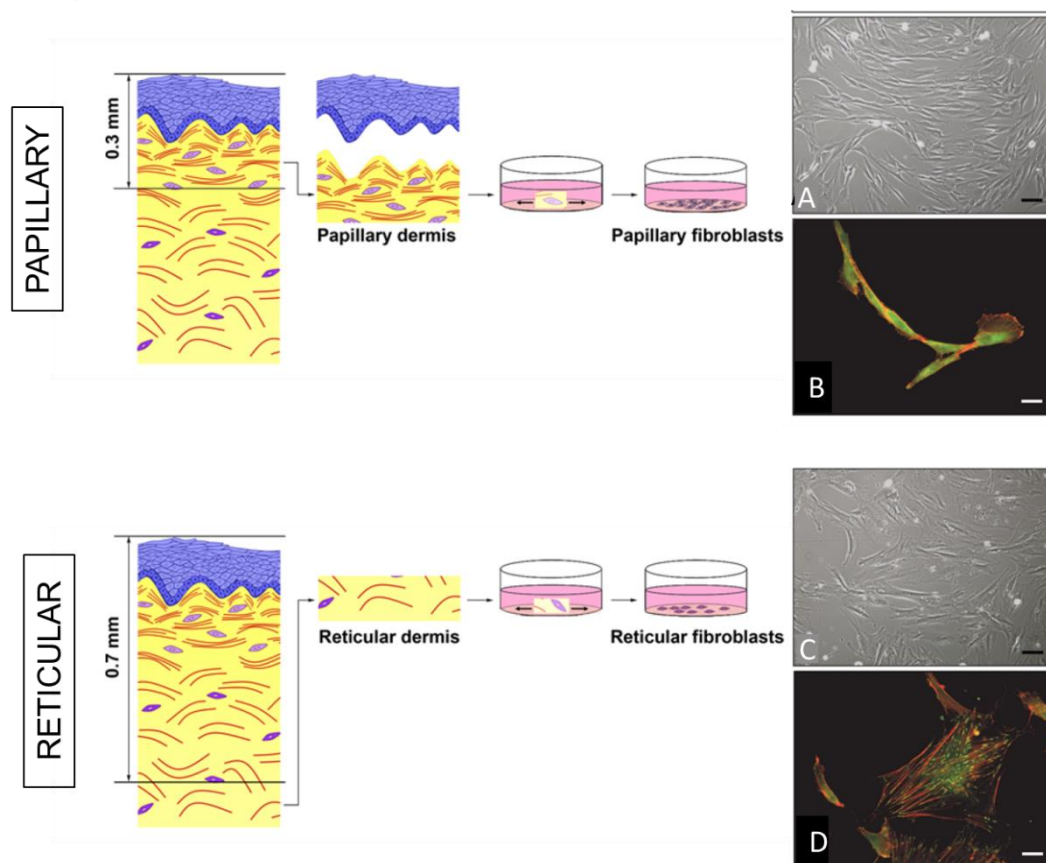
It has been presumed for a long time that dermis was composed of fibroblasts with distinct identities, since papillary and reticular dermal layer already had different histological characteristics and the morphology of the residing fibroblasts was histologically different (Harper and Grove, 1979). Mouse lineage tracing leads to the discovery that dermal fibroblasts all originate from a common progenitor (Driskell et al., 2013). They then differentiate into two lineages during embryonic development that will further generate the upper part and the lower part of the dermis, respectively (Figure 26). The upper lineage will give rise to two fibroblasts subtypes: dermal papilla fibroblasts and papillary fibroblasts. Dermal papilla fibroblasts can further differentiate into dermal sheath cells and/or arrector pili muscle cells; and regulate hair

follicle formation and hair growth (Driskell et al., 2013; Sennett and Rendl, 2012). The lower lineage differentiates into reticular fibroblasts and preadipocytes that will further differentiate into adipocytes in the hypodermis. Each subtype of fibroblast mediates specific functions and is essential for skin homeostasis (Driskell and Watt, 2015). In human skin, markers for upper and lower lineages have also been identified (Korosec et al., 2019). However, it is important to note that mouse and human skin largely differ in terms of organization and molecular markers as it has been shown by Philippeos and collaborators (Philippeos et al., 2018).

By combining spatial and single cell transcriptomic approaches, four populations of dermal fibroblasts have been evidenced in human skin (Philippeos et al., 2018; Solé-Boldo et al., 2020). These two studies additionally showed that these four dermal populations are losing their identity with aging and that they do not always spatially segregate. The four subpopulations include the papillary and reticular fibroblasts. The two other subpopulations have been suggested to be pro-inflammatory fibroblasts and a pericyte-like population, respectively (Philippeos et al., 2018; Solé-Boldo et al., 2020). In addition, fibroblasts underlying the dermo-hypodermal junction have been recently characterized and defined as a new subpopulation of fibroblasts in human dermis (Haydont et al., 2020). It remains to be clarified whether or not this newly discovered fibroblast subpopulation is distinct or included into the four populations identified. The recent development of 'spatial-omics' methodology will undoubtedly help to better identify and characterize the fibroblast subpopulations of the human dermis (Bingham et al., 2020).

Among dermal fibroblast subpopulations, papillary and reticular fibroblasts have been by far the most studied and clearly possess distinct gene expression patterns as well as morphological and functional differences (Janson et al., 2013; Mine et al., 2008; Sorrell et al., 2004). Papillary fibroblasts are smaller, with low granularity and show a high proliferation rate compared to reticular fibroblasts (Figure 27) (Harper and Grove, 1979; Mine et al., 2008). When cultivated in living skin equivalent models, it has been shown that papillary fibroblasts are more prone to stimulate keratinocyte proliferation and terminal differentiation than reticular fibroblasts. This indicates that the two fibroblasts subtypes differ in cytokines and growth factors secretion and thus in their communication with the keratinocytes. Papillary fibroblasts seem to be more prone to produce and respond to biochemical signals, whereas reticular fibroblasts allow to counteract mechanical stresses (Nauroy et al., 2017). Additionally, global transcriptomic analyses have determined specific biomarkers of these two populations (Haydont et al., 2019c; Janson et al., 2012; Nauroy et al., 2017). Nauroy and collaborators showed that *COL7A1*, *LOXL3*, *MMP1* and *PDPN* are specific markers of papillary fibroblasts,

while *ELN*, *MGP*, *COL11A1*, *TGM2* and *ACAN* are specifically upregulated in reticular fibroblasts. The differential expression of most of these markers were recently confirmed by an independent study (Haydout et al., 2019c). Some of them were already identified using DNA microarray (Janson et al., 2012). The upregulation of ECM regulators (*LOXL3*, *MMP1*) confirm an active remodeling in the upper part of the dermis and the upregulation of the elastin gene (*ELN*) indicates active elastogenesis in the reticular dermis. The expression of *COL11A1* in reticular dermis was unexpected, since this collagen was known until then as a component of the cartilaginous ECM to respond to high mechanical tensions. It has been suggested to play a role in ECM fibrillogenesis and to contribute to the mechanical properties of the reticular dermis. Papillary and reticular fibroblasts have clearly distinct expression profiles suggesting distinct functions in the human dermis. As such, in wound healing, fibroblasts of the deep dermis lineage are first attracted to the wound site, differentiate into myofibroblasts to contract and secrete high levels of ECM components to close the wound and form the granulation tissue, whereas upper dermal fibroblasts are only recruited after re-epithelialization (Rossio-Pasquier et al., 1999).



**Figure 27. Papillary and reticular fibroblasts.**

Principle of papillary (Fp) and reticular (Fr) fibroblast isolation used in Mine et al., 2008. (A) and (C) Photographs of typical cultures of Fp and Fr observed with light microscopy. Scale bars = 10 mm. (B) and (D) Fluorescence photographs of Fp and Fr after actin and vinculin immuno-staining. Scale bars = 2.5 mm. Modified from Mine *et al.*, 2008.

Deciphering structural and functional properties of fibroblasts subpopulations remains a key challenge, but the accumulated data over the last decade already show some interesting functional differences between the fibroblasts supopulations that could be beneficial for skin therapies. As an example, since papillary fibroblasts have been shown to secrete high levels of collagen VII, which is the main components of anchoring fibrils of the epidermis into the dermis, they could be used for patients with skin blistering diseases as a therapeutic treatment (Nauroy et al., 2017; Nyström et al., 2013).

### 3. The transcription factor NRF2

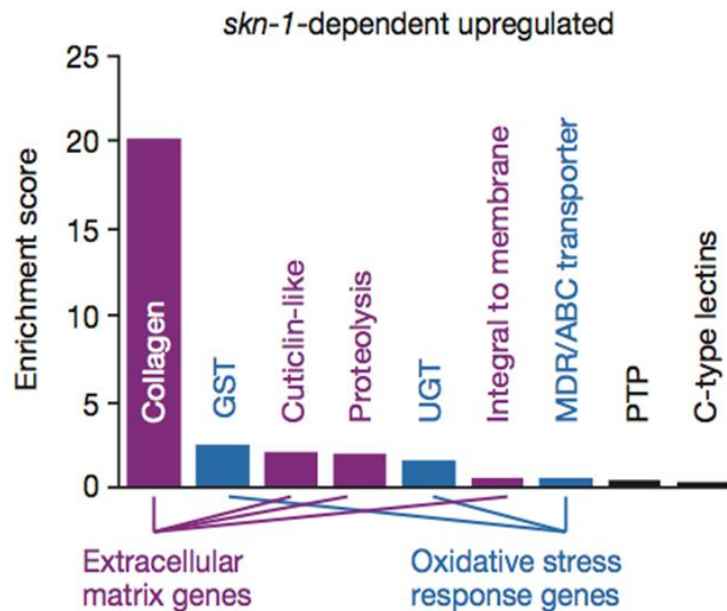
#### 3.1. The discovery of SKN-1 as regulator of ECM gene expression in *C.elegans*

As previously introduced, SKN-1 has been identified as a key longevity regulator in *C. elegans*. The fact that its activation leads to an unexpected massive upregulation of several collagens in worm (Ewald et al., 2015) was actually the starting point of my thesis. The exact mechanism through which SKN-1 controls longevity was not fully elucidated and seemed to depend on genetic and environmental parameters. It was suggested that SKN-1 may act through its antioxidant, detoxification or proteostasis 'conventional' functions (Blackwell et al., 2015; Li et al., 2011). However, Ewald and collaborators made the surprising discovery, that SKN-1 increased longevity mainly through the activation of expression of ECM genes, compared to which the detoxification/antioxidant gene response was only mildly upregulated. It has to be emphasized that this finding was made in mutant worms, displaying reduced insulin/IGF-1 signaling, although under conditions that the worms did not undergo the dauer stage, which is the diapause allowing worms to survive to low nutrient conditions (Ewald et al., 2015). Using a transcriptomic approach, they identified collagens amongst the upregulated ECM genes (Figure 28). Most of them are expressed in the worm cuticle. The cuticle is the exoskeleton of *C. elegans* that is vitally important for its morphology and mobility, interaction with the environment, and protection from external assaults, very similar to human skin functions. As a matter of curiosity for ECM scientists, there are many more collagens found in the *C.elegans* genome than in any mammalian genome. In total 181 genes encode collagen molecules, with 173 out of the 181 being predicted to be cuticle collagens and unique to the nematode (Teuscher et al., 2019). Collagens identified in this study were further shown to be required for the increase in longevity, since their down-regulation or upregulation was sufficient to change the lifespan of the worms (Ewald et al., 2015). This study has therefore demonstrated a key role for collagens in longevity, but most importantly for us, has demonstrated for the first time a potential new function of SKN-1 as a regulator of ECM gene expression (Ewald et al., 2015).

Moreover, it is interesting to note that ECM genes were also found upregulated in mice treated with the inhibitor of mTOR rapamycin and under caloric restriction. Both conditions increase mouse longevity and in both conditions, NRF2 activity was found to be upregulated (Ewald et al., 2015; Neff et al., 2013; Zhang et al., 2012). Similarly, rapamycin was also associated to an increase of NRF2 activity, and a delayed ageing in normal human fibroblasts (Lerner et al., 2013; Sodagam et al., 2017). These similarities suggest a conserved signaling



cascade for NRF2 with regard to ECM regulation. The transposition of NRF2/SKN-1 findings on ECM gene regulation from *C.elegans* to humans therefore represents an interesting, and challenging question to address.



**Figure 28. *skn-1* dependent upregulated genes in longevity-increased worms reveals collagen genes as the most enriched gene set.**

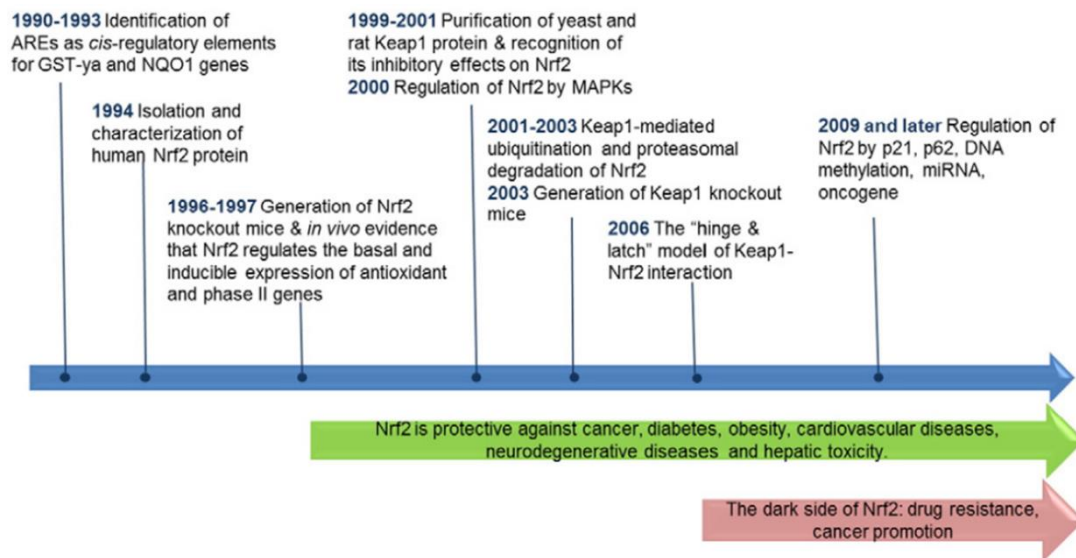
Functional categories enriched in SKN-1 upregulated *daf-2(-)* gene sets, identified by the Database for Annotation, Visualization and Integrated Discovery (DAVID). Enrichment scores of at least 1.3 are shown. From Ewald et al., 2015.

### 3.2. The human transcription factor NRF2

#### 3.2.1. History of the central transcription factor, NRF2

The human homologue of SKN-1, the nuclear factor erythroid 2-related factor (NRF2 encoded by the *NFE2L2* gene), belongs to the mammalian cap'n'collar (CNC) subfamily of basic leucine zipper (bZIP) transcription factors. This subfamily of transcription factors was first discovered in 1989 with the identification of NF-E2 p45 (Mignotte et al., 1989) and comprises today six transcription factors that were discovered shortly thereafter: NRF1 (Chan et al., 1993), NRF2 (Moi et al., 1994), NRF3 (Kobayashi et al., 1999), and the more distantly related factors BACH1 and BACH2 (Oyake et al., 1996). NRF2 has become the most studied and best-known transcription factor of this subfamily, due to its role as a master regulator of the antioxidant stress response. It maintains cellular homeostasis by activating the expression of

cytoprotective genes involved in redox, metabolic and protein homeostasis. The NRF2 pathway also intersects with many other signaling pathways and to ensure its wide range of functions, hence it has to be tightly regulated. Its correct regulation has been shown to be essential for preserving human health, which has generated a lot of scientific studies over the past four decades (Figure 29) (Dodson et al., 2019).

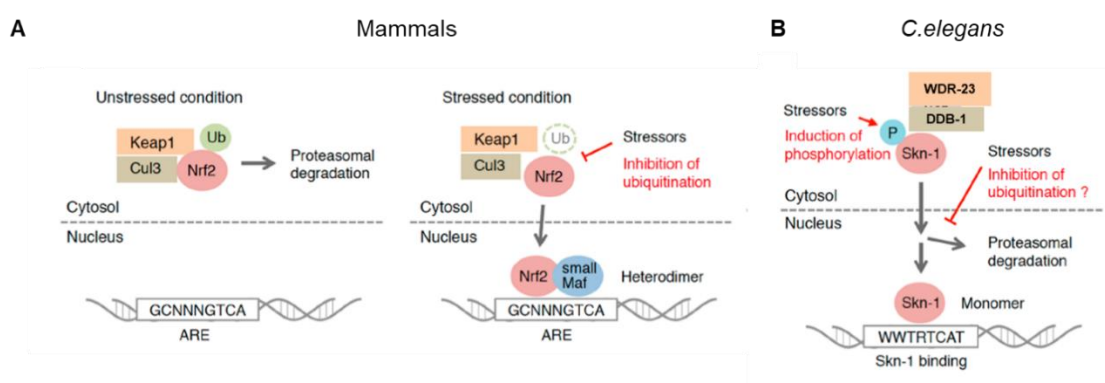


**Figure 29. Key discoveries in the NRF2 pathway.**  
From Huang et al., 2015.

NRF2 was identified in 1994 by Moi and collaborators (Moi et al., 1994), and first described as a regulator of  $\beta$ -globulin gene expression, similarly to the first discovered member of the family NF-E2 p45. Shortly after, NRF2 was identified as a master regulator of the oxidative stress response by heterodimerizing with sMaf (small musculoaponeurotic fibrosarcoma) proteins and regulating the expression of various target genes through the binding to an antioxidant response element (ARE) motif (Itoh et al., 1997). NRF2 was then shown to be negatively regulated at the protein level through a direct interaction with the dimeric redox sensor, Kelch-like ECH-associating protein 1 (KEAP1), which was originally identified by a yeast-two hybrid screen (Itoh et al., 1999). The negative regulation of NRF2 by KEAP1 was confirmed by the generation of KEAP1<sup>-/-</sup> mice, which showed a constitutive activation of NRF2 (Wakabayashi et al., 2003). KEAP1 functions as an adaptor protein for to the Cullin 3-Ring box 1 (CUL3-RBX1) E3 ubiquitin ligase, which ubiquitinylates NRF2 and targets it for proteasomal degradation (Kobayashi et al., 2004; Zhang et al., 2004). Scientific research about this transcription factor underwent two major shifts. The first one was in 2001, when NRF2 was discovered to be chemoprotective, as Nrf2<sup>-/-</sup> mice were unable to respond to

oltipraz that normally induces a chemoprotective response. This work raised the awareness that activating NRF2 could be beneficial to protect cells from chemical assaults. However, activating NRF2 can have a flipside by promoting survival of cells involved in the development of pathogenic conditions. In 2006, constitutive activation of NRF2 in KEAP1 mutated cells was found to promote carcinogenesis in non-small-cell lung (Singh et al., 2006). NRF2 implication in other severe pathologies including diabetes, chronic, neurodegenerative, cardiovascular, kidney and liver diseases has also emerged (Dodson et al., 2019). This reveals the 'dark side' and dual role of NRF2, highlighting the complexity of NRF2 pathway (Wang et al., 2008). The number of targets and functions of NRF2 keeps extending, which evidences the central role of NRF2 in cellular organisms.

Hence, the role and regulation of NRF2 have been extensively explored in diverse model organisms. It is of interest to note that the NRF2/KEAP1 system is ubiquitous and evolutionary conserved among mammals, ensuring a vital cell defense mechanism against endogenous and exogenous stresses through mostly well-known regulation pathways (Figure 30A). Model organisms, such as *Drosophila melanogaster*, *Danio rerio* or *C. elegans*, also possess orthologs of NRF2, with similar roles as an anti-stress system (Fuse and Kobayashi, 2017). Even though the regulation mechanism and DNA-binding of NRF2 orthologs are sometimes different from those in mammals, these model organisms allow elucidating new facets of the NRF2 pathway (Blackwell et al., 2015; Ewald et al., 2015). There is for example no true ortholog of KEAP1 in *C. elegans* but the functional homologs WDR-23 and DDB-1 (Figure 30B). As my PhD work was conducted in human cells, we will further describe the regulation and functions of NRF2 in humans.

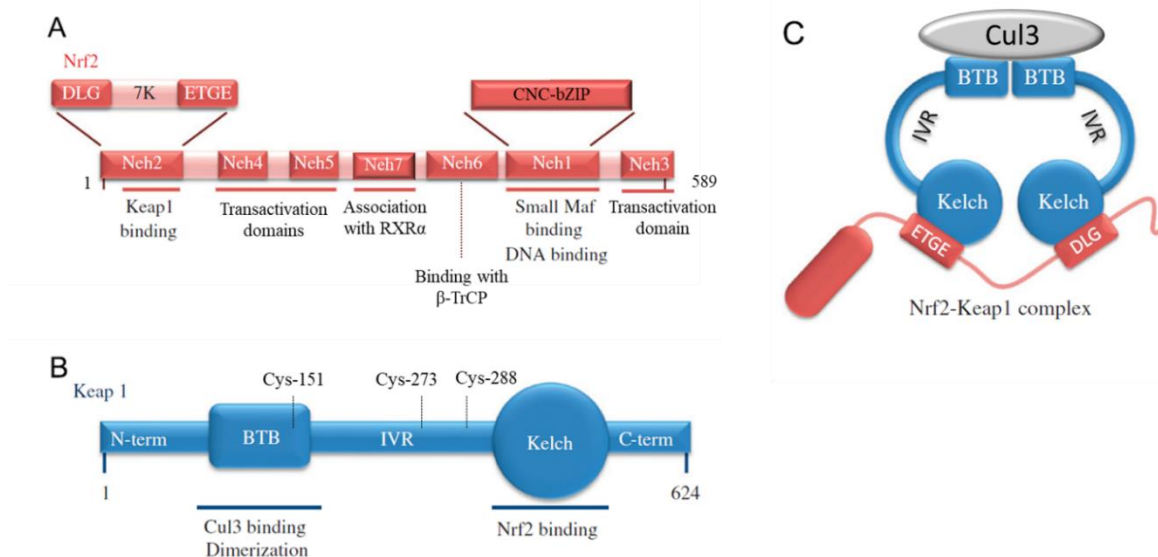


**Figure 30. Regulatory mechanisms of the NRF2 pathway across species.**

The mechanism is depicted in eukaryotes (A) and in *C.elegans* (B) KEAP1/CUL3 and WDR-23/DDB-1 are E3 ubiquitin ligase complex that regulate the turnover of NRF2/SKN-1 through proteasomal degradation. Modified from Fuse and Kobayashi 2017.

### 3.2.2. NRF2-KEAP1 structures and stress response pathway

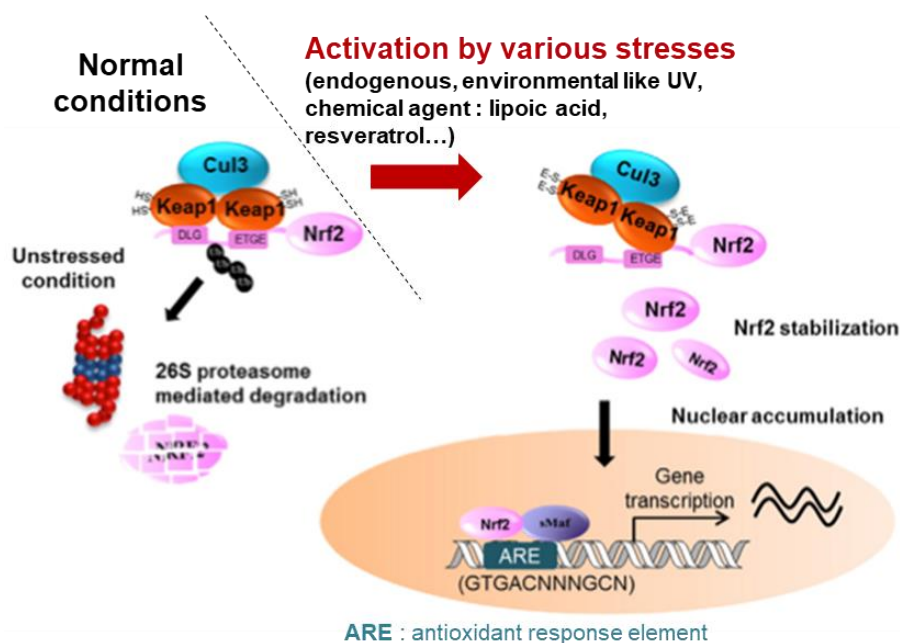
NRF2 is a modular protein, which is composed of seven Nrf2-ECH homology (Neh) domains (Neh1-7), all fulfilling specific functions (Figure 31A) (Hayes and Dinkova-Kostova, 2014). Neh1 contains the basic CNC-bZIP motif which both allows dimerization with the sMaf protein and DNA binding to the ARE sequence. This Neh1 domain also reveals, after being released from KEAP1 molecule, a specific nuclear localization signal required for NRF2 translocation to the nucleus (Bellezza et al., 2018). The N-terminal domain, Neh2, contains two amino acid motifs DLG and ETGE, which ensure the interaction with KEAP1; and seven lysine residues that are targeted for ubiquitylation and subsequent proteasomal degradation of NRF2 (Tong et al., 2006). Neh3, 4 and 5 are transactivation domains that enhance the transcription of NRF2 targets. Neh3 is located at the C-terminus and can recruit CDH6, a chromo-ATPase/helicase DNA binding protein (Nioi et al., 2005). Neh4 and 5 are able to bind the cAMP response element-binding protein (CREB)-binding protein (CBP) and to the Ras-related C3 botulinum toxin substrate 3 (RAC3) (Kim et al., 2013). Neh6 negatively regulates NRF2 stability since it promotes its proteasomal degradation in a KEAP1-independent manner through the direct binding of  $\beta$ -transducing repeat-containing protein ( $\beta$ -TrCP), which acts as an adaptor protein for the SCF E3 ubiquitin ligase (Chowdhry et al., 2013). Neh7 domain also promotes a repression of NRF2 by allowing a physical binding with the retinoid X receptor  $\alpha$  (RXR $\alpha$ ) (Wang et al., 2013).



**Figure 31. Structure of NRF2, KEAP1 and of the NRF2/KEAP1 complex.**

(A) Structure of NRF2 with its seven Neh domains with their respective roles. (B) Structure of KEAP1 with the most commonly modified cysteine residues and the five structural domains. (C) Association of NRF2 to KEAP1 in basal conditions. Modified from Kansanen et al., 2013.

KEAP1 is made of five domains: three broad complex-tramtrack-bric à brac (BTB) at the N-terminal; one intervening region (IVR) and two C-terminal glycine repeat domains (DGR), also named Kelch repeat domain (Figure 31B). Additionally, in humans, KEAP1 is a cysteine rich-protein with 27 cysteine residues, which are sparsely distributed in the different domains. The BTB domains contain a cysteine residue (cys-151) that mediates both the homodimerization of KEAP1 and the interaction with the cullin-3 E3 ligase. The IVR domain contains two other cysteine residues (cys-273 and cys-288) and a further binding site for the cullin-3 E3 ligase. The Kelch repeat motifs are able to bind to the DLG and ETGE motifs of NRF2 (Figure 31C). The cysteine residues of KEAP1 are mainly responsible for its stress-sensing ability since they are highly reactive to oxidants and electrophiles. Specific cysteine residues appeared to be reactive to specific stressing molecules, which led to the emergence of a 'cysteine code' concept (Canning et al., 2015). Indeed, cysteines -151, -273 and -288 were shown to sense electrophilic Nrf2-inducing chemicals (Saito et al., 2016), whereas cysteines -226, -613, -622 and -624 have been recently identified as specific H<sub>2</sub>O<sub>2</sub> sensors (Suzuki et al., 2019). The exact mechanism by which KEAP1 cysteine residues are modified leading to the activation of NRF2 still remains unknown. However, it is known that oxidation or cross-linking of these cysteines residues induce a conformational change of KEAP1, thereby disrupting its binding to NRF2, which is released and can no longer be ubiquitinated.



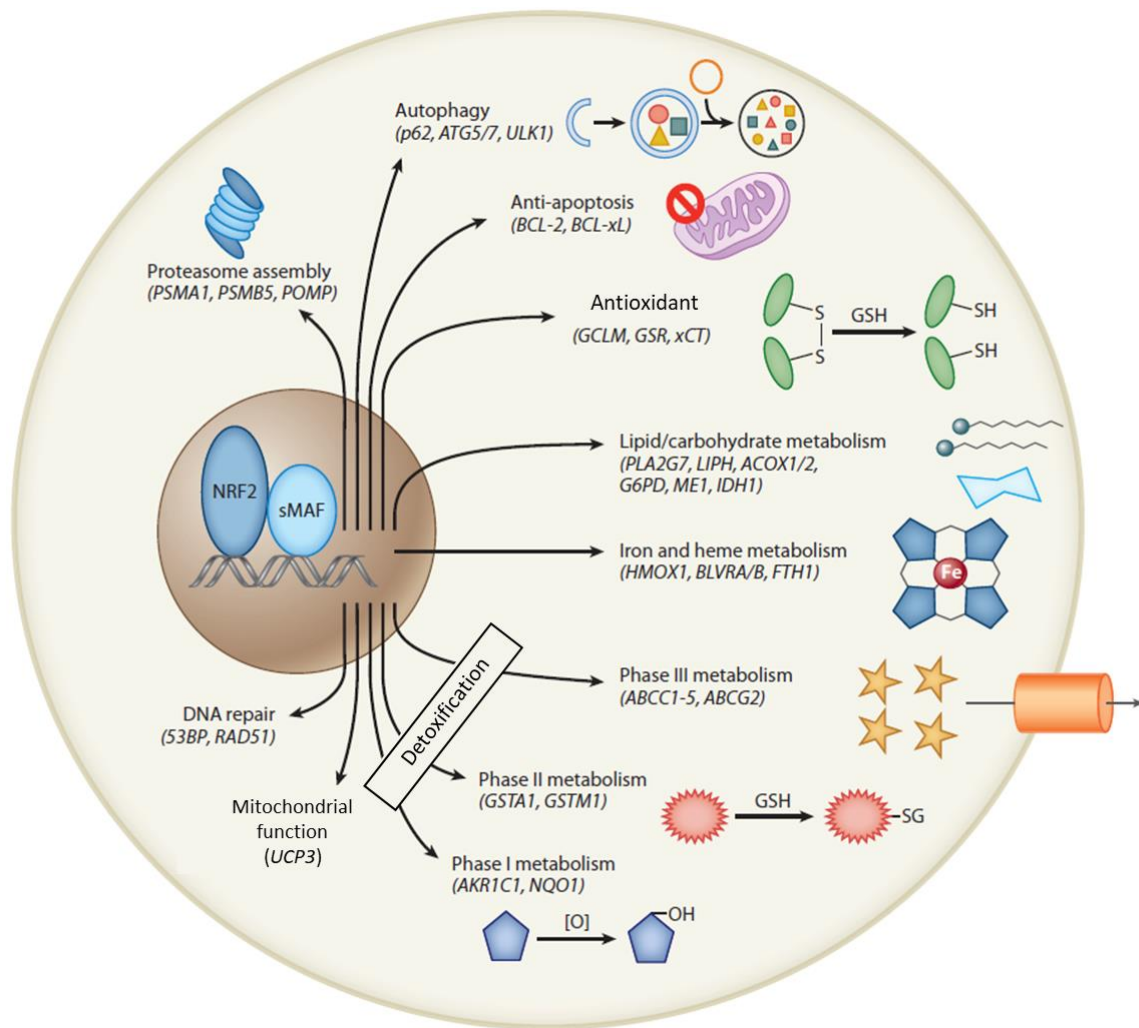
**Figure 32. NRF2 signaling pathway.**  
Modified from Huang et al., 2015

Under basal conditions, very low levels of cytoprotective genes are required. Therefore, NRF2 is mainly located in the cytoplasm where it is bound to KEAP1, which destines it for proteasomal degradation (Figure 32). NRF2 has a rapid turnover with a half-life-time of about 10 to 30 minutes, which allows both maintenance of low basal levels in normal conditions and a quick synthesis of a new pool of proteins in response to stress (Tonelli et al., 2018). The presence of endogenous stressors, such as elevated harmful levels of reactive oxygen species (ROS) or exogenous stressors including electrophiles, toxins or chemical drugs, induces modifications of the KEAP1 cysteine residues, which in turn inactivate its binding activity to NRF2. Hence, NRF2 is successively released from its repressor, stabilized and translocated into the nucleus where it heterodimerizes with one of the sMaf proteins (MafG, MafK or MafF) (Figure 32). sMaf proteins are crucial to activate the transcription of NRF2-target genes. Indeed, neither *Nrf2*<sup>-/-</sup> mice nor triple knockout mice for MafG, MafK and MafF were able to activate any of the cytoprotective genes upon stimulation (Katsuoka et al., 2005; Motohashi et al., 2004). The NRF2/sMaf complex activates the expression of about 200 genes by binding to a specific DNA binding motif (5'-TGACNNNGC-3') (Bellezza et al., 2018; Hayes and Dinkova-Kostova, 2014). This DNA binding motif was first identified with the discovery of NF-E2 p45, which was binding to a DNA binding sequence containing an activator-protein 1 (AP-1) motif. This NF-E2/AP-1 sequence was thereupon identified in the rat glutathione-S-transferase (*GST-Ya*) gene and was further renamed antioxidant response element (ARE) (Rushmore and Pickett, 1990). Since then, studies have continuously expanded the number of ARE-containing genes and have shown that they are regulated by NRF2 (Itoh et al., 1997). Interestingly, the global mapping of NRF2 DNA binding sites through different ChIP-seq analysis revealed that most of the NRF2 binding sites are distal and localize outside the proximal promoter region (Chorley et al., 2012; Hirotsu et al., 2012; Lou et al., 2006).

### 3.2.3. NRF2 target genes and main functions

While its initial functions were related to oxidative stress, it is now well admitted that NRF2 responds to a variety of other stressors. Malhotra and collaborators have classified NRF2 targets in three main categories: the NRF2 basal targets expressed in unstressed conditions, the NRF2 stress-induced targets activated upon different types of stimuli and the NRF2 common targets, the expression of which is activated both in basal and stress conditions (Malhotra et al., 2010). Whereas stress-induced targets include diverse signaling pathways, NRF2 basal targets have been found to be mainly involved in cell proliferation. The expression of NRF2 target genes strongly depends on NRF2 protein levels. Microarray analysis data from

livers of *Nrf2*<sup>-/-</sup> mice, *Keap1*<sup>-/-</sup> mice and mice with reduced expression of *Keap1* underline the sensitivity of target gene expression to NRF2 levels. (Wu et al., 2011). Hence, depending on the type of the stimuli (internal or external) perceived by the cell but also depending on the intensity of this stimuli, NRF2 is able to regulate the transcription of numerous target genes in a dose-dependent manner. These target genes are involved in wide range of biological processes, for example detoxification, antioxidant defense, energy metabolism, and NADPH regeneration that will be further presented and that are represented in Figure 33.



**Figure 33. NRF2 cellular functions.**

By heterodimerizing with a sMAF protein, NRF2 activates the expression of genes involved in detoxification; antioxidant processes; lipid/carbohydrate metabolism with the regeneration of NADPH; iron and heme metabolism; proteostasis through autophagy and proteasomal assembly; DNA repair and anti-apoptosis and mitochondrial function. Representative target genes are below each function. Modified from Dodsden et al., 2019.

- Antioxidant defense system

NRF2 controls the expression of the main components of the glutathione (GSH) and thioredoxin (TXN) antioxidant systems, of which GSH is a major cellular antioxidant. NRF2 regulates the expression of the catalytic and modulatory subunits (*GCLC* and *GCLM*) of the glutamate cysteine ligase (GCL), which catalyzes the rate-limiting step of GSH biosynthesis. Additionally, NRF2 controls the transcription of glutathione peroxidases (*GPX2* and *GPX4*) and peroxiredoxins (*PRDX1* and *PRDX6*), which both reduce peroxides with and without GSH, and glutathione reductases (*GSR*), which reduces the oxidized form of GSH (GSSG). GSH intracellular levels are thus regulated and maintained through NRF2 activity (Hayes and Dinkova-Kostova, 2014). The reduction of oxidized protein thiols is ensured by both thioredoxin 1 (*TXN1*) and thioredoxin reductase 1 (*TXNRD1*), and by the import of cysteine into cells through the xCT transporter (*SLC7A11*), which all are NRF2 target genes (Lu and Holmgren, 2014).

- ROS and xenobiotic detoxification

NRF2 counteracts endogenous and exogenous stressors, such as drugs or toxins by controlling the expression of drug-metabolizing enzymes from all three phases of the detoxification process. Phase I enzymes reduce ROS and xenobiotics into active metabolites that are conjugated to GSH or glucuronic acid by enzymes from the phase II, which are further exported out of the cell by the phase III membrane transporters. Phase I enzymes include aldo-keto reductases, which are encoded by the following NRF2 targets such as *AKR1C1*, *AKR1B1*, *ALDH1A1*, *ALDH3A1*, *ALDH7A1* and *NQO1*. Phase II enzymes controlled by NRF2 comprise both glutathione S-transferases (*GSTA1-4*, *GSTM1-4* or *MGST1*) and UDP glucuronosyltransferases (*UGT1A1* or *UGT2B7*). ATP-binding cassette family members (*ABCC 1-5* and *ABCG2*) are phase III drug enzymes, the expression of which is controlled by NRF2 activity (Dodson et al., 2019; Hayes and Dinkova-Kostova, 2014).

- Heme and iron metabolism

To protect the cell from ferroptosis, programmed cell death depending on iron, NRF2 activates the expression of the heme oxygenase (*HMOX1*) which degrades heme molecules and generates free Fe<sup>2+</sup> ions (Alam et al., 2003; Gozzelino et al., 2010). However, the free Fe<sup>2+</sup> ions can further damage the cell through the Fenton reaction, which is the oxidation of the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by Fe<sup>2+</sup> into highly damaging OH radicals. To prevent the formation of free radicals through the Fenton reaction when H<sub>2</sub>O<sub>2</sub> levels are high, NRF2 simultaneously



activates *HMOX1* expression and components of the ferritin complex (*FTL1*, *FTH1*, *FECH*), which store the released  $\text{Fe}^{2+}$  in its structure. The Fenton reaction is thus inhibited (Kerins and Ooi, 2018).

- NADPH regeneration, carbohydrate and lipid metabolism

NADPH is a required co-factor for many antioxidant and drug-metabolizing enzymes, such as NQO1 or TXNRD1. NRF2 supports the antioxidant and detoxification processes by positively regulating NADPH-generating enzymes, which include the glucose-6-phosphate dehydrogenase (*G6PD*), the isocitrate dehydrogenase (*IDH1*) and malic enzyme (*ME1*) (Wu et al., 2011).

Additionally, NRF2 regulates metabolic enzymes required for the anabolic metabolism of glucose and fatty acids. NRF2 is involved in the pentose phosphate pathway (PPP) by regulating G6PD, 6-phosphogluconate dehydrogenase (PGD), transaldolase (TALDO1) and transketolase (TKT) (Hayes and Dinkova-Kostova, 2014; Mitsuishi et al., 2012). NRF2 is also involved in lipid metabolism in mouse, by regulating fatty acid oxidation and lipid catabolism through the transcriptional regulation of *Acot7*, *Acot8*, *Lipf*, to name a few.

- Protein homeostasis: role in proteasomal activity and autophagy

NRF2 is additionally involved in protein homeostasis or proteostasis by removing damaged or misfolded proteins through the induction of the proteasomal activity and autophagy. The expression of multiple components of the proteasome (proteasome subunit  $\alpha 1$  *PSMA1*, proteasome subunit  $\beta 5$  *PSMB5* and proteasome maturation protein *POMP*) is increased following treatment with NRF2 inducer (18 $\alpha$ -GA) in human fibroblasts and in mouse tissues (Jang et al., 2014; Kapeta et al., 2010; Kwak et al., 2003). It has also been suggested that NRF2 controls the expression of some autophagic genes, such as autophagy-related 5 and 7 (*ATG5*, *ATG7*), and unc-51-like autophagy activating kinases (*ULK1*, *ULK2*) (Pajares et al., 2016). The expression of the protein p62/SQSTM1, which targets ubiquitinated protein aggregates for autophagic degradation, is also controlled by NRF2 (Jena et al., 2018). Last but not least, NRF2 has been reported to directly control mTOR, which is a master regulator of protein biosynthesis and autophagy (Bendavit et al., 2016).

- DNA repair and prevention of apoptosis

NRF2 induces DNA repair and prevents cells from apoptosis by regulating the transcription of p53-binding protein (*53BP1*), DNA repair protein RAD51 homolog 1 (*RAD51*),

and two antiapoptotic proteins (*BCL2* and *BCXL*) (Dodson et al., 2019). The treatment of human colonic epithelial cells with an inducer of NRF2 prior to ionizing radiation significantly reduces chromosomal aberrations. This effect was abolished when NRF2 is knocked-down, suggesting that NRF2 as an activator of DNA repair signaling (Kim et al., 2012).

- Mitochondrial function

Although there is still not much evidence for direct targets of NRF2 in mitochondria, there is some evidence of its role in mitochondrial regulation (Holmström et al., 2016). *Nrf2*<sup>-/-</sup> mice display decreased ATP levels, mitochondrial depolarization and impaired respiration. On the opposite, genetic activation of *Nrf2* in mice leads to increased ATP levels, mitochondrial membrane potential and a higher respiration rate (Holmström et al., 2013). Two other recent papers link NRF2 to mitochondrial biogenesis: the first one demonstrated that dimethyl fumarate, which is known to activate the NRF2 pathway, induced mitochondrial biogenesis in human cells and in mice (Hayashi et al., 2017); the second is a review, which discusses an interplay between NRF2 and PGC-1 $\alpha$ , the key regulator of mitochondrial biogenesis (Gureev et al., 2019).

### 3.2.4. NRF2 additional functions through crosstalk with other signaling pathways

In addition to a direct regulation of the expression of hundreds of genes, there is accumulating evidence that NRF2 is involved in a variety of cell functions by cross talking with other major signaling cascades.

It has been shown in several studies that mainly during embryogenesis NRF2 seems to influence the NOTCH signaling pathway, which regulates cell fate and differentiation. (Wakabayashi et al., 2014). NRF2 and NOTCH were shown to concomitantly mediate cytoprotection, particularly in the context of liver regeneration (Sato et al., 2019; Wakabayashi et al., 2015).

NRF2 has been shown to interact with the aryl hydrocarbon receptor (AhR) nuclear pathway, that also regulates the response to exogenous stressors and specifically to the exposure to polycyclic aromatic hydrocarbons (Miao et al., 2005). While NRF2 activates the transcription of ARE-containing genes, the transcription factor AhR activates the expression of Xenobiotic Response Element (XRE)-containing genes. It has been shown that AhR is able to

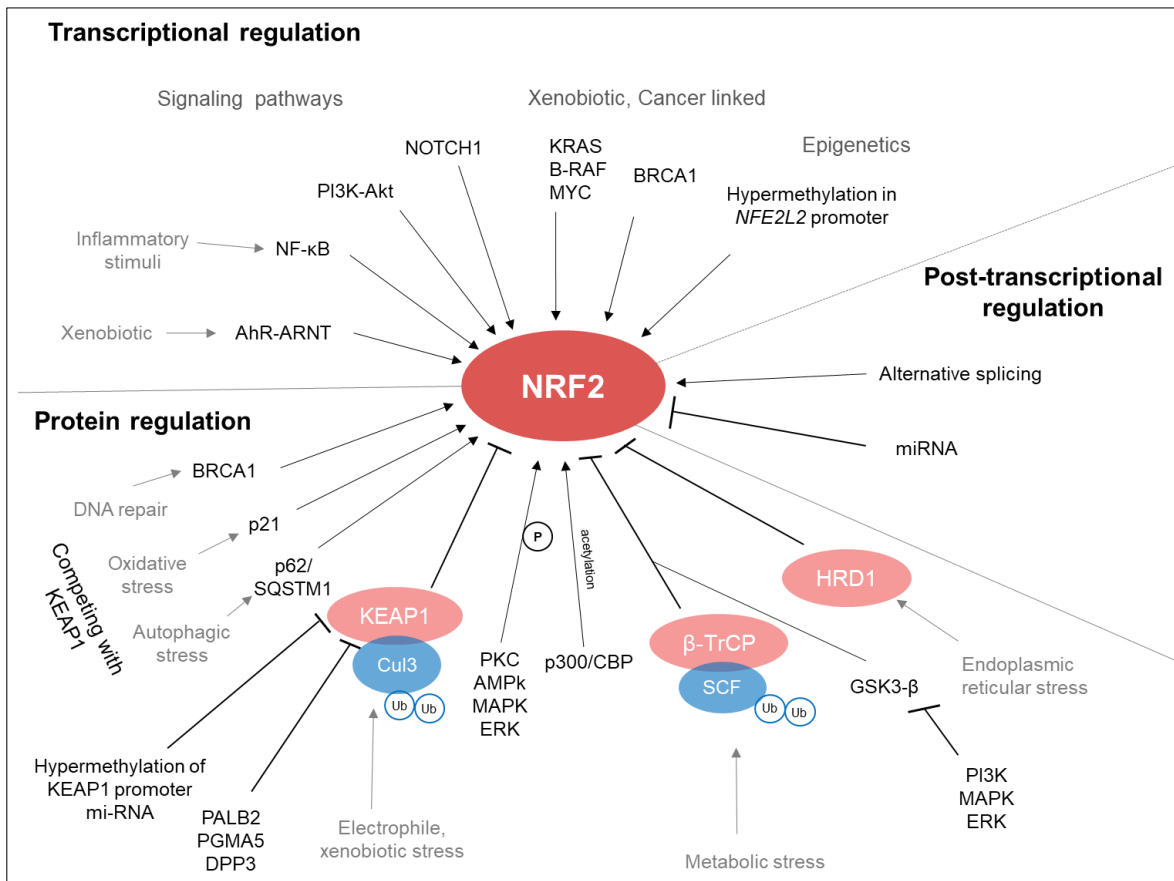
activate the transcription of *NFE2L2* and vice-versa (Shin et al., 2007). These two pathways either act together or inhibit each other to initiate appropriate defense responses (Aleksunes and Klaassen, 2012; Fuyuno et al., 2018).

NRF2 is also involved in diverse metabolic pathways. AMP activated protein kinase (AMPK), which is a master regulator of energy metabolism at the cellular and whole-body level, has been shown to phosphorylate the serine 550 of NRF2, which therefore enhances its nuclear translocation (Joo et al., 2016). Additionally, PI3K-AKT is able to phosphorylate GSK3- $\beta$ , which in turn activates NRF2 degradation by  $\beta$ -TrCP. This pathway was suggested to represent another level of regulation that fine-tunes NRF2 activity (Salazar et al., 2006; Wang et al., 2019).

There is also a bidirectional relationship between the NF- $\kappa$ B and NRF2 signaling pathways, therefore illustrating a role of NRF2 in the inflammation process (Wardyn et al., 2015). *Nrf2*<sup>-/-</sup> mice exhibited increased expression of NF- $\kappa$ B targets following stimulation with some pro-inflammatory stimuli (Thimmulappa et al., 2006). On the opposite, stimulation of the NRF2 pathway with sulforaphane has been shown to decrease levels of NF- $\kappa$ B target expression (Heiss et al., 2001). This crosstalk is particularly well evidenced in skin wound healing that will be further discussed (Ambrozova et al., 2017).

### 3.2.5. Regulation of NRF2 activity

Given the wide range of cellular functions controlled and modulated by NRF2, the regulation of its activity is quite complex and multifactorial. NRF2 activity is fine-tuned and regulated at the transcriptional, post-transcriptional and protein levels, which will be further described. The different regulators are summed up in the [Figure 34](#).



**Figure 34. Schematic representation of regulation of NRF2 activity.**

The mechanisms include transcriptional, post-transcriptional and protein regulations. Arrows indicate activating regulation and bars indicate inhibitory regulation. All text written in light grey are stimuli activating these regulators.

- Transcriptional regulation of NRF2

*NFE2L2* transcription is regulated by some of the signaling pathways previously presented: in presence of xenobiotics or polycyclic aromatic compounds, AhR binds to its binding partner, the AhR nuclear translocator (ARNT), and transactivates *NFE2L2* transcription by directly binding to its promoter (Miao et al., 2005); NF- $\kappa$ B is also able to directly bind to the *NFE2L2* promoter upon inflammatory stimuli (Wardyn et al., 2015). Furthermore, NOTCH1

signaling and PI3K-Akt signaling have been shown to increase *NFE2L2* transcription (Mitsuishi et al., 2012; Wakabayashi et al., 2014).

The oncogenic proteins KRAS, B-RAF, MYC are also able to directly activate *NFE2L2* transcription (DeNicola et al., 2011). Xenobiotic stress can induce *NFE2L2* transcription in human MCF10A mammary epithelial cells that harbor a modified breast cancer susceptibility-1 (*BRCA1*) gene (Kang et al., 2011). The activation of NRF2 expression through these oncogenic proteins promotes tumorigenesis, which illustrates the 'dark side' of NRF2.

Finally, recent work unraveled how NRF2 levels are modified by epigenetic regulation. Hypermethylation of the *NFE2L2* promoter has been shown to significantly decrease NRF2 activity in late-stage prostate cancer tumors (Khor et al., 2014). On the other hand, hypermethylation of the *KEAP1* promoter has been shown to increase NRF2 levels in arsenite-treated human keratinocytes (Wang et al., 2015).

- Post-transcriptional regulation of NRF2

NRF2 expression is mainly controlled at the post-transcriptional level by micro-RNAs (mi-RNAs). For instance, miR27a, miR-144 and miR-93 have been shown to significantly decrease *NFE2L2* mRNA translation (Wang et al., 2016; Xue et al., 2015; Zhou et al., 2016). mi-RNA regulation of both *NFE2L2* and *KEAP1* expression are also involved in aging and cancer development and progression (Silva-Palacios et al., 2018; Zimta et al., 2019). Alternative splicing of *NFE2L2* mRNA is another post-transcriptional regulation mechanism. In certain types of cancers, alternatively spliced *NEF2L2* variants have been detected. They encode proteins that lack the interaction domain with KEAP1 and therefore results in constitutive activation of NRF2 (Goldstein et al., 2016).

- Post-translational regulation of NRF2 level

Regulation of NRF2 activity is mainly controlled by maintaining NRF2 protein levels in cells, which are regulated by post-translational modifications and protein interactions. Post-translational modifications regulating NRF2 activity include phosphorylation, acetylation, methylation and SUMOylation. Phosphorylation of some specific serine residues (S-40, S-550 and others) of NRF2 by upstream kinases like PKC, AMPk or ERK, have been suggested to increase its nuclear translocation and thus its activity (Huang et al., 2002; Joo et al., 2016; Sun

et al., 2009). Acetylation of lysine residues of the Neh1 domain of NRF2 by p300/CBP increases NRF2 activity by enhancing its DNA-binding capacities (Kawai et al., 2011).

There are three E3 ubiquitin ligase complexes that inhibit NRF2 activity by ubiquitylation and subsequent proteasomal degradation. As the best-known, the cytosolic KEAP1/cullin-3/RBX1 complex actively leads to NRF2 degradation in unperturbed conditions and is inactivated by electrophilic and oxidative stress. The interaction between NRF2 and KEAP1 can be competitively inhibited by other NRF2 binding partners such as p62 under autophagy stress or DNA damage, p21 induced by p53 in response to oxidative stress, and BRCA1 for DNA repair (Chen et al., 2009; Gorrini et al., 2013; Komatsu et al., 2010). The interaction between NRF2 and KEAP1 can also be disrupted by proteins interacting with KEAP1, including dipeptidyl-peptidase 3 (DPP3) (Hast et al., 2013), phosphoglycerate mutase 5 (PGAM5) (Lo and Hannink, 2008), and the BRCAC2 partner and localizer (PALB2) (Ma et al., 2012).

The second protein inhibitor of NRF2 is  $\beta$ -TrCP, which is an adaptor protein for the SCF E3 ubiquitin ligase. GSK3- $\beta$  phosphorylates the Neh6 domain of NRF2 triggering  $\beta$ -TrCP/SCF complex binding to NRF2, which mediates its proteasomal degradation (Cuadrado, 2015). This degradation complex is sensitive to cellular metabolic stress. It is noteworthy that inhibition of GSK3- $\beta$  by kinases, including ERK, MAPK, PI3K and PKC, will therefore activate NRF2.

Finally, NRF2 can be tagged for degradation by the E3 ubiquitin ligase synoviolin (HRD1), which is activated upon endoplasmic reticulum (ER) stress (Wu et al., 2014).

## 4. NRF2 functions in skin

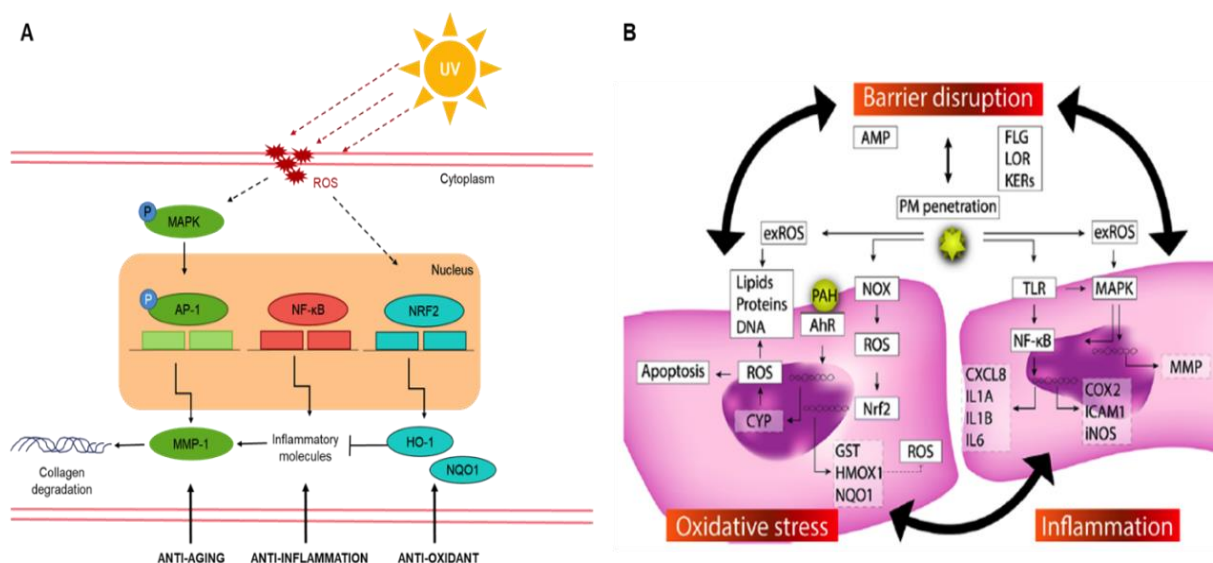
Thanks to its cytoprotective activity, NRF2 protects all main skin cell types (i.e. keratinocytes and melanocytes in the epidermis and fibroblasts in the dermis) from oxidative stress, which is generated by external aggressions as well as internal stimuli such as wound healing or epidermal renewal. Beyond its antioxidant and detoxification functions, NRF2 also plays an important anti-inflammatory role in skin (Gęgotek and Skrzydlewska, 2015). Besides, NRF2 is dysregulated in various skin diseases and its activation is foreseen as a promising treatment. However, its regulation needs to be tightly controlled as NRF2 has also been demonstrated to be pro-tumorigenic when constitutively or chronically overexpressed (Hennig et al., 2020).

### 4.1. NRF2 protects skin from external aggressions

The skin is constantly facing external assaults from pathogens and toxins, considered as xenobiotics. Moreover, it is exposed to harmful ultra-violet (UV) radiation and pollution, which will be further detailed.

UV radiation is the main environmental cause for skin cancer and skin aging. It induces excessive formation of ROS, DNA damage and mutations. UVs are composed of the short-wave UVB (280-320 nm), which only reaches the epidermis and the long-wave UVA (320-400 nm), which penetrates deeper into the skin and affects the dermis. UVB is responsible for sunburns and the majority of skin cancers, whereas UVA is the main cause of skin photo-aging (age spots and wrinkles). NRF2 activity has been shown to protect skin cells and to decrease overall cytotoxicity of both UV types (Figure 35A). UVA and UVB activate NRF2 in keratinocytes and melanocytes (Marrot et al., 2008), whereas in fibroblasts NRF2 translocation is only induced upon UVA stimuli (Hirota et al., 2005). Upon stimulation by UVs, NRF2 activation decreases intracellular ROS levels and apoptosis through the transcriptional activation of detoxification phase II enzymes and the cysteine and glutathione antioxidants (Hseu et al., 2018; Marrot et al., 2008; Schäfer et al., 2010). It also inhibits proper degradation of collagen I by metalloproteinases (MMP-1 and 3), which is a main cause of skin aging, through cross-talk with the MAPK/AP-1 signaling cascade (Chaiprasongsuk et al., 2017; Hseu et al., 2018; Hwang et al., 2018). Finally, NRF2 activation decreases inflammation in the epidermis when irradiated with UVB (Saw et al., 2014).

In addition to UV, skin is highly exposed to pollutants including ozone and particulate matter (PM) containing polycyclic aromatic hydrocarbons (PAH). A recent review summarized the effect of PM on human skin from epidemiology and *in vitro* studies that have been conducted so far, in order to better assess the potential risks of pollutants on human health (Dijkhoff et al., 2020). PM disrupt the skin barrier function by increasing the levels of antimicrobial peptides (AMP) and reducing the levels of key differentiation and epidermal markers such as filaggrin and keratins. PM are also provoking the massive production of both exogenous and endogenous ROS leading to increased oxidative stress and elevated inflammatory markers that in turn activate NRF2, MAPK and NF- $\kappa$ B pathways (Figure 35B). Effects of pollutants have been associated with skin barrier dysfunctions pathologies such as atopic eczema (Dijkhoff et al., 2020; Marrot, 2018). However, skin damaging effects of pollutants and precise mechanisms are still a matter of investigation. It is important to note that the concentration of PAHs in living skin hasn't been measured so far. However, it is known that the concentration in blood is in the nanomolar range. As shown by Soeur and collaborators, physiological levels of PAHs combined with sunlight exposure have a synergistic effect and perturb skin homeostasis. (Soeur et al., 2017). To counteract these different pollutants effects, the activation of NRF2 has been suggested as a possible strategy (Marrot, 2018).



**Figure 35. Effects of the UV radiation (A) and air pollutants (B) on skin cells.**

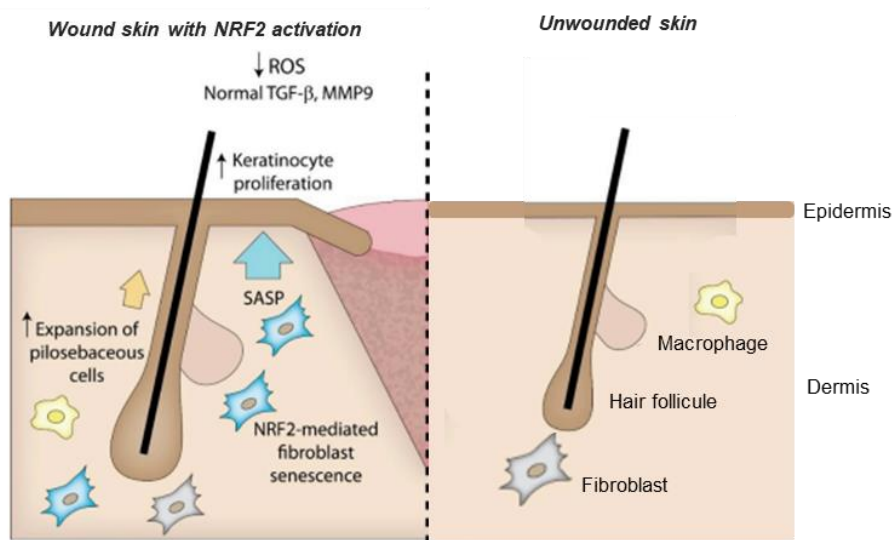
(A) NRF2 is induced by UV radiations to activate its anti-oxidant and anti-inflammatory functions to reduce skin aging induced by sunlight. Reproduced and modified from Hwang et al., 2018. (B) Schema illustrating the underlying mechanism of the effects of PM exposure on human skin. Abbreviations: antimicrobial peptides, AMP; filaggrin, FLG; loricrin, LOR; keratins, KER; exogenous ROS, exROS; endogenous ROS, ROS; nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes, NOX); Toll-like receptor, TLR; tumor necrosis factor, TNF; interleukin 1 alpha, IL1A; interleukin 1 beta, IL1B; C-X-C motif chemokine ligand 8, CXCL8; intercellular adhesion molecule 1, ICAM1; cyclooxygenase 2, COX2 and inducible nitric oxide synthase, iNOS. From Dijkhoff et al., 2020.



## 4.2. NRF2 regulates cutaneous wound healing

Given the importance of the skin barrier function, mammals have developed a sophisticated and complex wound healing response to efficiently and rapidly repair injured skin. Skin wounds generate large amounts of ROS, which mediate various cell signaling events to eliminate pathogens, to control the inflammatory response and to reconstruct the tissue. A variety of transcription factors are involved in this process, among which NRF2 has been identified as a key player (Hiebert and Werner, 2019). In addition, the interplay between NRF2 and NF- $\kappa$ B is of crucial importance in wound healing by controlling the skin immune response (Ambrozova et al., 2017). While NF- $\kappa$ B activates the innate immune response, proliferation and migration of cells by controlling secretion of cytokines and growth factors, NRF2 protects against harmful ROS and regulates the repair-associated inflammation process.

The NRF2 pathway has been found to be activated in skin wounds in mice (Eichenfield et al., 2016). Furthermore, soon after injury, *Nfe2l2* mRNA levels increase in hyperproliferative keratinocytes and macrophages, followed by a progressive decrease as the wound repairs (Braun et al., 2002). NRF2 loss- and gain-of-function models have been generated to study the mechanism by which NRF2 activity influences cutaneous wound healing. Surprisingly, even if the inflammatory response was slightly delayed, *Nrf2*<sup>-/-</sup> mice did not display any change in the wound closure rate (Braun et al., 2002; Hiebert et al., 2018). However, it was hypothesized that additional stresses or pathologic conditions may exacerbate the lack of NRF2 and affect the wound closure rate, as it is the case for streptozocin (STZ) induced diabetic *Nrf2*<sup>-/-</sup> mice, which displayed a delay in the wound healing process (Li et al., 2019, p. 2; Long et al., 2016). Moreover, treatment of those mice with NRF2 activating compounds significantly improved wound closure (Long et al., 2016; Rabbani et al., 2018). To study NRF2 gain-of-function in skin, constitutively activated mice for NRF2 (caNRF2) specifically in keratinocytes or fibroblasts were generated (Hiebert et al., 2018; Muzumdar et al., 2019). Both caNRF2 mice models displayed accelerated wound closure and re-epithelialization. The overall activation of NRF2 reduced oxidative stress, resulting in the suppression of the chronic inflammation and excessive production of MMP9 (Figure 36). NRF2 activation in keratinocytes (caNRF2) promoted the expansion of pilosebaceous cells, which provided a pool of keratinocytes for re-epithelialization. The caNRF2 fibroblasts displayed a Senescence-Associated Secretory Phenotype (SASP) which concomitantly activated proliferation of keratinocytes and accelerated wound closure (Figure 36) (Hiebert et al., 2018; Muzumdar et al., 2019).



**Figure 36. NRF2 activation in wound healing.**

Schema illustrating the characteristic features of chronic wounds, which may be improved by treatment with NRF2 activating compounds. Arrows pointing to the top indicate upregulation and arrows pointing to the bottom indicate downregulation. Modified from Hiebert and Werner, 2019.

These findings demonstrate the role of NRF2 in wound healing beyond its activity as a regulator of the anti-oxidant response and suggest that pharmacological short-term activation of NRF2 holds promise for the treatment of chronic wounds.

### 4.3. NRF2 in skin pathologies

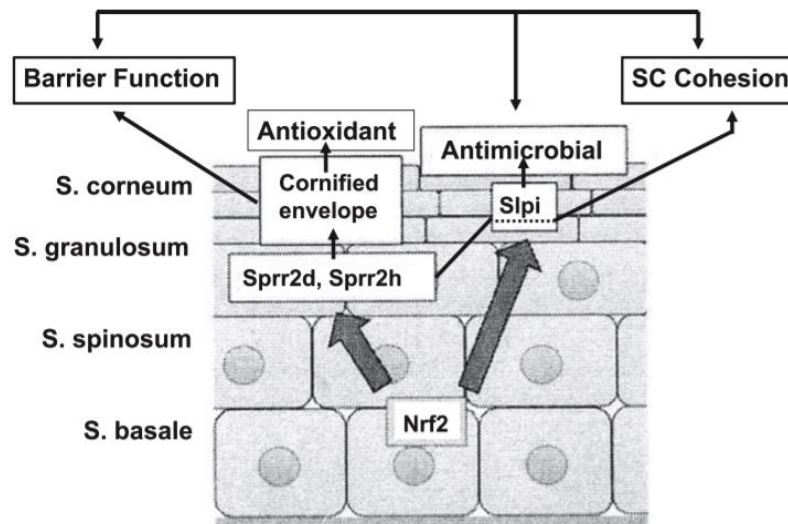
#### 4.3.1. NRF2 contributes to the maintenance of the epidermal barrier function

Of major importance for cutaneous homeostasis, the epidermal barrier is ensured by the stratum corneum and by the cohesion between keratinocytes through desmosomes, which are specialized junctional complexes mediating cell-cell interactions.

Beyond its antioxidant and detoxification role, NRF2 appears to be a direct player in the epidermal barrier function by affecting desmosome stability, corneocyte fragility and keratinocyte proliferation. NRF2 has been shown to activate the expression of two cornified envelope proteins, the small-proline-rich-protein 2 d and h (SPRR) and secretory leukocyte peptidase inhibitor (SLPI), the upregulation of which in mice with specific epidermal expression of caNRF2 led to epidermal thickening and hyperkeratosis (Figure 37) (Schäfer et al., 2012).

NRF2 also regulates the expression of keratins 6, 16 and 17, which are linked to keratinocyte hyper-proliferation (Yang et al., 2017). Moreover, it increases the expression of mi-RNA 29 a and b, which in turn affect the desmosome functions (Kurinna et al., 2014).

NRF2 activation has been suggested as a treatment for Netherton syndrome, atopic



**Figure 37. NRF2 regulates the epidermal barrier function.**

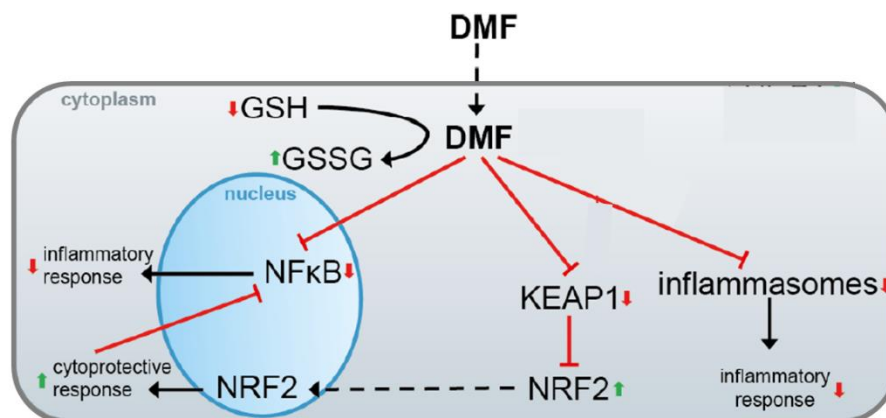
NRF2 upregulates the expression of *Spr2d* and *Spr2h*, which act as ROS scavengers and *Slpi*, which has antimicrobial activities. From Elias and Wakefield 2015, who modified the figure from Schäfer et al., 2012.

dermatitis (AD) and psoriasis, which are all disorders characterized by an impaired skin barrier function. Netherton syndrome is a severe, rare and monogenic disorder due to a mutation in the *SPINK5* gene, which encodes a kallikrein inhibitor (Chavanas et al., 2000). The uncontrolled kallikrein activity in these patients leads to premature desquamation of the stratum corneum, which in turn dramatically alters the epidermal barrier and further results in chronic cutaneous inflammation and systemic infections. Genetic activation of *Nrf2* in keratinocytes of *Spink5* mutant mice has improved their skin barrier defect by both reducing the expression of pro-inflammatory cytokines and upregulating *Slpi*, which inhibits kallikrein 7 and elastase 2 and thus increases the attachment of the stratum corneum by stabilizing desmosomes (Muzumdar et al., 2020).

AD and psoriasis are common skin inflammatory diseases characterized by itchy skin and skin barrier dysfunctions, that are mainly due to excessive T-cell activation and infiltration to the skin. These two pathologies differ in the type of skin-infiltrating T-cell subpopulation and cytokine production: while AD is driven by Th2 cells that secrete IL-4, IL13 and IL-31, psoriasis is mainly a Th17-mediated disease, in which the IL-17, IL-22 and TNF $\alpha$  are important

biomarkers (Sabat et al., 2019). Although different signaling pathways have been evidenced, up to 80% of the dysregulated genes are common between patients with AD and psoriasis (Tsoi et al., 2019). As oxidative stress promotes inflammation by upregulating the expression of pro-inflammatory cytokines, there have been accumulative evidence for an important role of ROS in both of these pathologies (Emmert et al., 2020). Suphraphane has shown beneficial effects on mice with AD thanks to the activation of NRF2-HO-1 axis (Wu et al., 2019). Treatment with dimethylfumarate (DMF), which belongs to the fumaric acid esters (FAEs) and is a known inducer of the NRF2 signaling pathway, has shown to be promising for the treatment of psoriasis (Brewer and Rogers, 2007; Lee et al., 2017; Linker et al., 2011; Ogawa et al., 2020). Once in the cell, DMF interacts with GSH to counteract elevated ROS levels and reduce inflammation. DMF then binds to the cysteine residues of KEAP1, therefore resulting in the activation of NRF2, which itself inhibits the NF- $\kappa$ B pathway (Hennig et al., 2020) (Figure 38). Altogether, DMF results in a decrease of inflammatory cytokine levels by accelerating the shift from pro-inflammatory Th1/Th7 response to an anti-inflammatory Th2 response. Despite intensive investigations to decode the underlying mechanism, there is still some information missing concerning the mechanism of action (Brück et al., 2018; Hennig et al., 2020).

In addition to psoriasis, DMF treatment has also beneficial effects on patients with systemic sclerosis, which is another skin inflammatory disease characterized by skin fibrosis that will be further presented.



**Figure 38. Proposed pathway for DMF action in skin keratinocytes.**

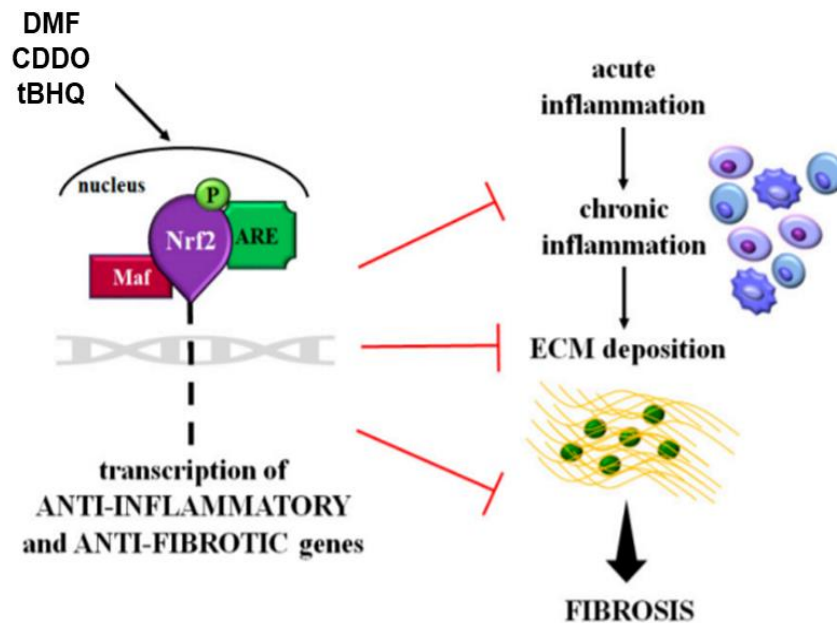
DMF enters the cell and activate NRF2 by the inhibition of KEAP1. DMF inhibits the NF- $\kappa$ B pathway by oxidation of specific cysteine residues in different NF- $\kappa$ B regulatory proteins. DMF can also inhibit inflammasome activation. Furthermore, DMF can react with GSH influencing the GSH/GSSG ratio, which in turn can affect the NRF2, NF- $\kappa$ B and inflammasome pathways. Modified from Hennig et al., 2020.

### 4.3.2. NRF2 activity is associated with skin fibrosis

Fibrosis is a pathological feature of most chronic inflammatory diseases, including pulmonary, cardiac fibrotic disorders and systemic sclerosis (SSc) (Weiskirchen et al., 2019). Fibrosis is characterized by an excessive production of ECM components, mainly of collagens produced by myofibroblasts. Development and progression of fibrosis is mediated by various growth factors and cytokines secreted by inflammatory cells. Increased oxidative stress has been identified as one of the driving force of fibrosis, which further prompts to the investigation of the role of NRF2 in fibrotic diseases. As one of the main regulator of the cellular oxidative stress response, NRF2 is dysregulated in fibrotic diseases (Figure 39) (El-Agamy et al., 2019; Wang et al., 2019). In addition, recent studies show a clear downregulation of NRF2 and its conventional targets HO-1 and GCL in skin fibroblasts of patients with SSc (Kavian et al., 2018; Wei et al., 2017). As previously introduced, SSc is a rare and severe connective disorder characterized by skin fibrosis and vascular alterations due to fibrosis of internal organs. These findings endow NRF2 with a key role in skin fibrosis.

Skin fibroblasts from SSc patients display elevated levels of ROS and decreased levels of the most powerful antioxidant, GSH. These cellular features have been connected to a decrease in the NRF2 signaling pathway activity. Two independent studies demonstrate that *Nrf2*<sup>-/-</sup> mice either treated with intradermal injections of hypochloric acid (HOCl) to mimic SSc or with the fibrotic inducer bleomycin, develop more severe fibrotic phenotypes with increased dermal thickness and collagen secretion than their respective controls (Kavian et al., 2018; Wei et al., 2017). These two studies further demonstrate that different NRF2 agonist-treatments, DMF and 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), were able to alleviate the fibrotic phenotype of their respective models, probably by blocking canonical TGF- $\beta$ /Smad signaling (Kavian et al., 2018; Wei et al., 2017). Hence, DMF treatment decreases fibrosis in SSc mouse models by restoring GSH intracellular levels and decreasing oxidative stress, which further decrease the expression of collagen and inflammatory markers (Kavian et al., 2018).

In addition, the antioxidant compound cinnamaldehyde has also been shown to reduce fibrosis in dermal fibroblasts. Cinnamaldehyde upregulates NRF2 activity, which results in decreased ROS levels, which in turn inhibit TGF- $\beta$ 1 and IL-13 pathways and periostin expression, the latter being established as a novel key player in the progression of fibrosis (Mitamura et al., 2018). NRF2 activation has therefore been foreseen as a promising treatment for skin fibrosis in several studies.



**Figure 39. Schematic representation of the role of NRF2 in fibrosis.**

Activation of NRF2 in fibrosis through different NRF2-agonists (DMF, CDDO or tBHQ) alleviates fibrosis through the regulation of inflammatory, redox and fibrotic genes. Modified from Pompili et al., 2019.

Despite intensive investigation, the mechanism by which NRF2 regulates fibrosis is not fully elucidated yet. DMF has been shown to induce NRF2 expression in fibroblasts derived from patient with multiple sclerosis (Hammer et al., 2018; Kavian et al., 2018). The increase of NRF2 has been suggested to result in the decrease of ROS levels *via* the induction of GSH. This in turn induces the phosphorylation of MAPK, which decreases fibroblasts proliferation and SMAD signaling, provoking a decrease in collagen synthesis (Kavian et al., 2018). DMF has also been suggested to directly inhibit the TGF- $\beta$  pathway through the inhibition of the PI3K/Akt pathway and the downregulation of YAP/TAZ transcriptional targets (Toyama et al., 2018). Finally, Wei and collaborators' study suggests that NRF2 inhibits the TGF- $\beta$  and SMAD pathways (Wei et al., 2017).

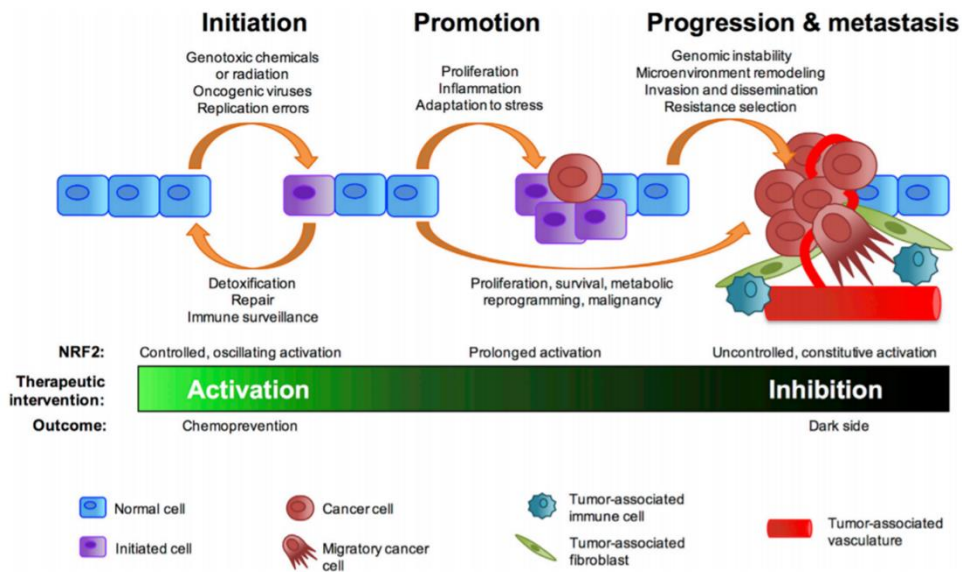
Although the role of NRF2 in skin fibrosis has been clearly evidenced, the full elucidation of the underlying anti-fibrotic mechanism of action requires additional investigation.

### 4.3.3. The dual role of NRF2 in skin cancer

While NRF2 was first demonstrated to have chemoprotective effects and to prevent the onset of skin tumor development, it was later found that 6.3% of human cutaneous squamous cell carcinomas (SCC) were caused by mutations upregulating NRF2 expression and activity (auf dem Keller et al., 2006; Kim et al., 2010). These findings highlight the dual role of NRF2 as both a pro- and anti-tumorigenic factor in skin cancer, which is suggested to be context-dependent (Hiebert and Werner, 2019).

In healthy skin cells, NRF2 reduces cellular stress and counteracts pathogens and carcinogens *via* its ROS, xenobiotic detoxifying and anti-inflammatory activities, which therefore promote cellular homeostasis and prevent skin cancer onset. This has been illustrated with mice studies lacking functional NRF2, which display increased chemically induced tumor multiplicity (auf dem Keller et al., 2006; Xu et al., 2006). Hyper-activation of NRF2 in mice and humans also protected them from UV radiation-induced skin cancer (Knatko et al., 2015). Additionally, constitutive activation of *Nrf2* in mouse keratinocytes (caNRF2) reduces the susceptibility of these mice to develop chemically-induced skin tumors (Rolfs et al., 2015).

Concomitantly, a study shows that constitutive activation of *Nrf2* in a mouse model of skin tumorigenesis (genetic mouse model of Human Papilloma Virus 8-induced skin cancer) dramatically increases tumor incidence and multiplicity (Rolfs et al., 2015). The pro-tumorigenic potential of NRF2 has also been observed in murine skin fibroblasts with constitutive activation of NRF2. Constitutive activation of *Nrf2* in mouse fibroblasts (caNRF2) express the cancer-associated-fibroblast (CAF) gene expression signature and promotes tumor growth in grafted human squamous cell carcinoma cells (Hiebert et al., 2018). Altogether, these data show that a hyper-activation of NRF2 can become detrimental to patients as it confers proliferative advantages to tumoral cells and increase their chemotherapy resistance (Figure 40).



**Figure 40. Dual role of NRF2 in cancer.**

The action of NRF2 is context dependent: in healthy cells, controlled activation NRF2 will prevent skin tumorigenesis; whereas chronic activation of NRF2 should be avoided as it can promote cancer development, progression, and metastasis. NRF2 activation in the early phase of tumor promotion and in malignant cells should be avoided and can be antagonized by inhibition of NRF2. From de la Vega et al., 2018.

To conclude, the role of NRF2 in skin cancer is very complex and further investigations are needed to better characterize its dual role and the underlying mechanisms of action. Even though pharmacological NRF2-activating treatments seem promising for several skin diseases and pathological wound healing defects, they should not be administrated chronically or in case of skin cancer suspicion.



## 5. Objectives

As presented in the preamble and in the scientific context of the introduction, ECM integrity is fundamental for the mechanical properties and functions of connective tissues, including dermis. ECM alteration is one of the major signs of skin aging (Sparavigna, 2020). Thus, the identification of ECM regulators and the characterization of the underlying mechanisms of ECM regulation are subjects of intensive investigation.

The starting point of my PhD thesis was to investigate if the principle of longevity based on signaling pathways discovered in *C. elegans* could be transposed to human skin. I thus began my PhD work by studying the possible role of the longevity factors AAK-2/AMPK and SKN-1/NRF2 in dermal fibroblasts to investigate their potential implication in the regulation of ECM components, as shown previously in *C. elegans*. AMPK results will be presented only in the additional data section since I mainly focused my PhD work on the SKN-1/NRF2 transcription factor that was the first to show early and promising results. Several studies have shown a link between oxidative stress and ECM regulation, most probably mediated by NRF2 and this has been exemplified in several physiological and pathological conditions. However, Ewald and collaborators were the first to suggest a direct regulation of collagen genes by SKN-1/NRF2. (Blackwell et al., 2015). More recently, the work of Hiebert and collaborators showed that NRF2 is involved in the regulation of ECM gene expression in mouse fibroblasts (Hiebert et al., 2018).

Hence, my thesis project intended to further advance our knowledge on the direct implication of NRF2 in the regulation of ECM composition in primary human dermal fibroblasts.

## **II. Results**

## 1. Part 1

**“The transcription factor NRF2 regulates matrisome gene expression and collagen fibrillogenesis in human skin fibroblasts “**

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*Manuscript in preparation*

## **The transcription factor NRF2 regulates matrisome gene expression and collagen fibrillogenesis in human skin fibroblasts**

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## Abstract

Fibroblasts are the major producers and organizers of collagen-rich tissues and, as such, play a key role in dermis homeostasis. SKN-1, the nematode homologue of the transcription factor NRF2, is a master regulator of longevity that, under specific metabolic conditions, was found to act through the activation of expression of collagen genes. Here, we investigated whether NRF2, regarded as a master regulator of anti-oxidative response in humans, may also play a yet undiscovered role in the regulation of extracellular matrix (ECM) gene expression in human skin fibroblasts. RNA-seq transcriptomic analysis of NRF2 silenced primary human dermal papillary fibroblasts revealed that, in addition to NRF2 conventional targets, matrisome genes were the most represented gene set. Our data show that in our conditions the impact on extracellular matrix components was independent of a Reactive Oxygen Species (ROS) mediated mechanism. Composition and topology of the ECM produced by shNrf2 dermal fibroblasts was analyzed using a combination of light and electron microscopies (Second Harmonic Generation, confocal microscopy, Transmission Electron Microscopy and Atomic Force Microscopy). We showed that silencing NRF2 in human skin fibroblasts reduces collagen expression and impacts collagen fibril formation and organization. Our data pointed to direct and indirect mechanisms of action. The latter may involve a transcriptional regulatory cascade mediated by ZNF469, a regulator of collagen expression involved in connective tissue disease, that we have identified as a novel target of NRF2. NRF2 can thus be considered as a novel positive regulator of ECM gene expression in human skin fibroblasts and, in that, represents a new target to maintain dermal homeostasis.

## Introduction

As the outermost and largest organ of the human body, skin is vital for protection from external aggressions and water loss. These roles are actively ensured by its two main constitutive layers: the epidermis and the dermis, which are supported by the subcutaneous adipose tissue, called hypodermis (Tobin, 2017). In contrast to the epidermis, which is composed of multiple cell layers, the dermis is only sparsely settled with cells that are surrounded by an abundant extracellular matrix (ECM). Fibroblasts constitute the main cell type found in the dermis, and they are responsible for the production of the dermal ECM. This complex macromolecular network is composed of interlaced fibrils formed by collagens and elastin, in which proteoglycans and glycoproteins fill the space to form a hydrated structure able to withstand tensile and compressive forces (Frantz et al., 2010; Nyström and Bruckner-Tuderman, 2019). It is also a highly dynamic structure that undergoes constant remodeling.

Although the ECM was first believed to be only a structural scaffold for its surrounding cells, it is now clear that it also ensures important biological functions by regulating fundamental cell functions, such as, cell proliferation, migration or differentiation (Frantz et al., 2010). Dysregulation or alteration of the ECM can lead to a wide range of pathologies including cancers and fibrosis (Taha and Naba, 2019; Theocharis et al., 2019) and structural changes in the dermal ECM are the prime causes of skin aging signs (Haydont et al., 2019a; Sparavigna, 2020). In addition to conferring strength and resilience to the skin, dermal ECM is also actively involved in the communication between keratinocytes and fibroblasts, which has been shown to play a crucial role in maintaining skin integrity (Russo et al., 2020; Shephard et al., 2004; Werner et al., 2007). The proper regulation of the dermal ECM is therefore of paramount importance for a healthy skin homeostasis and of the many factors contributing to ECM regulation, transcription factors have been extensively investigated (Shin et al., 2019).

Among all the described ECM regulators, an elegant study by Ewald and collaborators identified the worm transcription factor SKN-1, the *C. elegans* ortholog of the mammalian nuclear factor E2-related factor (NRF2), as a new regulator of ECM genes (Ewald et al., 2015). Known for its key role in cellular antioxidant and detoxification processes, SKN-1 has been characterized as a master regulator of longevity in *C. elegans* (Blackwell et al., 2015; Tullet et al., 2008). Ewald and collaborators made the surprising finding that, under specific metabolic conditions, the SKN-1 longevity effect mainly depends on the activation of collagen gene expression instead of the activation of antioxidant genes (Ewald et al., 2015).

In humans, NRF2 (encoded by the *NFE2L2* gene) is also very well established as a master regulator of the intracellular antioxidant response and xenobiotic metabolism, as it activates the expression of cytoprotective genes under basal and stress conditions (Hayes and Dinkova-Kostova, 2014; Huang et al., 2015; Tonelli et al., 2018). Normal conditions require only low levels of cytoprotective proteins and NRF2 is located mainly in the cytoplasm where it is rapidly turned-over by binding to its repressor Kelch-like ECH-associating protein 1 (KEAP1) that destines it for proteasomal degradation. Therefore, NRF2 basal levels remain low, but under oxidative or xenobiotic stress-conditions, (ROS) and electrophiles are able to induce a conformational change of KEAP1 through the oxidation of reactive cysteine residues, which disrupts its binding to NRF2 (Suzuki et al., 2019). NRF2 is thus stabilized and translocates into the nucleus where it heterodimerizes with a member of the sMaf protein family. There, it initiates the transcription of its target genes by binding to an antioxidant response element (ARE) DNA binding motif. The complexity of the NRF2 pathway is illustrated by its hundreds of target genes mainly involved in the antioxidant glutathione (GSH) homeostasis (*SLC7A11*, *GCLC*, *GCLM*), ROS and xenobiotic detoxification (*NQO1*, *ALDH3A1*, *MGST1*, *UGT1A1*), and heme and iron metabolism (*HMOX1*, *FECH*), to give the main examples (Hayes and Dinkova-Kostova, 2014).

In skin cells, NRF2 plays a central role in the maintenance of the redox balance and protects the main skin cell types (i.e. fibroblasts, keratinocytes and melanocytes) from external insults, such as UV or pollution (Gęgotek and Skrzydlewska, 2015; Hseu et al., 2018; Marrot, 2018; Schäfer et al., 2010; Soeur et al., 2017). Moreover, NRF2 is involved in the wound healing process and in the skin immune response, mainly by clearing oxidative stress and inflammation (Ambrozova et al., 2017; Hiebert and Werner, 2019; Kobayashi et al., 2016). Activation of NRF2 improved the skin diseases psoriasis and systemic sclerosis (SSc), the latter being an autoimmune connective tissue disease characterized by skin fibrosis (Brück et al., 2018; Kavian et al., 2018; Sekiguchi et al., 2019; Wu et al., 2020). Whereas several studies caution against constitutive activation of NRF2 in skin as it may promote tumorigenesis (Hiebert et al., 2018; Rolfs et al., 2015; Schäfer et al., 2012), the role of NRF2 in protecting skin against external (UV, pollution, chemicals) and internal (wound healing process) stimuli that induce oxidative stress is well established.

Recently, Hiebert and collaborators reported that genetic upregulation of NRF2 – independent from elevated ROS levels – modulates structural ECM gene expression (Hiebert et al., 2018). These findings support the hypothesis of a conserved role of SKN-1/NRF2 in mammals. Additionally, NRF2 has been linked to aging in mammals as decreased NRF2

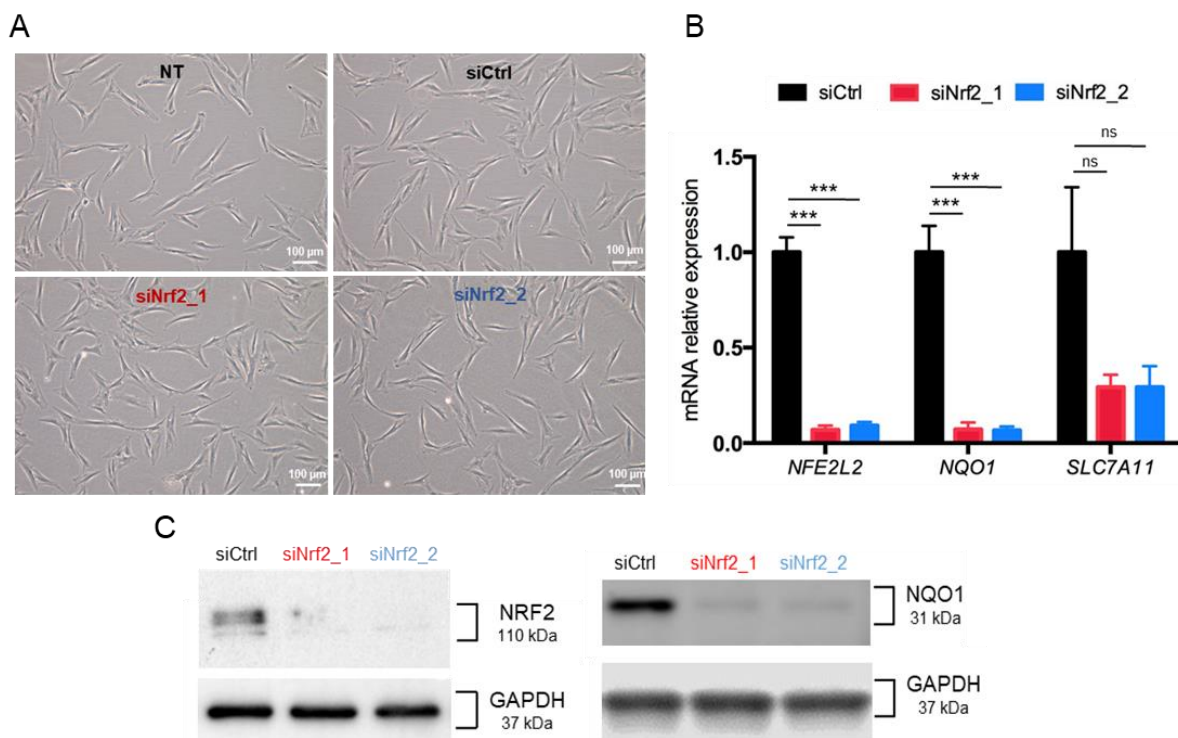
expression was shown to contribute to the aging process (Bruns et al., 2015; Schmidlin et al., 2019). In the present study, we further investigated the potential role of NRF2 in the ECM regulation and synthesis in human dermal papillary fibroblasts. We demonstrated that knockdown of NRF2 altered the expression of a variety of ECM genes, and most importantly, that NRF2 controls collagen I expression levels and collagen fibrillogenesis and thus ECM structure and properties.



## Results

### Transcriptomic profiling of siNrf2 primary dermal fibroblasts highlights NRF2 conventional targets as the most highly differentially expressed genes

We first investigated the potential role of NRF2 in ECM regulation by using an unbiased transcriptomic analysis of human primary dermal fibroblasts silenced for NRF2. To obtain statistically meaningful results we used transient transfection of two different siRNAs against NRF2 into primary papillary fibroblasts derived from three young female donors. Dermal fibroblasts can be derived from the papillar or reticular dermis (Haydont et al., 2019b; Nauroy et al., 2017). Papillary fibroblasts were chosen here for their higher proliferation rate than the reticular fibroblasts. To mimic the decrease in NRF2 expression generally observed in aged tissues (Schmidlin et al., 2019), we opted for RNAi strategy.



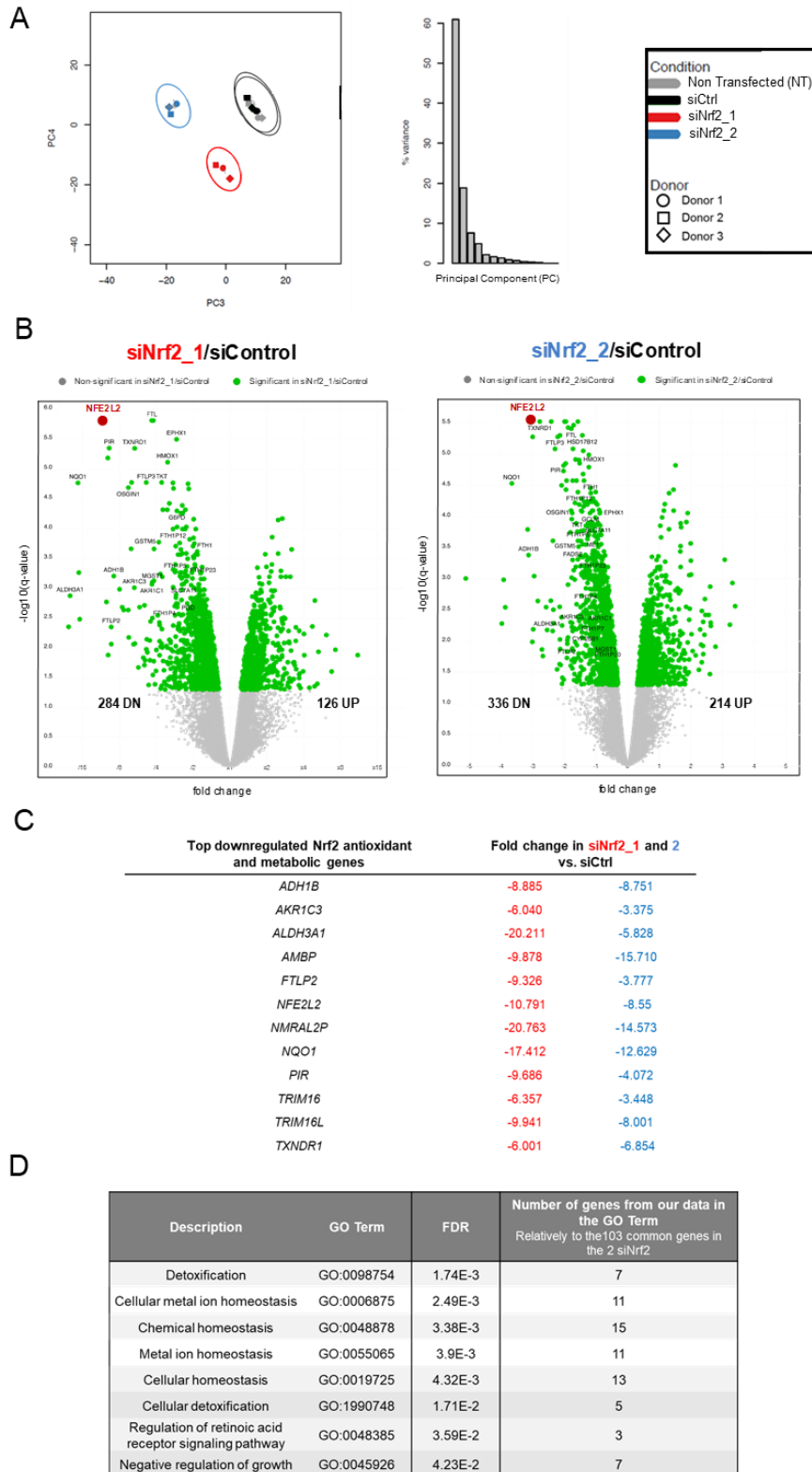
**Figure 1: NRF2 silencing in human skin papillary fibroblasts.**

(A) Representative bright field images of human dermal fibroblasts at 72h post-transfection. (B) qPCR analysis of *NFE2L2*, *NQO1*, *SLC7A11* at 72h post-transfection. Statistics were performed using the ordinary one-way ANOVA test ( $n_{\text{donor}} = 3$ ). Asterisks indicate p-value:  $*** \leq 0.001$ , ns: non-significant. (C) Western blots with anti-NRF2 and anti-NQO1 antibodies of total protein extracts 72h after transfection. Representative Western blot membranes are shown (one donor).

As NRF2 is a stress sensing and a stress sensitive factor, we evaluated the morphology of the cells at the end of the transfection with bright field microscopy to rule out any obvious

stress induced by transfection. Fibroblasts did not exhibit any visible sign of stress and showed the normal spindle shape of papillary fibroblasts in all conditions (Figure 1A). We then validated NRF2 specific silencing by the siRNAs at the mRNA level before proceeding to RNA-seq analysis. NRF2 is the master regulator of 200 target genes involved in cell responses to oxidative stress and cell detoxification. We showed that *NFE2L2* and its well-known basal targets *NQO1* and *SLC7A11* were efficiently silenced by each of the two different siRNAs in cells from the three donors compared to siControl (siCtrl) (Figure 1B). Downregulation of NRF2 and NQO1 were also confirmed at the protein level (Figure 1C).

Principal component analysis (PCA) of the RNA-seq data showed that the values clustered by donor on the first two axes (data not shown) and then by condition when plotted on the PC3 and PC4 axis (Figure 2A). These two latter axes account for 15% of the total variance. Importantly, the non-transfected (NT) and siCtrl conditions were almost identical since they clustered together on the PC3/PC4 axis. Moreover, differential analysis between NT and siCtrl conditions did not reveal any significantly differentially expressed genes between the two conditions (data not shown) indicating that the transfection itself does not introduce significant experimental bias. On the contrary, statistical analysis of RNA-seq data from siNrf2 and siCtrl fibroblasts displayed hundreds of significantly differentially expressed genes. We identified respectively 410 and 550 differentially expressed genes in siNrf2\_1 and siNrf2\_2 cells compared to their control condition (fold change set up at 2, p-value<0.05). Volcano plots (Figure 2B) showed that *NFE2L2* is significantly downregulated in both siNrf2 compared to siCtrl. Moreover, many of its conventional targets involved in the antioxidant and detoxification responses were found as the most highly differentially expressed genes in both siNrf2 vs siCtrl (named on the volcano plots in Figure 2B). Top downregulated genes include NRF2 detoxification target genes, including *NQO1* or *ALDH3A1*, but also target genes involved in heme and iron metabolism or proteostasis (Figure 2C). Interestingly, our study also confirmed the strong downregulation of new NRF2 target genes, that have been identified in other cell types (Figure 2C). We investigated further TRIM16, which is involved the regulation and stability of NRF2, KEAP1, and p62. The downregulation of TRIM16 upon NRF2 silencing suggests a regulatory feedback loop between TRIM16 and NRF2 (Jena et al., 2018). Downregulation of *TRIM16* expression was validated at the protein level (Figure S1). The complete list of the downregulated NRF2 targets is shown as Supplementary data (Table S1).



**Figure 2: Transcriptomic analysis of siNrf2 fibroblasts**

(A) Principal component analysis of the transcriptomic data. (B) Volcano plots of differentially expressed genes in siNrf2 versus siCtrl fibroblasts. Green dots correspond to genes significant differentially expressed (fold change  $\geq 2$  or  $\leq -2$  and the p-value  $< 0.05$ ). Named genes are NRF2 targets already described in literature. Grey dots are not significant genes. (C) Top target genes of NRF2 downregulated (FC  $< -6$ , p-value set at 0.05). (D) Gene Ontology analysis on the genes commonly differentially expressed in both siNrf2.

A Gene Ontology (GO) analysis of the commonly differentially expressed genes in siNrf2 fibroblasts was then performed (Figure 2D). The most enriched GO terms are “detoxification” and “cellular metal ion homeostasis”, confirming well-known pathways regulated by NRF2. Overall, the six top GO terms did not reveal any new function of for NRF2. More unexpectedly, our results suggest that NRF2 is involved in the regulation of the retinoic acid receptor signaling pathway and cell growth, which we have not explored any further.

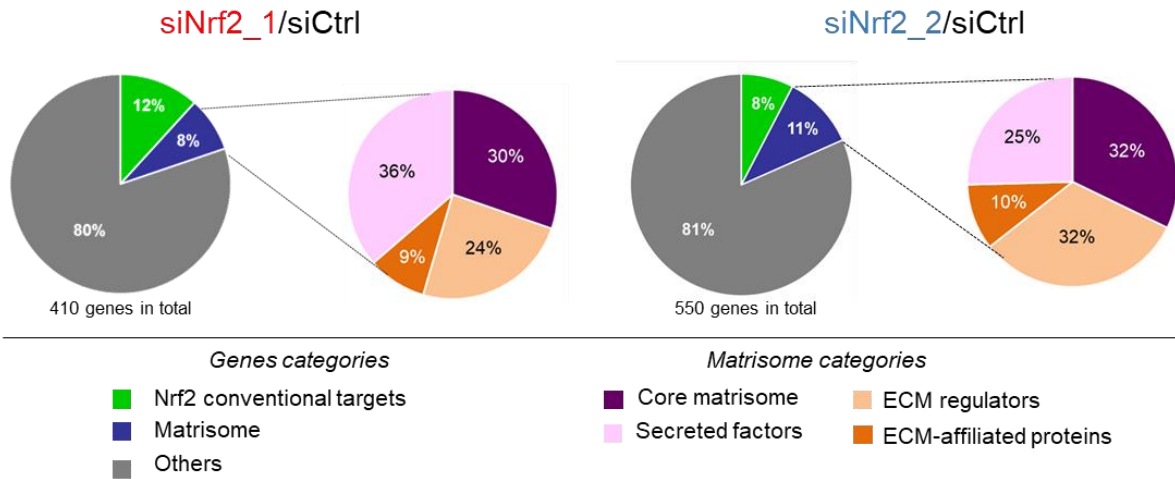
To conclude, NRF2 silencing using 2 different siRNA in human dermal primary fibroblasts was effective since we identified in both RNA-seq data *NFE2L2* and many of its conventional targets as the top downregulated genes.

### **NRF2 knockdown affects matrisome gene expression profile in human dermal fibroblasts**

In order to highlight changes in ECM gene expression and to counter the lack of a gene ontology term including all extracellular matrix components, we used the matrisome gene list characterized by Naba and collaborators (Naba et al., 2012b). The matrisome is subdivided into four categories: the core matrisome, secreted factors, ECM regulators and ECM-affiliated proteins. The first category includes all structural ECM proteins and the last three cover the proteins associated with ECM regulation and signaling.

Interestingly, we found as many matrisome genes as NRF2 conventional target genes, about 10% for each, affected by the downregulation of NRF2 in human dermal primary fibroblasts (Figure 3A). A deeper analysis of the matrisome categories revealed that genes coding for the “core matrisome”, “secreted factors”, and “ECM regulators” categories were the most significantly impacted by NRF2 silencing. Relevantly, both siNrf2 displayed similar distribution of matrisome gene categories (Figure 3A). However, there was only a small subset of matrisome genes commonly impacted by both siNrf2, in contrast to what was found for the NRF2 conventional targets. To even out potential differences in the regulation of gene expression levels between the two siRNAs, we lowered the fold change threshold to 1.4 (adjusted p-value <0.05). This led to the identification of 36 commonly differentially expressed genes (Table S2). Only core matrisome genes affected by NRF2 silencing are listed in Figure 3B.

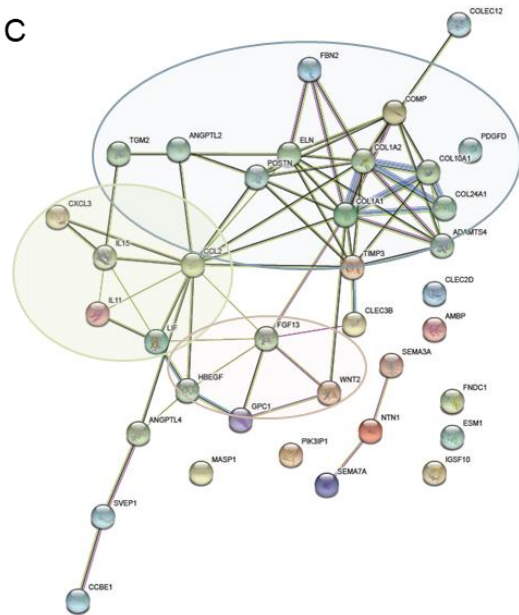
A



B

Core matrisome	Gene	Fold change in siNrf2_1 and 2 vs. siCtrl		
Collagens	COL24A1	-4.61	-4.67	DOWN
	COL1A2	-1.46	-1.45	DOWN
	COL1A1	-1.38	-1.47	DOWN
	COL10A1	4.7	3.2	UP
ECM Glycoproteins	FBN2	-2.79	-1.49	DOWN
	NTN1	-2.52	-1.57	DOWN
	IGSF10	-1.67	-1.83	DOWN
	FNDC1	-1.52	-1.55	DOWN
	SVEP1	-1.61	-2.67	DOWN
	ELN	1.52	1.72	UP
	COMP	1.81	1.50	UP
	POSTN	1.55	1.65	UP
Proteoglycans	ESM1	3.16	8.37	UP

C



**Figure 3: Expression of matrisome genes is altered in siNrf2 dermal fibroblasts.**

(A) Pie charts of the differentially expressed genes in siNrf2\_1 and siNrf2\_2 dermal fibroblasts showing the proportion of matrisome and NRF2 target genes (FC set up at 2 and p.value <0.05). (B) Core matrisome genes commonly differentially expressed in both siNrf2 (FC set up at 1.4. p.value <0.05). (C) STRING analysis of matrisome proteins identified in both siNrf2 fibroblasts (FC set up at 1.4. p.value <0.05).

We then analyzed the molecular links between these 36 differentially expressed matrisome encoded proteins using the STRING database (Figure 3C). This revealed a major hub made of structural core matrisome proteins and ECM regulators, such as TGM2 or COMP. Structural proteins affected by NRF2 silencing include collagens type I, X and XXIV (encoded by COL1A1, COL1A2, COL24A1 and COL10A1) and components of elastic fibrils (ELN and FBN2). We also identified two additional smaller hubs both composed of secreted factors. The first one consists in secreted factors mainly involved in the inflammation process and the second one contains growth factors regulating a variety of biological processes, including

development, cell growth, tissue repair, and wound healing (Figure 3C). Whereas most of the collagens are downregulated, there is a slight increase in the gene expression of elastin (*ELN*), periostin (*POSTN*) and cartilage oligomeric protein (*COMP*). Surprisingly, *COL10A1* and *COL24A1* were the most downregulated among the collagen genes even though their expression was not expected in primary papillary fibroblasts, while fold changes in the expression of the dermal fibroblast markers *COL1A1* and *COL1A2* were less elevated.

Recently, Haydont and collaborators proposed another approach to study the matrisome by looking at the impact of the ECM on cellular functions (Haydont et al., 2019b). They defined the ‘tissue skeleton’ as the network connecting the ECM to the nuclear DNA. The ‘tissue skeleton’ is composed of seven subcategories: the ECM, secreted factors, focal adhesion proteins, the cytoskeleton, transduction pathways, the LINC complex and the nucleoskeleton (Figure S2A). Among the differentially expressed genes (FC set up at 2, p-value <0.05), the ‘tissue skeleton’ genes respectively represent 29% and 35% in siNrf2\_1 and siNrf2\_2 fibroblasts compared to control. (Figure S2B). We then subdivided these genes according to the seven different categories (Figure S2C). This analysis confirmed our previous findings that gene expression of “ECM” and “secreted factor” categories, that are equivalent to the matrisome, are the most dysregulated by NRF2 silencing. Additionally, we found that focal adhesion and cytoskeleton gene sets are highly affected in both siNrf2 fibroblasts, suggesting that changes in ECM gene expression may impact fibroblasts behavior. However, they were only a few genes commonly differentially expressed between the two siNrf2 fibroblasts, even though the ‘tissue skeleton’ includes a large subset of genes.

Oxidative stress affects the expression of extracellular matrix components (Avantaggiato et al., 2014; Spadoni et al., 2015). Therefore, we next investigated if NRF2 silencing could lead to a redox imbalance in fibroblasts, which in turn can impact ECM gene expression. We measured ROS levels using a fluorescent probe in the transiently transfected siNrf2 fibroblasts (Figure S3A). Silencing of NRF2 did not generate elevated ROS levels since fluorescence peaks were almost identical for the siNrf2 and siCtrl fibroblasts. In addition, known ROS-induced genes (Murphy-Marshman et al., 2017; Spadoni et al., 2015; St-Pierre et al., 2006) were not upregulated in siNrf2 fibroblasts *versus* siCtrl in our RNA-seq data (Figure S3B).

The differences observed between the two siRNAs may be due to kinetic effects when using transient transfection, indicating a possible indirect regulation of the ECM genes by NRF2. Our data showed that silencing of NRF2 does not generate elevated levels of ROS

under normal culture conditions, confirming that our results are not skewed by an indirect effect. Hence, we conclude that ECM genes are *bona fide* NRF2 targets.

### **NRF2 silencing alters collagen fibril diameter and organization in human dermal fibroblasts**

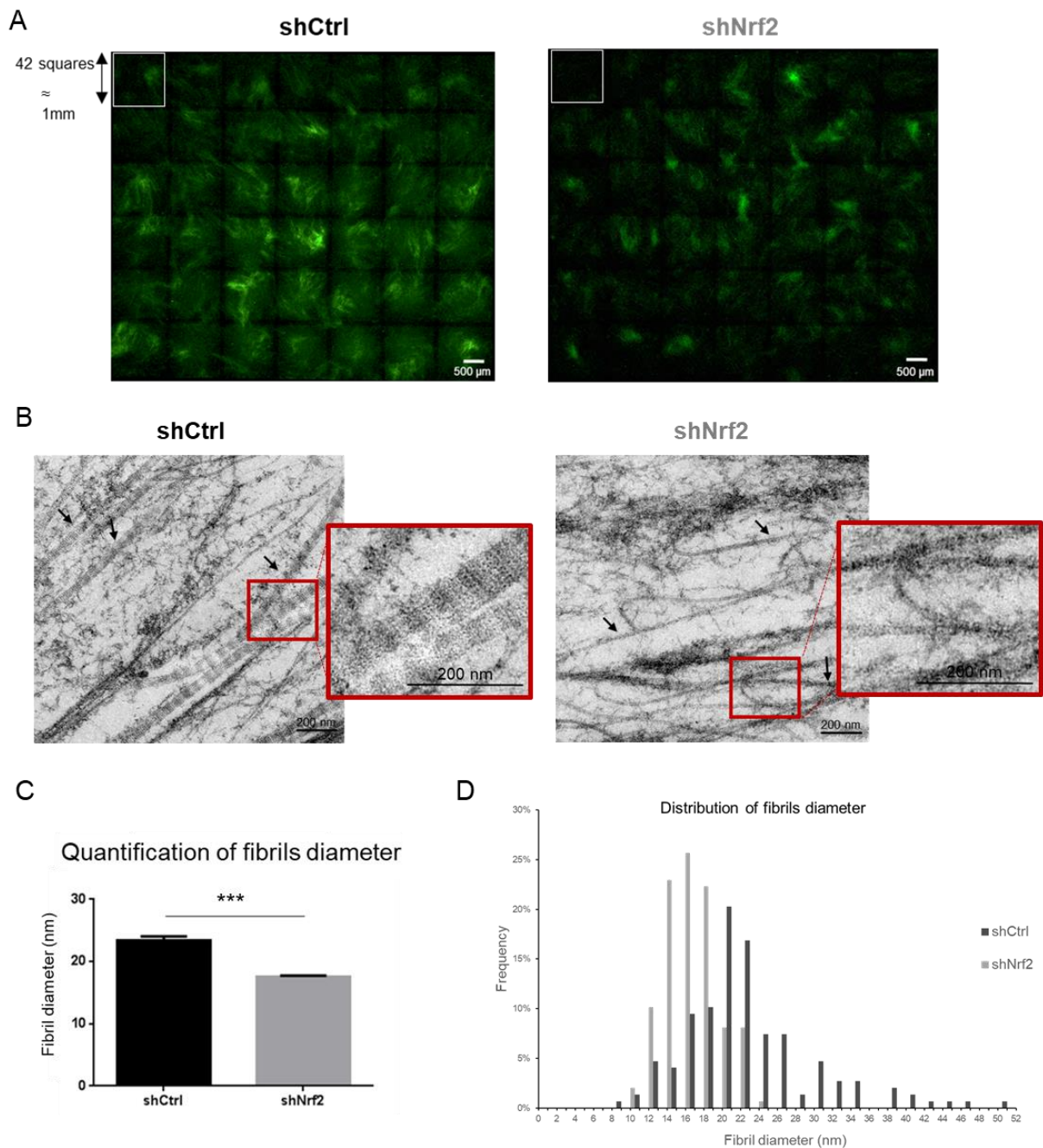
Next, we aimed to explore the impact of NRF2 silencing on the ECM at the protein level. Production and deposition of ECM by cells requires several days of cell-culture, which cannot be achieved with transiently transfected fibroblasts. We therefore established primary fibroblast lines stably silenced for NRF2 to circumvent any potential kinetic effect compared to transient transfection and enable the analysis of the ECM. To that end, we used lentiviral infection of shRNA particles targeting NRF2 (shNrf2) and subsequent selection of the fibroblasts by FACS thanks to the expression of the RFP fluorescent protein (Figure S4). The effective silencing of NRF2 in human dermal primary fibroblasts (shNrf2 fibroblasts) was confirmed at the transcriptional and protein levels using RT-qPCR and WB respectively (Figure S4D and E). In addition, NRF2 knockdown also affected gene expression of the conventional antioxidant targets *NQO1* and *SLC7A11*, confirming that NRF2 activity is blunted in the shNrf2 cells.

As a first global approach to visualize the ECM, we used Second Harmonic Generation (SHG) microscopy after 8 days of culture in presence of sodium ascorbate. This technique allows the detection of collagen fibers when well-formed and of large diameter (>30nm) (Bancelin et al., 2015). SHG images showed a substantial decrease in fluorescence indicating that NRF2 knockdown hindered the formation of collagen fibrils in dermal fibroblast ECM (Figure 4A). We then used transmission electron microscopy (TEM) to analyze in more details the structure of the collagen fibrils produced by fibroblasts silenced for NRF2. We confirmed the presence of numerous striated large collagen fibrils in shCtrl fibroblasts interspaced with proteoglycans aggregates and other filamentous ECM proteins (Figure 4B). In striking contrast to the shCtrl fibroblasts, the collagen fibrils observed in shNrf2 fibroblasts appeared thinner with the banded pattern barely visible and numerous patches of disorganized unidentified ECM components (Figure 4B and C). These observations were confirmed by the quantification of the fibril diameter showing that the shNrf2 fibril diameter was significant smaller than that in shCtrl. The collagen fibril distribution patterns of shNrf2 fibroblasts and shCtrl fibroblasts were also different (Figure 4D). Whereas shCtrl collagen fibrils were heterogenous and displayed a wide range of fibril diameters (from 10 to 50 nm), shNrf2 showed a predominance of regular narrow-diameter fibrils that peaked at 14-18 nm with no fibrils with a diameter > 25 nm. This

explains the apparent scarcity of collagen fibrils observed in shNrf2 fibroblasts with SHG. We conclude that NRF2 knockdown resulted in a dysfunctional regulation of fibril formation and lateral growth. Together, these results indicate that NRF2 silencing does not seem to affect the structure of the collagen fibrils, but clearly impacts the collagen fibril network by decreasing the diameter of the individual fibrils.

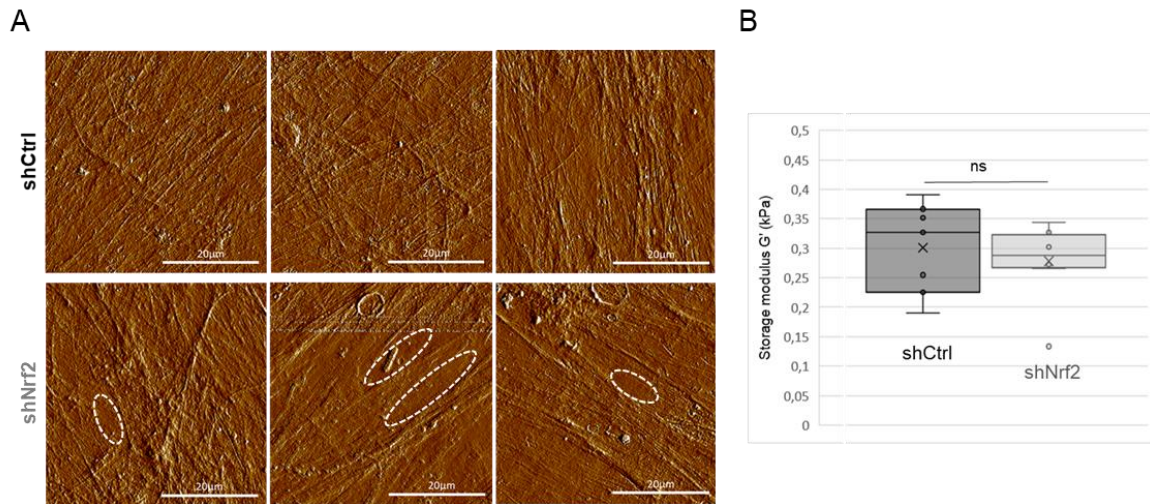
As change in collagen fibril diameter may affect ECM rigidity, we thus decided to perform measurements of the mechanical properties of the ECM produced by fibroblasts using AFM. To that end, we decellularized our fibroblast cultures to facilitate direct measurements of the ECM layer that is mostly located beneath the fibroblasts. AFM images of shCtrl and shNrf2 ECM revealed differences in ECM topology between the two conditions. shCtrl collagen fibrils covered most of the fields selected for observations, whereas “empty” spaces were clearly visible in shNrf2 ECM (Figure 5A, white dotted circles). This indicates that shNrf2 fibroblasts produced fewer collagen fibrils than the control fibroblasts. Detection of fewer fibrils by SHG (Figure 4A) can thus be due to a combined effect of a decreased production and thinner collagen fibrils. Measurements of the storage modulus ( $G'$ ) did not reveal statistically significant differences between in mechanical properties of shCtrl and shNrf2 secreted ECMs (Figure 5B). However, there is a clear tendency to lower values obtained for the shNrf2 ECM, suggesting a decrease in the rigidity of the ECM produced by shNrf2 fibroblasts compared to the control. Moreover, the results reflect the higher heterogeneity of the collagen fibrils diameter in control fibroblasts compared to shNrf2 fibroblasts and thus confirmed our TEM data (Figure 4D).





**Figure 4: Stable silencing of NRF2 in fibroblasts (shNrf2) affects collagen fibrils.**

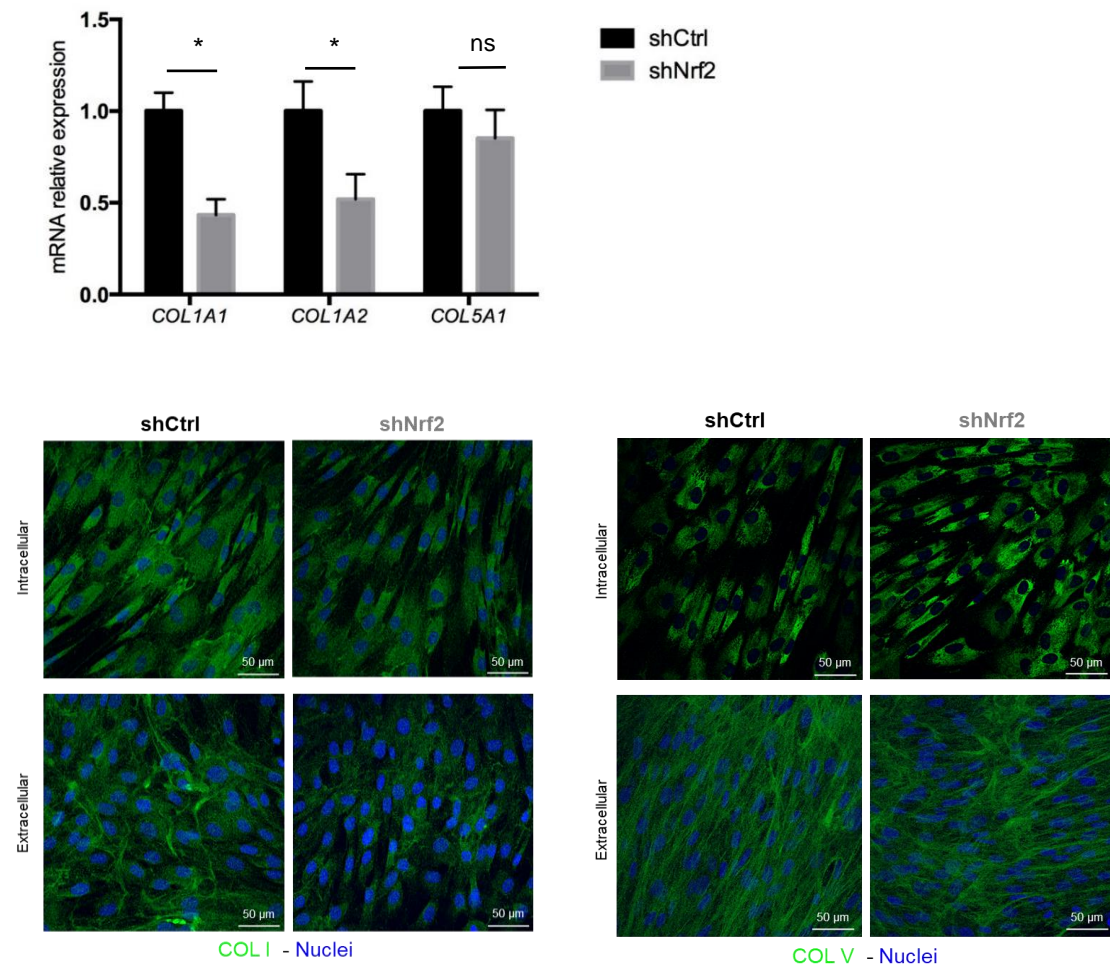
(A) Second Harmonic Generation (SHG) large field images of collagen fibers produced by shCtrl and shNrf2 fibroblasts, after 8 days of culture in presence of vitamin C. (B) Transmission Electron Microscopy (TEM) images of ECM produced by shCtrl (B) and shNrf2 (C) fibroblasts after 8 days of culture with vitamin C. Collagen fibers are shown with the arrows. (C) Quantification of collagen fibrils diameters. Statistics were done using an unpaired t-test ( $n_{\text{fibrils}} = 148$ ). Asterisks indicate p-value  $*** \leq 0.001$ . (D) Frequency histogram of collagen fibril diameter (C).



**Figure 5: Decellularized matrices produced by shCtrl and shNrf2 fibroblasts differ in topology and in rigidity.**

(A) Atomic force microscopy (AFM) images showing the topography of shCtrl (upper line) and shNrf2 (lower line) decellularized matrices secreted by. (B) Representation of the storage modulus  $G'$  (kPa), which correlates with the rigidity of the sample, measured on shCtrl and shNrf2 decellularized matrices acquired on 7 and 8 zones respectively of 3 independent experiments. 10,000 measurements were acquired per area. Histogram represents average values of each area. Statistics were done using a non-paired t-test ( $n_{\text{zones}} = 7$  or 8). Asterisks indicate p-value: \*  $\leq 0.05$ , \*\*  $\leq 0.01$ , \*\*\*  $\leq 0.001$ , ns: non-significant.

Fibril size is mainly determined by the composition of fibrillar collagens (for review, (Ruggiero et al., 2005)). To better understand how NRF2 can regulate the thickness of collagen fibrils, we assessed in the shNrf2 and shCtrl fibroblasts the expression levels of collagen I genes and of the main fibril-forming collagen regulator the collagen V. It has been shown that the ratio between collagen I and V directly correlates with the diameter of collagen fibril (Birk, 2001; Ricard-Blum and Ruggiero, 2005). Interestingly, expression levels of *COL1A1* and *COL1A2* (encoding respectively the  $\alpha 1(I)$  and  $\alpha 2(I)$  chains of the collagen I molecule) were significantly decreased in shNrf2 fibroblasts cultured during 5 days compared to control fibroblasts, whereas expression of *COL5A1* (encoding the  $\alpha 1(V)$  chain) remains unchanged (Figure 6A) indicative of a potential change in collagen I/collagen V ratio. We thus performed intracellular and extracellular immunofluorescence staining to confirm RT-qPCR results at the protein level. A substantial decrease of intracellular collagen I staining was observed, and the drop was even more pronounced in the extracellular space (Figure 6B). Fibrils of collagen I were clearly detected in shCtrl fibroblasts, whereas they were almost not visible in the shNrf2 fibroblasts. In contrast, no change or a slight increase in collagen V immunoreactivity was observed in shCtrl vs shNrf2 fibroblasts (Figure 6B).



**Figure 6: Collagen expression and production is affected by NRF2 stable silencing (shNrf2).**

(A) qPCR analysis of *COL1A1*, *COL1A2* and *COL5A1* in shCtrl and shNrf2 fibroblasts. Statistics were done using a paired t-test (n\_experiments = 3). Asterisks indicate p-value: \*  $\leq 0.05$ , \*\*  $\leq 0.01$ , ns : non-significant. (B) Immunofluorescence staining of collagen I at intracellular (upper line) and extracellular (lower line) level in shCtrl and shNrf2. Fibroblasts were culture during 8 days without vitamin C for the intracellular staining and for 6 days with vitamin C for the extracellular staining. These images are representative of 3 independent experiments. (C) Immunofluorescence staining of collagen V at intracellular (upper line) and extracellular (lower line) level in shCtrl and shNrf2. Fibroblasts were culture during 8 days without vitamin C for the intracellular staining and with vitamin C for the extracellular staining. These images are representative of 3 independent experiments.

We conclude that knockdown of NRF2 in human dermal fibroblasts affects collagen I gene expression that results in a decrease in collagen I protein synthesis and an imbalanced ratio between collagens I and V, which can at least partly explain the decrease in collagen fibrils diameter observed in shNrf2 fibroblasts.

### How NRF2 acts on the ECM gene expression

Although there is evidence for a role of NRF2 in ECM gene regulation (our data; (Ewald et al., 2015; Hiebert et al., 2018)) the molecular mechanism remains elusive. NRF2 may directly regulate ECM gene expression by binding to ARE motifs in ECM genes promoters or indirectly *via* a transcriptional regulatory cascade.

In an attempt to get insights into this yet unknown mechanism, we first searched for ARE motives in the proximal promoters of the most characterized ECM targets so far, the collagen genes. Public NRF2 ChIP-seq data obtained in human lung fibroblasts (IMR-90) ((ENCODE Project Consortium, 2012); project GSE91565 in the NCBI-GEO database) revealed NRF2 binding peaks for *COL1A1* and *COL1A2* located at the TSS for *COL1A1*, and 147 bp downstream of the TSS for *COL1A2* (Figure S5). We then performed bioinformatics analyses to search for potential antioxidant response element (ARE) motif in the -1500/+100 bp promoter sequence of collagen I genes *COL1A1* and *COL1A2* (Figure S5). No ARE motifs were detected in any of the two sequences using p-value set at 0.0001. However, by being less stringent and setting the p-value at 0.00017 for *COL1A1* and 0.00018 for *COL1A2*, ARE were located respectively 817 and 1150 bp upstream of the transcription start site (TSS) (Figure S5).

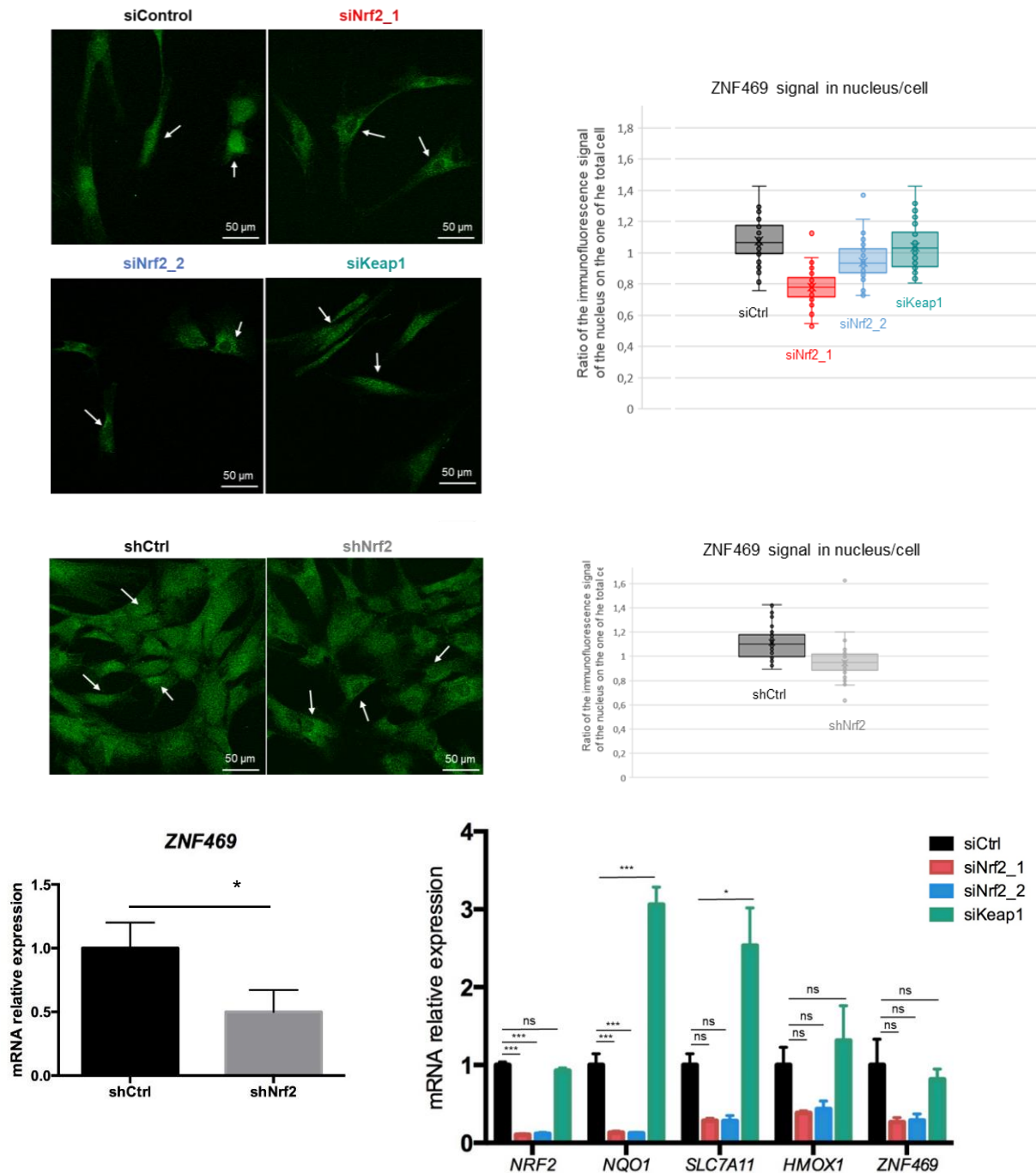
In parallel, none of the matrisome target genes were found within the 25 top commonly differentially expressed genes, therefore suggesting possible transcriptional regulatory cascades that may result in a delay in cell response. However, among the top commonly differentially regulated genes in both siNrf2, we identified *ZNF469*, which encodes the zinc finger protein 469 and was significantly downregulated. *ZNF469* is described as a transcription factor or an extra-nuclear regulator and has been suggested to regulate extracellular matrix genes, particularly fibrillar collagens through a yet unknown pathway (Burkitt Wright et al., 2011; Rohrbach et al., 2013).

To further investigate *ZNF469* downregulation in siNrf2 fibroblasts, we performed immunofluorescent confocal microscopy with anti-*ZNF469* antibodies. We did not observe noticeable difference in the overall staining intensity between siNrf2 and control fibroblasts (Figure 7A), but surprisingly our observations revealed distinct subcellular staining patterns. *ZNF469* signal in the nucleus was noticeably decreased when NRF2 is transiently silenced in dermal fibroblasts (Figure 7A). In addition, when fibroblasts were silenced for the NRF2 repressor KEAP1, leading to an upregulation of NRF2 protein levels, no change in *ZNF469* staining intensity or localization was observed compared to controls (Figure 7A). Quantification of the *ZNF469* signal in the nucleus compared to the total intracellular signal confirmed our

observations (Figure 7B). Furthermore, these results were nicely confirmed in shNrf2 fibroblasts (Figure 7C and quantification in D) in which *ZNF469* expression was also significantly decreased compared to control fibroblasts (Figure 7E).

As no change in *ZNF469* localization was observed, we next checked that *KEAP1* was efficiently silenced in dermal fibroblasts, i.e. provoked an elevation of NRF2 protein level that in turn induced an upregulation of common targets (i.e. basal and stress-induced targets) such as *NQO1* and *SLC7A11* but not basal target such as *HMOX1* (Malhotra et al., 2010). We indeed found that expression levels of *NQO1* and *SLC7A11* were significantly upregulated in siKeap1 fibroblasts, while *HMOX1* and *ZNF469* expression levels remained unchanged (Figure 7F). We conclude that *ZNF469* is a basal target of NRF2 and its nuclear translocation or nuclear tethering depends on NRF2 expression. ECM gene regulation induced by NRF2 can result from a transcriptional cascade involving *ZNF469*.

Altogether, with our bioinformatics data suggesting *COL1A1* and *COL1A2* as potential direct transcriptional targets of NRF2, we here provide direct and indirect potential regulatory clues that can be challenging to further dissect.



**Figure 7: ZNF469 expression is changed at the gene and the protein level in NRF2 silenced (siNrf2 and shNrf2) fibroblasts but not in Nrf2 upregulated fibroblasts.**

(A) Immunofluorescence staining of ZNF469 in the siRNA fibroblasts. (B) Quantification of ZNF469's signal in the nucleus and in the total cell in the siRNA conditions;  $n_{\text{cells}} = 50$ . (C) Immunofluorescence staining of ZNF469 in the shRNA fibroblasts. (D) Quantification of ZNF469's signal in the nucleus and in the total cell in the shRNA conditions for the shRNA experiment;  $n_{\text{cells}} = 75$ . (E) qPCR analysis of *ZNF469* in shNrf2 fibroblasts. Statistics were done using a paired t-test ( $n_{\text{experiments}} = 3$ ). Asterisks indicate p-value: \*  $\leq 0.05$ . (F) qPCR analysis of *NFE2L2*, *NQO1*, *SLC7A11*, *HMOX1* and *ZNF469* in siNrf2 and siKeap1 conditions. Statistics were done using the ordinary one-way ANOVA test ( $n_{\text{donor}} = 3$ ). Asterisks indicate p-value: \*  $\leq 0.05$ , \*\*  $\leq 0.01$ , \*\*\*  $\leq 0.001$ , ns : non-significant.

## Discussion

Our transcriptomic analysis of human dermal fibroblasts silenced for NRF2 revealed that the most dysregulated gene sets are NRF2 conventional targets and matrisome genes, including a variety of genes involved in the ECM composition and regulation. Hence, this work identified NRF2 as a potential positive regulator of dermal ECM homeostasis. Most of the downregulated NRF2 conventional targets are involved in the well-known antioxidant, detoxification and metabolic functions of NRF2, reinforcing the robustness of our data (Dodson et al., 2019; Hayes and Dinkova-Kostova, 2014). These latter processes have to respond quickly to internal and external assaults to ensure cell survival and must therefore be highly inducible at the transcriptional level. In contrast to that, matrisome genes are encoding structural components and even though the ECM undergoes continuous remodeling in response to external stimuli, these processes are mainly controlled at the post-translational level (Vallet and Ricard-Blum, 2019; Yamauchi and Sricholpech, 2012). Difference of inducibility between the antioxidant and ECM response might partly explain the difference of fold changes, since ECM genes were not found as the most dysregulated genes in our study. As NRF2 is a regulator of stress response, it could also indicate that NRF2 may fine-tune matrisome gene expression in response to mechanical or metabolic stress. When using the stably silenced shNRF2 fibroblasts the downregulation of *COL1A1* and *COL1A2* genes was confirmed. The fold change decrease in the collagen I gene expression was more prominent than what was observed in transiently transfected dermal fibroblasts, suggesting that the transcriptional downregulation of matrisome genes is not an acute response to NRF2 silencing. This implies that even a gradual decrease of NRF2 activity over a longer period of time, as observed in several tissues with aging (Bruns et al., 2015; Schmidlin et al., 2019), may significantly impact ECM composition.

ROS signaling and inflammation have been identified as driving forces of ECM synthesis causing skin fibrosis (Kavian et al., 2018; Wu et al., 2020). NRF2 can improve skin fibrotic conditions by suppressing oxidative stress and attenuating inflammation. Importantly, we were able to exclude a ROS-dependent mechanism in our study. We found that in the NRF2 silenced skin fibroblasts, ROS levels were not upregulated compared to the controls. This indicates that the residual NRF2 activity in our cell system was sufficient to assure basal antioxidant defense, which was not the case in NRF2 loss-of-function models (Hiebert et al., 2018; Jódar et al., 2011; Kovac et al., 2015).

Using constitutively activated NRF2 (caNRF2) in mouse skin fibroblasts, Hiebert and collaborators also observed changes in the matrisome gene expression profile (Hiebert et al., 2018). Interestingly among those genes interestingly, 25 were also identified in the transcriptomic data (out of the 36 matrisome genes identified, Table S2). This finding is striking and supports a general role for NRF2 in ECM regulation in fibroblasts. These commonly identified core matrisome genes, substantiate that NRF2 regulates the expression of the collagens *COL1A1*, *COL1A2*, *COL10A1* and the glycoproteins *ELN*, *FBN2* and *POSTN*. The expression of *COL10A1* seems surprising since it is generally described as a marker of hypertrophic chondrocyte and mineralized cartilage (Chen et al., 2019; Lu et al., 2014). However, expression of *COL10A1* was also previously identified in independent transcriptomic data of human dermal fibroblasts (Haydont et al., 2020; Nauroy et al., 2017).

Most importantly, we showed here that changes in ECM gene expression upon NRF2 silencing was accompanied by abnormal collagen fibrillogenesis. Although the ECM produced by silenced NRF2 dermal fibroblasts contained distinguishable collagen fibrils, they are significantly thinner and sparser compared to the larger well-striated collagen fibrils found in the ECM of the control fibroblasts. ECM secreted by the shNrf2 fibroblasts display a slightly reduced rigidity and the cells are embedded in an amorphous extracellular material that is not observed in the control condition. Dermal collagen fibers result from the tightly regulated assembly of three main fibrillar collagens: collagen I (80%), collagen III (10%) and collagen V (<5%), which together orchestrate fibrillogenesis (Shin et al., 2019). Collagen V is the initiator of collagen fibrillogenesis and it acts as a negative regulator of fibril diameters (Sun et al., 2011; Wenstrup et al., 2004). Fibril diameter is inversely proportional to type V/type I collagen ratio (Chanut-Delalande et al., 2004). We showed that NRF2 silencing reduced collagen I expression at the mRNA and protein level in human dermal fibroblasts, while collagen V expression was unchanged. The unbalanced ratio between these two fibril-forming collagens is thus likely the cause of the appearance of thinner collagen fibrils in fibroblasts silenced for NRF2. Nevertheless, we cannot rule out that the dysregulation of other factors also played into the equation. We found that *COL24A1* expression was also dysregulated in siNrf2 fibroblasts. Collagen XXIV was suggested to regulate the diameter of the fibrils assembled in the corneal stroma (Koch et al., 2003). However, because of the poor quality of available antibodies against collagen XXIV, we were not able to validate the observed dysregulation of *COL24A1* expression at the protein level.

Collagens are not the only regulators of fibril diameter and fibrillogenesis is controlled by a number of additional ECM proteins, including small leucine rich repeat proteoglycans



(SLRPs) and matricellular proteins (Bella and Hulmes, 2017). The most abundant matricellular proteins in skin are thrombospondin-1, tenascin-C and periostin (*POSTN*) (Nyström and Bruckner-Tuderman, 2019). As previously reported by (Mitamura et al., 2018) we found that *POSTN* gene expression is significantly upregulated when NRF2 is silenced. Periostin upregulation has been observed as a driving force for fibrosis in various skin diseases such as melanoma or atopic dermatitis (Kuwatsuka and Murota, 2019; Mitamura et al., 2018). Thinner collagen fibrils are formed in *Postn*<sup>-/-</sup> mice, whereas human skin fibroblasts silenced for periostin synthesized larger collagen fibrils (Egbert et al., 2014; Norris et al., 2007). Despite these conflicting results, periostin seems to be involved in the regulation of collagen fibril diameter and might contribute to the effect we observed in shNrf2 fibroblasts.

The present study clearly demonstrates that NRF2 is a prominent regulator of ECM gene expression with consequences in ECM organization. Even though we could not completely elucidate the underlying mechanism, our analyses brought some interesting leads that need to be further investigated. CHIP data analysis and the bioinformatics analyses that we further conducted identified some potential ARE DNA binding motives for NRF2 in the proximal promoters of the *COL1A1* and *COL1A2* human genes, together suggesting that NRF2 regulates collagen I expression through a direct mechanism. It has to be underlined though that the CHIP binding peaks and the NRF2 ARE DNA binding sites did not co-localize. However, combined global NRF2 ChIP-seq analysis and microarray analysis of mouse embryonic fibroblasts derived from *Nrf2*<sup>-/-</sup> mice also identified *COL1A2* as a basal target of NRF2 (Malhotra et al., 2010). This reinforces the assumption of a potential direct regulation of collagen I genes by NRF2. Moreover, we identified in our transcriptome data *ZNF469* as a novel target of NRF2 activity. ZNF469, also known as *KIAA1858* or *BCS*, was first identified in 2008 by Abu *et al.* as a large zinc finger protein of 3925 amino acids (Abu et al., 2008). ZNF469 mutations have later been established as the main cause for Brittle Cornea Syndrome (BCS), an autosomal recessive genetic disorder that affects cornea thickness and strength (Rohrbach et al., 2013). ZNF469 has been suggested to act as a regulator of fibril-forming collagen expression and collagen fibrillogenesis even though the underlying molecular mechanism remains unknown (Hoehn et al., 2012; Vithana et al., 2011). Strikingly, silencing NRF2 in human fibroblasts had a very similar effect on collagen expression and organization to that observed in ZNF469 mutated fibroblasts, notably collagen I downregulation (Burkitt Wright et al., 2011). These data strengthened our hypothesis that ZNF469 is involved in the NRF2 transcription regulatory cascade controlling ECM gene expression and ECM organization, potentially shed light on the mechanism of BCS caused by mutations in ZNF469.

In conclusion, we showed that NRF2 acts on expression of matrisome genes notably by regulating collagen I gene expression that is the main collagen of human dermis. Reduced activity of NRF2 had functional consequences on the structure of the ECM network. Collagen I downregulation and abnormal collagen fibrils are the major modifications found in aged skin (Baumann, 2007; Shin et al., 2019). As a decrease in NRF2 expression levels contributes to the aging process (Bruns et al., 2015; Schmidlin et al., 2019), NRF2 could represent an interesting target for skin anti-aging strategies.

## **Material and methods**

### **Fibroblast isolation and cell culture**

Full-thickness skin biopsies were purchased from suppliers accredited by the French Minister of Research: Icelltis (Toulouse, France), Alphenyx (Marseille, France) and Biopredic (Saint-Grégoire, France). Dermal papillary fibroblasts were extracted from biopsies of three healthy caucasian female donors aged from 22 to 31 y.old, who were undergoing breast reduction plastic surgery. After removal of the hypodermis, the papillary dermis was harvested with a dermatome. Skin samples were cut at the depth of 300  $\mu\text{m}$  thus separating the epidermis and papillary dermis from the reticular dermis. The epidermis was then removed by a treatment with 2.4 U/ml dispase (Roche, France) and by mechanical dissection. Papillary fibroblasts were then extracted from the tissue by digestion with type II collagenase 0.2% (Gibco, France).

Papillary fibroblasts were cultured in Modified Eagle's Medium (MEM) (Gibco, France) supplemented with 10% of Fetal Bovine Serum (FBS) (HyClone, UK), non-essential amino acid solution (NEAA) (Gibco, France), 1 mM of sodium pyruvate (Gibco, France), 2 mM of glutamine (Gibco, France), 20 U/ml of penicillin/streptomycin (Merck Millipore, Germany) and antibiotic-antimycotic (Gibco, France) in a 90% humidified incubator with 5%  $\text{CO}_2$  at 37°C. Cells were seeded at 4000 cell/ $\text{cm}^2$ , unless stated differently, and cultivated until the end of passage 7 (P7).

### **Transfection with siRNA**

Fibroblasts transfection was performed using the HiPerfect transfection reagent (Qiagen, Germany) and the Stealth RNAi siRNA (Thermo Fischer Scientific, France) technologies. Briefly, fibroblasts were trypsinized and resuspended in a low serum cell culture media (MEM, 2% FBS, NEAA, sodium pyruvate, glutamine, penicillin/streptomycin and antibiotic-antimycotic). In parallel, transfection complexes were generated by gently mixing HiPerfect transfection reagent and the siRNA previously diluted in the low serum cell culture media, according to the manufacturer's instructions. Transfection complexes and cells were then seeded at the same time at a cell density of 5600 cell/ $\text{cm}^2$  in flasks or wells and incubated for 72h in a 90% humidified incubator with 5%  $\text{CO}_2$  at 37°C. Sequences of the anti-NRF2 and anti-Keap1 siRNAs are: 5'-CAAGCUGGUUGAGACUACCAUGGUU-3' for the siNrf2\_1; 5'-CAAUGAUUCUGACUCCGGCAUUUC-3' for the siNrf2\_2 and 5'-UGUGUGACGUCACACUGCAGGUCAA-3' for the siKeap1. The Stealth RNAi™ siRNA Negative Control, Med GC was used as the siRNA control in all experiments.

### **Lentiviral-based shRNA transduction**

Five MISSION® shRNA plasmids targeting NRF2 (shNrf2) and shRNA control (shCtrl) (SHC016) in pLKO.1-U6-shRNA-CMV-TAGRFP were purchased from Sigma-Aldrich (MO, USA). shRNA sequences are listed in Table S3. Plasmids were then packaged into lentiviral particles by the lentivectors production facility from the SFR Biosciences Gerland (UMS 3444/US8, Lyon Sud, France). These five shRNA against NRF2 were first tested in primary fibroblasts to select the best shRNA sequence with the highest knock-down efficacy in our system to conduct further experiments. Data only with the selected bold sequences are presented. Primary papillary fibroblasts from one of the female donor previously described, were transduced at a 60% stage of confluency with highly concentrated shNrf2 or shControl lentiviral particules at the multiplicity of infection (MOI) of 5, with 8 µg/ml of polybrene for 8h. The infection was then stopped and cells were left in the incubator to recover with fresh cell culture media during 40h. Cell sorting was then performed using a BD FACSAria™ III flow cytometer (BD Biosciences, CA, USA) to enrich the population of cells with the RFP-positive transduced cells. Population enriched either with shCtrl or shNrf2 transduced fibroblasts were further cultivated through 2 passages. These passages allow them to recover a normal doubling time. shCtrl and shNrf2 fibroblasts were finally harvested for cell banking at the end of passage 6 (P6). Fibroblasts were then thawed at the beginning of each experiment to perform the different analyses at P7.

### **RNA extraction, cDNA synthesis and quantitative real time PCR**

Total RNA was extracted from fibroblasts cell culture either at the end of the 72h for the siRNA experiments or at an 80% stage of confluence for the shRNA experiments after a full medium renewal 24h before. All RNA extractions were performed using the RNeasy kit (Qiagen, Germany), according to the manufacturer's instructions. RNA concentration and quality were assessed using NanoDrop 2000 (Thermo Fischer Scientific, France). cDNA was then synthesized using the iScript cDNA Synthesis kit (Bio-Rad, CA, USA) with 500 ng of total RNA according to manufacturer's instructions. qPCR primers were either manually designed to obtain amplicons between 100 and 150 bp or the QuantiTec primers purchased from Qiagen (Germany). qPCR primers used are listed in Table S4. qPCR was performed according to the manufacturer's instructions using the iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) and a CFX96 Real Time PCR detection system (Bio-Rad, CA, USA). Data were finally analyzed using the  $\Delta\Delta C_t$  method and normalized to three housekeeping genes *rplp0*, *hprt1* and *PPIB*.

## **Transcriptomic analysis by RNA sequencing**

RNA sequencing experiments were performed by the IGFL sequencing platform. RNA samples were prepared as previously described and considering three biological replicates (i.e. 3 donors) by condition (NT, siControl, siNrf2\_1 and siNrf2\_2). Concentration and quality of the RNA samples were assessed using Qubit 2.0 (Thermo Fischer Scientific) and TapeStation 2200 (Agilent Technologies) respectively. All RNA samples used in this study were of high quality with RNA Integrity Number (RIN) higher than 9.4. Barcoded libraries were built using SENSE mRNA-seq Library Prep Kit V2 (Lexogen) following the manufacturer's instructions. Libraries were pooled in equimolar ratios and sequenced on Illumina NextSeq 500 sequencing platform in single end (75 bp). At least 35 million reads per sample were obtained. AltraBio company (Lyon, France) performed the data analyses and generated the results in a friendly format under their proprietary interactive tool, WikiBioPath. Roughly, reads were mapped to the human genome reference GRCh38.p12 using STAR (Dobin et al., 2013). Then, differential gene expression (DGE) analysis was performed using DESeq2 package (Anders and Huber, 2010) with a filtering step (genes that did not have more than 0.339 count per million counts in at least 2 samples were filtered out) and corrections (to remove library size and technical effects). The differentially expressed (DE) genes were further analysed considering various thresholds. Only DE genes with adjusted p-value <0.05 were considered as significantly expressed. The fold change was computed as the ratio of mean expression for siNrf2 and siControl conditions (siNrf2/siControl). Reciprocal ratio was taken for values lower than 1 and reported as negative. Significant DE genes with absolute fold change either >2 or >1.4 were considered for further investigations and according to the analyses. The GOrilla web tool (<http://cbl-gorilla.cs.technion.ac.il/>) was used to identify enriched Gene Ontology (GO) terms of the Biological Process category for the significant DE genes. Finally the functional enrichment analysis (protein-protein interaction networks) was realized using the online STRING database v11.0 (<https://string-db.org/>).

## **Protein extraction and Western Blot**

Total proteins were extracted by scrapping fibroblasts cell culture on ice, in a RIPA lysis buffer (Sigma-Aldrich, MO, USA) supplemented with Halt Protease & Phosphatase inhibitor cocktail (Thermo Fischer Scientific, France). Cell lysates were then left at 4°C for 30 min and centrifuged at 15000 rpm at 4°C during 15 min. Supernatants were collected and protein concentrations were assessed using the BCA protein assay kit (Thermo Fischer Scientific, France). 10 µg of proteins were separated on a 10% precast PROTEAN® TGX™ gel (Bio-Rad, CA, USA) and then transferred to a PVDF membrane (Merck Millipore, Germany) overnight at 4°C. Membranes were further saturated during 1h at RT and incubated with the following

primary antibodies: NRF2 rabbit polyclonal antibody (1/400<sup>e</sup>, PA5-27882, Thermo Fischer Scientific, France), NQO1 monoclonal mouse antibody (1/500<sup>e</sup>, sc-32793, Santa Cruz, US), TRIM16 (1/500<sup>e</sup>, PA5-54847, ThermoFischer, France), and GAPDH rabbit polyclonal antibody (1/1000<sup>e</sup>, 2118S, Cell Signalling, USA). Anti-rabbit and anti-mouse HRP-coupled secondary antibodies (1/10000<sup>e</sup>, Bio-Rad, CA, USA) and the Clarity<sup>TM</sup> Western ECL substrate (Bio-Rad, CA, USA) were finally used to reveal the proteins using a ChemiDoc MP system (Bio-Rad, CA, USA). When needed, membranes were stripped by incubating with the Antibody Stripping buffer (Gene Bio-Application L.T.D, Germany) during 30 min at RT.

### **Redox status analysis**

Redox status was analyzed by measuring ROS levels with dihydrorhodamine 123 which is a reactive oxygen species (ROS) indicator that is oxidized to cationic rhodamine 123 in mitochondria and thus exhibits fluorescence. Transfected fibroblasts are trypsinized and then incubated for 30 min in dark at 37°C, 5%CO<sub>2</sub> with 5µM of dihydrorhodamine 123. Cells are then centrifuged and resuspended in PBS for the analysis by an Epics XL-MCL flow cytometer (Beckman Coulter, CA, USA).

### **Immunofluorescence staining**

Fibroblasts were cultivated on glass cover slips during 4 days for ZNF469, 5 days for extracellular staining of collagen I and 8 days for intracellular staining of collagen I and staining of collagen V. The cell culture media was supplemented with 1 mM of vitamin C (Sigma-Aldrich, MO, US) for the extracellular staining of collagen I and V. Cells were fixed using a 4% paraformaldehyde solution (Thermo Fischer Scientific, France) for 1h at RT; then permeabilized 10 min with 0.1% triton X-100 solution (Sigma-Aldrich, MO, USA) and saturated 20 min in a 3% BSA solution (Sigma-Aldrich, MO, USA). Cells were incubated 1h at RT with the following primary antibodies: rabbit polyclonal antibodies to human ZNF469 (1/25<sup>e</sup>, HPA069784, Sigma, MO, USA), rabbit polyclonal antibodies to human collagen I (1/200<sup>e</sup>, Novotec, Lyon, France), and home-made monoclonal antibody to human collagen V $\alpha$ 1 (Bonod-Bidaud et al., 2012). After 4 washes, incubation of 30 min with secondary antibodies Alexa Fluor 488 coupled (1/1000<sup>e</sup>, Thermo Fischer Scientific, France) and Hoechst 1/2500<sup>e</sup> (Invitrogen, USA) was performed. Glass cover slips were finally washed and mounted in Dako fluorescent mounting medium (Agilent Technologies, France). Observations were performed using a confocal Leica SP8 microscope (Leica, Germany). For ZNF469 immunofluorescence, quantifications of ZNF469 signal in nucleus and total cell were performed using Image J software (Gaussian Blur filter and Yen threshold).

### **Second Harmonic Generation (SHG) microscopy**

Transduced fibroblasts (shCtrl and shNrf2) were cultivated for 8 days in a cell culture medium supplemented with 1 mM Vitamin C (Sigma-Aldrich, MO, US) in 60 mm Petri dishes. Cultures were observed on a Leica SP8 coupled to a Spectra Physics Insight femto second laser, with an HCX APO L 10x/0.3 W objective (Germany). SHG signal emitted from the collagen fibers was acquired by illuminating the samples with a laser at 760 nm and collecting the signal with a non-descanned detector at 380/14 nm. Images were extracted using Image J software. Controls without Vitamin C were also prepared and analyzed, as no collagen fibers were detected, data are not shown.

### **Transmission Electron microscopy (TEM)**

Transduced fibroblasts (shCtrl and shNrf2) were cultivated for 8 days in a cell culture medium supplemented with 1 mM Vitamin C (Sigma-Alrich, MO, US) in 35 mm Petri dishes. Cell cultures were fixed in 2% PFA and 2.5% glutaraldehyde diluted in 0.2 M cacodylate buffer (pH 7.4) 20 min at RT and then overnight at 4°C. Samples were then washed with 0.2 M cacodylate buffer (pH 7.4) (5 min, 3 times). After post-fixation in 1% osmium tetroxide in 0.1 M cacodylate buffer, samples were gradually dehydrated in successive baths of ethanol (from 30% to 100%) and embedded in epoxy resin. 70nm-ultra-thin sections of shCtrl and shNrf2 fibroblasts cultures were performed and stained with 7% uranyl acetate in methanol and lead citrate. Sections were observed with a Philips CM120 electron microscope, equipped with a Gatan Orius 200 2Kx2K camera (Centre Technologique des Microstructures, LyMiC, Université Lyon1, France).

### **Decellularization and Atomic Force Microscopy (AFM)**

For AFM experiments on ECM produced either by shCtrl or shNrf2, samples needed to be decellularized. shCtrl and shNrf2 fibroblasts were cultivated for 8 days in a cell culture medium supplemented with 1 mM Vitamin C (Sigma-Alrich, MO, US) in 35 mm Petri dishes (3 dishes/condition). Samples were washed three times in PBS and then decellularized using the following lysis solution: 0.5% Triton X-100 and 20 mM NH<sub>4</sub>OH in PBS during 5 min at RT. Lysis was followed under the microscope and stopped by adding gently 3 ml of PBS. Samples were washed three times with PBS, fixed in a 4% paraformaldehyde solution (Thermo Fischer Scientific, France) during 1h at RT and then conserved at 4°C in PBS until measurements. AFM experiments were performed by BioMeca (Lyon, France). AFM experiments were carried out with a Bioscope Resolve (Bruker Nano Surface) mounted on a fluorescent microscope (DMi8, Leica, Germany). Elastic modulus measurements were acquired using the PeakForce QNM (Quantitative Nanomechanical Mapping) AFM mode. The foundation of material property

mapping with PeakForce QNM is the ability of the system to acquire and analyze the individual force curves from each tap that occurs during the imaging process. The spring constant of the cantilever used was of 0.37 N/m with a radius < 10nm, according to the manufacturer. The deflection sensitivity of cantilevers was calibrated against a clean silicon wafer. AFM pictures were acquired with a cantilever with a spring constant of 6 N/m. Elastic modulus measurements were made in 1x PBS at room temperature and AFM pictures on dried samples. Each AFM measurement consisted in the analysis of force curves (FC) extracted from 50  $\mu\text{m}$   $\times$  50  $\mu\text{m}$  areas. Each FC is constituted of 10 000 measurements from which the elastic modulus was derived by using the Hertz–Sneddon model (Sneddon, 1965). Three FC were realized per Petri dish, resulting in 9 FC per condition. Aberrant values were eliminated for significant statistical analyses.

## **Bioinformatics analysis**

### *Identification of NFE2L2 motifs in DNA promoter sequences*

DNA sequences of proximal promoters of *NFE2L2* target genes (-1500/+100 bp from the transcription start site (TSS)) were obtained from R Bioconductor packages TxDb.Hsapiens.UCSC.hg19.knownGene, GenomicFeatures and Biostrings. Sequences were scanned for matches to human NFE2L2 motifs (MA0150.1, JASPAR database <http://jaspar.genereg.net/>) using the MEME-FIMO on-line tool (<http://meme-suite.org/tools/fimo>). Only motif occurrences with p-value < 0.0002 were selected.

### *Public ChIP-seq data*

ChIP-seq data targeting *NFE2L2* in human IMR-90 cells were downloaded from the ENCODE data portal (<https://www.encodeproject.org/>) (ENCODE Project Consortium, 2012) (project GSE91565 in the NCBI-GEO database) GRCh38 genome, ENCFF474PPT file giving optimal IDR thresholded peaks, IDR<0.05). Annotation of the 17298 peaks was made the R package ChIPseeker leading to 8932 genes associated with *NFE2L2* ChIP-seq peaks.

## **Statistical analysis**

Results are given as the mean  $\pm$  SEM. Statistics were performed using GraphPad Prism 6 (La Jolla, California, USA). Comparison of qPCR or protein expression used either ANOVA or Student t-test according to the number of conditions to compare. ANOVA for repeated measures and paired t-test were used when appropriate.



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# Supplementary data

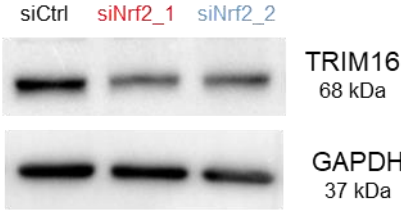
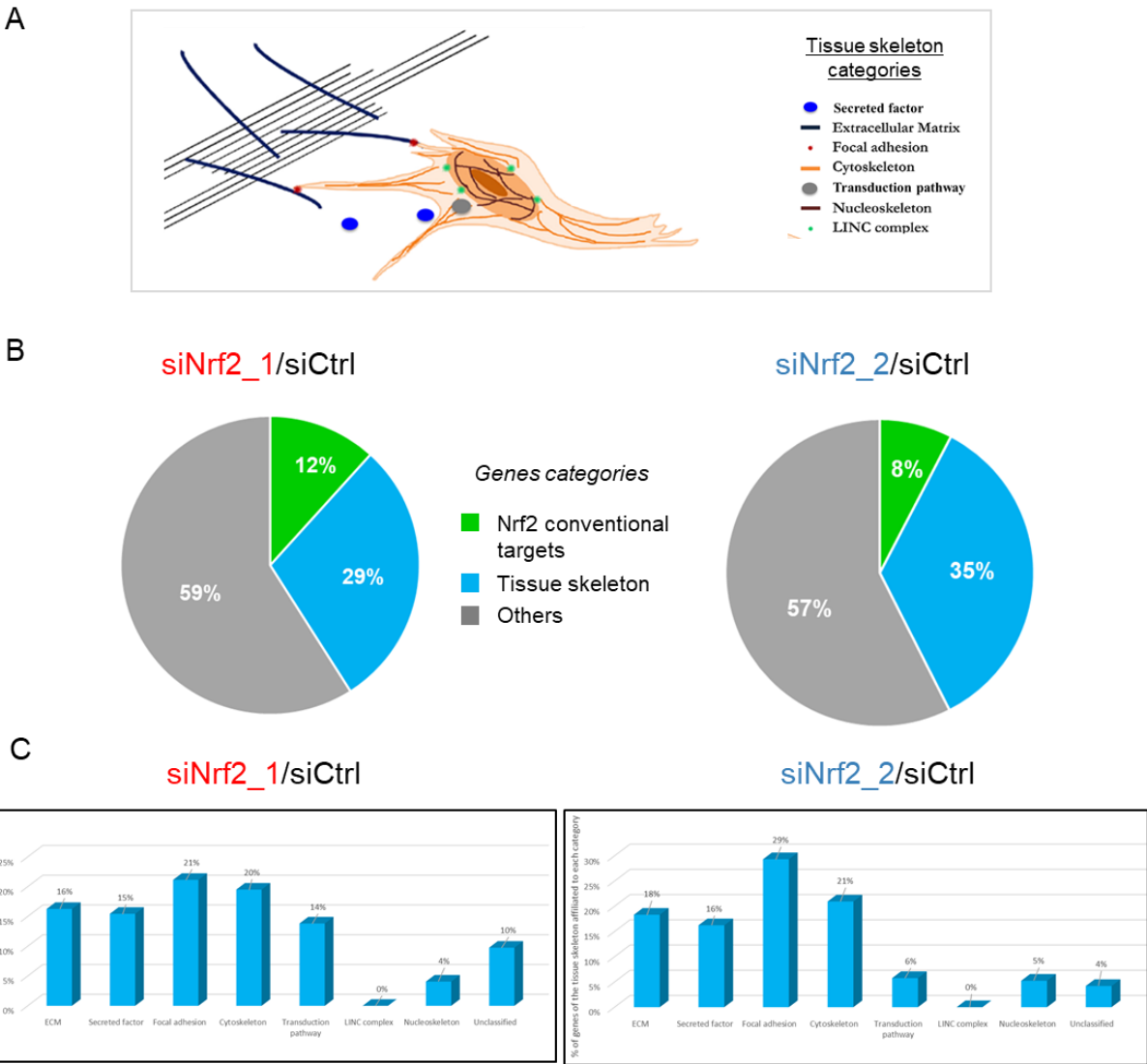
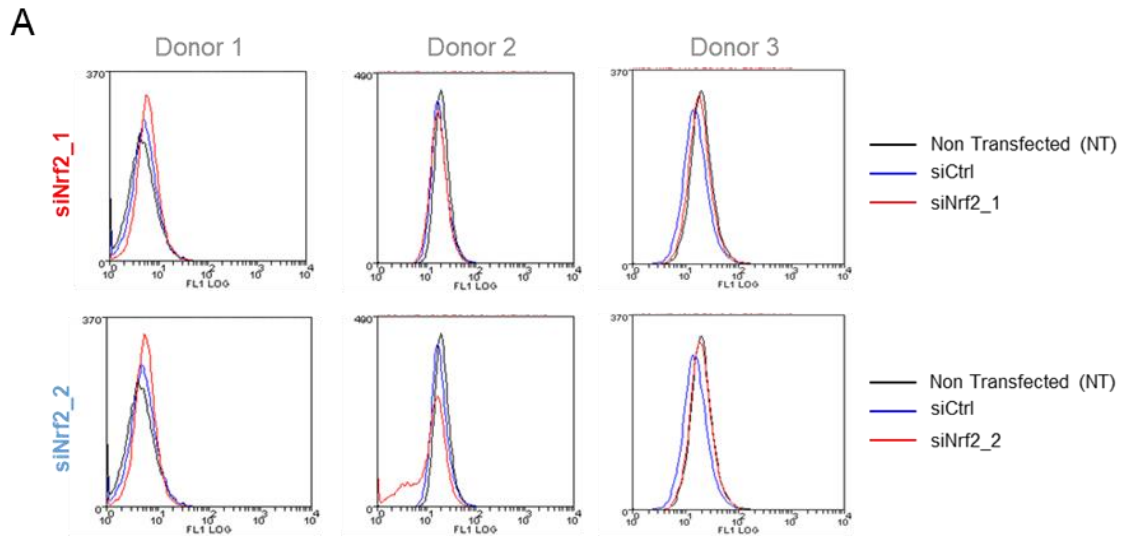


Figure S1: TRIM16 is effectively down-regulated at the protein level by siNrf2. Western blot with anti-TRIM16 antibody on total protein extracts 72h after transfection. Representative WB shown for one donor.



**Figure S2: Expression of 'tissue skeleton' genes are altered in siNrf2 fibroblasts.** (A) Schematic representation of the different tissue skeleton categories, adapted from Haydont *et al.*, 2019 (B) Pie charts in siNrf2\_1 and siNrf2\_2 vs. siCtrl fibroblasts. (C) Tissue skeleton analysis of the genes that are significantly differentially expressed genes in siNrf2\_1 and siNrf2\_2 vs. siCtrl.

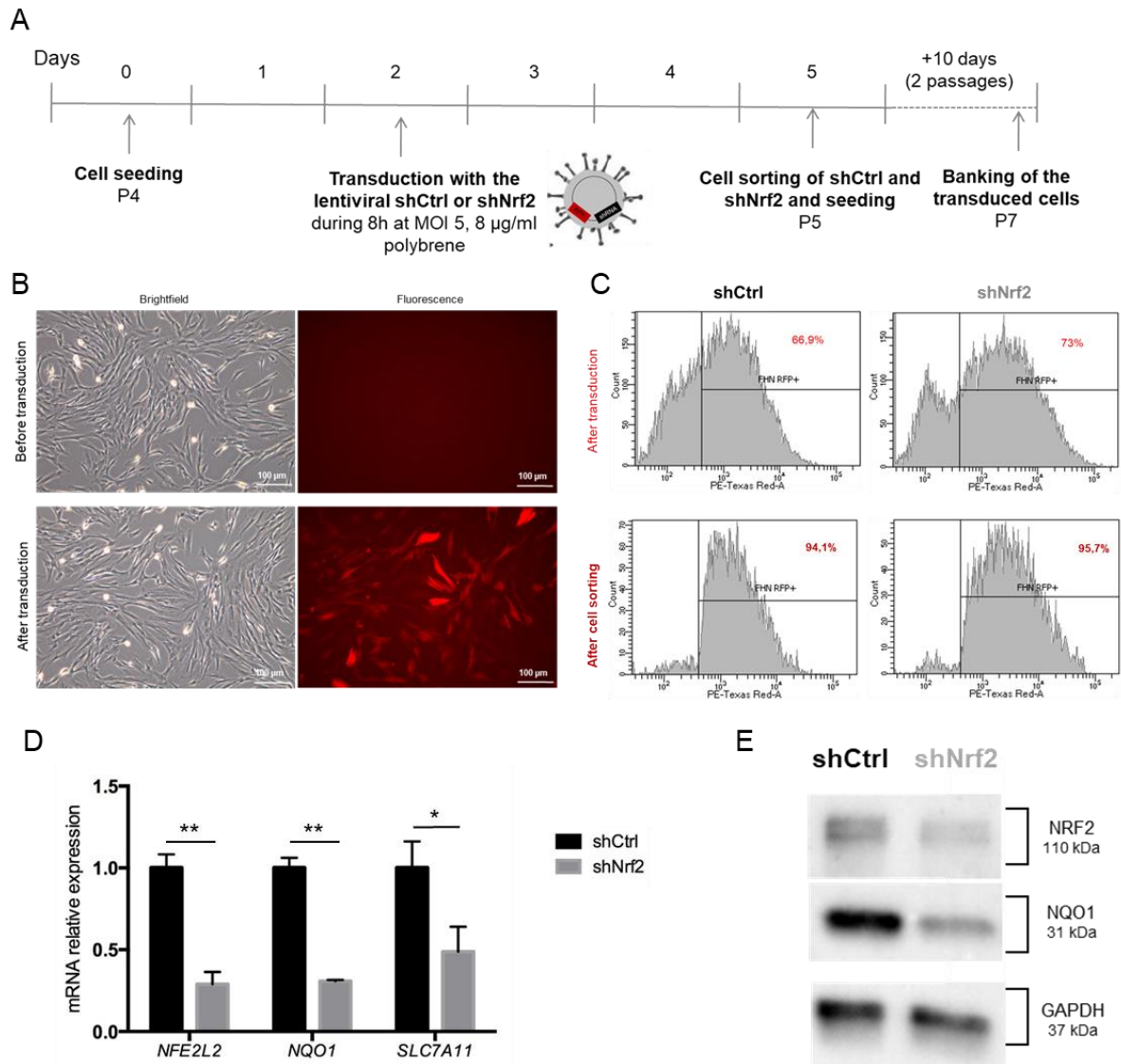




**B**

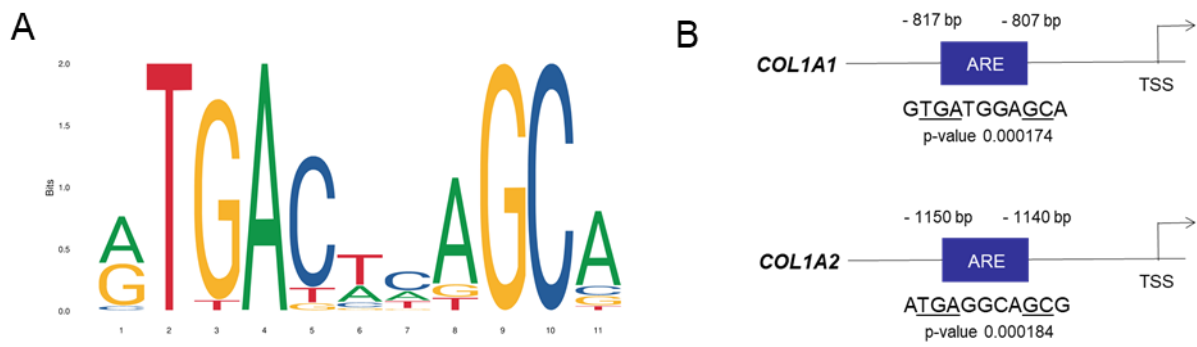
ROS Genes References	Fold change in siNrf2_1 and 2 vs. siCtrl (p-value)		
	CAT	1.096 (0.419)	-1.103 (0.397)
	PPARGC1A	-1.026 (0.963)	-2.676 (0.0685)
St Pierre <i>et al.</i> , 2006	SOD1	1.268 (0.134)	1.207 (0.242)
	SOD2	-1.121 (0.550)	1.072 (0.728)
	SOD3	-1.798 (0.0128)	-1.481 (0.0644)
	GPX1	-1.156 (0.253)	-1.178 (0.209)
Spadoni <i>et al.</i> , 2015	NOX4	1.174 (0.537)	1.5 (0.0929)
Murphy-Mashman <i>et al.</i> , 2017	NOX5	1.280 (0.634)	1.656 (0.268)

**Figure S3: Redox status of siNrf2 fibroblasts remains unchanged compared to the controls.**  
 (A) Intracellular ROS levels of the transfected fibroblasts were determined by flow cytometry using the dihydrorhodamine 123 free radical sensor at 72 hours post-transfection. (B) List of ROS system-genes and their fold changes extracted from our RNA-seq data.



**Figure S4: Lentiviral infection of shNrf2 established a stable fibroblast line effectively downregulated for NRF2.**

(A) Timeline of the protocol used to establish the shCtrl and shNrf2 fibroblasts. (B) Pictures of the cells before and after transduction. (C) Percentage of transduced fibroblasts at the end of the transduction (upper line) and at the end of the cell sorting (lower line) analyzed by flow cytometry. (D) qPCR analysis of *NFE2L2*, *NQO1* and *SLC7A11* in shNrf2 fibroblasts. Statistics were done using a paired t-test ( $n_{\text{experiments}} = 3$ ). Asterisks indicate p-value:  $* \leq 0.05$ ,  $** \leq 0.01$ . (E) Western blots with anti-NRF2 and anti-NQO1 antibodies on total protein extracts in shCtrl and shNrf2 fibroblasts.



**Figure S5: Potential binding sites of *NFE2L2* on *COL1A1* and *COL1A2* genes.**

(A) Human *NFE2L2* transcription motif (MA0150.1) extracted from JASPAR database. (B) Predicted ARE binding sites on *COL1A1* and *COL1A2* human genes.

Nrf2 function categories	Gene	Fold change siNrf2_1/siControl	Fold change siNrf2_2/siControl
Detoxication : phase I and II proteins	<i>NFE2L2</i>	-10.791	-8.550
	<i>ADH1B</i>	-8.885	-8.751
	<i>AKR1C1</i>	-4.336	-2.590
	<i>AKR1C2</i>	-4.523	-2.267
	<i>AKR1C3</i>	-6.040	-3.375
	<i>ALDH3A1</i>	-20.211	-5.828
	<i>ALDH4A1</i>	-2.064	-1.234
	<i>CYP26B1</i>	-3.066	-2.513
	<i>CYP27B1</i>	1.233	2.509
	<i>CYP7B1</i>	-2.286	-1.371
	<i>EPHX1</i>	-2.729	-1.841
	<i>NMRAL2P</i>	-20.763	-14.573
	<i>NQO1</i>	-17.412	-12.629
	<i>MGST1</i>	-4.300	-2.216
Heme and iron metabolism	<i>AMBP</i>	-9.878	-15.710
	<i>FTH1</i>	-2.002	-2.244
	<i>FTH1P10</i>	-1.155	-2.132
	<i>FTH1P11</i>	-1.740	-2.042
	<i>FTH1P12</i>	-2.914	-2.886
	<i>FTH1P20</i>	-2.010	-2.242
	<i>FTH1P23</i>	-2.296	-2.139
	<i>FTH1P3</i>	-1.437	-2.281
	<i>FTH1P4</i>	-2.615	-2.494
	<i>FTH1P5</i>	-2.806	-2.965
	<i>FTH1P7</i>	-1.905	-2.142
	<i>FTL</i>	-4.346	-3.435
	<i>FTLP2</i>	-9.326	-3.777
	<i>FTLP3</i>	-4.825	-4.904
<i>HMOX1</i>	-3.227	-2.895	
<i>PIR</i>	-9.686	-4.072	
Carbohydrate metabolism and NADPH regeneration	<i>G6PD</i>	-2.709	-1.980
	<i>ME1</i>	-1.829	-2.135
	<i>PGD</i>	-2.190	-1.644
	<i>TKT</i>	-3.611	-2.494
Lipid metabolism	<i>ACOT4</i>	-2.018	-1.067
	<i>FADS2</i>	-1.979	-2.520
Gluthation metabolism	<i>GCLM</i>	-1.775	-2.256
	<i>GSTM5</i>	-3.810	-2.926
	<i>SLC7A11</i>	-2.382	-2.757
TXN based system	<i>TXNRD1</i>	-6.001	-6.854
	<i>SQSTM1</i>	-2.197	-1.554
Proteostasis	<i>TRIM16</i>	-6.357	-3.448
	<i>TRIM16L</i>	-9.941	-8.001

**Table S1: Significantly differentially expressed classical targets of NRF2 in siNrf2\_1 and siNrf2\_2 compared to their control.**

Genes have been considered as significantly differentially expressed when the fold change  $\geq 2$  or  $\leq -2$  and the p-value  $< 0.05$ . Genes and their associated fold changes have been classified into the different function ensured by NRF2 according to the literature (Hayes and Dinkova-Kostova, 2014).

Matrisome categories	Gene	Fold change in siNrf2_1 and 2 vs.siCtrl		
Collagens	<i>COL24A1</i>	-4.61	-4.67	DOWN
	<i>COL1A2</i>	-1.46	-1.45	DOWN
	<i>COL1A1</i>	-1.38	-1.47	DOWN
	<i>COL10A1</i>	4.7	3.2	UP
ECM Glycoproteins	<i>FBN2</i>	-2.79	-1.49	DOWN
	<i>NTN1</i>	-2.52	-1.57	DOWN
	<i>IGSF10</i>	-1.67	-1.83	DOWN
	<i>FNDC1</i>	-1.52	-1.55	DOWN
	<i>SVEP1</i>	-1.61	-2.67	DOWN
	<i>ELN</i>	1.52	1.72	UP
	<i>COMP</i>	1.81	1.50	UP
	<i>POSTN</i>	1.55	1.65	UP
Proteoglycans	<i>ESM1</i>	3.16	8.37	UP
ECM regulators	<i>AMBP</i>	-9.88	-15.71	DOWN
	<i>MASP1</i>	-1.55	-3.87	DOWN
	<i>TIMP3</i>	1.56	2.05	UP
	<i>ADAMTS4</i>	1.71	2.18	UP
	<i>TGM2</i>	2.38	2.00	UP
Secreted factors	<i>PIK3IP1</i>	-1.50	-2.15	DOWN
	<i>WNT2</i>	-1.58	-1.67	DOWN
	<i>CCL2</i>	-1.96	-1.90	DOWN
	<i>FGF13</i>	-1.91	-1.83	DOWN
	<i>ANGPTL2</i>	-1.42	-2.17	DOWN
	<i>CXCL3</i>	-7.97	-4.43	DOWN
	<i>IL15</i>	-1.74	-1.82	DOWN
	<i>PDGFD</i>	-1.67	-2.06	DOWN
	<i>CCBE1</i>	-1.49	-1.41	DOWN
	<i>IL11</i>	1.99	1.91	UP
	<i>HBEGF</i>	2.23	1.64	UP
	<i>LIF</i>	3.11	2.38	UP
	<i>ANGPTL4</i>	2.52	2.70	UP
ECM-affiliated proteins	<i>GPC1</i>	-1.75	-1.55	DOWN
	<i>SEMA3A</i>	-1.54	-2.12	DOWN
	<i>COLEC12</i>	-1.65	-1.68	DOWN
	<i>CLEC3B</i>	-1.79	-1.48	DOWN
	<i>CLEC2D</i>	1.96	1.84	UP
	<i>SEMA7A</i>	2.09	2.01	UP

**Table S2: Significant differentially expressed matrisome genes affected by transient NRF2 silencing with siNrf2\_1 (red) and siNrf2\_2 (blue).**

Genes have been considered as significantly differentially expressed when the fold change  $\geq 1.4$  or  $\leq -1.4$  and the p-value  $< 0.05$ . Genes and their associated fold changes have been classified into the different matrisome subcategories defined by Naba and collaborators (Naba et al., 2012a).

	Mission® Custom Plasmid TRCN number	DNA sense sequence (5' to 3')	Vector
<b>shControl</b>	<b>Non Target shRNA</b>	<b>GCGCGATAGCGCTAATAATTT</b>	pLKO.1-U6-shRNA- CMV-TAGRFP
<b>shNrf2 - 1</b>	<b>TRCN0000284998</b>	<b>GCTCCTACTGTGATGTGAAAT</b>	
shNrf2 - 2	TRCN0000273552	CTTGCAATTAATTCGGGATATA	
shNrf2 - 3	TRCN0000284999	CCGGCATTTCACATAAACACAA	
shNrf2 - 4	TRCN0000007556	GCACCTTATATCTCGAAGTTT	
shNrf2 - 5	TRCN0000273494	AGTTTGGGAGGAGCTATTATC	

**Table S3: List of the different shRNA against NRF2 tested and selected (in bold) in primary papillary fibroblasts**

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>NFE2L2</i>	GCAACAGGACATTGAGCAAG	TGGACTTGAACCATGGTAGT
<i>NQO1</i>	ACTGCCCTCTTGTGGTGCAT	GCTCGGTCCAATCCCTTCAT
<i>SLC7A11</i>	GCGTGGGCATGTCTCTGAC	GCTGGTAATGGACCAAAGACTTC
<i>HMOX1</i>	GCCTGGAAGACACCCTAATGTG	GGCCGTGTCAACAAGGATACTT
<i>COL1A1</i>	CACTCCTTCCCAAATCTG	GAGCATTGCCTTTGATTG
<i>COL1A2</i>	GAGGAGAGCCTGGCAACA	GGTCCCTGAGCACCATTG
<i>COL5A1</i>	CCGGATGTCGCTTACAGAGT	CTGCCTTTCTTGGCTTTCAC
<i>PPIB</i>	TGTGGTGTGGCAAAGTTC	GCTTCTCCACCTCGATCTTG
<i>HPRT1</i>	GACCAGTCAACAGGGGACAT	CCTGACCAAGGAAAGCAAAG
<i>RPLP0</i>	GTCAGTGTGCCAGCCCAGAA	TCAATGGTGCCCCTGGAGAT
<i>ZNF469</i>	QT00202566	

**Table S4: List of RT-qPCR primers.**  
Sequences or supplier reference are provided.

## 2. Part 2

**“Role of AMPK in dermis”**

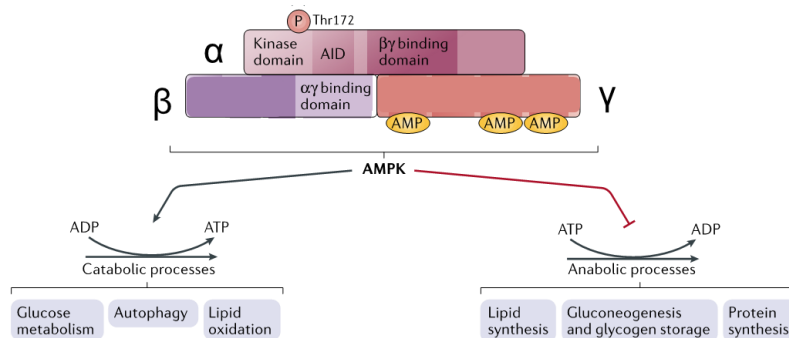
*Additional results*

## Role of AMPK in dermis

As introduced in the preamble, we started to investigate the role of two longevity regulators of *C.elegans* in human dermis. In addition to SKN-1/NRF2, we also explored the role of AAK-2/AMPK in dermis.

### 2.1. Introduction: AMPK, a master regulator of energy metabolism and potential regulator of dermal aging

AMP-activated protein kinase (AMPK) is considered as a master regulator of energy metabolism at the cellular and whole-body level. AMPK is activated during metabolic stress when cell energy levels are low, indicated by a high AMP/ATP ratio. Activated AMPK then readjusts ATP levels by stimulating a number of energy producing metabolic pathways and by inhibiting those requiring energy (Figure 41) (Hardie and Ashford, 2014). It is therefore often referred to as a cellular “fuel gauge”. AMPK is a heterotrimer composed of the catalytically active  $\alpha$  subunit and the regulatory  $\beta$  and  $\gamma$  subunits (Figure 41). Significant activation of AMPK requires phosphorylation at threonine 172 in the AMPK $\alpha$  subunit by an upstream kinase (Hardie et al., 2016). Two isoforms of the AMPK $\alpha$  subunit exist with partially overlapping and cell dependent functions, AMPK $\alpha$ 1 and AMPK $\alpha$ 2 (Viollet et al., 2009).

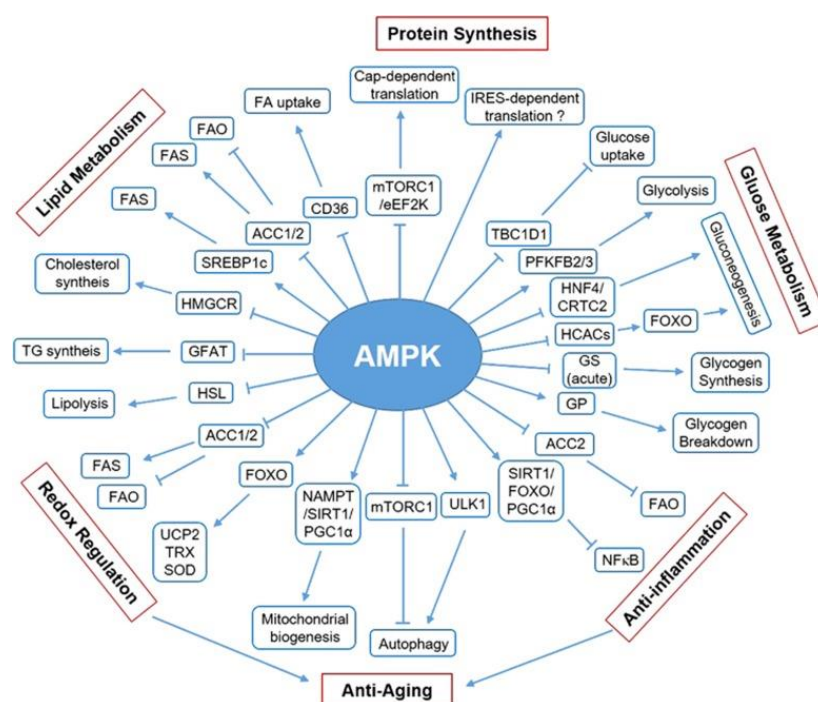


**Figure 41. AMPK structure and activation.**

Domain structure of the AMP-activated protein kinase (AMPK) trimer, showing the catalytic  $\alpha$ -, regulatory  $\beta$ - and  $\gamma$ -subunits. The  $\gamma$ -subunit is responsible for the AMP binding, therefore directly involved in the energetic sensor function of AMPK. The effect of AMPK activation is to rewire metabolism to decrease anabolic processes (that is, ATP consumption) and increase catabolism (that is, ATP production) to restore a more favourable energy balance. AID stands for auto-inhibitory domain. Modified from Herzig and Shaw, 2017.



Numerous studies in model organisms and different cell types have substantiated the importance of AMPK function in general aging and in age-associated or genetic metabolic disorders (Carling, 2017; Reznick et al., 2007; Salminen and Kaarniranta, 2012). As previously introduced, the *C.elegans* homologue of AMPK $\alpha$ , AAK-2 is a master regulator of longevity since its up and downregulation increases and decreases the lifespan of the worm, respectively (Apfeld et al., 2004; Greer and Brunet, 2009). Moreover, in AAK-2 over-expressing worms collagen genes were differentially regulated (Ewald et al., 2015), which suggests a link between AMPK activity and ECM production. Based on additional studies in different model organisms, the proposed anti-aging activity of AMPK is more largely based on its interconnection with signal transduction pathways known to be involved in the aging process, such as the mTOR pathway, the sirtuin 1 pathway, and mitochondrial biogenesis (Figure 42) (Burkewitz et al., 2014). Moreover, AMPK also regulates the redox balance through several mechanisms (Figure 42) (Garcia and Shaw, 2017), including FOXO3A signaling (Ido et al., 2015) and NRF2 activation by direct phosphorylation (Joo et al., 2016). The link between AMPK and NRF2 has indeed already been shown in *C. elegans*, endothelial cells as well as, in rodents (Onken and Driscoll, 2010; Salminen and Kaarniranta, 2012; Wang et al., 2019). Altogether, this prompted us to question the role and status of AMPK in skin aging.



**Figure 42. Metabolic functions of AMPK.**

A schematic summarizing the mechanisms underlying AMPK-induced regulation of diverse metabolic pathways. Arrow indicates activation, and bar-headed line indicates inhibition. From Jeon 2016.

Interestingly, the level of phosphorylated AMPK, measured by western blotting, has shown to be decreased in aged skin (Ido et al., 2015). Moreover, activation of AMPK prevented oxidative stress induced senescence and proliferative dysfunction in a model of primary keratinocytes culture (Ido et al., 2015, 2012). Additional investigations confirmed that AMPK activity decreases in both aged epidermis and dermis layers by performing immunofluorescence staining of on frozen skin sections to evaluate the phosphorylation level of the AMPK target, Acetyl-CoA Carboxylase (ACC) (data not shown).

Brought together, these data reinforced our hypothesis that *C.elegans* longevity regulators could regulate the aging process in human skin. We thus investigated if AMPK downregulation in fibroblasts could mimic the dermal aging process by changing the functional and mechanical properties of dermal ECM. To that end, we used primary dermal fibroblasts that we silenced for AMPK using lentiviral shRNA constructs that were already available and validated in primary human keratinocytes by L'Oréal researchers.

## 2.2. Material and methods

### Fibroblast cell culture

Primary papillary-enriched fibroblasts from breast reduction surgery of a 17 years old woman were purchased at passage 3 (P3) from Episkin (Lyon, France). These primary papillary-enriched fibroblasts were cultivated in Dulbecco's Modified Eagle's Medium (DME)/Ham's Nutrient Mixture F-12 (D8062) (Sigma-Aldrich, MO, USA) supplemented with 10% of Fetal Bovine Serum (Dutscher, France) and 1% of Zell Shield (Minerva Biolabs, Germany) in a 90% humidified incubator with 5% CO<sub>2</sub> at 37°C. Cells were seeded at 3800 cell/cm<sup>2</sup>, unless stated differently, and cultivated until the end of passage 6 (P6).

### shRNA particles

The first lentiviral particles targeting AMPK that we tested were purchased from Vectalys (Toulouse, France). These particles used the expression construct pLV1.H1.shRNA.EF1.Puro.P2A.ZsGreen, in which an shRNA targeting AMPK $\alpha$ 1 and AMPK $\alpha$ 2 was inserted in the shAMPK particles (shAMPK-A); and a shRNA sequence that does not target any known human gene was inserted in the control particles (shCtrl-A).

For the second lentiviral particles that we used to silence AMPK, Custom MISSION<sup>®</sup> shRNA plasmids targeting AMPK $\alpha$ 1 and AMPK $\alpha$ 2 (shAMPK-B) and shRNA control (shCtrl-B)

(SHC016) in pLKO.1-U6-shRNA-CMV-TAGTurboGFP were purchased from Sigma-Aldrich (MO, USA). All shRNAs and loops are listed in [Table 1](#).

	DNA sense sequence (5' to 3')	Loop	Vector
shAMPK-A	ATGATGTCAGATGGTGAATTT	AAGTTCTCT	pLV1.H1.
shCtrl-A	CAACAAGATGAAGAGCACCAA	CTCGAG	shRNA.EF1.Puro.P2A.ZsGreen
shAMPK-B	ATGATGTCAGATGGTGAATTT	CTCGAG	pLKO.1-U6-shRNA-CMV-
shCtrl-B	GCGCGATAGCGCTAATAATTT	CTCGAG	TAGRFP

**Table 1: List of the different shRNA against AMPK tested in primary papillary fibroblasts**

### **Fibroblast transduction**

Primary papillary fibroblasts were transduced at a 60% stage of confluency with highly concentrated shAMPK or shCtrl lentiviral particules at the multiplicity of infection (MOI) of 5 or 10, with 8 µg/ml of polybrene for 8 or 18h. The infection was then stopped and cells were left in the incubator to recover with fresh cell culture media during 40h. Selection, to enrich the population of cells with the GFP-positive transduced cells, was either realized using 1,5 µg/ml of puromycin (InvivoGen, CA, US) during 48 hours or by FACS using a BD FACSAria™ III flow cytometer (BD Biosciences, CA, USA). Cells were either directly frozen at the end of P5 after puromycin selection or further cultivated through 2 passages and frozen at the end of P6 when cell sorting was used. Fibroblasts were thawed at the beginning of each experiment to perform the different analyses.

### **RNA extraction, cDNA synthesis and quantitative real time PCR**

Total RNA was extracted from fibroblasts cell culture at an 80% stage of confluence after a full medium renewal 24h before. All RNA extractions were performed using the RNeasy kit (Qiagen, Germany), according to the manufacturer's instructions. RNA concentration and quality were assessed using NanoDrop 2000 (Thermo Fischer Scientific, France). cDNA was synthesized using the iScript cDNA Synthesis kit (Bio-Rad, CA, USA) with 500 ng of total RNA according to manufacturer's instructions. qPCR primers were either manually designed to obtain amplicons between 100 and 150 bp or QuantiTec primers were purchased from Qiagen (Germany). qPCR primers used are listed in [Table 2](#). qPCR was performed according to the manufacturer's instructions using the iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) and a CFX96 Real Time PCR detection system (Bio-Rad, CA, USA). Data were analyzed using the  $\Delta\Delta C_t$  method and normalized to two housekeeping genes *hprt1* and *PPIB*.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>PRKAA1</i>	AAAGTCGGCGTCTGTTCCAA	TGCAGCATAGTTGGGTGAGC
<i>PRKAA2</i>	ACCAGCTTGCAGTGGCTTAT	ACATCTTGCTTTGGGGCTGT
<i>PPIB</i>	TGTGGTGTTTGGCAAAGTTC	GCTTCTCCACCTCGATCTTG
<i>HPRT1</i>	GACCAGTCAACAGGGGACAT	CCTGACCAAGGAAAGCAAAG

**Table 2: List of RT-qPCR primers.**

### **Protein extraction and Western Blot**

Cultured adherent fibroblasts were washed three times with cold PBS buffer on ice. The cells were scraped on ice in a RIPA lysis buffer (Sigma-Aldrich, MO, USA) supplemented with Halt Protease & Phosphatase inhibitor cocktail (Thermo Fischer Scientific, France). Cells were lysed at 4°C for 30 min and then centrifuged at 15000 rpm at 4°C during 15 min. Supernatants were collected and protein concentrations were assessed using the BCA protein assay kit (Thermo Fischer Scientific, France). 10 µg of proteins were separated on a 10% precast PROTEAN® TGX™ gel (Bio-Rad, CA, USA) and then transferred in a liquid transfer system using CAPS 1X (Sigma-Aldrich, MO, USA) supplemented with 20% of ethanol as transfer buffer overnight at 4°C to a PVDF membrane (Merck Millipore, Germany). Membranes were saturated during 1h at RT with 5% milk in PBS buffer and incubated with the following primary antibodies: AMPKα1 rabbit polyclonal antibody (1/1500<sup>e</sup>, 2795, Cell Signaling, USA), AMPKα2 rabbit polyclonal antibody (1/1000<sup>e</sup>, GTX103487, GeneTex, US), acetylated tubulin mouse monoclonal antibody (1/10000<sup>e</sup>, T6793, Sigma-Aldrich MO, USA) and actin monoclonal mouse antibody (1/7000<sup>e</sup>, MAB1501, Merck Millipore, Germany). Anti-rabbit and anti-mouse HRP-coupled secondary antibodies (1/10000<sup>e</sup>, Bio-Rad, CA, USA) and the Clarity™ Western ECL substrate (Bio-Rad, CA, USA) were used to reveal the proteins using a ChemiDoc MP system (Bio-Rad, CA, USA).

### **MTT proliferation assay**

Fibroblasts were plated in a 96-well plate at a density of 5000 cell/well and incubated in a 90% humidified incubator with 5% CO<sub>2</sub> at 37°C. Cells were cultured during 7 days, and the cell culture medium was renewed every two days. Cell proliferation was measured on day 1, 3, 5 and 7. For each of these measurements, cell culture medium was discarded and 100 µL of fresh medium were added in the well with 10 µL of the MTT solution (V-13154, Vybrant MTT Cell Proliferation Assay Kit, Thermo Fischer, France) and incubated during 4 hours at 37°C. Formazan crystals were solubilized by adding 50 µL of DMSO per well and incubating

the plate during 10 min at 37°C. The color product was quantified spectrophotometrically by measuring absorbance at 540 nm using a plate reader (EnSpire Multimode Plate Reader, Perkin-Elmer, UK).

### **Reconstruction of skin equivalent and analyses**

The reconstruction of the three skin models and their respective analyses have been realized by Episkin (Lyon, France). Briefly, 1) a dermis-equivalent was reconstructed by cultivating  $5 \cdot 10^5$  fibroblasts in a collagen lattice during 18 days in RealSkin™ culture medium, with a medium renewal every two days; 2) a second dermis-equivalent was reconstructed by seeding  $2 \cdot 10^6$  fibroblasts into a polystyrene scaffold during 21 days in an appropriate cell culture medium, with a medium renewal every two days; 3) a full-skin model was reconstructed by seeding  $2 \cdot 10^6$  fibroblasts into a polystyrene scaffold during 14 days in an appropriate cell culture medium, with a medium renewal every two days. Normal human primary keratinocytes (NHK) were cultured for 11 days on the dermal equivalent at the air-liquid interface in an appropriate cell culture medium, with a medium renewal every two days.

For the contractility test using skin model 1), pictures of the collagen lattices were taken at day 1, 4, 5, 11 and 18 of culture and fibroblast contractility capacities were measured by the diameter of the lattice, that was realized using Image J software. Rheological parameters: the storage modulus ( $G'$ ) in Pascal and the phase angle ( $\delta$ ) in degrees, of model 2) were measured using a Malvern rheometer (Malvern Panalytical, Germany). The full thickness model 3) was analyzed at day 28 with Optical coherence tomography (OCT, according to the manufacturer's instructions) to measure epidermal thickness and hematoxylin and eosin staining (HES) to visualize global morphology.

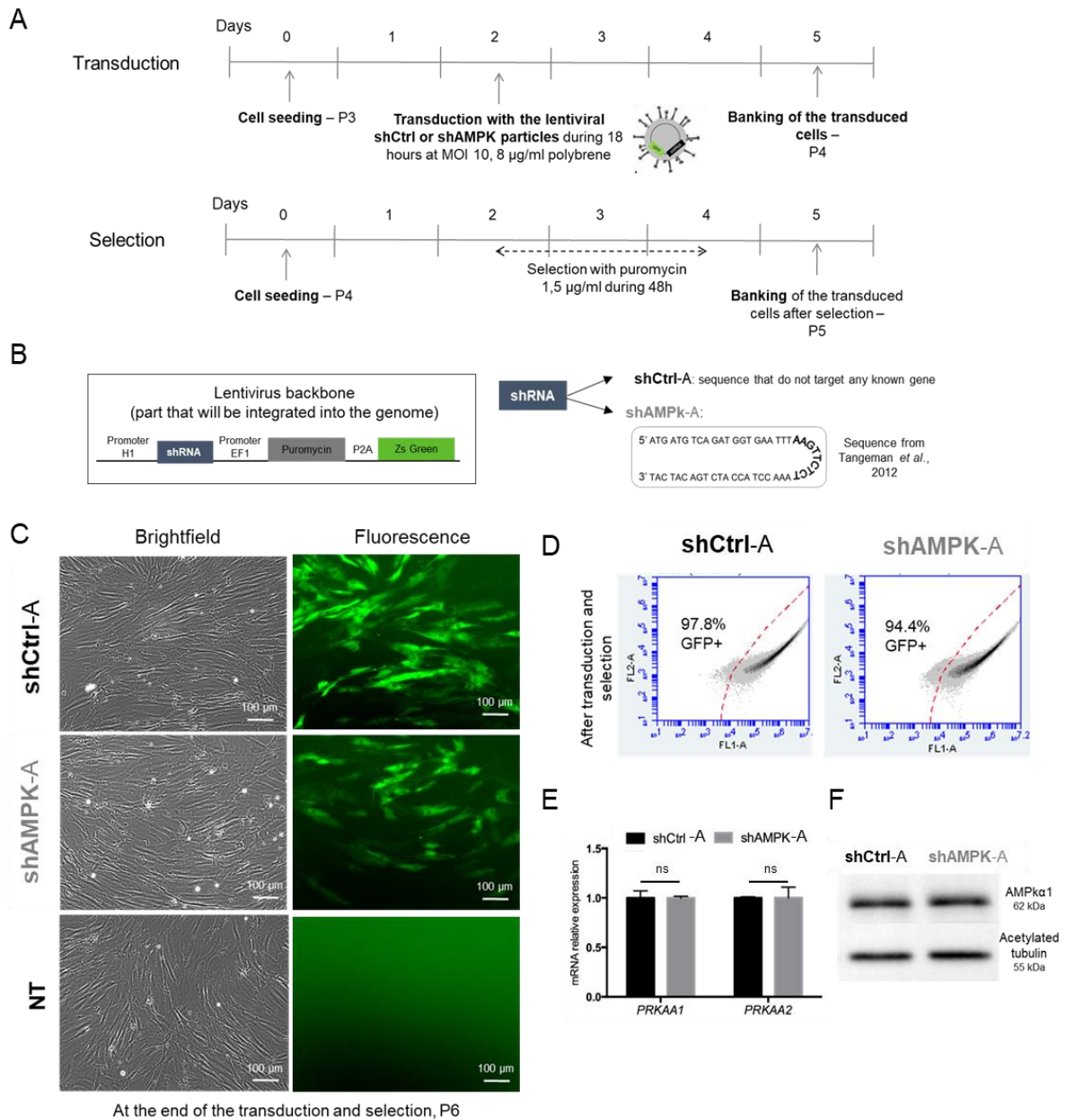
### **Statistical analysis**

Results are given as the mean  $\pm$  SEM. Statistics were performed using GraphPad Prism 6 (La Jolla, California, USA). Comparison of qPCR were done using Student t-tests.

### 2.3. Results

#### The shRNA construct silencing AMPK in keratinocytes is not effective in fibroblasts

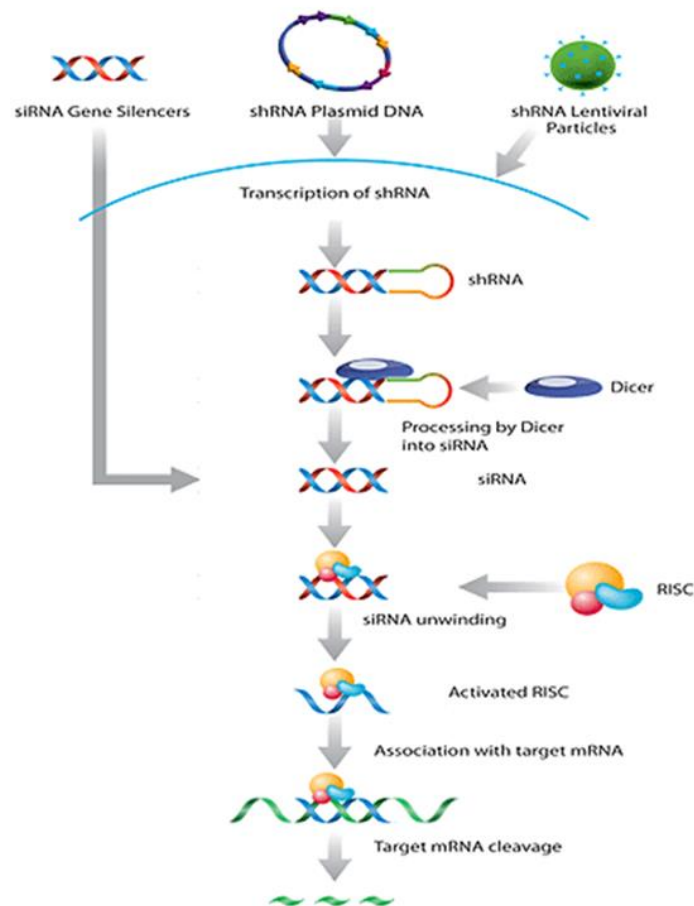
We used lentiviral particles containing shRNA targeting both the  $\alpha 1$  and  $\alpha 2$  catalytic subunits of the AMPK (shAMPK-A), which are respectively encoded by the *PRKAA1* and *PRKAA2* genes (Tangeman et al., 2012). We transduced primary papillary-enriched skin fibroblasts with these particles and selected the cells that integrated the lentiviral backbone into their genome by using puromycin (Figure 43A). Zs Green protein was used as a fluorescent marker to validate the transduction by fluorescence microscopy and Fluorescence-Activated Single Cell Sorting (FACS) (Figure 43B). Transduced fibroblasts were highly fluorescent under the microscope compared to the control cells (Non Transduced, NT) (Figure 43C). Additionally, the FACS analyses revealed that 97.8% and 94.4% of the fibroblast population respectively transduced with shCtrl-A and shAMPK-A particles were GFP-positive (Figure 43D), confirming a high transduction efficiency. However, neither AMPK $\alpha 1$  nor AMPK $\alpha 2$  was down-regulated at the mRNA level as measured by RT-qPCR (Figure 43E). Since we identified that AMPK $\alpha 1$  as the major catalytic isoform in human skin fibroblasts (data not shown) we analyzed its protein expression by western blotting. We did not detect any change in AMPK $\alpha 1$  protein levels between extracts from shAMPK-A and shCtrl-A fibroblasts (Figure 43F). Hence, although validated in primary human keratinocytes, these lentiviral particles cannot be used to silence AMPK in primary human skin fibroblasts.



**Figure 43: Lentiviral infection of shAMPK-A in primary papillary-enriched fibroblasts does not silence AMPK.**

(A) Timeline of the transduction and selection protocol to generate the shCtrl-A and shAMPK-A fibroblasts. (B) Cartography of the part of the lentivirus backbone that will be integrated into the transduced cell, and shRNA sequences. (C) Pictures of the fibroblasts at the end of the transduction and selection (P6). (D) Percentage of transduced fibroblasts at the end of the transduction and selection, at P6, analyzed by flow cytometry. (E) qPCR analysis of *PRKAA1* and *PRKAA2* in shCtrl-A and shAMPK-A fibroblasts at P6. Statistics were done using an unpaired t-test ( $n_{\text{experiments}} = 3$ ).  $p\text{-value} > 0,05$ : ns. (F) Western blots with anti-AMPK $\alpha$ 1 antibody on total protein extracts from shCtrl-A and shAMPK-A fibroblasts at P6.

We developed two main hypotheses to explain our negative results: 1) the promoter might not be adapted for primary fibroblasts and 2) the shRNA loop might not allow successful shRNA processing in fibroblasts. The H1 promoter and the human U6 small nuclear promoter (U6) are the two most commonly used promoter for the transcription of short RNA sequences, as they specifically recruit the RNA Polymerase III. It has been previously shown that the U6 promoter is a stronger promoter than the H1 promoter (Mäkinen et al., 2006; Singer and Verma, 2008). We reasoned that effective silencing in fibroblasts might require the U6 promoter upstream of the shRNA sequence. The shRNA loop is another key parameter in competent gene silencing (Pang et al., 2018; Schopman et al., 2010). After transcription, the shRNA is processed by Dicer, which digests the loop and transforms the shRNA into a siRNA. The siRNA is further unwound by the RNA-Induced Silencing complex (RISC complex), which targets it to the specific mRNA destined for degradation. This finally results in the inhibition of the protein expression (Figure 44). Hence, the recognition of the loop by Dicer directly controls the shRNA performance. These two hypotheses guided us to design new lentiviral particles.



**Figure 44. Mechanism of shRNA based gene silencing.**  
From <https://www.scbt.com/fr/whats-new/gene-silencers>.

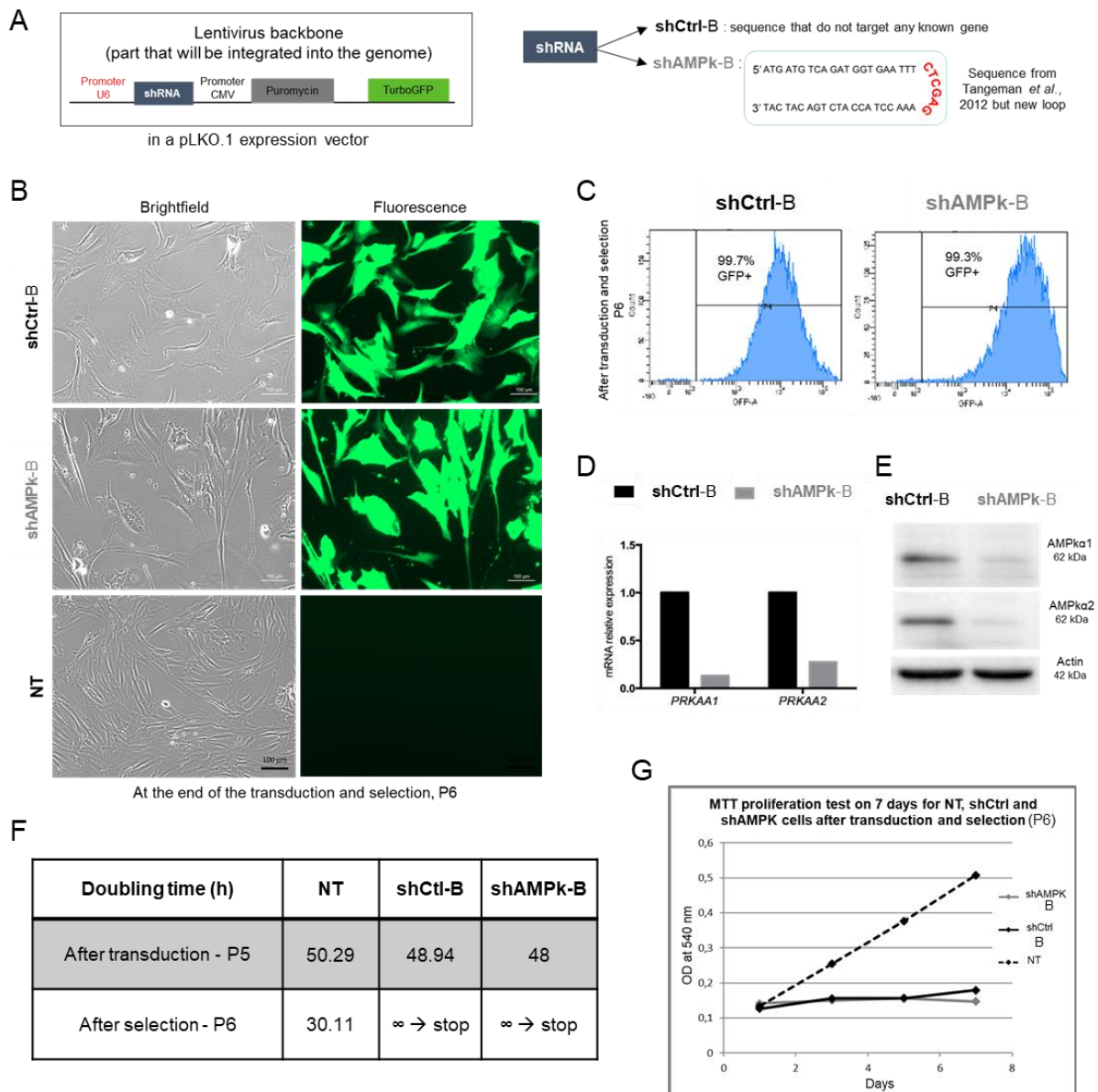


## **Newly designed lentiviral particles effectively silence AMPK expression but induce cellular toxicity**

We thus designed new lentiviral particles comprising an U6 promoter and the same shRNA sequence but fused to a different loop (Figure 45A). These new constructs expressed turbo GFP as a fluorescent marker, and will be named shCtrl-B and shAMPK-B lentiviral particles.

Using the same protocol as before (Figure 45A), we transduced and selected primary dermal fibroblasts. The transduction and selection resulted in almost 100% GFP-positive cells in shCtrl-B and shAMPK-B transduced fibroblasts (Figures 45B and C). AMPK $\alpha$ 1 and AMPK $\alpha$ 2 genes were effectively silenced at the mRNA (Figure 45D) and protein levels (Figure 45E), which confirmed the success of our strategy to improve the efficiency of the constructs.

Unfortunately, the shCtrl-B and shAMPK-B fibroblasts rapidly ceased proliferating after selection indicating that this procedure conferred cytotoxicity to primary dermal fibroblasts. We confirmed this observation by calculating the cell doubling times (Figure 45F) and by performing MTT test (Figure 45G). Hence, we further tried to optimize the transduction conditions in order to maintain an effective silencing of AMPK without inducing cytotoxicity.



**Figure 45: Lentiviral infection of shAMPK-B in primary skin fibroblasts silences AMPK but induces toxicity.**

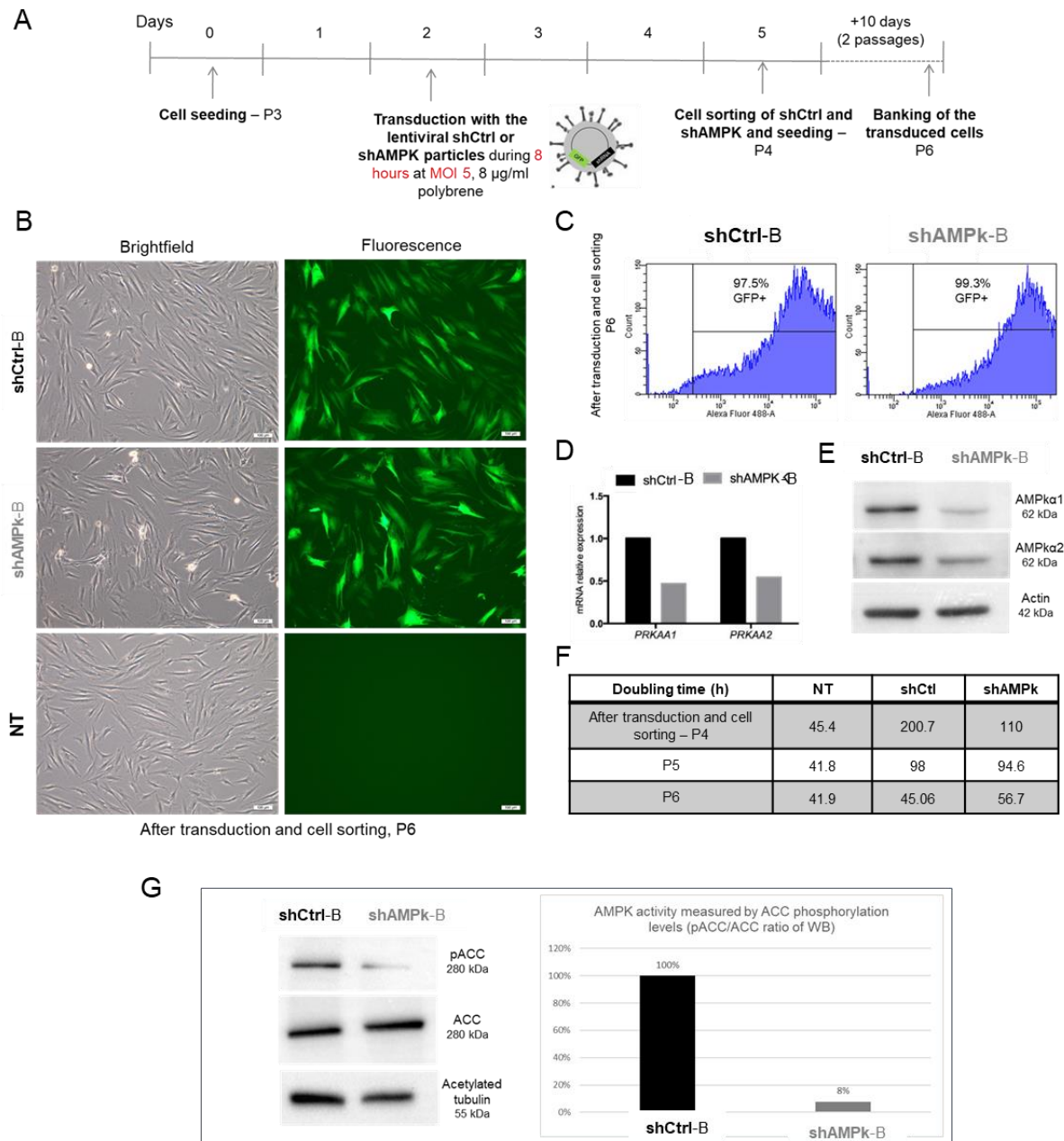
(A) Cartography of the part of the lentivirus backbone that will be integrated into the genome of the transduced cells with the new promoter U6 in red, and the shRNA sequence with the new shRNA loop in red for the shAMPK lentiviral particles. (B) Pictures of the fibroblasts at the end of the transduction and selection at P6. (C) Percentage of transduced fibroblasts at the end of the transduction and selection, at P6, analyzed by flow cytometry. (D) RT-qPCR analysis of *PRKAA1* and *PRKAA2* in shCtrl-B and shAMPK-B fibroblasts after selection at P6,  $n_{\text{experiment}}=1$ . (E) Western blots with anti-AMPK $\alpha_1$  and  $\alpha_2$  antibodies on total protein extracts from shCtrl and shAMPK fibroblasts after selection, at P6. (F) Doubling times of shCtrl and shAMPK fibroblasts compared to the control (Non Transduced, NT). (G) MTT proliferation test of shCtrl-B and shAMPK-B fibroblasts compared to the NT fibroblasts on 7 days, after transduction and selection at P6.

## Optimization of the transduction conditions to maintain effective but non-toxic AMPK silencing in human skin fibroblasts

Several critical experimental parameters in the transduction protocol can contribute to cytotoxicity issues: the infection period; the multiplicity of infection (MOI); and the concentration of polybrene, which facilitates the penetration of the lentiviruses into the cells by neutralizing the charge repulsion between virions and the cell surface. We tried several protocols to optimize these parameters. We finally selected a protocol with an unchanged concentration of polybrene, while we decreased the MOI from 10 to of 5 and the transduction time from 18 hours to 8 hours (Figure 46A). Additionally, we selected the transduced primary fibroblasts by FACS to better standardize our protocol.

Using this new protocol, shCtrl-B and shAMPK-B fibroblasts were still highly enriched in GFP-positive cells, after transduction and cell sorting (Figure 46B and C). We then confirmed that expression level of AMPK $\alpha$ 1 and AMPK $\alpha$ 2 mRNAs was reduced by about 50%, and the protein levels by 60% (Figure 46D, E quantification not shown). Importantly, although shCtrl-B and shAMPK-B fibroblasts displayed lower proliferation rates just after transduction and cell sorting compared to controls, they recovered to an almost normal proliferation rate with a doubling time of 45h and 57h for shCtrl-B and shAMPK-B fibroblasts at P6, respectively, compared to 42h doubling time of P6 NT fibroblasts (Figure 46F).

We further confirmed the functionality of the AMPK silenced fibroblasts by analyzing the phosphorylation of ACC by western blotting. The results showed that AMPK silencing also reduces AMPK activity as phosphorylation of ACC was decreased in shAMPK-B cells compared to controls (Figure 46G).



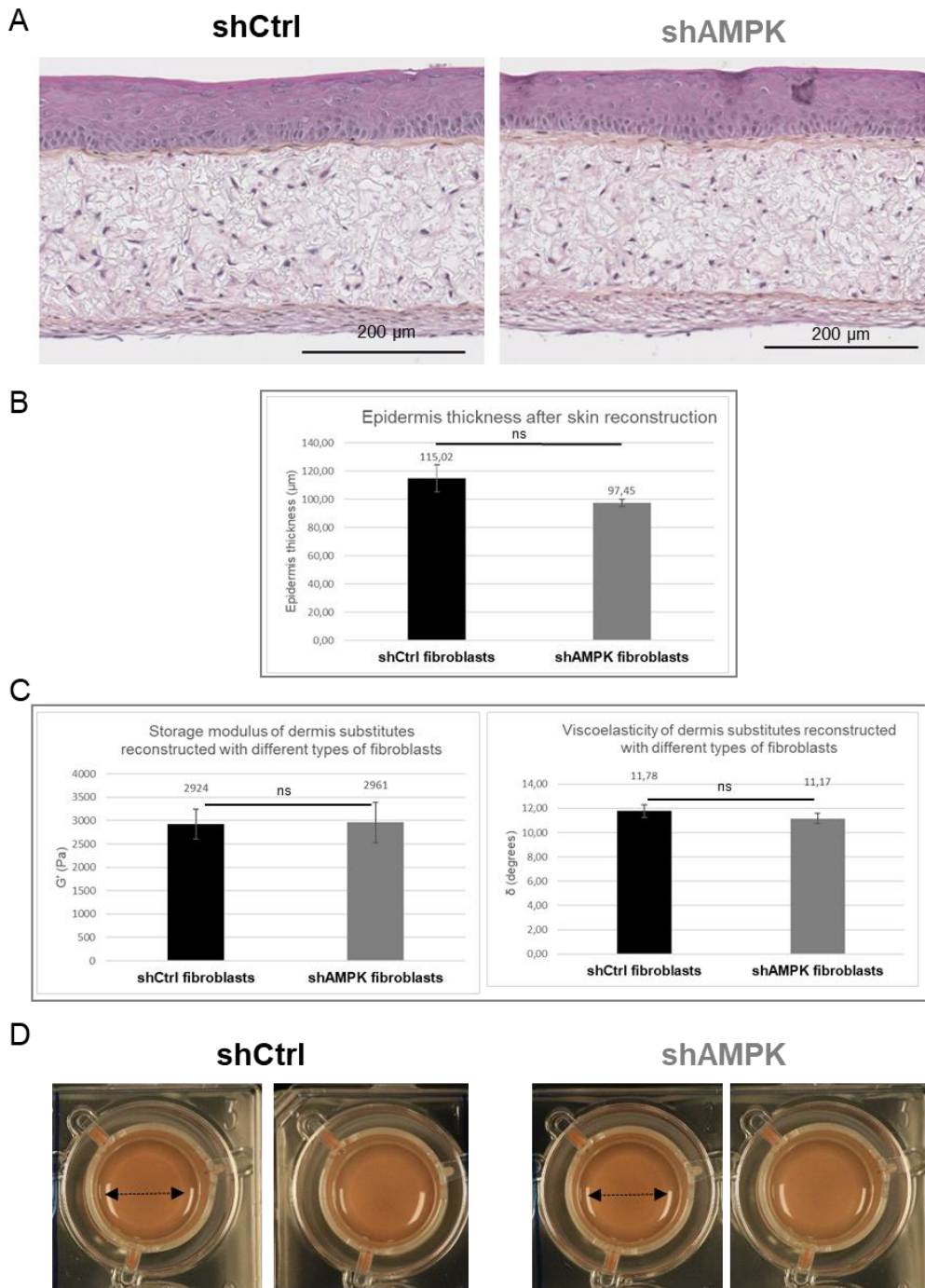
**Figure 46: Generation of a stable shAMPK-B primary dermal fibroblast line.**

(A) Timeline of the newly defined transduction protocol to generate shCtrl-B and shAMPK-B fibroblasts. (B) Pictures of the fibroblasts after transduction and cell sorting at P6. (C) Percentage of transduced fibroblasts after transduction and cell sorting (P6), analyzed by flow cytometry. (D) RT-qPCR analysis of PRKAA1 and PRKAA2 in shCtrl-B and shAMPK-B fibroblasts after transduction and selection (P6),  $n_{\text{experiment}} = 1$  (E) Western blots with anti-AMPK $\alpha$ 1 and  $\alpha$ 2 antibodies on total protein extracts from shCtrl and shAMPK fibroblasts after transduction and selection at P6. (F) Doubling times of shCtrl and shAMPK fibroblasts compared to the NT control. (G) Activity of AMPK measured through phosphorylation levels of ACC. Densitometry of western blotting signals of pACC and ACC.

## Living skin equivalents containing AMPK-silenced fibroblasts did not display an apparent aging phenotype

We further used our shCtrl-B and shAMPK-B fibroblast lines (for an easier reading shCtrl-B and shAMPK-B will further be named shCtrl and shAMPK respectively) to reconstruct three different skin models: 1) a dermis-equivalent composed of a collagen lattice, 2) a dermis-equivalent composed of a polystyrene matrix allowing the fibroblasts to secrete their proper ECM, and 3) a full thickness skin equivalent using the polystyrene matrix. For the latter, the transduced fibroblasts were seeded into the polystyrene matrix for dermal reconstruction. Then, normal human primary keratinocytes were seeded on top of the dermis equivalents to develop an epidermis. As the communication between fibroblasts and keratinocytes is known to be affected by aging (Cole et al., 2018; Jevtić et al., 2020; Marionnet et al., 2006), we analyzed, the epidermis formation and keratinocyte morphology in shAMPK skin equivalents compared to the shCtrl ones as a readout of the potential “aging” effect of AMPK silencing in primary dermal fibroblasts. Hematoxylin-eosin stainings (HE) did not reveal any obvious histological differences. In both models, the epidermis was properly differentiated (Figure 47A). However, we observed a slight albeit not significant decrease in the epidermal thickness of the shAMPK skin equivalents compared to shCtrl ones (Figure 47B). As epidermal thinning is one of the characteristics of aged skin (Mine et al., 2008), this result warrants further investigation.

There was no obvious difference in the HE staining of the dermal compartments of the full thickness skin equivalents from the two conditions. Hence, AMPK silencing in fibroblasts does not strikingly affect ECM synthesis and organization. Moreover, we found no difference in the rigidity and viscoelasticity properties, respectively measured by the  $G'$  and  $\delta$  parameters, between the dermal equivalents reconstructed with shCtrl *versus* shAMPK fibroblasts in the polystyrene matrix (Figure 47C). We conclude that AMPK silencing does not affect the biomechanical properties of the dermal equivalent. Finally, shCtrl and shAMPK fibroblasts were evaluated for their capacity to contract a collagen gel, which has been shown to increase with aging for papillary fibroblasts (Mine et al., 2008). There was no obvious difference in lattice diameter when they are seeded with shAMPK or shCtrl fibroblasts, therefore suggesting no difference in contraction capacities between shCtrl and shAMPK fibroblasts (Figure 47D).



**Figure 47: Skin equivalent reconstructed with shCtrl and shAMPK fibroblasts do not display any significant morphological difference nor alteration in their biomechanical properties.**

All of these experiments were conducted with shCtrl-B and shAMPK-B. (A) Hematoxylin-eosin staining of reconstructed skin models with shCtrl and shAMPK fibroblasts and normal human keratinocytes. (B) Quantification of the epidermal thickness after skin reconstruction on the skin models showed in A. Statistics were done using an unpaired t-test ( $n_{\text{measurements}} = 9$  on 3 different tissues),  $p\text{-value} > 0,05$ : ns. (C) Rheology measurements of the storage modulus ( $G'$ ) and viscoelasticity ( $\delta$ ) properties of dermal equivalents reconstructed with shCtrl and shAMPK fibroblasts. Statistics were done using an unpaired t-test ( $n_{\text{tissues}} = 5$ ),  $p\text{-value} > 0,05$ : ns. (D) Pictures of fibroblasts in a collagen gel. The diameter (double arrow line) of the collagen gel, or lattice, is measured to assess fibroblasts contractile capacities.

## 2.4. Conclusions

Together, these results do not indicate any obvious differences between shCtrl and shAMPK fibroblasts regarding their ECM production or contractile capacities. However, we cannot exclude that we partly lost silencing of AMPK in the skin models, since we did not validate AMPK silencing in skin equivalent samples. It would be interesting to pursue the analysis though using immunofluorescence stainings on specific ECM markers. The slight effect observed on epidermal thickness should be further confirmed by additional experiments, as, it is a really interesting lead that deserves further investigation. However, as 3D models are time consuming and expensive, I was not able to repeat these preliminary results during the course of my thesis. These preliminary data should be further completed to be able to state about AMPK status in dermal aging.

### **III. General discussion and perspectives**



Being first considered as a structural component only, ECM has been clearly established now as a mediator of various fundamental biological functions such as cell proliferation, adhesion, and differentiation, and it provides a unique platform for cell signaling (Frantz et al., 2010; Theocharis et al., 2016). The ECM is vital since it ensures the cohesion and homeostasis of all mammalian organs and tissues. ECMs are highly dynamic structures that display diverse compositions, structures, stiffness and abundance. ECM in skin and particularly in the dermis ECM is a very good example of this. The most abundant ECM proteins are members of the collagen superfamily, on which we had the great opportunity to write a review (Salamito et al., in press) that can be found in annex of this manuscript. Dysregulation of collagen expression and/or alteration of collagen-containing networks are associated with acquired disorders (fibrosis, cancer) and genetic diseases called collagenopathies but also with chronological and biological aging (Salamito et al., in press); (Theocharis et al., 2019). Hence, deciphering the role of individual ECM components and their networks in tissues as well as identifying their main regulators are paramount in fundamental and biomedical research.

During my PhD, we identified the transcription factor NRF2 as potent regulator of ECM gene expression in human skin primary fibroblasts. NRF2 is well known for its main role in cellular defense against oxidative and xenobiotic stresses in humans and many other species. However, NRF2 functions and targets keep on extending, and ECM regulation in non-stressed conditions was recently suggested as one of them (Blackwell et al., 2015; Dodson et al., 2019). It was originally demonstrated by Ewald and collaborators that SKN-1, the worm homologue of NRF2, regulates collagen genes expression under specific metabolic conditions and this results in an increased longevity of the worms (Ewald et al., 2015). Further work using constitutive overexpression of NRF2 in mouse skin fibroblasts substantiated this finding in a mammalian system (Hiebert et al., 2018). Along this line, my PhD work further established the role of NRF2 in the regulation of matrisome genes in human skin primary fibroblasts. I showed using different RNA interference approaches, that NRF2 silencing in these fibroblasts impacts matrisome genes, and more specifically decreases expression and production of the fibrillar collagen I, which is the most abundant collagen in the dermis. This decrease further affects the organization and biomechanical properties of the ECM, since fibroblasts with a reduced NRF2 activity produced thinner collagen fibrils resulting in a matrix with slightly reduced rigidity properties compared to control fibroblasts (Figure 48). The thinner diameter of the fibrils assembled in the shNRF2 fibroblasts extracellular space could be explained by an altered COL1 to COLV ratio, a key factor of the collagen fibril diameter regulation in chicken corneal fibroblasts (Marchant et al., 1996) and in mice models for classical EDS (Chanut-Delalande et al., 2004; Wenstrup et al., 2004). The present work unravels a critical role for NRF2 in human

dermal ECM homeostasis. My PhD data further point to possible direct and indirect mechanisms of action of NRF2 on ECM gene though the underlying mechanisms still remain elusive and require further investigation (Figure 48). As such, our transcriptomic analysis identified ZNF469 as a target of NRF2 in dermal fibroblasts, a poorly documented factor that has been shown to act as a potential ECM regulator. This avenue clearly needs to be further explored.

Hence, as the main perspective of this work, I will discuss our current hypotheses about potential mechanism of action that underpins ECM gene expression; and then put our results and learning into the larger context of the NRF2 and ECM fields.

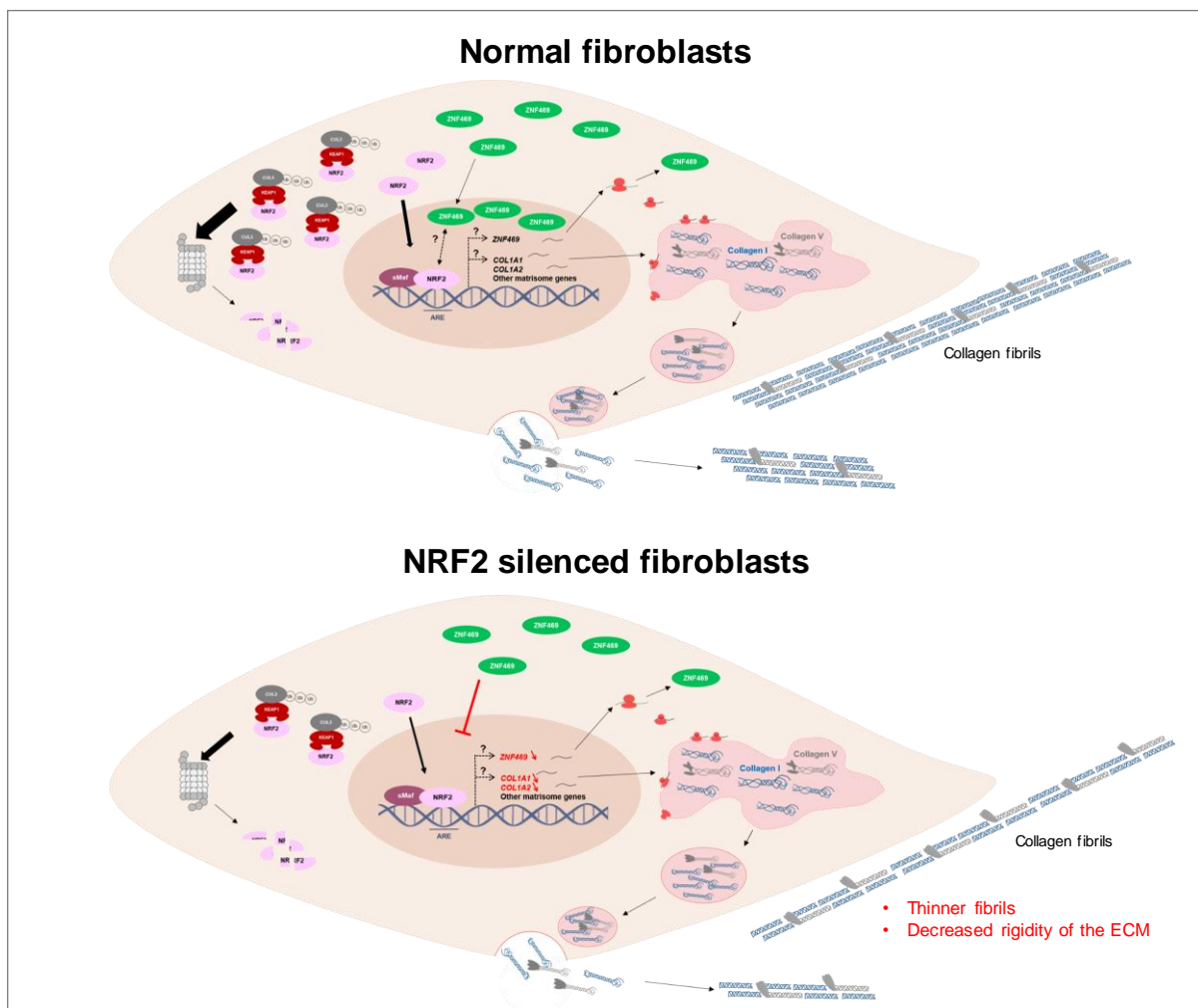


Figure 48: Schema illustrating the different results obtained and the remaining questions.

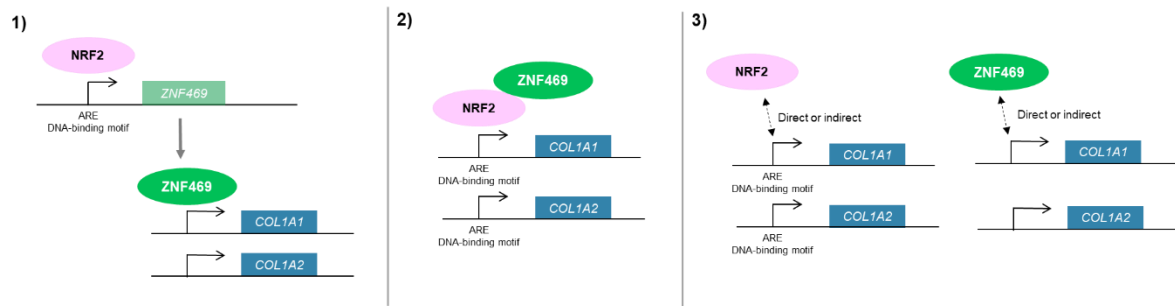
## 1. Mechanism of action of NRF2 on ECM genes: what should we do next

We showed that NRF2 is able to regulate the expression of ECM genes and especially of collagen I genes, *COL1A1* and *COL1A2*, and that the observed effect was independent of ROS. Our analysis of public NRF2-ChIP data in human IMR-90 fibroblasts and bioinformatics analyses identified NRF2 binding peaks and NRF2 DNA binding sequences in *COL1A1* and *COL1A2* gene proximal promoters. This therefore suggests collagen I genes as potential direct NRF2 target genes. In addition, combined global NRF2 ChIP-seq and microarray analyses of mouse embryonic fibroblasts derived from *Nrf2*<sup>-/-</sup> mice also identified *COL1A2* as a basal target of NRF2 (Malhotra et al., 2010). However, we cannot exclude other possibilities because no specific binding site could be pinpointed from analyses of these public data due to differences in the localization of ARE DNA binding domains and ChIP-binding peaks for NRF2 in collagen I gene promoters.

Another possibility is that ZNF469 is part of the NRF2 pathway cascade and directly regulate some of the ECM genes. ZNF469 was among the top 25 genes commonly differentially expressed in our transcriptomic analysis. Silencing of NRF2 (siNrf2 and shNrf2) strongly decreased ZNF469 mRNA levels and, more unexpectedly, changed its subcellular localization from the nucleus to the cytosol. ZNF469 is a very large zinc-finger protein, the function of which still remains elusive. It is still predicted to be a transcription factor or an extra-nuclear regulator of fibrillar collagen expression and fibrillogenesis (Abu et al., 2008; Rohrbach et al., 2013). Immunostaining of ZNF469 in the nucleus supports the assumption that it has transcriptional activity as suggested in the literature. Interestingly, similar to what we observed in the silenced NRF2 fibroblasts, cultured dermal fibroblasts derived from Brittle Cornea Syndrome (BCS) patients carrying ZNF469 mutations showed a reduction in the amount of collagen I in intra- and extra-cellular immunostaining (Burkitt Wright et al., 2011). Moreover, ZNF469 was reported to be upregulated in human bronchial epithelial cells in contact with ambient vapors which upregulate the NRF2-ARE pathway (Shinkai et al., 2014). These results support our assumption of a possible transcriptional regulatory cascade mediated by ZNF469.

Hence, three hypotheses to explain how NRF2 acts on fibrillar collagen I gene expression still remain open (Figure 49): 1) an indirect mechanism by which NRF2 activates ZNF469 expression, which in turn stimulates collagen I gene expression; 2) a direct mechanism by which NRF2 acts as a transcription factor and ZNF469 as a specific cofactor; and 3) NRF2 and

ZNF469 separately control collagen I gene transcription through direct or indirect mechanisms. In the following paragraph, I will discuss the experiments that could help to elucidate the mechanism.



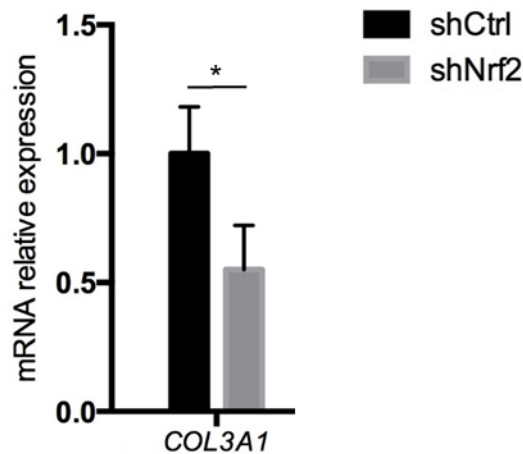
**Figure 49: Different scenarii of mechanisms of action by which NRF2 regulates *COL1A1* and *COL1A2* gene expression.**

From left to right: 1) a cascade mechanism mediated by ZNF469, 2) a direct mechanism of NRF2 with ZNF469 as a cofactor, 3) two independent direct or indirect mechanisms of NRF2 and ZNF469.

The first point would be to clarify the link between ZNF469 and collagen I gene expression. As already introduced, ZNF469 mutations have been identified as the main cause for BCS, which is an autosomal recessive genetic disorder characterized by extreme thinning and fragility of the cornea thought to be due to defects in the collagen network formation and organization. BCS patients have a high risk for cornea perforation, eventually leading to blindness (Abu et al., 2008; Rohrbach et al., 2013). BCS is often associated with other symptoms including keratoconus, blue sclerae and/or deafness. BCS patients also commonly display joint hypermobility or skin hyperelasticity, which are common clinical features of EDS disorders. As such, BCS was at first confused with classical EDS (Al-Hussain et al., 2004; Al-Owain et al., 2012).

Even though the role of ZNF469 in normal cornea development has been confirmed by different genome wide association studies, the mechanisms by which it regulates the corneal organization and thickness still remains unclear (Burkitt Wright et al., 2011; Hoehn et al., 2012; Vithana et al., 2011). Nevertheless, studies on the second most frequent BCS causing gene, *PRDM5*, revealed that *PRDM5* and ZNF469 are part of the same regulatory pathway and seem to control the expression of some ECM components (Burkitt Wright et al., 2011). The expression of some collagen genes (*COL4A1*, *COL11A1*) and some ECM regulators and stabilizers genes (*HAPLN1*, *TGFB2*, *EDIL3*, *CLU*, *GPC6*, *PCOLPE2* and *THBS1*) have been shown to be altered at the mRNA level in both *PRDM5* and ZNF469 mutant skin fibroblasts from BCS patients (Burkitt Wright et al., 2011; Rohrbach et al., 2013). Additionally, decreased protein levels of fibrillar collagens, fibronectins and integrins were found in skin fibroblasts from

patients carrying *ZNF469* or *PRDM5* mutations. A substantial decrease in collagens I and III protein levels was specifically observed in BCS skin fibroblasts caused by *ZNF469* mutations whereas COLV protein level remained unchanged (Burkitt Wright et al., 2011). While we did not identify in our transcriptomic analysis the genes dysregulated by *ZNF469* mutations, this latter finding is in agreement with our observations in shNrf2 cells (Figure 6 of the paper), which led us to the hypothesis that NRF2 and *ZNF469* may be functionally linked. Additionally, using RT-qPCR, we checked the expression levels of *COL3A1* encoding the fibrillar collagen III in our shNrf2 fibroblasts and shCtrl fibroblasts and found that NRF2 silencing significantly decreases *COL3A1* expression as reported for fibroblasts with *ZNF469*-related BCS at the protein level (Burkitt Wright et al., 2011) (Figure 50).



**Figure 50: NRF2 silencing decreases *COL3A1*.**

qPCR analysis of *COL3A1* in shCtrl and shNrf2 fibroblasts. Statistics were done using a paired t-test (n\_experiments = 3). Asterisks indicate p-value: \*  $\leq$  0.05.

Even if our results have to be confirmed at the protein level using immunofluorescent staining or western blotting, they are in favor of the assumption that NRF2 and *ZNF469* are part of the same molecular pathway that regulates fibrillar collagen gene expression. Interestingly, *PRDM5* which has been shown to be involved in the same regulatory pathway as *ZNF469*, also controls the transcription of collagen I genes (*Col1a1* and *Col1a2*) and eventually collagen fibrillogenesis in mouse preosteoblastic cultured cells (Galli et al., 2012).

Therefore, the next step should be to examine whether *ZNF469* directly regulates gene expression of the fibrillar collagens types I, III and V in skin fibroblasts. This could be achieved by silencing *ZNF469* in skin primary fibroblasts. In addition to silencing *ZNF469*, we aim to use *ZNF469* related BCS primary fibroblasts since it represents a more physiological cell system. Dr Fransiska Malfait (University of Ghent, Belgium) kindly agreed to provide us with *ZNF469*-

mutated fibroblasts from BCS patients. If silencing ZNF469 or ZNF469 mutations do not impact collagen gene expression, the assumption that ZNF469 may have a direct transcriptional activity to regulate collagen I genes or is part of NRF2 pathway could be excluded (scenario 1 and only NRF2 would stay in the scenario 3 of [Figure 49](#)).

To further analyze the link between NRF2 and ZNF469 and test the second scenario of [Figure 49](#) with ZNF469 acting as a cofactor of NRF2, a possible physical interaction between the two factors has to be investigated. This can be achieved using the Proximity Ligation Assay (PLA) or by co-immunoprecipitation. It will also be important to see whether a preferential cytosolic localization of ZNF469 is also observed in BCS fibroblasts as we observed in NRF2 silenced fibroblasts. This will further support the idea that the interplay of ZNF469 and NRF2 is required for the transcriptional regulation of the collagen genes.

Last but not least, it would be interesting to check it should also be clarified whether or not NRF2 can binds directly to the promoters of the promoter of the genes coding for collagen I genes by using a NRF2 ChiP assay with the specific amplification of *COL1A1* and *COL1A2* by qPCR. This would favor scenario 1 and 3 left, with NRF2 as a direct regulator of collagen I genes.

All these experiments will help identifying the molecular mechanisms by which NRF2 regulates collagen I gene expression. The experiments using skin fibroblasts from patients with ZNF469 related BCS, ZNF469 silenced fibroblasts and PLA to analyze a potential physical interaction between NRF2 and ZNF469 are designed and almost ready to be launched.

## 2. Outlook

NRF2 could be a regulator of collagen I genes in non-stressed conditions in human skin fibroblasts. But is NRF2 an activator or a repressor of collagen I expression in skin fibroblasts? This has to be clarified. Hiebert and collaborators showed that constitutive activation of NRF2, results in downregulation of the collagen I gene expression (Hiebert et al., 2018), while in our study NRF2 silencing gave similar results. Even though we cannot entirely explain this discrepancy, there may be two contributing factors. It is known that the regulation of NRF2 targets is context specific and fine-tuned (Tonelli et al., 2018; Wu et al., 2011), and our experimental conditions were fundamentally different from those used in Hiebert and collaborators. We identified the down-regulation of the collagen I gene expressions with NRF2 knock-down whereas Hiebert et al. used mice with constitutive active NRF2 expression in the

dermis. Another major difference is that Hiebert et al. did not investigate a specific fibroblasts subtype while we focused our study on papillary fibroblasts. It has been shown that different fibroblasts subtypes display different gene expression profiles and specifically papillary and reticular dermal fibroblasts show distinct gene expression signatures (Haydont et al., 2019; Nauroy et al., 2017).

Would NRF2 be able to regulate collagen I genes in other tissues/organs than skin? This is clearly an open question. Collagen I is the predominant collagen type in the human body ECMs, it is the main constituent of dermal ECM but also the one of tendons and bones, blood vessel walls, cornea etc. 85% of mutations in *COL1A1* or *COL1A2* genes are responsible for a severe bone disorder, called Osteogenesis Imperfecta (OI) (Marini et al., 2017). NRF2 is a critical regulator of bone homeostasis: its deficiency promotes osteoclast differentiation and activity which eventually turns into bone resorption (Sun et al., 2015). Moreover, *Nrf2*<sup>-/-</sup> mice display impaired fracture healing compared to wildtype mice (Lippross et al., 2014). In these studies, the role of NRF2 in bone regulation was attributed to its protective role against oxidative stress. However, in light of our results and also data from literature, it would be interesting to analyze the role of NRF2 in osteoblasts under non-stressed conditions, as we did, but also to explore in more details a possible regulation of collagen I genes by NRF2 in bones.

It would be also interesting to investigate the action of NRF2 on other collagen genes, such as *COL3A1* but also on the collagen IV which have been found to be dysregulated in NRF2 different studies with NRF2 genetic modifications. Malhotra and collaborators have identified *Col4a1* as a strong target of NRF2 in mouse embryonic fibroblasts as revealed by CHIP-seq experiments (Malhotra et al., 2010). Moreover, Hiebert and collaborators also identified in their transcriptomic analysis, the six collagen IV genes (*Col4a1-6*) encoding respectively the  $\alpha$ 1–6(IV) chains, as dysregulated in caNRF2 mice (Hiebert et al., 2018). We did not observe any dysregulation in collagen IV expression gene in our transcriptomic analysis. But, as said above, NRF2 pathway activation is fine-tuned through a yet unclear mechanism and a transient knock-down of NRF2 as in our study and a complete lack of NRF2 might not affect the same NRF2 targets (Tonelli et al., 2018; Wu et al., 2011). Being one of the main basement membrane components, and as such of the DEJ in skin, it would be very interesting to address the question of whether or not NRF2 affects the DEJ in skin.

Besides collagens, NRF2 seems also to regulate ECM glycoproteins such as *ELN* and *FBN2*. Interestingly, these 2 genes were identified in our siNrf2 fibroblasts transcriptome and

that of caNRF2 mouse fibroblasts (Hiebert et al., 2018). Both proteins, elastin and fibulin-2 are major components of the elastic fibers, and their dysregulation when NRF2 is deficient may impact elastogenesis and consequently the biomechanical properties and homeostasis of the skin (Kielty et al., 2002; Thomson et al., 2019).

We have identified ZNF469 as a new NRF2 target. As already discussed above, BCS is a rare autosomal recessive connective disorder caused by mutations in ZNF469 or PRMD5 with only about 60 patients reported so far in the literature. Today, there are only limited treatment options available (Walkden et al., 2019). Our results question the possibility of using NRF2 activating treatments, such as sulphoraphane or DMF, in BCS patients. These two molecules are currently under clinical evaluation for the treatment of chronic obstructive pulmonary disease and cystic fibrosis (Dodson et al., 2019).

Finally, going back to the initial question, we can now clearly state that the role of SKN-1/NRF2 in collagen gene regulation originally reported in *C.elegans* (Ewald et al., 2015) can be transposed to human skin. Even if NRF2 acts on ECM genes in human skin fibroblasts, the results have not been discussed yet from the perspective of longevity *versus* aging, that raises yet more questions. NRF2 silencing results in a decrease of collagen I gene expression at the transcript and protein levels in dermal fibroblasts and in the dysregulation of other ECM genes, which is one of the main skin aging feature (Shin et al., 2019; Sparavigna, 2020). This clearly opens avenues for future research.

In conclusion, my PhD work identifies NRF2 as a new transcriptional regulator of ECM genes, including *COL1A1* and *COL1A2* but not only, in human skin fibroblasts. The mechanism of action still requires further investigation that we plan to do in the coming weeks. As collagen I is widely expressed in the human body, these findings could be of major importance in skin aging and, to a great extent, in the biomedical field.





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# Annex



## Annex

### **The Collagen Superfamily: everything you always wanted to know.**

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## **Abstract**

Collagens are the most abundant extracellular matrix proteins in multicellular organisms. In humans, the collagen superfamily counts 28 proteins encoded by 44 genes whose functions are far from being completely elucidated. Their primary function is to provide structural support and strength to cells in order to maintain biomechanical integrity of tissues. However, collagens are no longer considered just as structural proteins and there is an extensive literature that documents the high diversity of collagens functions in cell behavior, integrity and tissue homeostasis. A number of common diseases are directly linked to an imbalance between collagen synthesis and degradation that lead to tissue dysfunction. In addition, collagens are associated with a broad spectrum of heritability-related diseases known as “collagenopathies” that affect a multitude of organs and tissues including sensorial organs. However, the particular complexity and diversity of the collagen nomenclature, structure, biosynthesis and molecular assembly often repel scientists and clinicians who are not in the field. This chapter aims at providing a glossary of the collagen superfamily that allows readers to locate piece of information they need without alienating them.

**Abbreviations:** BM, basement membrane; COL, collagenous domain; ECM, extracellular matrix; EDS, Ehlers-Danlos syndrome; ER, endoplasmic reticulum; FACIT, Fibril-Associated Collagens with Interrupted Triple helices; MACIT, Membrane-Associated Collagens with Interrupted Triple helices; NC, non-collagenous domain; OI, Osteogenesis Imperfecta; P4H, Prolyl-4-hydroxylases ; UCMD, Ullrich congenital muscular dystrophy

## 1. Introduction

Cells use a thousand genes to produce, assemble and remodel tissue-specific extracellular matrices (ECM) that show a remarkable versatility and permeate all aspects of cellular function in health and disease. Among them, 44 genes code for collagens in humans. Collagens account for one third of the total protein mass in mammals and are the prevalent component of ECM. The collagen superfamily comprises 28 trimeric proteins that display large diversity in their structure, supramolecular organization and function. Initially collagens have been given Roman numerals (I-XXVIII) in the order of their discovery). Reaching the number XXVIII, which was certainly not expected when the first collagen types were discovered, become somewhat annoying for easy identification of samples and easy reading of papers. The classic (and still recommended) nomenclature has the tendency to include now Arabic numbers not only for collagen genes but also the proteins (for example, Col18 instead of ColXXVIII). In some animal models, the mutant names are still used instead of the gene name (for example *stumpy* in zebrafish is referring to *col19a1* gene). For didactic learning of collagen structure and nomenclature, the reader is referred to a collagen lexicon in [Figure 1](#).

Collagens were first described as banded fibrils and famous for providing structural and tensile properties in connective tissues such as skin or cartilage. They are also key components of the basement membrane (BM), a specialized ECM structure that physically separates and anchors cell sheets to the underlying connective tissue and ensures molecular filtration and tissue homeostasis. Collagens act as extracellular modulators of signaling events and serve critical regulatory roles in various cell functions during embryonic development and adult homeostasis. The later identification of novel members in the early 21<sup>st</sup> century, after the completion of the human genome sequencing together with the discovery that bioactive fragments are released from most of the collagen molecules, led to new exciting biological functions including cell differentiation, behavior and integrity as well as biological processes such as tissue regeneration, wound-healing, inflammation, angiogenesis and many others.

A number of common diseases (microenvironmentally-driven tumors, fibrotic disorders, neurodegenerative diseases...) are directly linked to an imbalance between collagen synthesis and degradation that lead to tissue dysfunction. Thus, not surprisingly, these multifunctional and broadly expressed proteins are also associated with a wide spectrum of heritability-related diseases known as “collagenopathies”. Thousands of mutations in more than half of the collagen genes affect a multitude of organs and tissues including sensorial organs. This chapter aims at providing an overview of the fascinating collagen superfamily and giving specific insights into

the collagens molecules that are critical for the understanding of collagenopathies and their underlying mechanisms, that will be further detailed in the chapters of this book.

## **2. Collagens belong to the core matrisome**

For a long time, ECM was typically viewed as a physical support for tissues that chiefly comprises 3 major types of macromolecules: collagens, proteoglycans and glycoproteins. However, the knowledge accumulated during the last decades told us that this was definitively a narrow view of what the extracellular space is exactly made of. In addition to the structural proteins, the ECM serves as a reservoir for growth factors, cytokines and ECM regulators. This said, the full characterization of the diverse ECM landscape was not an easy task to tackle. First, ECM composition and topology are highly tissue-specific which do not facilitate the identification of all ECM molecules. Second, ECM constantly undergoes remodeling as a result of matrix proteases activity and generates smaller fragments that could be difficult to identify [1]. Third, ECM proteins display distinct physicochemical property profiles that make total protein extraction technically challenging. However, the recent emergence of new “omics” technologies offered potential powerful approaches to identify the complete landscape of ECM components.

ECM proteomes were first established by Naba and collaborators [2]. They collected samples from normal or pathological mouse or human tissues and the most insoluble fraction was sequenced by mass spectrometry. The ensemble of all ECM and ECM-associated proteins was defined as the matrisome [2–5]. An *in silico* approach was then used to define the “core matrisome” that comprises collagens, proteoglycans and glycoproteins. It was based on defining domains that characterized an ECM protein, or excluding domains, never present in ECM molecules [2,4]. The proteins present in the extracellular space but not considered as “pure” matrix proteins were then categorized as “matrisome-associated” proteins. The matrisome-associated category includes: (1) the “ECM regulators” such as ECM-modifying enzymes, matrix proteases and their inhibitors; (2) the “secreted factors” corresponding to the cytokines, growth factors or signaling molecules of the extracellular space and (3) the “ECM-affiliated proteins” containing other secreted factors and proteins that were regularly found in ECM-enriched preparation [5].

The characterization of the matrisome revealed that organisms used up to a thousand genes to build tissue-specific ECMs. The human matrisome is composed of 1027 genes while 1110 genes encode the mouse matrisome [5]. Both the mouse and human core matrisome comprises 274 genes but the number of matrisome-associated genes within the different subcategories

varies considerably between the two species, even between the different subcategories [5]. Although human and mouse both have 44 collagen genes (Table 1), the *COL21A1* gene does not exist in mice and the *COL6A4* gene is not functional in humans due to a chromosome inversion [6,7]. It is worth noting that the ECM receptors such as integrins or Discoidin Domain Receptors were not included in the matrisome. ECM transmembrane receptors are responsible for signaling cross talks between ECM and cells and, thereby, trigger specific cell responses as diverse as cell adhesion, migration, proliferation, survival and death, to name but a few. The complete characterization of the integrin adhesion complex components (adaptors, enzymes and cytoskeleton) was termed adhesome [8–10] ([www.adhesome.org](http://www.adhesome.org)).

### 3. Collagen, a singular protein that should be read as plural

By essence, collagens are large trimeric molecules containing at least one triple helical domain and secreted in the extracellular space (Figure 2A). However, the latter definition does not entirely include transmembrane collagens that exist as full-length transmembrane proteins with soluble ectodomains that are proteolytically released from cell surface by sheddases. Collagen trimers consist in the association of three polypeptide chains, called  $\alpha$ -chains (Figure 2A). Each of the so-called  $\alpha$ -chain is composed of Gly-Xaa-Yaa triplet repeats, in which Xaa is often a proline and Yaa a hydroxyproline. This motif allows the formation and the stabilization of the triple helix. Interchain hydrogen bonds and electrostatic interactions are holding the three  $\alpha$ -chains together. Glycines (Gly) are strictly present as every third residue to fit into the triple helix as it is the least bulky amino-acid. As such, a single substitution of a Gly residue into any other residue can lead to a distortion of the triple helix. The generation of pathogenic triple helix micro-unfoldings can further provoke cascading pathological scenario by causing disturbance of collagen stability, biosynthesis and supramolecular aggregates thus impacting tissue biomechanics and/or interactions with cells or binding partners.

The triple helical region of collagens, named collagenous domain (COL), can be either large and uninterrupted in some molecules (e.g. collagen I, III or VII) or short and interrupted (e.g. collagen IV, XII, or the transmembrane collagen XIII). Consequently, the COL domains represent 96% of the collagen I structure whereas they represent less than 10% in collagen XII (Figure 2B). Besides this structural collagen hallmark, collagens also possess non-triple helical domains, called non-collagenous domains (NC) (Figure 2B and C). The NC domains are globular domains when located at the collagen molecule extremities and/or short linker regions of about 20 residues when located between domains. The NC domains alternate with COL domains and are numbered starting from the C-terminus (e.g. NC1, NC2 and NC3 alternate with COL1, COL2

and COL3). NC linkers provoke short interruptions within COL domains that allow flexibility to the rod-shaped molecule conferred by the triple helix and increase sensibility to extracellular enzymes. NC domains are the most frequently engaged in molecular interactions with other ECM proteins and/or cell receptors. They can be proteolytically shed from the parental collagen as a bioactive molecule called matricryptin which functions, once released, are distinct from the ones of the entire collagen. Like a string of beads, NC domains are often repeated within a collagen (18 Type III Fibronectin domains in collagen XII  $\alpha 1$  chain – [Figure 2 B and C](#)) and can be present in other ECM proteins. The most frequent domains in collagens are the von Willebrand like domain which is involved in protein-protein interactions; Type III Fibronectin domains and the thrombospondin N-terminal like domain while the Kunitz and Frizzled domains are only present in collagen VII and collagen XVIII respectively ([Figure 2B and C](#)). The functions of these domains are not yet fully elucidated and what is even more obscure is how important is the number and order of NC domain repetition in collagen molecules.

Further diversity of the collagens occurs with the different existing subunits for a given collagen type. In humans there are 44 collagen genes, encoding for 44  $\alpha$ -chains, and 28 collagen types, that can be explained by the fact that (i) one collagen type can comprise up to 6 different  $\alpha$ -chains ([Table 1](#)) and (ii) collagens can form homotrimers (when the three  $\alpha$ -chains are identical) or heterotrimers (when at least one  $\alpha$ -chain is different), making several  $\alpha$ -chains combinations possible and thus several molecules. Most of the collagens are homotrimeric molecules, thus resulting from the assembly of three  $\alpha 1$ -chains encoded by one single gene, as for collagen types II [ $\alpha 1(\text{II})$ ]<sub>3</sub> and III [ $\alpha 1(\text{III})$ ]<sub>3</sub>. It is also the case for most of collagen types with the highest numbers (collagen types XII to XVIII – [Table 1](#)). On the top of that, rare hybrid collagen molecules resulting from the association of  $\alpha$ -chains from two different collagen types can be found in tissues. So far it has been described only for collagen V and the cartilaginous collagen XI, two structurally and functionally closely related collagens. An explanation for this rare event is, as previously suggested [16], that collagen V/XI  $\alpha$ -chains were initially wrongly attributed to two distinct collagen types due to the exclusive tissue-specific chain combinations despite the fact that they were found as closely structurally and functionally related. Finally, some collagens possess glycosaminoglycans chains and are thus by definition collagen/proteoglycan hybrids. Collagen IX carries chondroitin sulfate chains, collagen XVIII possesses heparan sulfate chains whereas collagen XV is decorated by chondroitin sulfate chains and, in a less extent heparin sulfate chains. This high molecular diversity results in the formation of a variety of supramolecular aggregates ([Figure 2D](#)) that are responsible for tissue-specific mechanical properties such as stiffness, deformability, tensile force or shear resistance but also to a wide range of biological functions through chemical and/or mechanical signals.

With the expansion of the collagen superfamily, collagen types have been classified into subfamilies according to similarities in their molecular or supramolecular organizations. This classification gave rise to 6 subsets of the collagen superfamily (Table 1 and Figure 2D). The “supramolecular organization” subsets comprise the “fibril-forming collagens” (7 members); the “network-forming collagens” (5 members). This subset includes collagen VI that assembles into “beaded filaments” and collagen VII that forms “anchoring fibrils”. The “molecular organization” subsets include the FACIT (Fibril-Associated Collagens with Interrupted Triple helices) comprising 8 members; the MACIT (Membrane-Associated Collagens with Interrupted Triple helices) that are transmembrane collagens (4 members) and the multiplexins (for collagens with “multiple triple helix domains with interruptions”; 2 members). Two collagens remain unclassified, collagens XXVI and the last discovered collagen type XXVIII. Whereas the function of collagen XXVI is still unclear, type XXVIII collagen was found to be chiefly expressed in the peripheral nervous system and mutations in *COL28A1* are responsible for the Charcot-Marie-Tooth disease, a disease that primarily impairs nerve myelination [17]. For further and more in-depth analysis on the collagen subfamilies we refer to previous comprehensive reviews [18–20].

It is here worthy to note that besides the “genuine” collagen molecules, a sizeable number of proteins that do contain one or several collagenous domains were not included in the collagen superfamily. This holds true for the members of the collectin family, the acetylcholinesterase, emilins, adiponectin, C1q, ficolin, and more recently the gliomedin and ectodysplasins A, many of them just because they were first reported by “non-matrix biologists” (reviewed in [18]).

#### **4. Collagen biosynthesis: a complex and multistep process**

Fibrillar collagen biosynthesis has been extensively studied but regarding to the extreme diversity of collagens, it would be naïve to assume that their biosynthesis, that includes supramolecular assembly, is identical for the other collagen family subtypes. They do share similarities in some early post-translational modifications, but even the initiation of the triple helix formation differs from one type to another. Yet, collagens IV, VI, VII display important biosynthetic specificities. For further information about these collagens biosynthesis, we invite the reader to refer to the chapters in this book. Here, we will discuss the biosynthesis of fibril-forming collagens only as it represents a textbook case of collagen biosynthesis. The full elucidation of this multistep process is crucial for the understanding of the pathogenesis of collagenopathies and further development of effective treatment for affected patients.

Fibrillar collagen biosynthesis comprises a succession of intracellular and extracellular events from the formation of the triple helix and its secretion to the supramolecular organization into fibrils in the extracellular space. As for all secreted proteins, fibrillar collagens are synthesized as pro $\alpha$ -chains by membrane bound ribosomes and are then translocated into the lumen of the endoplasmic reticulum (ER) where they assemble into triple helix molecules (Figure 2A). Collagen pro $\alpha$ -chains then undergo numerous and complex post-translational modifications in the ER among which the most important are: (1) cleavage of the signal peptides; (2) hydroxylation of most of the prolines by the Prolyl-4-hydroxylases (P4H) at the Yaa position of the Gly-Xaa-Yaa triplets. *Prolyl-3-hydroxylases also hydroxylate selected prolines residues in several collagens but not all* [21]; (3) hydroxylation of some lysines and (4) glycosylation of hydroxylysines generated in step (3). These post-translational modifications are followed by an important step of collagen biosynthesis, that is the specific assembly of the newly synthesized collagen pro $\alpha$ -chains into a triple helix. This occurs in a zipper-like manner, starting from the C-terminus of the molecule and propagating towards the N-terminus (Figure 2A). Collagen C-propeptides play a major role for the selection of the pro $\alpha$ -chains and procollagen folding. C-propeptides association depends on specific recognition sequences and protein disulfide isomerase (PDI) activity to create disulfide bonds between three individual C-propeptides that facilitate the initiation of the triple helix formation [22–24]. *In vivo*, the presence of hydroxyprolines (about 10% in fibrillar collagens) allows the binding of HSP47 chaperon protein and the subsequent procollagen trimer stabilization together with the formation of inter-chain disulfide-bonds within the C-propeptides discussed above [25].

The molecule is further translocated to the Golgi apparatus where accurate procollagen glycosylation and trafficking is ensured. During their transport to the extracellular space, procollagens start to aggregate laterally within Golgi to plasma membrane carriers. When procollagens reached the plasma membrane, carriers connect to the ECM through deep projections of the plasma membrane, called “fibripositors” [26,27]. The precise nature of these vesicles is still a matter of considerable debate. Specifically, the question of how large rod-shape molecules of up to 700 nm in length can fit into secretory vesicles remains puzzling. Collagen trafficking is far from being completely understood and we refer the reader to comprehensive reviews on this topic [27–29].

Once secreted in the extracellular space, N- and C-propeptides of fibrillar procollagens are entirely (e.g. collagens I and III) or partially (e.g. collagens V and XI) removed by specific matrix enzymes including BMP-1, mTLD and TLL-1 for the C-propeptide and ADAMTS family members for the N-propeptide. Mature collagens spontaneously self-assembled into fibrils that result from



end-overlap alignments of collagen molecules, in a quarter-staggered packing pattern (Figure 2A and D). Fibrils structure is strengthened by intra- and inter-molecular crosslinks catalyzed by lysyl oxidases between lysine and hydroxylysine residues [30–33]. Heterotypic fibrils made of two or three different fibrillar collagen types are the most common structure found in tissues (e.g. collagen I, III and V in skin, collagen II and XI in cartilage) while homotypic fibrils made of collagen III only can be found in blood vessel walls [34] and homotypic collagen V fibrils were described in embryonic tissues and in specific location in skin and cornea [16,35]. The fact that banded collagen fibers could be formed *in vitro* directly from purified fibril-forming collagens without the contribution of cells has been overlooked and is still hotly disputed.

## 5. Collagens across species

However, although mice were historically used as a preferred model organism for human connective tissue diseases studies and preclinical trials, scientists cannot conduct research using only one animal model. Therefore, additional organisms such as zebrafish, drosophila and *Caenorhabditis elegans* have rapidly emerged as reliable model organisms to study collagen function and are now also extensively used to investigate the pathogenesis and molecular mechanisms of collagenopathies. Accordingly, their matrisomes were established thanks to fruitful collaborative work with Naba's lab, the matrisome's maker, and ECM experts in zebrafish (our lab, [11]); *C. elegans* (Ewald's lab, [12]) and drosophila (Horne-Badovinac's lab, [13]). Complete matrisome gene lists are available on The Matrisome Project website (<http://matrisomeproject.mit.edu>).

As the zebrafish underwent an additional whole genome duplication compared to mammals, a large number of collagen genes are duplicated and referred as “a” and “b” in the nomenclature. The *in silico* approach used to define the zebrafish matrisome retrieved 58 collagen genes [11]. Among them, the orthologue of the human *COL21A1* gene, whose function is still unknown, is present in the zebrafish genome but absent in the mouse genome as mentioned before. A second gene encoding for an additional chain of collagen XVIII that was lost during evolution in mammals exists in zebrafish [14]. The duplicated collagen genes in zebrafish are submitted to different evolutionary processes such as non-functionalization of one copy, subfunctionalization, neofunctionalization or dosage selection. Previous work that aimed at deciphering the function of collagen genes using zebrafish revealed gene specification of collagen paralogues [15].

To establish the *C. elegans* matrisome, the authors combined gene orthology and protein structure analysis such as, for example, the presence of Gly-Xaa-Yaa repeats (a structural

hallmark of collagens). The *C. elegans* matrisome contains 719 proteins including 181 collagens [12]. However, except 8 collagen genes, most of them are restricted to the cuticle and do not share orthology with human collagen genes. To clarify the *C. elegans* collagen classification, the authors created four different groups based on the structural organization of the molecules. The first group contains the vertebrate-like collagens that are conserved in the nematode including the BM collagens *emb-9* and *let-2* (orthologs of human collagen IV genes); a single multiplexin gene *cle-1* and a transmembrane collagen (MACIT), *col-99*. The second group is composed of proteins that contain a collagenous domain and resemble mammalian gliomedins and collectins. Non-cuticular *C. elegans* collagens with no orthology with mammalian genes are in the third group and 173 cuticular collagens with unknown function form the last group that is itself divided into five different clusters.

Using similar biocomputational approaches, the drosophila matrisome was established and contains 641 genes including only 4 collagens that are all BM collagens [13]. Three of them possess mammalian orthologs while only one (pericardin) is specific to the drosophila. Contrary to the mammalian matrisomes, the drosophila matrisome has an additional category that is called “Apical Matrix” representing about 50% of the total drosophila matrisome and containing chitins-based ECM proteins secreted by the apical side of epithelial tissues.

## **6. An introduction to collagenopathies**

Collagens are the most abundant proteins in humans and are broadly expressed in tissues. They display diverse structural features, as discussed above, and are known to fulfill diverse mechanical and biological functions. It is thus not surprising that defects in collagen synthesis and assembly lead to a large variety of common and genetic disorders that affect various tissues and organs (Figure 3). A number of common diseases (microenvironmentally-driven tumors, fibrotic disorders, neurodegenerative diseases...) are directly linked to an imbalance between collagens synthesis and degradation that lead to tissue dysfunction. In addition, collagens are associated with a broad spectrum of heterogeneous genetic diseases now known as “collagenopathies” that affect a multitude of organs and tissues including sensorial organs. Mutations in genes of a single collagen type, such as collagen IV genes, can be responsible for different diseases; and conversely tissues as skin or cartilage can be affected by mutations in different collagen types. Both of these cases will be illustrated in the coming chapters.

Among the 44 human collagen genes, up to 29 genes coding for 17 distinct collagen types are associated to collagenopathies! Intuitively, it would have been assumed that genes responsible

for collagenopathies would be the ones highly expressed in the tissue. But genes responsible for collagenopathies are often expressed at low levels in the affected tissues or do not affect at all the tissues in which the corresponding protein is highly present. For instance, collagen V is highly expressed in cornea and minorly expressed in skin, but mutations in *COL5A1* or *COL5A2* genes primary result in defects in skin and joints in which this collagen is expressed in low levels (see chapter 3 of this book). Collagen XVIII isoforms are localized in various BM and the long form is highly expressed in liver, however mutations in *COL18A1* are responsible for a rare condition called Knobloch syndrome characterized by severe vision problems (high myopia, retinal detachment) as well as skull defect (occipital encephalocele) [36]. In addition, there are growing evidence for diseases with overlapping symptoms and clinical presentation between different conditions. Collagen XII was first discovered in the early 90's and knock-out mice for this gene showed a bone phenotype [37]. Mutations in *COL12A1* was reported in 2013 with patients displaying a novel Ehlers-Danlos syndrome (EDS)/myopathy syndrome, named myopathic EDS in the recent classification of EDS [38,39]. *Clinical and morphological overlaps* between the Ullrich congenital muscular dystrophy (UCMD) caused by collagen VI gene mutations and classic EDS have also been reported [40,41].

Among collagens, collagen I is by far the most abundant collagen in vertebrates particularly in bone and skin providing them with structural support. This is thus not surprising that mutations in collagen I genes are associated with several connective tissue disorders. Mutations in *COL1A1* and *COL1A2* genes have been early reported to be responsible for a severe bone disorder called Osteogenesis imperfecta (OI). OI is an inherited connective tissue disorder mainly characterized by bone fragility and deformity leading to severe skeletal abnormalities and growth deficiency. In the last 15 years, it appeared that mutations in collagen I genes explain only 85%-90% of OI and that other molecules involved in collagen I synthesis, processing or maturation can cause OI [42]. As collagen I-related OI were already extensively reviewed in the literature, chapter 2 by Omari *et al.* focuses on the newly discovered molecules responsible for skeletal disorders. Based on the recent discovery that OI and other skeletal disorders can be caused by defects in the protein secretory pathway, the authors provide a complete review on the importance of procollagen folding, trafficking from the ER to the Golgi and degradation for bone and other connective tissue homeostasis.

Together with the fibril-forming collagens III and V, collagen I deficiency was also identified as responsible for a heterogeneous group of connective tissue disorders named Ehlers-Danlos syndrome (EDS) as briefly introduced above. The EDS common features are fragile hyperextensible skin with abnormal wound healing and joint hypermobility but the important

clinical heterogeneity of EDS prompted the clinicians to classify them into subcategories. An update of the Villefranche nosology was provided in 2017 and resulted in a list of 13 different EDS types which involve mutations in 20 different ECM genes [43]. In chapter 3 Malfait and collaborators discuss the impact of mutations in other ECM proteins or collagen-modifying enzymes that were recently found to cause new EDS types or to augment the list of genes causing one of the already characterized EDS types. The authors have gathered the more recent information obtained from EDS patients, cells and animal models of EDS to provide the reader with a clear review of the various and complex molecular defects causing the different subtypes of EDS and their supramolecular consequences.

Collagen II is the major component of the hyaline cartilage and the territorial matrix contains not less than 6 different collagens. Contrary to most of the members of the collagen superfamily, collagens II, IX and XI are expressed only in cartilage with the exception of the vitreous in eye. They co-polymerize into collagen II/IX/XI heterotypic fibrils in which collagen II is the major component while collagens IX and XI are minor components. Nevertheless, these minor components play important role in fibril formation and structure by regulating fibrillogenesis. Mutations in collagens II or XI, two fibrillar collagens, or in collagen IX, a member of the FACIT collagen subfamily, have been associated with a large spectrum of cartilage disorders among which osteoarthritis. Common clinical features of cartilage disorders are disproportionate stature, dwarfism, short limbs and premature osteoarthritis. As some of the cartilaginous collagens are also expressed in the vitreous in eye, patients with cartilage-related disorders also often have eye defects. In chapter 4, Uwe Hansen outlines all aspects of the physiological and pathological roles of cartilaginous collagens and cartilage-related disorders.

Collagen IV is together with laminins, perlecan and nidogen, one of the major components of all BM, a.k.a. BM tool-kit [44]. BM are thin and highly organized ECM structures that underline all epithelia and endothelium and surround a few individual cell types as for instance muscle cells, adipocytes or Schwann cells [45]. Contrary to interstitial connective tissues, they are conserved across species and can be found in most multicellular organisms. Thereby, mutations in the six human genes encoding collagen IV lead to a wide spectrum of clinical signs and diseases ranging from vascular defects to eye and kidney disorders. Chapter 5 by Dean and Van Agtmael starts by an in-depth description of the distinctive organization of collagen IV genes, the complex biosynthesis of collagen IV molecules and their expression patterns. The authors then carefully described the different genetic collagen IV related diseases among which Alport and Goodpasture syndromes are surely the most well-known. Importantly they also gave insights

into the underlying mechanisms of those diseases and reported on the development of novel therapeutic strategies.

ECM components, and more specifically collagens, are critical for the development and homeostasis of the neuromuscular system. Twelve different collagens are expressed in skeletal muscle, including the neuromuscular junction and the myotendinous junction. Among them, collagen VI is definitely the most studied collagen and its deficiency underlies the so-called *collagen VI-related myopathies* that encompass a *spectrum* of disorders ranging from the milder Bethlem myopathy to the severe UCMD [46]. These disorders are characterized by muscle weakness and atrophy, distal joint hyperlaxity and proximal joint contractures. Chapter 6 by Tonelotto *et al.* carefully describes collagen VI and collagen VI-related disorders, but also highlights the important contribution that mouse and zebrafish models brought to the understanding of collagen VI-related collagenopathies and describe the role of other collagens in muscle-associated disorders.

Skin contains a large number of different collagen types, and consequently this tissue is highly subjected to collagenopathies. In chapter 7, Nyström *et al.* emphasize the role of collagens VII and XVII on skin homeostasis. Both collagens are critical components of the dermo-epidermal junction as they ensure the cohesion of the epidermis with the underlying dermis either by forming anchoring fibrils for collagen VII, or by being a component of hemidesmosomes for collagen XVII. Mutations in *COL7A1* or *COL17A1* genes lead to severe genetic and autoimmune skin blistering disorders such as epidermolysis bullosa [47]. The authors give a comprehensive overview of collagen VII and XVII physiology and of diseases associated with *COL7A1* or *COL17A1* mutations. They also described the different therapeutic approaches that are currently under evaluation to treat patients affected with these collagenopathies.

The past decades have seen the emergence of the role of ECM in the nervous system. Chapter 8 by Keikkinen *et al.* emphasizes the critical role of collagens in the development, homeostasis and repair of the different morphological compartments of the central and peripheral nervous systems. Unexpectedly, mutations in collagens, that originally were not even thought to be expressed in the nervous system, were found to cause various neurodevelopmental, degenerative and psychiatric disorders. Through the description of *in vitro* and *in vivo* data from several mouse models, this chapter gives a thorough and comprehensive overview of this expanding type of collagenopathies.

## 7. Outlook

There is little question that the number of collagen superfamily members will further increase, the identification of *COL6A5*, a novel chain of collagen VI, being wrongly attributed to the discovery of collagen XXIX [48]. But the full landscape of collagen functions is far from being completely elucidated. This is already evidenced by the new findings and concepts in which collagens are involved in cell stem niche, cell signaling, growth factor bioavailability, mechanotransduction, regulation of metabolism...

Mice have been instrumental in furthering our understanding of collagenopathies and for pre-clinical studies but zebrafish has recently joined the mouse as a powerful new model [15]. The development of diverse animal models can be very helpful in the understanding of the disease mechanisms and in the identification of candidate genes for unresolved collagenopathies. Mouse and zebrafish models have pinpointed a critical role of collagen XXII and XV in the development of the neuromuscular system, and, as such, these studies have revealed *COL15A1* and *COL22A1* as candidate genes for orphan neuromuscular disorders [51–53].

Nonetheless, there is already evidence that the list of collagenopathies will rapidly increase. Mutations in *COL8A2* are responsible for Fuchs' corneal dystrophy, a progressive degenerative disease of cornea [54], recessive mutations in *COL25A1* were shown to cause Congenital Cranial Dysinnervation Disorder [55] and mutations in *COL27A1* were described in patients with Steel syndrome [56,57]. *COL21A1*, a collagen gene remaining poorly documented, could be associated to nonsyndromic orofacial cleft [58]. With the widespread use of whole exome sequencing and whole genome sequence in clinical diagnostics, identification of new mutations involved in collagen-related diseases will definitely be shortly unraveled, an issue of utmost importance for the development of therapies for various collagenopathies.

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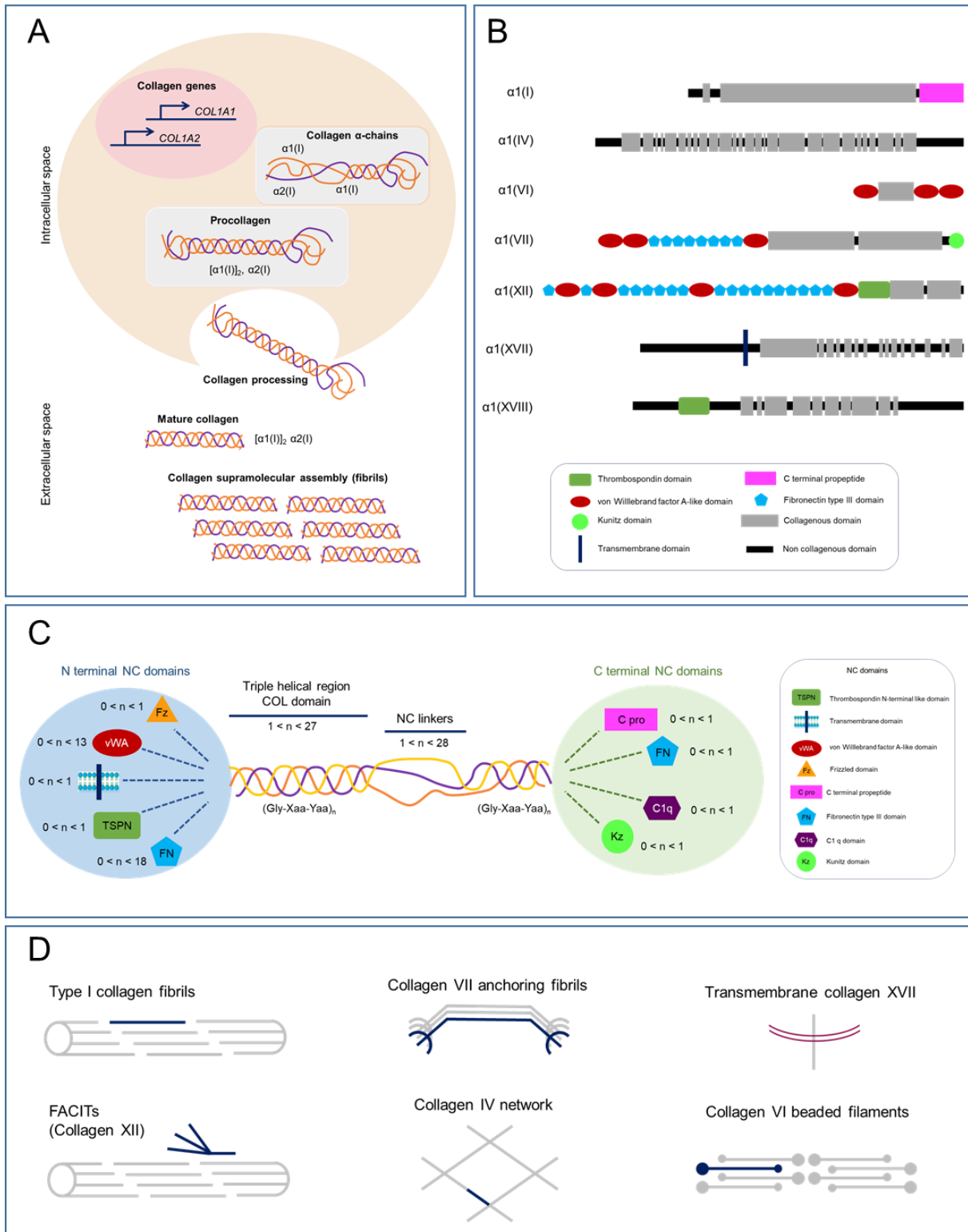
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## Figures and table

<b>COLLAGEN LEXICON</b>		
Genes	<p><math>COLN_n</math> in which <math>N</math> is the number of the collagen type; <math>n</math> the number of collagen chain</p> <p>E.g.: The human <i>COL5A1</i> gene encodes collagen V<math>\alpha</math>1 chain; <i>COL4A2</i> encodes collagen IV<math>\alpha</math>2 chain</p>	<i>COL24A1</i>
Chains	<p>Chains are numbered with Arabic numerals based on their electrophoretic pattern</p> <p>Three collagen <math>\alpha</math> chains are needed to form a collagen molecule (homotrimer, heterotrimer or hybrids)</p> <p>E.g.: Collagen XV homotrimer is written <math>[\alpha 1(XV)]_3</math>; collagen I heterotrimer is written <math>[\alpha 1(I)]_2\alpha 2(I)</math></p> <p>Collagen <math>\alpha</math>-chain contains NC (non collagenous – linkers or domains) and COL (collagenous) domains numbered from the C-term to the N-term</p> <div style="text-align: center;"> <p style="text-align: center;">N-term ← ————— C-term</p> <p style="text-align: center;">NC4 COL3 NC3 COL2 NC2 COL1 NC1     <math>\alpha</math>-chain</p> </div>	$[\alpha 1(XXIV)]_3$
Collagen	<p>Collagen types are numbered with Roman numerals based on the chronology of their discovery</p> <p>A collagen type can comprise one or several chains encoded by distinct genes</p> <p>E.g.: Collagen XXVIII is encoded by the <i>COL28A1</i> gene</p> <p>Collagen IV is encoded by 6 different genes that give rise to 3 collagen IV types resulting from 3 different chain assemblies</p>	COLXXIV Col24*
Aggregates	<p>Collagens form supramolecular aggregates in the extracellular space on their own or by forming alloys with other collagens or extracellular matrix molecules</p> <p>E.g.: Collagen fibers made of fibrillar collagens are generally heterotypic fibrils</p> <p>Beaded filaments are made with collagen VI only</p>	fibrils

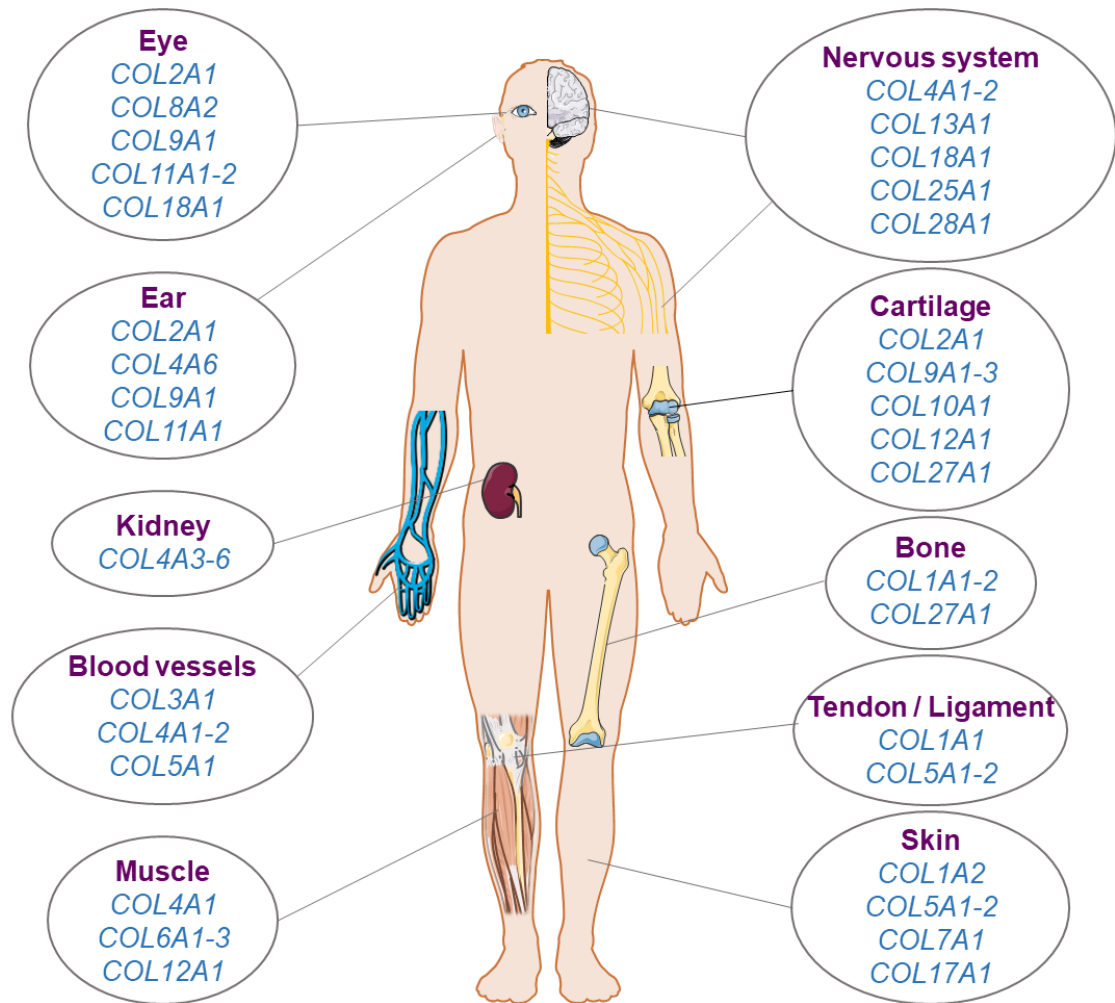
**Figure 1: The collagen lexicon.**

The collagen lexicon shows the different levels of collagen organization from the synthesis of individual  $\alpha$ -chains to the supramolecular aggregates found in the extracellular space, and indicates the commonly used nomenclature for each organizational level (left panel uses collagen XXIV as an example). \* The recommended nomenclature has the tendency to include now Arabic numbers not only for collagen genes but also the proteins.



**Figure 2: The collagen superfamily: biosynthesis, molecular structure and extracellular aggregates.** (A) Collagen I biosynthesis as a textbook case. *COL1A1* and *COL1A2* genes are translated respectively in  $\alpha 1(I)$  and  $\alpha 2(I)$  collagen  $\alpha$ -chains that start to assemble as a triple helix through their C-terminal domain in the endoplasmic reticulum. Procollagen  $[\alpha 1(I)]_2\alpha 2(I)$  transits through the Golgi apparatus and is secreted into the extracellular space for C- and N-terminal processing. Mature  $[\alpha 1(I)]_2\alpha 2(I)$  collagen assembles in supramolecular assembly to form collagen fibrils. (B) Examples of the high structural diversity of collagens that contain COL and NC domains of varying length and number (C) Prototype of a collagen molecule. All

collagens contain at least one and up to 27 triple helical regions (also named collagenous domain - COL) that are composed of  $(\text{Gly-Xaa-Yaa})_n$  repeats where Gly is a glycine, Xaa often a proline and Yaa often a hydroxyproline. Non-collagenous (NC) regions are intercalated between collagenous domains. Several others NC domains can be present at the C- or N-terminal part of the molecule and repeated up to 18 times as for the Fibronectin type III domain. (D) Collagen supramolecular aggregates. Mature collagens can assemble into specific homotypic or heterotypic supramolecular aggregates to fulfil their functions as exemplified here. Some collagens do not exhibit any particular supramolecular organization.



**Figure 3: Major tissues and organs of the human body affected by collagenopathies.**

For clarity, only primary affected functions of tissues and organs by a given collagen mutation are highlighted. This figure was created using Servier Medical Art under a Creative Commons Attribution 3.0 Unported License.



**Table 1:** Atlas of the 28 members of the collagen superfamily: genes, chain assembly, bioactive domains and supramolecular assemblies. Collagen types and genes for which mutations have been associated with human diseases are in bold.

Collagen type	Gene	$\alpha$ -chains	Released domain / Matricryptins	$\alpha$ -chains assembly	Supramolecular assembly
Fibrillar collagens					
<b>Collagen I</b>	<b>COL1A1</b> <b>COL1A2</b>	$\alpha_1(I)$ $\alpha_2(I)$	Trimer propeptide (C3) N-propeptide	$[\alpha_1(I)]_2, \alpha_2(I)$ $[\alpha_1(I)]_3$	Collagen fibril
<b>Collagen II</b>	<b>COL2A1</b>	$\alpha_1(II)$	Chondrocalcin N-propeptide	$[\alpha_1(II)]_3$	
<b>Collagen III</b>	<b>COL3A1</b>	$\alpha_1(III)$	N-propeptide C-propeptide	$[\alpha_1(III)]_3$	
<b>Collagen V</b>	<b>COL5A1</b> <b>COL5A2</b> <b>COL5A3</b>	$\alpha_1(V)$ $\alpha_2(V)$ $\alpha_3(V)$	TSPN <sup>a</sup>	$[\alpha_1(V)]_2, \alpha_2(V)$ $[\alpha_1(V)]_3$ $\alpha_1(V)\alpha_2(V)\alpha_3(V)$	
<b>Collagen V/XI</b>	<b>COL5A1</b> <b>COL11A1</b> <b>COL2A1</b>	$\alpha_1(V)$ $\alpha_2(XI)$ $\alpha_3(XI)^b$		$\alpha_1(XI)\alpha_1(V)\alpha_3(XI)$	
<b>Collagen XI<sup>b</sup></b>	<b>COL11A1</b> <b>COL11A2</b> <b>COL2A1<sup>b</sup></b>	$\alpha_1(XI)$ $\alpha_2(XI)$ $\alpha_3(XI)^b$	TSPN <sup>a</sup>	$\alpha_1(XI)\alpha_2(XI)\alpha_3(XI)$	
Collagen XXIV	<b>COL24A1</b>	$\alpha_1(XXIV)$		$[\alpha_1(XXIV)]_3$	
<b>Collagen XXVII</b>	<b>COL27A1</b>	$\alpha_1(XXVII)$		$[\alpha_1(XXVII)]_3$	
Fibril-associated collagens with interrupted triple helices (FACIT)					
<b>Collagen IX</b>	<b>COL9A1</b> <b>COL9A2</b> <b>COL9A3</b>	$\alpha_1(IX)$ $\alpha_2(IX)$ $\alpha_3(IX)$	TSPN <sup>a</sup>	$\alpha_1(IX), \alpha_2(IX),$ $\alpha_3(IX)$	Fibrils Associated Collagens
<b>Collagen XII</b>	<b>COL12A1</b>	$\alpha_1(XII)$		$[\alpha_1(XII)]_3$	
Collagen XIV	<b>COL14A1</b>	$\alpha_1(XIV)$		$[\alpha_1(XIV)]_3$	
Collagen XVI	<b>COL16A1</b>	$\alpha_1(XVI)$		$[\alpha_1(XVI)]_3$	
Collagen XIX	<b>COL19A1</b>	$\alpha_1(XIX)$	NC1 $\alpha_1(XIX)$	$[\alpha_1(XIX)]_3$	
Collagen XX	<b>COL20A1</b>	$\alpha_1(XX)$		$[\alpha_1(XX)]_3$	

Collagen XXI	<i>COL21A1</i>	$\alpha_1(\text{XXI})$		$[\alpha_1(\text{XXI})]_3$	
Collagen XXII	<i>COL22A1</i>	$\alpha_1(\text{XXII})$		$[\alpha_1(\text{XXII})]_3$	
Network forming collagens					
Collagen IV	<b><i>COL4A1</i></b>	$\alpha_1(\text{IV})$	Arresten	$[\alpha_1(\text{I})]_2, \alpha_2(\text{IV})$ $\alpha_3(\text{IV}), \alpha_4(\text{IV}),$ $\alpha_5(\text{IV})$ $[\alpha_5(\text{I})]_2, \alpha_6(\text{IV})$	Hexagonal network
	<b><i>COL4A2</i></b>	$\alpha_2(\text{IV})$	$\alpha_1(\text{IV})$ 1263-1277		
	<b><i>COL4A3</i></b>	$\alpha_3(\text{IV})$	$\alpha_1(\text{IV})$ CB3		
	<b><i>COL4A4</i></b>	$\alpha_4(\text{IV})$	$\alpha_1(\text{IV})$ 531-543		
	<b><i>COL4A5</i></b>	$\alpha_5(\text{IV}),$	anstatin		
	<b><i>COL4A6</i></b>	$\alpha_6(\text{IV})$	Tumstatin Oncothanin Tetrastatin 1-3 Pentastatin 1-3 Hexastatin 1-2 NC1 $\alpha_6(\text{IV})$		
Collagen VI <sup>c</sup>	<b><i>COL6A1</i></b>	$\alpha_1(\text{VI}),$		$\alpha_1(\text{VI})\alpha_2(\text{VI})\alpha_3(\text{VI})$ $\alpha_1(\text{VI})\alpha_2(\text{VI})\alpha_5(\text{VI})$ $\alpha_1(\text{VI})\alpha_2(\text{VI})\alpha_6(\text{VI})$	Beaded filaments
	<b><i>COL6A2</i></b>	$\alpha_2(\text{VI})$			
	<b><i>COL6A3</i></b>	$\alpha_3(\text{VI})$			
	<i>COL6A5</i>	$\alpha_5(\text{VI})$			
	<i>COL6A6</i>	$\alpha_6(\text{VI})$			
Collagen VII	<b><i>COL7A1</i></b>	$\alpha_1(\text{VII})$		$[\alpha_1(\text{VII})]_3$	Anchoring fibrils
Collagen VIII	<i>COL8A1</i>	$\alpha_1(\text{VIII}),$ $\alpha_2(\text{VIII})$	Vastatin	$[\alpha_1(\text{VIII})]_3$ $[\alpha_2(\text{VIII})]_3$ $[\alpha_1(\text{VIII})]_2, \alpha_2(\text{VIII})$ $\alpha_1(\text{VIII}), [\alpha_2(\text{VIII})]_2$	Hexagonal network
	<b><i>COL8A2</i></b>				
Collagen X	<b><i>COL10A1</i></b>	$\alpha_1(\text{X})$		$[\alpha_1(\text{X})]_3$	
Transmembrane collagens or MACIT for Membrane-Associated Collagens with Interrupted Triple helices)					
Collagen XIII	<b><i>COL13A1</i></b>	$\alpha_1(\text{XIII})$		$[\alpha_1(\text{XIII})]_3$	
Collagen XVII	<b><i>COL17A1</i></b>	$\alpha_1(\text{XVII})$		$[\alpha_1(\text{XVII})]_3$	
Collagen XXIII	<i>COL23A1</i>	$\alpha_1(\text{XXIII})$		$[\alpha_1(\text{XXIII})]_3$	
Collagen XXV	<i>COL25A1</i>	$\alpha_1(\text{XXV})$		$[\alpha_1(\text{XXV})]_3$	
Multiplexins					
Collagen XV	<i>COL15A1</i>	$\alpha_1(\text{XV})$	Restin	$[\alpha_1(\text{XV})]_3$	Basement membrane associated collagens
Collagen XVIII	<b><i>COL18A1</i></b>	$\alpha_1(\text{XVIII})$	Endostatin Neostatin 7 and 14 Frizzled module	$[\alpha_1(\text{XVIII})]_3$	
Others					
Collagen XXVI	<i>COL26A1</i>	$\alpha_1(\text{XXVI})$		$[\alpha_1(\text{XXVI})]_3$	
Collagen XXVIII	<b><i>COL28A1</i></b>	$\alpha_1(\text{XXVIII})$		$[\alpha_1(\text{XXVIII})]_3$	

<sup>a</sup> TSPN is released from the N-terminal extremity of the collagen molecules by BMP1 activity and has no known function so far; <sup>b</sup>  $\alpha_3(\text{XI})$  is encoded by *COL2A1* gene. The  $\alpha_3(\text{XI})$  incorporated in the collagen XI molecule is overmodified compared to  $\alpha_1(\text{II})$  that forms the collagen II molecule; <sup>c</sup> In contrast to its murine ortholog, the human *COL6A4* gene has been converted into pseudogene by a chromosomal break.

## Abstract

The extracellular matrix (ECM) provides structural support for cells and tissues but it also functions as signaling platforms that can influence almost every decision made by the cells through chemical and mechanical signals. Dysregulation or deficiency of the ECM components can lead to various acquired and inherited disorders. Structural changes in the skin ECM also represent one of the hallmarks of the aging process. In that context, my PhD aimed at identifying new regulators of the dermal ECM synthesis and organization.

Over the past years, *Caenorhabditis elegans* has emerged as a powerful genetic model organism with predictive values in mammals to study the aging process. Our attention was particularly drawn to two genes: *aak-2* and *skn-1* for their regulation of *C.elegans* longevity through upregulation of several collagen genes. Collagens are the major proteins of the ECM. We therefore investigated if AMPK and NRF2, which are the mammalian orthologs of AAK-2 and SKN-1 respectively, could also regulate ECM gene expression in human skin primary fibroblasts, which are the main producers of ECM in skin.

As with aging AMPK and NRF2 activities decline, I used siRNA and/or shRNA to silence AMPK and NRF2 to mimic dermal aging and to investigate potential changes in functional and mechanical properties of the secreted ECM. Since the first set of data obtained with NRF2 was the most promising, I further focused my PhD work on the transcription factor NRF2.

NRF2 is a master regulator of the oxidative stress response and regulates the transcription of numerous cytoprotective genes. RNA-seq analysis of siNrf2 fibroblasts revealed that, additionally to NRF2 conventional targets, ECM genes were the most represented differently expressed genes. Composition and topology of the ECM produced by shNrf2 dermal fibroblasts were analyzed using a combination of light and electron microscopies. We showed that NRF2 silencing in human skin fibroblasts reduces collagen I expression and impacts collagen fibril formation and organization. Our data suggest that ZNF469, a zinc finger protein identified in our study as a new target of NRF2, is involved in the collagen I transcriptional regulatory cascade triggered by NRF2. ZNF469 is indeed involved in connective tissue diseases and has been suggested as a regulator of collagen expression.

In conclusion, this PhD work identifies NRF2 as a positive regulator involved in ECM regulation and homeostasis in human skin fibroblasts and in that, represents a promising new target in anti-aging strategies.

**Keywords:** human dermal fibroblasts, extracellular matrix, NRF2, collagens, fibrillogenesis, RNA-seq, RNA silencing strategies