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Deciphering the role of oncofetal fibronectin isoforms in matrix assembly and cellular function

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Georgios Efthymiou. Deciphering the role of oncofetal fibronectin isoforms in matrix assembly and cellular function. Molecular biology. COMUE Université Côte d'Azur (2015 - 2019), 2019. English. NNT : 2019AZUR6024 . tel-02971506

HAL Id: tel-02971506

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THÈSE DE DOCTORAT

Etude d'assemblage et des fonctions des isoformes oncofoetales de la fibronectine

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**Présentée en vue de l'obtention
du grade de docteur en Biologie d' Université
Côte d'Azur**

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Soutenue le : 19 Décembre 2019 à Nice

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Etude d'assemblage et des fonctions des isoformes oncofoetales de la fibronectine

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Titre

Etude d'assemblage et des fonctions des isoformes oncofoetales de la fibronectine

Resumé

La matrice extracellulaire (MEC) constitue une plateforme fibrillaire intégrant l'action des signaux chimiques et mécaniques de l'environnement. La fibronectine (FN), un composant majeur de la MEC, est au centre de cette plateforme de biorégulation et module de nombreuses actions biologiques telles que l'adhésion et la motilité cellulaires, la prolifération et la différenciation, ainsi que la structure et le dépôt de la MEC. La FN se présente sous deux formes : la FN plasmatique (pFN) et la FN cellulaire (cFN), dite « oncofoetale ». Cette dernière est nommée ainsi pour son expression uniquement au cours du développement et dans certaines conditions physiopathologiques (réparation tissulaire, inflammation et cancer). La différence entre les deux est l'existence dans la cFN des domaines supplémentaires EDB et EDA, qui résultent d'un épissage alternatif. Comment la présence de ces « extra-domaines », EDA et EDB, régit l'assemblage des FN et comment les variantes assemblées régulent le comportement des cellules est en grande partie inconnu. Des études de délétion ciblées d'un seul domaine chez la souris ont révélé le rôle de l'EDA dans des phénomènes très variés dont la morphogenèse des valves lymphatiques, l'athérosclérose et la cicatrisation / fibrose. Au niveau moléculaire, l'inclusion de l'EDA élargit le répertoire des récepteurs cellulaires de la FN (intégrines $\alpha4\beta1$, $\alpha7\beta1$ et TLR4). A ce jour, aucun récepteur n'a été rapporté pour l'EDB. De ce fait il a été proposé que sa présence modifierait la conformation du site de liaison de FN aux cellules et faciliterait ainsi l'assemblage fibrillaire induit par les intégrines via des mécanismes qui restent à établir.

Le but des travaux de cette thèse était de décoder les rôles de l'EDA et de l'EDB de la FN, et plus précisément de :

1) étudier l'impact de la présence des domaines EDA et EDB sur l'assemblage fibrillaire de la FN à la surface de cellules compétentes pour l'assemblage, et

2) déterminer comment la présence de l'EDB et de l'EDA influence les propriétés biochimiques, physiques et fonctionnelles de la matrice, qui à leur tour affectent le comportement des cellules qui y sont attachées.

Dans un premier temps l'équipe a développé un ensemble d'outils biologiques composé de : i) vecteurs lentiviraux hébergeant la séquence codante complète du gène de la FN humaine contenant l'un, les deux ou aucun des « extra-domaines » alternativement épissés, ii) des variants de FN recombinants purifiés, iii) des fibroblastes embryonnaires de souris FN -/- (Fn1 -/- MEF), et iv) des MEF exprimant des variantes de FN. Cette batterie d'outils uniques a été utilisée pour étudier l'assemblage spécifique de variantes par les fibroblastes et les effets de réseaux de cFN homogène sur le comportement cellulaire.

Nos résultats ont montré que les « extra-domaines » de la FN sont responsables au niveau des fibroblastes du réglage fin de l'amplitude de plusieurs réponses dont l'assemblage de la matrice, la croissance et le métabolisme énergétique. En utilisant une approche informatique « non-biaisée », nous avons démontré que la présence des « extra-domaines » modifie la structure de la matrice de la FN déposée par les fibroblastes. Ceci témoigne d'événements de signalisation cellulaire différents, qui sont susceptibles de modifier aussi bien les réponses précoces que tardives induites par la FN. Les matrices variant-spécifiques que nous avons développées représentent des outils très puissants pour décrypter les fonctions des « extra-domaines » de la FN dans les multiples types de cellules impliquées non seulement dans des réponses physiologiques mais également dans des situations pathologiques.

Mots clés : matrice extracellulaire, fibronectine, extra-domaines, EDB, EDA, intégrines, fibroblastes embryonnaires de souris, cancer

Title

Deciphering the role of oncofetal fibronectin isoforms in matrix assembly and cellular function

Abstract

The Extracellular Matrix (ECM) constitutes a fibrillar platform that integrates the action of chemical and mechanical cues from the environment. Fibronectin (FN), a major component of the ECM, is at the center of this bioregulatory stage, modulating numerous biological procedures such as cell adhesion and motility, cell proliferation and differentiation, as well as ECM deposition and structure. FN is found in two forms: plasma FN (pFN) and cellular FN (cFN). cFN differs from pFN by the presence of alternatively spliced Extra Domains, namely EDB and EDA. Each of these alternatively spliced regions is encoded by a single exon, the regulation of which is strictly regulated and limited to embryonic tissues, as well as pathophysiological conditions such as wound healing, inflammation, and cancer. The term “oncofetal” was coined in order to describe FN isoforms harboring either of the Extra Domains, that are normally absent in adult tissues.

How the presence of EDA and EDB regulates FN assembly and how the assembled variants regulate cell behavior is largely unknown. Single Extra Domain-targeted deletion studies in the mouse have revealed roles for EDA in the morphogenesis of lymphatic valves, atherosclerosis, and wound healing/fibrosis. Mechanistically, the inclusion of EDA expands the repertoire of cellular FN receptors ($\alpha4\beta1$, $\alpha7\beta1$ integrins and TLR4). To date, no ligand has been reported for EDB. Rather, its presence has been postulated to alter the conformation of the cell-binding domain of FN and to facilitate integrin-driven fibrillar assembly by mechanisms that remain to be established.

The aim of this dissertation is to provide more insight regarding the roles of the FN Extra Domains EDA and EDB, and more specifically:

- 1) To investigate how the presence of alternatively spliced EDA and EDB domains affect the fibrillar assembly of FN at the surface of assembly-competent cells, and

2) To determining how the presence of EDB and EDA influences the biochemical, physical, and functional properties of the matrix that in turn affect the behavior of cells attached to it.

Here we present a biological toolset composed of : i) lentiviral vectors harboring the full-length coding sequence of human cFN containing one, both, or none of the alternatively spliced Extra Domains, ii) purified recombinant FN variants, iii) FN-null mouse embryo fibroblasts (MEFs) and iv) FN variant-expressing MEFs. These unique tools were used to study variant-specific assembly by fibroblasts and the effects of homogeneous cFN networks on cell behavior.

Utilizing an unbiased computational approach, we document that the presence of the Extra Domains results in a distinct pattern of FN deposition and assembly that in turn influences both early and late signaling events that control cell proliferation as well as cell contractility. Finally, we present a series of novel data that point towards a FN-impacted control of cell energetics.

We conclude that FN Extra Domains are responsible for the fine-tuning of the extent of several cellular processes that can reflect strict regulation in pathophysiological procedures such as tissue repair, fibrosis, and tumor progression.

Keywords: extracellular matrix, fibronectin, extra domains, EDB, EDA, integrins, mouse embryo fibroblasts, cancer

To all those who helped me get where I am now.
In their own personal way.

Acknowledgments

At the time that these lines are being written, it has been almost four years since I started my PhD back in 2015, just two months after being recruited in the highly competitive SIGNALIFE PhD program. Four years are definitely not a long period, but it is long enough for knowledge and experience to be obtained, and bonds to be formed among people, the gratitude to whom is hard for me to put into words.

I would like to start by thanking all the people involved in the Labex SIGNALIFE Program. A large family who has worked together in order to create an outstanding platform that has functioned as a springboard for me and other young researchers, providing the funds needed for the smooth completion of our studies. I would like to acknowledge the efforts of Konstanze Beck who has been the guide and companion for me and so many other SIGNALIFE Students since our arrival in France, as well as Martine Avella, the Labex SIGNALIFE Project Officer, and Anna Bliznyuk, the Labex SIGNALIFE Assistant.

At this point, it is also of major importance for me to thank the ARC Scientific Committee who deemed me worthy of one extra year of funding, which has proved both useful and necessary.

My greatest thanks goes to a group of people that have been my second family for the last four years: the team “Adhesion Signaling and Stromal Reprogramming in the Tumor Microenvironment” led by Dr Ellen Van Obberghen-Schilling. My words can in no way describe how grateful I am to Ellen. First for accepting me in the group and secondly for trusting me throughout all these years. You have been a real mentor and an inspiring scientist, who considered me a colleague. I thank you for always being available to discuss, search, troubleshoot, and for always hearing my opinions and proposals. The outcome of those four years is the result of fruitful communication and real teamwork between us. For that, I truly thank you, and I hope that all my future professional partnerships will be like this.

A big “merci” to Dominique Grall. As I used to say “Sans labo, Dominique existe. Sans Dominique, labo n’existe pas”! Do, you were the first person who came in direct

contact with my initial ignorance and clumsiness. Thanks to you, I learned so much in such a short time. Thank you for all your technical help in the lab, your patience, and your support. Thank you for our discussions and for believing in me.

Delphine Ciais has played a special part in my life in the lab. You have always been the toughest to convince and through this, you taught me how to be perseverant, coherent, concise, having a critical mind, and distinguish the difference between “doing experiments” and “finding the right approach to answer a question”. We have not really *worked* together, but our interaction has been of catalytic importance to me.

I feel more than grateful for having met and worked side by side with so many people that have been members of this beautiful family. Primarily, Agata Radwanska, who was the initiator of the project that eventually became my own. I owe you so much. Special thanks to Angélique Saint, the “médecin traitant du labo”, as well as Michael Ruff, “notre petit Michael”, for their help, support, and insight, but also for making the everyday routine more pleasant! I would also like to thank all the “younger” members of the lab: young students, master students, interns. Each one of them has contributed, even in some small way, to “surviving the thesis”!

During those four years, I have been lucky to work and interact with people from different laboratories as well. I greatly appreciate the invaluable insight, help, and sharing of knowledge and expertise provided to me by Laurent Counillon and all the members of the team “Ionic Transport Pathophysiology” at the Laboratory of Molecular Physiomedicine (LP2M), with a great emphasis to the contribution of Mallorie Poet. Additionally, I am indebted to Didier Pisani for his priceless help with the Seahorse analyzer, and Sabrina Pisano for all the AFM microscopy sessions and analysis she has performed for me.

It would be a serious omission if I did not include our collaborators in the i3S (Laboratoire d'Informatique, Signaux et Systèmes de Sophia Antipolis), namely Laure Blanc Feraud and Xavier Descombes, as well as the SIGNALIFE student Anca Ioana Grapa. Thank you very much for our nice collaboration. Many of the results in this manuscript would not have been possible without your aid and effort.

Of great importance has been the help I received from the different core facilities of the institute. Thus, I would like to thank all the people that took part in my efforts, directly and indirectly. More specifically, I would like to express my thanks to Maurice Hattab and the Biochemistry and Molecular Biology Facility for taking care of the purification of the proteins I have been working with all these years. To the Imaging facility and its personnel for all the trainings, assistance, analyses, and troubleshooting, especially Magali Mondin, Sébastien Schaub, and Simon Lachambre. I also thank in the Histopathology facility, Samah Rekima and Belinda Desrues, as well as Agnès Loubat for her assistance in the Cytometry facility. Finally, I would like to thank all the members of the institute, students, researchers, engineers, administration personnel, and the wonderful ladies in the common services who have collectively made my lab life and work so much easier. Thank you all!

A special thanks to the members of the scientific committee, for accepting to review and evaluate my work, Dr. Corinne Albigès-Rizo and Dr. Patricia Rousselle, for taking the time to come to Nice for my thesis defense. I also express my thanks to Dr. Robert Arkowitz for being my internal advisor during these four years, and for presiding the thesis committee.

This endeavor would not have been successful if not for several people who have been and have become part of my life outside of the lab. I would like to thank my close friends, my friends and my acquaintances in Nice and anywhere else in the world for all the moments we have shared together. You have formed me in so many ways.

Finally, I would like to thank my family. Εσάς, που με ενθαρρύνετε πάντα και δεν έχετε σταματήσει λειπτό να πιστεύετε σε μένα και στις δυνατότητές μου. Σας ευχαριστώ για την υποστήριξή σας σε όλες τις στιγμές της ζωής μου έως τώρα, και για την παρουσία σας σε κάθε στιγμή, ευχάριστη ή λιγότερο ευχάριστη. Ότι έχω καταφέρει έως τώρα το οφείλω εξίσου σε σας. Ακόμη κι αν δεν το δείχνω, σας ευχαριστώ.

Abbreviations

67LR: 67 kDa laminin receptor

ADMIDAS: adjacent to metal-ion-dependent adhesion site

AFM: atomic force microscopy

AMPK: 5' adenosine monophosphate-activated protein kinase

B-CAM: basal cell adhesion molecule

BM: basal membrane

cFN: cellular fibronectin

CS: chondroitin sulfate

CTGF: connective tissue growth factor

DDR: discoidin domain receptor

DGC: dystrophin glycoprotein complex

DS: dermatan sulfate

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ERK: extracellular signal-regulated kinase

ESE: exonic splicing enhancer

FACITs: fibril associated collagens with interrupted triple helix

FAK: focal adhesion kinase

FERM: protein 4.1, ezrin, radixin, moesin

FKBP12: FK506 binding protein 12

FN: fibronectin

GAGs: glycosaminoglycans

GARP: glycoprotein A repetitions predominant

GPVI: glycoprotein VI

GS domain: Gly-Ser-rich domain

HA: hyaluronic acid

Hep: heparin sulfate (may also refer to the respective binding site on FN)

HIF1: hypoxia inducible factor 1
HRS: hepatic arginine-serine rich protein
HS: heparan sulfate
IAP: integrin-associated protein
ILK: integrin linked kinase
ISE: intronic splicing enhancers
JAK-STAT: Janus kinases – Signal transducer and activator of transcription proteins
JNK: c-Jun N-terminal kinase
KS: keratan sulfate
LAIR-1: leukocyte-associated immunoglobulin-like receptor-1
LETS: large, external transformation-sensitive
LGD: laminin globular domain
LLC: large latent complex
LOX: lysil oxidase
LRRC32: leucine-rich repeat-containing protein 32
LTBP: latent TGF- β binding protein
Lu: Lutheran
MACITs: membrane associated collagens with interrupted triple helices
MAPK: mitogen activated protein kinase
MIDAS: metal-ion-dependent adhesion site
mTOR: mechanistic (or mammalian) target of rapamycin
PAI1: plasminogen activator inhibitor 1
PDGF: platelet derived growth factor
PDGFR: platelet derived growth factor receptor
pFN: plasma fibronectin
PGs: proteoglycans
PI3K: phosphoinositide 3-kinase
PIPKI γ : phosphatidylinositol phosphate kinase type I γ
PKB: protein kinase B, also known as Akt
PSI: plexin-semaphorin-integrin

PTB: phospho-tyrosine binding
RIAM: Rap1-GTP-interacting adaptor molecule
ROS: reactive oxygen species
RSV: Rous Sarcoma Virus
RTK: receptor tyrosine kinase
SDS: sodium dodecyl sulfate
SF-A: fibroblast surface antigen
SLC: small latent complex
SMAD: SMA (small work phenotype) and MAD (mothers against decapentaplegic) family of proteins
SRSF5: serine and arginine rich splicing factor 5
TGFBI: TGF- β inducible gene
TGF β RI: transforming growth factor receptor I
TGF β RII: transforming growth factor receptor II
TGF- β : transforming growth factor β
TIAM1: T-cell lymphoma invasion and metastasis 1
TMD: transmembrane domain
uPARAP: urokinase-type plasminogen activator associated protein
VBS: vinculin binding site
VEGFR: vascular endothelial growth factor receptor
 α -SMA: alpha smooth muscle actin

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Prologue

It has been almost 300 years since Carl von Linné coined the term *biologi* in his work *Bibliotheca botanica* in 1736. A term that has evolved as much as the scientific field it describes: “The objects of our research will be the different forms and manifestations of life, the conditions and laws under which these phenomena occur, and the causes through which they have been effected. The science that concerns itself with these objects we will indicate by the name *biology* or the doctrine of life” – Gottfried Reinhold Treviranus.

Biology (βιολογία), the study of (-λογία > λόγος) life (βίος), is a relatively recent scientific field despite its ancient roots. It was Antonie van Leeuwenhoek’s improvement of the microscope that led biology to its dramatic development. From the mere observation of unicellular organisms to protein purification, and from the discovery of the genetic material to gene manipulation, biology has been by far one of the most rapidly developing scientific fields.

Biological research initiated as an approach to simplify and elucidate the complex phenomena that govern life. Nowadays, biology is a scientific field that feeds from several domains (mathematics, physics, informatics) providing us with an explosive amount of information and new data on the origins of life, the function of complex intertwined cell communication, and the building of entire organisms and ecosystems. The simultaneous study of all known biological systems is impracticable. This could have possibly been the case 150 years ago. That is why it is important to break down the different biological systems in order to be able to extract useful information, but always bearing in mind that no oversimplified system makes sense except in the light of its entirety, paraphrasing the title of the well-known essay by Theodosius Dobzhansky (Dobzhansky 1973).

In this work, I will try to compile known data, as well as to enrich current knowledge with novel results regarding a major aspect of molecular cell biology in multicellular organisms (and more specifically in vertebrates, mainly focusing on mammals) that is “Cells in their Social Context”. Tissues in multicellular organisms are not made up solely of cells. They are also composed of a remarkably complex

network of macromolecules, collectively known as the extracellular matrix (ECM). These macromolecules, secreted by the cells, provide structural and functional cues to the adjacent cells, resulting in the formation of the highly organized multicellular structure. The target ECM component of this dissertation is fibronectin, which will be discussed extensively in terms of structure, function, expression patterns in health and disease, and how its different forms affect cell behavior.

The following pages consist of an introduction on the nature of the extracellular matrix, and the key players that mediate cell-ECM interactions: the integrins. It is beyond the scope of this manuscript to present all the knowledge obtained about the ECM, the various ECM components, and the integrin structure and function. Basic background information will be presented instead, in order to set the stage before proceeding to a detailed description of “fibronectin, the extracellular glue” (Zollinger and Smith 2017).

1. Introduction

In the macroscopic scale, no organism or population can be viewed as an individual entity. On the contrary, it has to be considered as a part of a dynamic ecosystem composed of other individuals and/or populations, as well as the surrounding environment. This notion, initially described by Plato in his Dialogues, is also reflected in the microscale: each cell is part of a tightly regulated community of cells and extracellular material, the extracellular matrix or ECM.

1.1 The Extracellular Matrix

The ECM is an ensemble of high molecular weight macromolecules expressed by the underlying cells that forms an astonishingly elaborate and dynamic structure that constantly interacts with cells in the vicinity. This cell-derived biomaterial had not been fairly characterized until recently when a set of 1056 genes was identified and described by Naba and colleagues (Naba et al. 2012).

The extracellular matrix is a major component of all tissues in every organ, and it provides mechanical support and physical scaffolding, as well as a wide range of crucial biochemical and biomechanical cues regulating the behavior of the residing cells. This way, the ECM influences their survival, development, migration, proliferation, shape, and function, thus guiding multiple facets of different processes such as morphogenesis and tissue homeostasis (Frantz, Stewart, and Weaver 2010; Shaohe Wang et al. 2017; Trapani, Bonaldo, and Corallo 2017).

Depending on the tissue, the extracellular matrix can possess different inherent properties, characteristic of that tissue. It can become calcified to form the rock-hard structures of bone and teeth, it can form the transparent substance of the cornea, or it can adopt the ropelike organization that gives tendons their enormous tensile strength. This extensive variety of different matrix types is reflected in the numerous interactions among the components of the matrix (collectively known as the *matrisome*) and the components that are not necessarily part of the matrix but plausibly do associate with it. Such components are growth and secreted factors, ECM-modifying enzymes and other proteins (R. O. Hynes and Naba 2012). This interplay as

well as with the cells themselves, mainly through a family of cell receptors known as integrins (Richard O Hynes 2002), results in the ECM diversity across tissues.

1.1.1 ECM Types

Just as the same genome gives rise to different cell types in an organism, the same matrix generates different matrix types depending on cell type and localization, even within the same tissue. In a broad sense, two types of ECMs exist varying in composition and structure: i) the interstitial matrix and ii) basement membranes (BMs) or basal laminae (**Figure 1**). Interstitial matrices surround cells, while BMs lie in the interphase between the epithelial cells of the parenchyma and the mesenchymal tissue, the latter being characterized by the presence of interstitial matrix.

The BM is a thin (40-120nm), tough, flexible sheet of matrix molecules that lines all epithelia, but also surrounds individual muscle fibers, fat cells, and Schwann cells. Although small in volume, it plays a critical role in body architecture. The basal lamina separates cells

and epithelia from the underlying connective tissue and forms the mechanical connections between them. In a different setting, a basal lamina can serve as a filtering barrier, as is the case for the kidney glomerulus. Basal membranes can also serve to determine cell polarity, influence cell metabolism, organize the proteins in adjacent plasma membranes, promote cell survival, proliferation and differentiation, and serve as highways for cell migration (Alberts 2015).

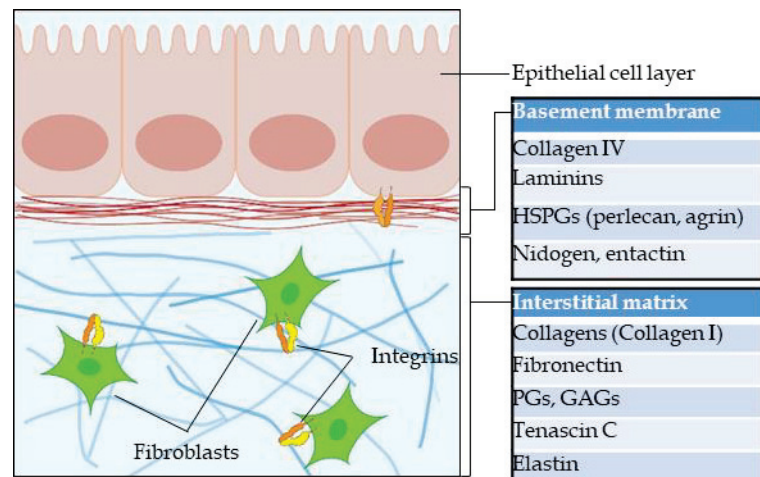


Figure 1 Epithelial cells reside on a rigid basement membrane (BM) that separates them from the mesenchymal part of the tissue where mesenchymal cells, like fibroblasts, produce and deposit the interstitial matrix. Each matrix type is composed of a set of different components that influence their physical properties and functions. Adapted from *Bonnans et al. 2014*.

1.1.2 ECM Components

Any typical matrix is composed of a large variety of macromolecules that share characteristic modular domains. Based on this feature, Naba and colleagues bioinformatically defined ECM components by screening for proteins containing domains characteristic of ECM proteins, ECM-affiliated proteins, ECM modifiers, and secreted factors. At the same time, they eliminated proteins that shared one or more of the defining domains without being part of the ECM (**Figure 2**).

In silico results were supported by biochemical analyses on normal and tumor tissues resulting in the establishment of the core matrisome, comprising 278 genes giving rise to proteoglycans (PGs), collagens, and glycoproteins. Furthermore, a second gene set, denoted as matrisome-associated components was established, containing 778 genes that produce ECM-affiliated components, ECM regulators, and secreted factors (Naba et al. 2012). The matrisome is mainly produced locally by cells in the matrix, which also help to organize the matrix through forces generated by the cytoskeleton influencing the orientation of the matrix components outside of the cell.

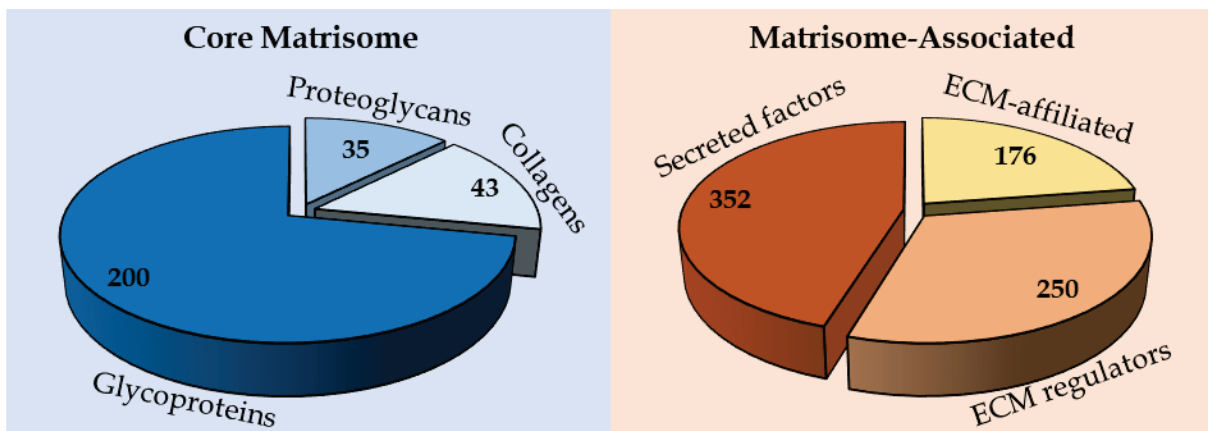


Figure 2 In silico definition of ECM components identified 1056 genes comprising two distinct groups. The core matrisome, composed of 278 genes, is responsible for the generation of the structural components of the ECM. The second set is composed of matrisome-associated genes that give rise to proteins linked to the ECM, proteins that regulate ECM form and function, and soluble factors that are known or believed to bind to the ECM.

1.1.2.1 Glycosaminoglycans and Proteoglycans

Glycosaminoglycans (GAGs) or mucopolysaccharides are composed of several repeats of a disaccharide unit, which is characteristic of each GAG. One of the two sugars in the repeating unit is always an amino sugar (N-acetylglucosamine or N-acetylgalactosamine), while the second is an uronic acid (glucuronic or iduronic) or

galactose. GAGs are subject to extensive modification: sulfation, carboxylation, deacetylation, and epimerization (Townley and Bülow 2018), and they form stiff, highly hydrophobic chains that cannot compact to form globular structures. Thus, GAGs adopt extended conformations with a very high volume to mass ratio, attracting Na⁺ and other cations that, in turn, capture large amounts of water forming highly hydrated gel-like structures with high viscosity and elasticity. These structures fill most of the extracellular space, and can withstand massive compressive forces (Alberts 2015; Sararman et al. 2016). A characteristic example of such matrix is the cartilage that lines the knee joint.

Six classes of GAGs are distinguished by their sugars, the type of oligosaccharide linker they contain, and the number and location of sulfate groups: chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS), heparin sulfate (Hep), keratan sulfate (KS), and hyaluronan or hyaluronic acid (HA) (Sararman et al. 2016). Two of the aforementioned classes, CS and DS, contain N-acetylgalactosamine as one of the repeating sugars, while the second sugar in the unit is either glucuronic acid or iduronic acid. HS and HA are copolymers of N-acetylglucosamine and glucuronic acid. The disaccharide unit in Hep is N-acetylglucosamine and iduronic acid, while KS is composed of units containing N-acetylglucosamine and galactose.

As mentioned earlier, all GAGs are characterized by long and unbranched, extensively modified sugar chains, and by the covalent attachment to a core protein, giving rise to proteoglycans (PGs). The only exception is HA. Its sugar chains are not sulfated, and it is not covalently linked to any core protein. Instead, it interacts with PGs via hyaluronan-binding motifs in a non-covalent fashion (Sararman et al. 2016).

PGs can be classified based on localization, gene/protein homology, and the utilization of specific protein modules within their respective protein cores (**Table 1**). These criteria result in the generation of four major families with distinct forms and functions: the intracellular, cell-surface, pericellular and extracellular PGs (Iozzo and Schaefer 2015).

PGs interact with numerous growth factors, cytokines and chemokines, cell surface receptors and ECM components through either their core proteins, or their

GAG chains, thus participating in key regulatory cell processes such as signaling, differentiation and proliferation, adhesion and migration, and apoptosis (Iozzo and Schaefer 2015; Theocharis et al. 2016). Additionally, they can interact both with cells and with other components of the ECM, playing an important role in ECM scaffolding and remodeling, and in cell distribution within it.

Thus, they affect normal physiology as well as the development of various diseases [reviewed in detail in (Townley and Bülow 2018; Sasarman et al. 2016; Iozzo and Schaefer 2015; Schaefer and Schaefer 2010; Theocharis et al. 2010; Afratis et al. 2012; Theocharis et al. 2014)].

Intracellular PGs	Cell Surface PGs	Pericellular & BM PGs	Extracellular PGs			Other (part-time PGs)
			Hyalectans (4)	SLRPs (18)	SPOCKs (3)	
Serglycin	Syndecans 1 - 4	Perlecan	Aggrecan	Class I	Testican	Prg4/lubricin
	CSPG4/NG2	Aggrin	Versican	Class II	SPARC/ Osteonectin CWCV	Endocan
	Betaglycan	Collagens XVIII & XV	Neurocan	Class III	Kazal-like domain PGs	Leprecan
	Phosphacan		Brevican	Class IV		Collagens IX & XII
	Glypicans 1 - 6			Class V		

Table 1 Proteoglycans as described in *Iozzo and Schaefer 2015*.

1.1.2.2 Collagens and Glycoproteins

Apart from the GAGs (connected to a core protein or not), there are almost 250 proteins, 43 of which are distinct collagen subunits, and around 200 glycoproteins constituting the matrisome (R. O. Hynes and Naba 2012). An extensive description of every ECM constituent is beyond the scope of this dissertation, so only a brief overview of the major collagens and glycoproteins follows ahead.

Collagens

The collagens are a family of fibrous and non-fibrous proteins found in all metazoa (**Table 2**). Collagens can form fibrils or networks, they can be laterally associated to other fibrils or serve as core proteins for proteoglycans, or they can be found docked through the plasma membrane. They are secreted in large quantities by connective-tissue cells, and they constitute the main structural element of the ECM.

	Type	Composition	Distribution
Fibrillar Collagens	I	$\alpha 1(I)_2\alpha 2(I)$	Bone, dermis, tendon, ligament, cornea, etc
	II	$\alpha 1(II)_3$	Cartilage, vitreous
	III	$\alpha 1(III)_3$	Co-distributed with collagen I, skin, blood vessels, intestine
	V	$\alpha 1(V)_3$	Co-distributed with collagen I, bone, dermis, cornea, placenta
		$\alpha 1(V)_2\alpha 2(V)$	
		$\alpha 1(V)\alpha 2(V)\alpha 3(V)$	
	XI	$\alpha 1(XI)\alpha 2(XI)\alpha 3(XI)$	Co-distributed with collagen II, cartilage, intervertebral disc
XXIV		Bone, cornea	
XXVII		Cartilage	
Network-forming collagens	IV	$\alpha 1(IV)_2\alpha 2(IV)$	Basement membranes
		$\alpha 1(IV)\alpha 4(IV)\alpha 5(IV)$	
		$\alpha 5(IV)_2\alpha 6(IV)$	
	VIII	$\alpha 1(VIII)_3$	Dermis, brain, heart, kidney
		$\alpha 2(VIII)_3$	
		$\alpha 1(VIII)_2\alpha 2(VIII)$	
X	$\alpha 1(X)_3$	Cartilage	
FACITs	IX	$\alpha 1(IX)\alpha 2(IX)\alpha 3(IX)$	Co-distributed with collagen II, cartilage, vitreous, cornea
	XII	$\alpha 1(XII)_3$	Found with collagen I, dermis, tendon
	XIV	$\alpha 1(XIV)_3$	Found with collagen I, bone, dermis, cartilage
	XVI		Dermis, kidney
	XIX		Basement membrane
	XX		Cornea
	XXIV		Stomach, kidney
	XXII		Tissue junctions
MACITs	XIII		Neuromuscular junctions, skin, endothelial cells, eye, heart
	XVII	$\alpha 1(XVII)_3$	Hemidesmosomes in epithelia
	XXIII		Heart, retina
	XXV		Brain, heart, testis
Anchoring fibrils	VII	$\alpha 1(VII)_2\alpha 2(VII)$	Dermal-epidermal junction, bladder
Beaded-filament-forming collagens	VI	$\alpha 1(VI)\alpha 2(VI)\alpha 3(VI)$	Bone, cartilage, cornea, dermis, muscle
		$\alpha 1(VI)\alpha 2(VI)\alpha 4(VI)$	
	XXVI		Testis, ovary
XXVIII		Dermis, sciatic nerve	
MULTIPLEXINS	XV		Basement membranes, eye, muscle, capillaries, testis, heart, kidney
	XVIII		Basement membranes, liver

Table 2 Types and composition of the currently known collagens, as well as examples of the tissues where they are most commonly found. Table adapted from *Theocharis et al, 2016*.

Collagens are found in the fibers of tendons, in the matrices of bone and cartilage, in the laminar sheets of basement membranes, in the viscous matrix of the vitreous humor, and in the interstitial ECM of the dermis and of capsules around the organs. They are the most abundant protein family representing 25 - 30% of the total protein mass (Frantz, Stewart, and Weaver 2010; R. O. Hynes and Naba 2012; Alberts 2015).

The collagen superfamily comprises 28 members designated with Roman numerals in vertebrates [I - XXVIII, reviewed in (Ricard-Blum 2011)]. The primary typifying feature of a collagen molecule is the presence of the repeating triplet Gly-X-Y, where X is frequently proline and Y is frequently 4-hydroxyproline, though they can be any of the 20 amino acids in proteins (Heino 2007). This repeating unit gives rise to stiff, trimeric coiled coils, in which three collagen polypeptide chains, also known as α chains, are wound around one another in a rod-like superhelix.

The length of the superhelix can vary significantly ranging from most of the molecule structure (96% in collagen I) to less than 10% of it (collagen XII), and it correlates with the localization within the tissue, as well as with ECM organization and structure [see (Ricard-Blum 2011) and references therein]. For example, the original type I collagen of bones and tendons consists almost entirely of a long and rigid uninterrupted collagen triple helix that spans approximately 1000 amino acids (R. O. Hynes and Naba 2012; Heino 2007). Each trimer assembles into higher-order fibrils and fibers after being crosslinked by various enzymatic and non-enzymatic reactions, and it is found in all interstitial matrices of the organism.

Other collagen types have interruptions in the repeating sequence (Gly-X-Y), thus adding flexibility into the molecules. An extra level of complexity is added because of the existence of several molecular isoforms for the same collagen type, and of hybrid isoforms comprised of α chains belonging to two different collagen types, distinguished by the use of Arabic numerals (Ricard-Blum 2011). For example, collagen XI is comprised of three α chains assembled into a heterotrimer, but the α (XI) chain forms type V/XI hybrid collagen molecules by assembling with the $\alpha 1$ (V) chain in the vitreous humor (Mayne et al. 1993) and cartilage (J.-J. Wu et al. 2009).

The criteria to name a protein collagen are not well defined. Many proteins contain the triple helical domain but do not belong to the collagen family. At the same time, all collagen genes encode additional non-collagenous domains, some of which are characteristic collagen N and C pro-domains, while others are domains shared with other ECM proteins.

Fibrillar collagens are the most abundant collagens in vertebrates where they play a structural role by contributing to the molecular architecture, shape, and mechanical properties of tissues such as the tensile strength in skin and the resistance to traction in ligaments, as also mentioned previously. However, as stated before for the ECM, the various matrix components are “not just pretty fibrils” (Richard O. Hynes 2009).

Indeed, it is now well documented that fibrous collagens, as well as fibril-associated collagens, alongside many other ECM constituents, play important roles in tissue homeostasis, growth factor signaling, differentiation, development, and disease. **Table 3** displays a non-exhaustive list of functions that depend on various collagens and their receptors. For details, reader can refer to the following reviews and references therein (Frantz, Stewart, and Weaver 2010; Ricard-Blum 2011; Heino 2007;

Function/Role		Receptor(s)	Reference
Platelet activation		$\alpha 2\beta 1$, GPVI	(Chen et al. 2002; He et al. 2003; Edelson et al. 2004)
Inflammation and immunity		$\alpha 1\beta 1$, $\alpha 2\beta 1$, LAIR-1	(Lebbink et al. 2006; Rubio et al. 1995; Fiorucci et al. 2002; Ray et al. 2004)
Angiogenesis	Cancer	$\alpha 1\beta 1$, $\alpha 2\beta 1$	(Pozzi et al. 2000; Senger et al. 2002)
	Wound healing	$\alpha 2\beta 1$	(Grenache et al. 2007; Zweers et al. 2007)
Mesenchymal cell proliferation		$\alpha 1\beta 1$, DDR2	(Ekholm et al. 2002; Zemmyo et al. 2003; Pozzi et al. 1998; Labrador et al. 2001)
Matrix remodelling	Collagen synthesis	$\alpha 1\beta 1$, $\alpha 2\beta 1$	(Ivaska et al. 1999)
	MMP expression	$\alpha 2\beta 1$, DDR2	(W. Vogel et al. 1997; Ravanti et al. 1999)
	Collagen endocytosis/phagocytosis	$\alpha 2\beta 1$, Endo180	(Curino et al. 2005; Upla et al. 2004)
Development	Mammary gland	$\alpha 2\beta 1$, DDR1	(Chen et al. 2002; W. F. Vogel et al. 2001)
	Skeleton	$\alpha 10\beta 1$, DDR2	(Bengtsson 2005; Labrador et al. 2001)
	Periodontal ligament	$\alpha 11\beta 1$	(Popova et al. 2007)

Table 3 Role of collagens in various biological processes exerted by collagen receptors (see also Section 1.2.) Table based on *Heino 2007*.

Richard O. Hynes 2009; Daley and Yamada 2013; Watt and Huck 2013; Bonnans, Chou, and Werb 2014; Pickup, Mouw, and Weaver 2014; Boyd and Thomas 2017).

Elastin and elastic fibers

Fibrillar collagens, as described above, are responsible for providing tissues with tensile strength. Indeed, a collagen fiber can withstand 25 times stronger tensile strain compared to a steel fiber of the same diameter before fracture occurs (Buehler 2006). However, organ functionality depends also on the extendibility of the underlying tissue. Skin, blood vessels, lung, heart, and bladder tissues need not only be strong, but also elastic in order to function properly. This is where the network of elastic fibers comes into play, giving tissues the resilience to recoil after transient stretch.

Elastic fibers are the largest structures in the ECM and consist of two morphologically distinct components [reviewed in (Wagenseil and Mecham 2007)]. The principal component is elastin, a crosslinked polymer of the monomeric secreted form of the protein tropoelastin (Sandberg, Weissman, and Gray 1971), a 60-72kDa precursor protein characterized by short segments that alternate along the polypeptide chain (Alberts 2015; Theocharis et al. 2016; Wagenseil and Mecham 2007; Wise and Weiss 2009). Highly hydrophobic segments are responsible for the elastic properties of the molecule (Muiznieks, Weiss, and Keeley 2010), and alanine- and lysine-rich α -helical segments are crosslinked to adjacent molecules by covalent attachment of lysine residues (Wagenseil and Mecham 2007). This covalent modification is performed by lysyl oxidase (LOX) or LOX-like molecules (Lucero and Kagan 2006), eventually stabilizing the polymer rendering the network insoluble (Wagenseil and Mecham 2007; Muiznieks, Weiss, and Keeley 2010). **Figure 3** shows the organizations of elastin in the developing mouse lung.

While elastin arises from a single gene, the second component of elastic fibers, the microfibrils, is characterized by higher complexity. Microfibrils are, as their name states, small, 10-15nm fibrils that localize to the periphery of the elastic fiber in the adult tissues (Wagenseil and Mecham 2007). The major structural trait of microfibrils is provided by a set of proteins termed fibrillins, large (350kDa), cysteine-rich glycoproteins (L. Y. Sakai 1986; Kumra and Reinhardt 2018), that in combination with

other associated proteins (Kielty, Sherratt, and Shuttleworth 2002) perform structural and regulatory roles (Kumra and Reinhardt 2018; Sengle and Sakai 2015).

After secretion, fibrillin monomers go through several interactions with other molecules to form short microfibrils that are subsequently transferred onto FN fibers for stabilization (Kinsey et al. 2008). Elongation then occurs through interaction with other microfibril proteins (Sabatier et al. 2009).

Furthermore, fibrillins regulate transforming growth factor β (TGF- β) localization and activity by modulating the binding of latent TGF- β binding proteins [LTBPs (Dallas et al. 2000; Isogai et al. 2003)], that in turn regulate TGF- β latency (discussed in Section 1.3). More specifically, LTBP-1 is assembled in the ECM through the presence of FN (Dallas et al. 2005), but it is finally transferred to microfibrils as the ECM matures (Ono et al. 2009). Additionally, different LTBPs show preferential binding for ECM components with various resistance to strain (Zilberberg et al. 2012), thus reflecting a differential TGF- β activation depending on the ECM composition and state.

Laminins

As described earlier, BMs are thin-layered surfaces responsible for tissue architecture providing protection against stress, and serving as an interactive platform between cells and the microenvironment. BMs are quite heterogeneous in terms of composition and structure, and this heterogeneity depends on the tissue, but also on the localization within the same tissue. Typically, a mature BM consists of laminin, type IV collagen, nidogen, agrin, and perlecan (Alberts 2015; Theocharis et al. 2016; Durbeej 2010).

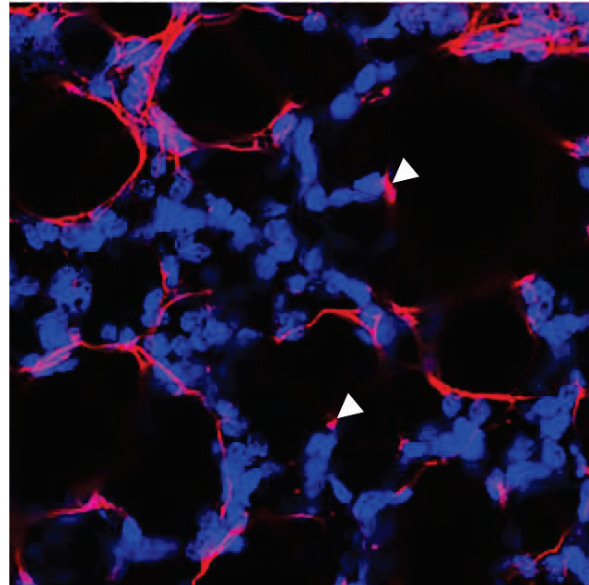


Figure 3 Immunofluorescence staining of elastin (magenta) in P7 mouse lung, showing elastin network and patches of elastin present at the tip of alveolar protrusions (arrowheads). Nuclei staining (blue) was used to visualize the structure of the lung tissue. Adapted from *Luo et al. 2018*.

Laminins are large (400 – 900kDa), trimeric, cross or T-shaped molecules with two or three short arms and one long arm (Aumailley et al. 2005). Short arms are composed of parts of one chain, while the long arm is formed by parts of each of the three chains. The three different polypeptide chains are termed α , β , and γ . Five α , three β , and three γ chains have been identified so far both for mouse and for human (Theocharis et al. 2016; Aumailley et al. 2005). All chains are glycosylated, while some of them have been shown to contain glycosaminoglycan side chains (Aumailley et al. 2005).

The members of the laminin family are generally characterized by the presence of an α -helical coiled coil domain, but they contain additional domains that contribute to laminin-cell and laminin-ECM interactions. Such domains comprise a large globular N-terminal domain, and five laminin globular (LG) domains at the C-terminal end (Theocharis et al. 2016; Durbeej 2010). Via their various domains, laminins can self-assemble to form networks in close association with the cells. This tight interaction is mediated via cell receptors like integrins or syndecans (See also Section 1.2).

1.1.2.3 Other Matrix-Associated Components

So far, only a small fraction of the elements that contribute to the formation of the ECM has been described. Many other components, members of the aforementioned matrisome, take an active part in matrix generation and assembly. Major examples are the glycoproteins fibronectin (the focus of this work, to be discussed shortly), and tenascins. Other members of the matrisome comprise non-structural extracellular modulators of cellular functions that facilitate cell-cell and cell-ECM interactions (Frantz, Stewart, and Weaver 2010; R. O. Hynes and Naba 2012; Theocharis et al. 2016).

There are also ECM-associated components such as modifiers of the ECM structure and function (transglutaminases, lysyl oxidases, hydrolases, metalloproteinases, ADAMs, etc.), and ECM-bound growth and secreted factors (VEGF, HGF, PDGF, TGF). This way the ECM acts as a reservoir of factors the bioavailability and/or activity of which is subject to the regulation of the cells surrounded by or assembling it (Naba et al. 2012; Frantz, Stewart, and Weaver 2010).

1.1.3 Fibronectin

1.1.3.1 The Early Days

In 1948 at Harvard University, Peter Morrison and colleagues were working on the identification and the properties of serum and plasma proteins. Their main interest was fibrinogen and its ability to form clots under the action of thrombin, reflecting a clinical importance considering blood coagulation. In their effort to optimize their fibrinogen isolation technique, they came to realize that they were able to obtain high amounts of fibrinogen from two distinct fractions. The first was separated from the bulk of non-clottable proteins by precipitation at low pH, while the second was obtained after centrifugation and the presence of ethanol at higher pH and low temperature (0°C). This second fraction, however (Fraction I-1) contained an unknown material of lower solubility than fibrinogen, which precipitated. The researchers introduced the term cold-insoluble globulin to denote the insoluble component, in the absence of a specific name (Morrison, Edsall, and Miller 1948).

It took more than 20 years to successfully purify to homogeneity the “cold-insoluble globulin” from plasma cryoprecipitate (Mosesson and Umfleet 1970). In the meantime, other researchers, independently, identified new features in a plasma component that had similar properties with the cold-insoluble globulin, without though being able to assign the described properties to a defined protein (Smith and Korff 1957; Wolff et al. 1967).

It was during the 1970s when a series of studies described a large glycoprotein found on the surface of fibroblasts [large, external, transformation-sensitive (LETS) protein or galactoprotein a, or cell surface protein (CSP)] that was lost after virus-induced transformation (Gahmberg and Hakomori 1973; R. O. Hynes 1973; Yamada and Weston 1974). Additionally, its presence varied depending on the growth state of normal cells (Richard O. Hynes 1974). During the same period, an antiserum was generated that recognized a fibroblast surface antigen [FS-A (Erkki Ruoslahti and Vaheri 1974)], that was also found to react with a serum antigen, later found to be the cold insoluble globulin (Erkki Ruoslahti and Vaheri 1975). This cell surface antigen

was recognized as the previously described transformation-sensitive protein LETS (Keski-Oja, Vaheri, and Ruoslahti 1976).

Despite the doubts generated by the limitations of the methods used at the time (not well-defined immunogens, not highly specific antiserum), several data converged towards the opinion that the cell surface glycoprotein and the cold-insoluble globulin were indeed the same protein. In the following years, several studies showed clearly the similarities between the cell-associated protein and the one purified from plasma. The use of antibodies to visualize stain patterns in cultured cells, the use of gel affinity chromatography to simply and rapidly purify the protein, as well as other biochemical approaches (solubility assays, SDS-polyacrylamide gel electrophoresis) resulted in the acceptance that the described proteins comprised a family of related proteins (Richard O Hynes 1990).

Those early studies, even if the researchers at the time could not fully explain their findings, provided information about the function of the molecules that are now known as fibronectins. The term was coined around the mid-1970s (from the latin *fibra* for fiber and the verb *nectere* which means to link, to connect), and was later adopted by all scientists in the field to describe the different forms of the protein fibronectin (FN).

1.1.3.2 Fibronectin: A Major ECM Building Block

Since its discovery, FN has been the subject of extensive studies that have importantly contributed to our knowledge regarding its structure, and the wide range of different functions it exerts. Product of a single gene, FN is expressed in two distinct forms with both additive and overlapping functions. The main source of FN is the liver, which secretes plasma FN (pFN) in a soluble form circulating through the blood plasma. Furthermore, cellular FN (cFN) is produced mainly by fibroblasts, as well as endothelial cells, chondrocytes, macrophages, platelets, and other cell types (Richard O Hynes 1990) both in vivo and in culture. cFN is mainly found in an insoluble form around the cells secreting it, where it is assembled into fibrils and fibers, resulting eventually in the formation of the underlying ECM.

Only a handful of human disease cases linked to FN mutations have been documented so far, and they concern either glomerulopathy with FN deposits (Castelletti et al. 2008; dos Reis Monteiro et al. 2019) or skeletal dysplasias (Costantini et al. 2019). This suggests that even point mutations can lead to deleterious or lethal effects. Indeed, a gene knockout study in the mouse has shown that inactivation of both FN alleles leads in early embryonic lethality resulting from severe defects in mesodermal migration, reflecting anomalies in cell adhesion, proliferation, and differentiation (George et al. 1993). Additionally, the highly conserved modular structure of the protein reinforces the notion that intact FN is indispensable for normal development and tissue homeostasis (Ni et al. 2003; J. Xu and Mosher 2011; Richard O Hynes 1990), functions that are mainly mediated via direct interactions with cell surface receptors, such as integrins (Richard O Hynes 1990; Richard O. Hynes 2009).

The most important property of FN is the fact that it drives the assembly of the ECM. Being among the very first components of the ECM (if not *the* first), FN is deposited and polymerized by assembly-competent cells, such as fibroblasts, in a provisional matrix that drives the deposition of other ECM components, such as collagens and other proteoglycans (Richard O. Hynes 2009). Hindering FN assembly *in vivo* results in delayed embryonic development (Darribère et al. 1990), while absence of FN in cultured fibroblasts impedes the formation of ECM (Cseh et al. 2010; Gopal et al. 2017). FN assembly will be discussed in more detail in Section 1.2.1.4.

1.1.3.3 FN Structure

FN is a high molecular weight glycoprotein composed of two similar subunits of 220-250 kDa (Engel et al. 1981; Erickson, Carrell, and McDonagh 1981). Every FN subunit has two carboxy-terminal cysteine residues that form two disulfide bonds with the cysteine residues of another FN subunit, giving rise to a functional FN dimer. FN is highly produced and secreted by the liver, or expressed locally by fibroblasts and other cell lines. In the first case, FN circulates in the blood plasma in a soluble form (plasma FN - pFN), while in the latter case FN forms an insoluble mesh that gives rise to the extracellular matrix (cellular FN, cFN). The linear structure of the subunits is highly repetitive, characterized by the presence of three distinct types of domains that

together constitute approximately 90% of the sequence [reviewed in (J. Xu and Mosher 2011)]. There are 12 type I repeats (hereafter FN_{I_x}, where subscript x denotes the number of the repeat), 2 type II repeats (hereafter FN_{II_x}), and 15 – 17 type III repeats (hereafter FN_{III_x}) (Richard O Hynes 1990). Apart from the repetitive domains, there is also a variable region (V or III_{CS}, type III connecting segment) that lies between FN_{III₁₄} and FN_{III₁₅}, which is subject to high alternative splicing, as well as short linker segments between different modules across the protein [reviewed in (J. Xu and Mosher 2011) and **Figure 4**].

The FN_I repeat is composed of 45 amino acids, four of which are cysteine residues that give rise to two disulfide bonds within the repeat. 3D structure studies of the FN_I repeat have shown that a scaffold of hydrophilic amino acids forms two compact β-sheets, with two and three strands, that enclose a hydrophobic core of aromatic residues (Baron et al. 1990; Potts et al. 1999).

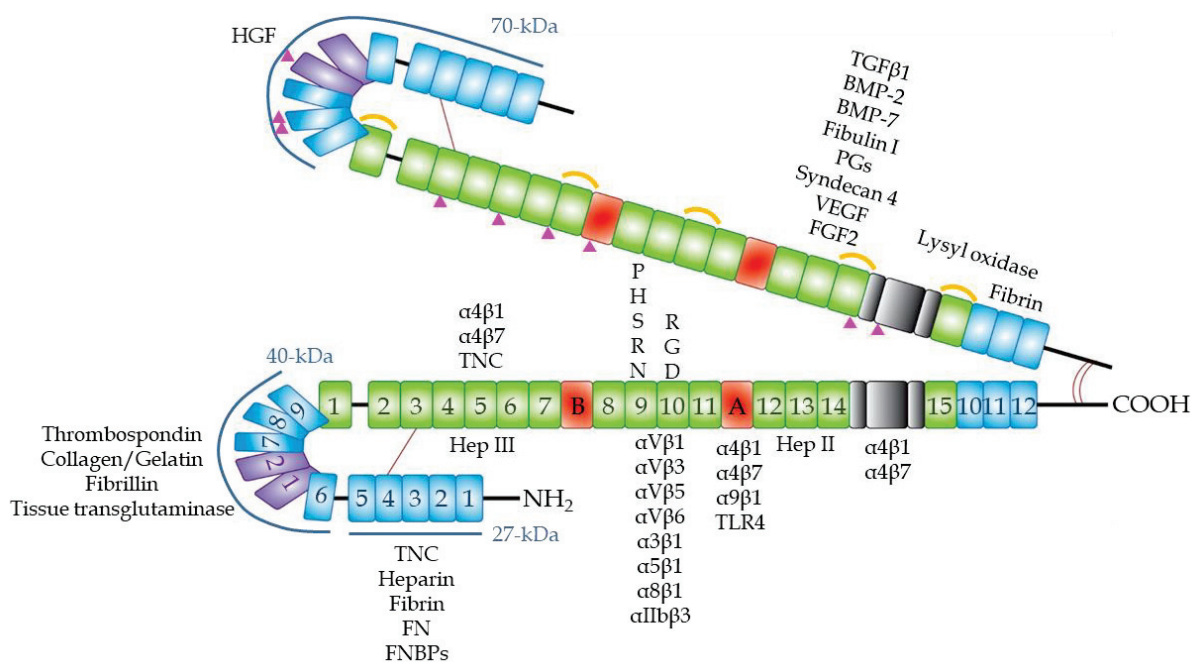


Figure 4 Graphic representation of the linear structure of FN showing the different FN modules. FN forms dimers linked in their C-terminal ends by two disulfide bonds. Each dimer is composed of 12 FN_I repeats (blue rectangles), two FN_{II} repeats (purple rectangles), and 15-17 FN_{III} repeats (green and red rectangles) two of which are subject to alternative splicing (EDB and EDA), a Variable region (gray), and short peptide segments that do not constitute parts of the modular domains (black lines). Different segments of FN result after proteolytic cleavage (70 kDa, 40 kDa, and 27 kDa segments). Glycosylation sites are indicated with pink triangles. The highly modular structure of the molecule permits an enhanced degree of folding resulting in the formation of cryptic sites (yellow arches) exposed by addition of extra domains, strain, and proteolysis. FN has multiple binding sites for cells (FN_{III}9-10 repeats) and other molecules. Adapted from Xu and Mosher 2011, and Van-Obberghen Schilling et al. 2011.

The FNII repeats have a somewhat similar structure with the FNI repeats. They are about 60 amino acids long and have two intra-domain disulfide bonds. Like FNI repeats, they consist of two antiparallel β -sheets each containing two strands, while the amino acid sequence is mainly characterized by the presence of aromatic residues (Constantine et al. 1992; Pickford et al. 1997). Unlike FNI repeats that are only present in vertebrates (Tucker and Chiquet-Ehrismann 2009), FNII repeats have also been identified in other, less complex organisms (Ozhogina and Trexler 2001), as well as in matrix metalloproteinases (Collier et al. 1988).

The FNIII repeat is a functionally conserved domain found in many other proteins including extracellular matrix components, cell surface receptors, and cytoskeletal proteins both in vertebrates and in invertebrates (Bork and Doolittle 1992). There are 15 standard FNIII domains present in every FN monomer, and two additional domains termed Extra Domains (EDB and EDA) that are included in the molecule under specific circumstances.

Each FNIII repeat is composed of 90 amino acids (though slight variations do exist across proteins and species) organized in seven β -strands [A, B, C, D, E, F, and G, (Figure 5)], further folding into two antiparallel β -sheets (like the other two types of FN repeats). One β -sheet is composed of three β -strands, while the other is composed of four, and they fold in a sandwich-like conformation the center of which is highly hydrophobic (Dickinson, Veerapandian, et al. 1994; Dickinson, Gay, et al. 1994; Leahy, Aukhil, and Erickson 1996; Main et al. 1992). Surprisingly, FNIII repeats lack any cysteine residues, thus they contain no intra-chain disulfide bonds. This affects the

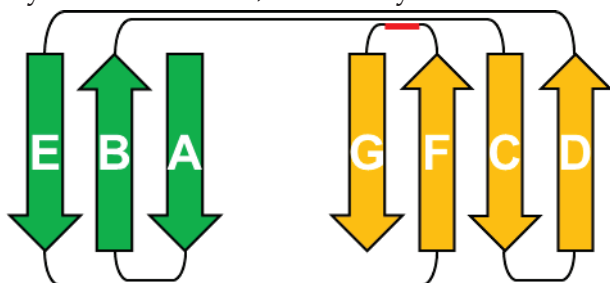


Figure 5 Linear representation of the structure of the FNIII₁₀ repeat. FNIII repeats are composed of seven β strands organized in two β sheets that fold in a sandwich-like structure with a highly hydrophobic core. In FNIII₁₀, between strands F and G, lies the cell binding sequence (RGD), shown here in red. See text for details.

form of the entire molecule, as the separate FNIII domains are prone to conformational changes resulting from mechanical forces (Krammer et al. 1999). These changes can greatly influence the folding state of the FNIII repeat while maintaining its structural integrity. Also the inclusion of additional type III

repeats, through alternative splicing, can impact FN conformation (Barbara Carnemolla et al. 1992).

The variable region (V) is not homologous to any other part of the protein. Several functional and structural studies have shown that the variable region is 120 amino acids long and it can be partly present or completely absent from the molecule, while most FN dimers naturally form with distinct variable regions in each subunit [see Figure 4, **Figure 6** and (J. E. Schwarzbauer 1989)]. The different forms of the V region arise from alternative splicing, which appears to be regulated in a tissue-dependent manner, and it changes throughout development and lifetime of the organism (Chauhan et al. 2004). The inclusion of different parts of the V region determines the repertoire of cell receptors that FN can interact with in order to promote cell attachment and spreading (Kocher, Kennedy, and Madri 1990), and controls FN dimer secretion and incorporation into fibrin clots and ECM in tissues [reviewed in (J. E. Schwarzbauer and DeSimone 2011)].

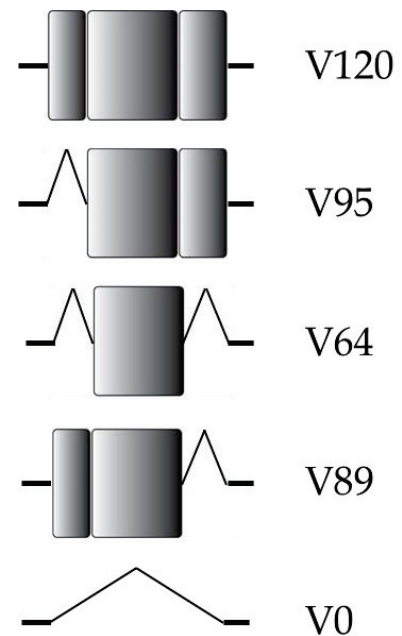


Figure 6 The variable region (V) or type III connecting segment (IIICS) is subject to alternative splicing and it is involved in fibronectin secretion and integrin binding. Five splice variants are found in human fibronectin. See text for details.

FN in solution is found in a compact conformation, which is mainly due to non-covalent interactions established within the FNIII domains (Ohashi and Erickson 2005), as well as an intramolecular interaction between FN_{I4} and FN_{III3} leading in the bending of the N-terminal region of both subunits [reviewed in (J. Xu and Mosher 2011)]. Furthermore, the ability of FN to self-associate through other regions has been documented, namely FN_{I1-5} (McKeown-Longo 1985), FN_{III1-3} (Aguirre, McCormick, and Schwarzbauer 1994; Hocking, Sottile, and McKeown-Longo 1994; Johnson et al. 1999), FN_{III12-14} (Bultmann, Santas, and Peters 1998), and FN_{III7} (Ingham et al. 1997). Another factor that largely contributes to FN conformation is the variety of post-translation modifications. FN can be phosphorylated, sulfated, and (as every

glycoprotein) glycosylated (Paul and Hynes 1984). The degree of glycosylation often depends on the tissue, though generally FN contains approximately 5% of carbohydrate chains either N-linked, or O-linked in distinct sites of both subunits [see Figure 4 and (J. Xu and Mosher 2011)]. Addition of glycans affects FN sensitivity to proteolysis, thermal stability, and binding affinity to other proteins such as collagen, thus modulating cell adhesion and migration (Olden, Pratt, and Yamada 1979; Bernard, Yamada, and Olden 1982; Jones 1986). Phosphorylation has been described in serine residues (Etheredge et al. 1985) enhancing cell attachment and mechanical cell functions (Yalak et al. 2019), while sulfation has been suggested to take place in the V region (Paul and Hynes 1984). Finally, citrullination, the conversion of arginine residues to citrulline, was recently described as another type of post-translational modification of FN. Twenty four sites of varied degree of citrullination were identified, impacting the FN attachment to both $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins, and reflecting a refined regulation of integrin clustering and cell contractility (Stefanelli et al. 2019).

1.1.3.4 Fibronectin Interactions and Function

The modular structure of FN, as well as its multiple post-translational modifications result in numerous interactions with a variety of proteins mediating cell attachment, ECM deposition and assembly, cell motility, cytoskeleton contractility, and host-pathogen interactions, to name just a few. The major family of proteins fibronectin interacts with is the family of integrins through which it exerts multiple roles in health and disease.

Many integrins have been shown to interact with FN. The “classic” FN receptor is integrin $\alpha 5\beta 1$ that binds FN through the tripeptide cell-binding site Arg-Gly-Asp (RGD) located within the flexible loop formed between the F and G strands in the FNIII₁₀ repeat [see Figure 5 and (Main et al. 1992; Pytela, Pierschbacher, and Ruoslahti 1985; Pierschbacher and Ruoslahti 1984; Pankov and Yamada 2002)]. This interaction is facilitated and further stabilized by the synergistic effect of the PHSRN site that is located in the adjacent FNIII₉ (Pierschbacher and Ruoslahti 1984). Binding of FN to $\alpha 5\beta 1$ results in activation of the integrin, subsequently leading in Rho-mediated contractility that in turn promotes assembly of fibronectin into a fibrillar matrix

(Danen et al. 2002; Zhong et al. 1998; Wennerberg et al. 1996). Apart from $\alpha 5\beta 1$, $\alpha v\beta 3$ also binds the RGD site as well as the integrins $\alpha 3\beta 1$, $\alpha 8\beta 1$, $\alpha v\beta 1$, $\alpha IIb\beta 3$, $\alpha v\beta 5$, and $\alpha v\beta 6$ [reviewed in (J. Xu and Mosher 2011)]. The importance of the RGD sequence has been demonstrated in mice expressing FN with a mutated cell-binding site. These mice die before birth and exhibit anomalies similar to those observed in mice with integrin $\alpha 5$ deletion (Takahashi et al. 2007).

Other integrins that have been demonstrated to interact with FN are integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$. These two integrin dimers can bind to the dedicated sequences KLDAPT found in FNIII₅ (Moyano et al. 1997), and IDAPS in FNIII₁₄ (Pankov and Yamada 2002). FN interaction with integrins results in the transmission of extracellular information to the cell interior inducing cytoskeletal re-organization, and activation of signaling pathways that regulate cell morphology, proliferation, and growth (Richard O Hynes 2002; Assoian and Schwartz 2001; Ginsberg 2014). Additionally, integrin-mediated cell contractility results in integrin-applied tension forces upon FN, which is subsequently stretched to an open conformation that reveals cryptic sites, further promoting the binding of other molecules or the assembly of FN into fibrils [reviewed in (Richard O Hynes 1990; J. Xu and Mosher 2011; Mao and Schwarzbauer 2005)].

Much effort has also been invested in the identification of integrins that bind the alternatively spliced regions of FN. More specifically, the sequence of EDA has been shown to contain the EDHIGEL site that was subsequently identified as a binding site for integrins $\alpha 4\beta 1$, $\alpha 4\beta 7$, and $\alpha 9\beta 1$ (Liao et al. 2002; Shinde et al. 2008). EDA has also been recognized as a ligand and activator of TLR4 (Okamura et al. 2001). No receptor has been identified so far for EDB, though a role has been suggested in osteoblast differentiation involving integrin $\beta 3$ (Sens et al. 2017). The V region presents an interesting integrin binding site, bearing the sequences LDV and REDV, both recognized by $\alpha 4\beta 1$ and $\alpha 4\beta 7$ (Komoriya et al. 1991; Mould et al. 1991; J. D. Humphries 2006).

A major regulatory domain of fibronectin is the collagen and gelatin-binding region that spans FNI₆ through FNI₉ repeats, including the two FNII repeats. FN binds locally unfolded collagen *in vivo*, mediating cell adhesion to denatured collagen and

contributing to the maturation of the extracellular matrix, as well as the blood vessel morphogenesis during embryonic development and pathological angiogenesis [reviewed in (J. Xu and Mosher 2011; Van Obberghen-Schilling et al. 2011)]. Interestingly, the collagen-binding domain is also a binding site for other ECM components such as fibrillin and thrombospondin (Dallas, Chen, and Sivakumar 2006).

One of the early-identified functions of FN was its role in wound healing and its incorporation in fibrin clots [reviewed in (Cho and Mosher 2006)]. This procedure is mediated via the three fibrin-binding domains of fibronectin. One comprises the first five FNI repeats (FNI₁ through FNI₅) and overlaps with another fibrin-binding site composed of FNI₄ and FN₅ (Williams et al. 1994; J. Xu and Mosher 2011). The third fibrin-binding site is located near the carboxy-terminus and spans FNI₁₀ through FNI₁₂ (J. Xu and Mosher 2011). Fibrin is covalently attached to FN via the crosslinking activity of Factor XIII transglutaminase, thus incorporating FN into the fibrin clot, subsequently regulating platelet growth and cell migration within the clot during the wound healing process (Cho and Mosher 2006).

FN interacts with several heparin sulfate proteoglycans via three heparin-binding domains (Hep I, II, and III). FNI₁ through FNI₅ constitute a weak Hep domain [Hep I, (Yamada et al. 1980; Richard O Hynes 1990), while Hep II spans FNIII₁₂ - FNIII₁₄ and presents the strongest heparin-binding site (Barkalow and Schwarzbauer 1991). Hep III lies within the region covered by FNIII₄ - FNIII₆ (Moyano et al. 1999), while a fourth heparin-binding domain has been identified within the V region (Mostafavi-Pour et al. 2001). Heparin binding domains are thought to co-operate with integrin-binding domains enhancing adhesion and spreading. It has been demonstrated, for example, that syndecan-4, among other proteoglycans, synergizes with $\alpha 5\beta 1$ binding to the RGD sequence in order to promote FN fibrillogenesis through the Hep II domain (Saoncella et al. 1999; Woods 2001; Cheng et al. 2016).

The ECM functions as a reservoir of growth factors, facilitating their bioavailability and activity in the cells of the underlying tissue (Frantz, Stewart, and Weaver 2010; R. O. Hynes and Naba 2012). FN interacts with several growth factors (see Figure 4)

regulating their function, such as members of the TGF- β superfamily (namely TGF- β 1, BMP-2, and BMP-7), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF). Apart from HGF that binds a region lying within the FNI₁ – FNI₉ repeats, all other growth factors bind the Hep II domain of FN (Sawicka et al. 2015). Direct interaction of FN with growth factors may result in enhanced cell migration, cell proliferation, survival signals, and angiogenesis, as downstream results of the activation of the different growth factors through mechanical or enzymatic activation (Richard O. Hynes 2009).

Finally, FN interacts with ECM components other than the ones mentioned above, such as tenascin-C (TNC), and ECM-associated enzymes, like tissue transglutaminase and lysyl oxidase (LOX) (Naba et al. 2012; R. O. Hynes and Naba 2012). Especially for TNC there are two distinct binding sites, one in the amino-terminal region of the molecule (FNI₁ – FNI₅) and another in the central part of the molecule (FNIII₄ – FNIII₆), and a crosstalk between FN and TNC has been described that fine-tunes cell adhesion and motility during angiogenesis, and tumor progression [reviewed in (Van Obberghen-Schilling et al. 2011)]. Additionally, FN can self-associate at the N-terminal domain between FNI₄ and FNIII₃ probably impacting assembly procedures (Aguirre, McCormick, and Schwarzbauer 1994).

We will close this section by referring to the role of FN as a binding partner of bacterial proteins. The amino-terminal region (FNI₁ – FNI₅) has been shown to be binding site for bacteria like *Staphylococcus aureus* and *Streptococcus pyogenes* [reviewed in (J. Xu and Mosher 2011)], while several bacterial proteins have been identified (FnBPs – fibronectin binding proteins) that bind that region facilitating FN-mediated cell adhesion and entry of the bacteria into host cells (Schwarz-Linek, Höök, and Potts 2004). Beside the N-terminal region, *Borrelia burgdorferi* protein BBK32 binds FNIII₁ – FNIII₃ (Harris et al. 2014), *Clostridium perfringens* binds FNIII₉ – FNIII₁₀ (Katayama et al. 2015), *Yersinia pestis* protein Ail binds to FNIII₉ (Tsang et al. 2012), *Pasteurella multocida* protein PM1665 to FNIII₉ – FNIII₁₀ (Mullen et al. 2008), and the *Salmonella enterica* protein ShdA binds FNIII₁₃ (Kingsley et al. 2004).

1.1.3.5 Oncofetal FN Variants

As previously mentioned, many different FN isoforms arise from a single gene through alternative splicing. In this section, we will focus on current knowledge on the oncofetal fibronectin isoforms and their role in health and disease. The term “oncofetal” was introduced to describe the reappearance of an embryonic FN splicing pattern during wound healing in adult tissues (Ffrench-Constant 1989; Jarnagin 1994). This splicing pattern involves the inclusion of Extra Domains B and A (**Figure 7** and **Figure 8**), that are under normal conditions only observed in embryonic development and are absent from the circulating plasma FN (pFN). Later it was documented that this embryonic splicing pattern not only reappeared during wound healing, but it also constituted a signature of tumorigenic transformation (B. Carnemolla 1989; P. Castellani 1986), with EDB and EDA FNs being markers of tumor angiogenesis and metastasis (Rybak et al. 2007; Patrizia Castellani et al. 1994; Ventura et al. 2010).

FN splicing is regulated by proteins of the SR family¹ (Serine- and Arginine-rich proteins), and depends on regulatory sequences that significantly vary between EDB and EDA. In the case of EDB, specific sequence repeats found in the downstream intron result in the inclusion of Extra Domain B in the FN mRNA. These intronic splicing enhancers (ISE) contain the sequence [TGCATG]_n, and are recognized by SRSF5 (Huh and Hynes 1993; 1994; Lim and Sharp 1998). An exonic splicing enhancer (ESE) has been found within the EDB sequence of rat regenerating liver that comprises a purine-rich stretch (Du et al. 1997). This sequence is recognized by HRS (hepatic arginine-serine rich protein), which is the rat homolog of SRSF5 (Screaton et al. 1995).

Extra Domain A is characterized by the presence of an exonic splicing enhancer (ESE) and an exonic splicing silencer (ESS) that form a stem-loop structure (Muro et al. 1999) that influence the binding of the splicing machinery by exposing the regulatory elements (Buratti et al. 2004). The ESE is comprised of the sequence GAAGAAGA and its absence results in constitutive exclusion of EDA in the FN mRNA (Caputi et al. 1994). On the other hand, the ESS comprises the sequence CAAGG, absence of which

¹ The nomenclature of the SR protein family members follows the scheme proposed by Manley and Krainer 2010.

leads in constitutive inclusion of EDA (Caputi et al. 1994). Whether EDA is included or not in the mature mRNA depends on a complex interplay between SRSF1, SRSF7, the promoter sequence and the physical properties of RNA pol II transcribing the gene (Caputi et al. 1994; White, Baralle, and Muro 2008).

Interestingly, the splicing factors that have been described so far as modulators of oncofetal FN expression, all belong in the same family (SR proteins). The expression of each family member depends on the cell type, while activity is regulated by SR protein kinase activity (Prasad and Manley 2003), extracellular factors (Shi and Manley 2007), while an Akt-dependent and growth-factor-dependent regulation of SR proteins has also been described (Blaustein et al. 2005). It is important to mention that several members of the SR protein family, namely SRSF5 and SRSF3, are subject to post-

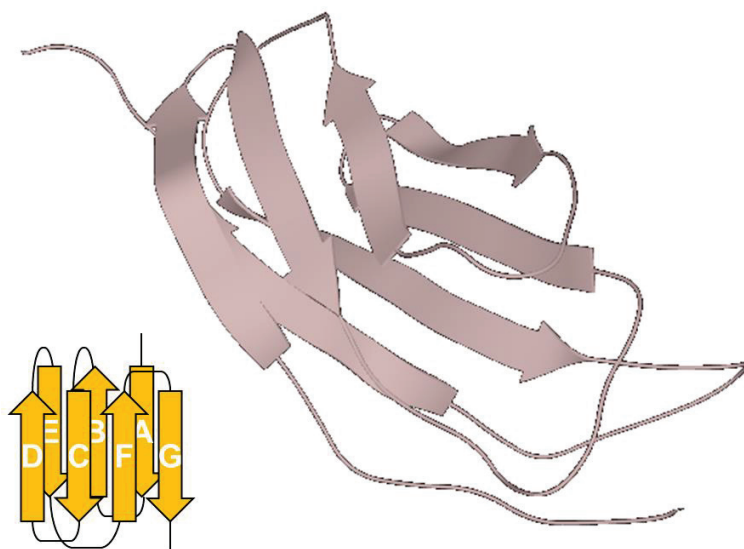


Figure 7 NMR-generated spatial structure of Extra Domain A according to Niimi et al 2001. The linear structure is also demonstrated for simplicity.

translational modifications, and differential expression, that highly correlate with specific cancers, like oral squamous cell carcinoma (Yuhan Chen et al. 2018; S. Yang, Jia, and Bian 2018). Importantly, inclusion of the Extra Domains in the FN molecule has been shown to be TGF- β -signaling-dependent (Balza et al. 1988; Borsi et al. 1990; Viedt,

Bürger, and Hänsch 1995). TGF- β stimulation of cultured fibroblasts has been shown to modulate SRSF protein family expression (Hallgren et al. 2012), though the precise mechanisms that generate EDB- and/or EDA-containing FNs remain unknown. Finally, microenvironment mechanosensing has been shown to regulate the inclusion of Extra Domains in the FN molecule. More specifically, in an in vitro study, Bordeleau and colleagues plated endothelial cells on substrates with increasing stiffness and

found that FN expression was enhanced with increasing stiffness. Interestingly, inclusion of EDB was significantly upregulated in both RNA and protein levels, and this effect was controlled by cell-tractility-mediated phosphorylation of SRSF5 involving the PI3K/ Akt pathway (Bordeleau et al. 2015). Similar results were obtained in a separate study in which authors report the increased inclusion of EDA in FN with increasing stiffness, followed by increased expression and matrix deposition of LTBP1, which in turn modulates TGF- β activity (Klingberg et al. 2018). These results might reflect a complex regulatory loop between TGF- β signaling and FN Extra Domain splicing.

1.1.3.6 Functional Roles of the Extra Domains

Despite extensive research, the precise functional properties of EDB and EDA have yet to be fully deciphered. A non-exhaustive list of in vitro and in vivo studies regarding the Extra Domains is presented in Annex 1. Early in vitro studies have shed some light to the role of EDA, suggesting that its inclusion results in enhancing cell adhesive capacity and migration on pFN. This is done in a complementary way (Xia and Culp 1995) by potentiating the interaction of full length FN with $\alpha 5\beta 1$ integrin through a conformational change facilitated by the presence of the Extra Domain resulting in the exposure of the RGD site (Manabe et al. 1997). In line with this is the fact that the presence of either Extra Domain leads in increased assembly of FN by cultured cells (Guan, Trevithick, and Hynes 1990), an event that is potentially reflected by the increase in EDA- (and EDB-) containing FN at the base of wounds inflicted in the flanks of adult rats (Ffrench-Constant 1989). Further supporting its role in wounds and injured tissue, EDA is constitutively upregulated in relation to the duration and the tissue stress inflicted (Satoi et al. 1999), and the effects are associated with inflammation induced by EDA-mediated TLR-4 activation (Okamura et al. 2001). Finally, it has been suggested that the increased cell adhesive capacity of EDA-containing FN compared to pFN and its increased cell receptor repertoire might partly explain its pro-thrombotic role, characterized by enhanced thrombosis and vascular occlusion (Chauhan et al. 2008).

Highlighting its role in matrix remodeling, EDA has been shown to induce MMP expression and reorganization of F-actin and these effects highly depend on the native state of FN or FN fragments that occur through proteolytic cleavage (Saito et al. 1999). Another early study suggested that the presence of EDA affects the targeted secretion of FN, with EDA-containing FN being preferentially secreted apically in a tracheal epithelial cell setting (A. Wang et al. 1991). In a similar context, it has been suggested that aberrant EDB and/or EDA splicing may reflect apico-basal polarity defects in endothelial cells, subsequently resulting in abnormal endothelial tube formation (Astrof, Crowley, and Hynes 2007; White, Baralle, and Muro 2008).

A key feature of EDA in cell differentiation was acknowledged when EDA-containing endothelial FN matrices induced the conversion of normal lipocytes to myofibroblast-like cells (Jarnagin 1994). This role in differentiation was subsequently reinforced when EDA-containing polymerized FN was shown to be necessary for TGF- β 1-mediated induction of myofibroblastic phenotype (Serini et al. 1998). The interplay between TGF- β -mediated myofibroblast differentiation and EDA implication in tissue fibrosis was presented in a study showing that absence of EDA-containing FN in an idiopathic pulmonary lung fibrosis mouse model resulted in less collagen deposition and fewer α -SMA expressing myofibroblasts. This effect correlated with diminished activation of TGF- β suggesting that EDA is implicated in latent TGF- β activation (Muro et al. 2008). Similar results were reported for EDB- and EDA-containing FN in glioblastoma-derived cells in culture, in which α SMA levels were significantly reduced, suggesting a role for oncofetal FNs in favoring EMT in a glioblastoma context (Ventura et al. 2018). In a different background, EDA and EDB were reported to promote osteoblast differentiation by activation of the α 4 β 1 integrin, or by enhancing RGD binding to β 3 integrins respectively (Sens et al. 2017).

A complex crosstalk between EDB- and EDA-FN and TGF- β activation and signaling has been postulated (Astrof and Hynes 2009; Gopal et al. 2017), with TGF- β regulation of FN expression and splicing having been described in several studies (Balza et al. 1988; Borsi et al. 1990; Viedt, Bürger, and Hänsch 1995; Ventura et al. 2018). Conversely, TGF- β can be regulated by the presence of EDB and EDA in the

underlying matrix (Ventura et al. 2018; Klingberg et al. 2018; Richard O. Hynes 2009). Additionally, EDA-containing FN was found potent in inducing G1-S phase transition by increasing the expression of Cyclin D1 and upregulation of integrin-mediated mitogenic signal transduction (Manabe, Oh-e, and Sekiguchi 1999), pointing towards a complex interplay of pathways activated by FN (Extra Domains).

A major breakthrough came with the targeted deletion or mutation of either the Extra Domains themselves or their splicing sites. In a novel approach, Muro A. and colleagues developed a mouse model in which engineered mice expressed FN with

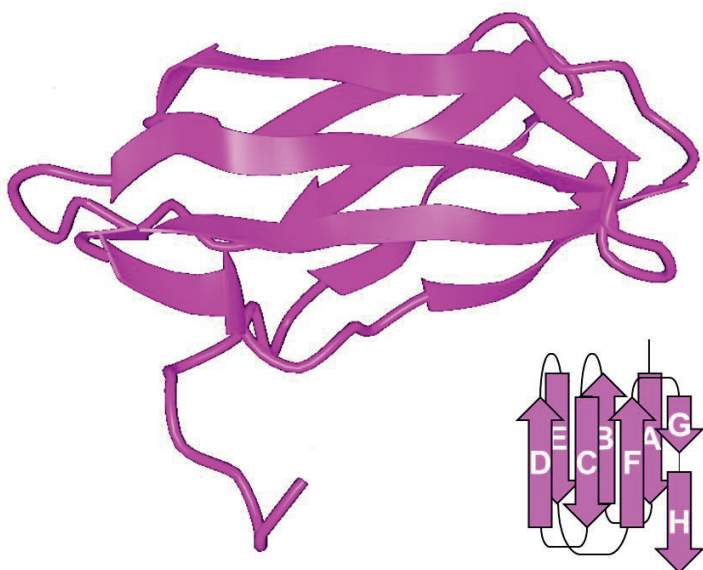


Figure 8 NMR-generated spatial structure of Extra Domain B according to *Fattorusso et al 1999*. The linear structure is also demonstrated for simplicity.

constitutively included or excluded EDA. Homozygous mouse strains were viable and developed normally independent of the presence or absence of EDA. However, mice lacking EDA displayed abnormal and delayed skin wound healing, while mice constitutively expressing EDA showed a pronounced decrease in the level of FN in all tissues. Interestingly, both mouse strains had a shorter lifetime compared to

control littermates (Muro et al. 2003). On the other hand, deletion of EDB displayed no significant phenotype in mouse development and fertility. However, fibroblasts extracted from mice lacking EDB grew more slowly in vitro, and were less efficient at depositing and assembling FN (Fukuda et al. 2002). It is evident that Extra Domains have overlapping functions during embryonic development, and at least one of the two is necessary for normal body growth since the absence of both Extra Domains is deleterious for the organism due to severe cardiovascular defects ranging from vascular leakage to defective angiogenesis and heart defects (Astrof, Crowley, and Hynes 2007).

Enhancing the notion that EDA might support various biological functions, several studies focused on the role of EDA- and EDB-containing FN in the arterial wall. Normally, the arterial wall is rich in FN, though no EDB or EDA FN is present (White, Baralle, and Muro 2008). In one study, increased levels of EDA-FN were found next to smooth muscle cells (Glukhova et al. 1989) resulting in the activation of TLR4 which can induce inflammation and atherogenesis (X. H. Xu et al. 2001). In a different approach the researchers removed EDA from FN in a mouse model that develops arterial wall lesions, and the mice not expressing EDA-FN displayed smaller lesions throughout the aortic tree suggesting that EDA modulates plasma lipoprotein metabolism (Tan et al. 2004). This hypothesis, however, was greatly opposed when subsequent analyses showed that mice bearing either variant of FN (constitutively including or excluding EDA) displayed similar reduction in atherosclerotic lesions, suggesting that strict regulation of EDA splicing is involved in atherosclerosis progression (Babaev et al. 2008).

1.2 Cell - ECM Interactions

In order for a multicellular organism to form and function properly, cells need to interact with each other as well as with the different components of the extracellular matrix. These interactions take place in a spatiotemporal manner starting at the very beginning of embryonic development and continue throughout the entire lifespan of the organism. Key players in these interactions are transmembrane protein complexes that link cells together (for example, epithelial cells lining the different organs, immune cells during antigen presentation), or with their extracellular environment (for example, epithelial cells bound on the basement membrane, or mesenchymal cells interacting with their supporting stroma).

ECM-binding cell receptors transmit signals bi-directionally. They induce intracellular signaling determined by the state (compositions and physical properties) of the ECM, and they participate in ECM deposition and dynamics driven by cytoskeletal rearrangements. ECM receptors are characterized by heterogeneity and specificity, with different receptors displaying binding preference for distinct ECM components. This specificity makes ECM receptors an attractive therapeutic target

against many pathological conditions such as inflammation, fibrosis, cardiovascular diseases, and cancer.

A major class of ECM receptors is the integrin family, a conserved family of heterodimer cell receptors that link the ECM with the cell interior by binding to different ECM molecules (collagens, FN, TNC, laminin) and to cytoskeletal proteins (actin, intermediate filaments). Integrins are described in detail below.

Syndecans (see also Section 1.1.2.1 and Table 1) comprise a family of four proteoglycans the core proteins of which are embedded in the cell membrane and are linked to HS or CS GAG chains (Morgan, Humphries, and Bass 2007; Cheng et al. 2016). Apart from binding to ECM components (both core matrisome and matrisome-associated components), syndecans can co-operate with integrins (**Figure 9**) to mediate cell adhesion and signaling as in the case of syndecan-4 and $\alpha 5\beta 1$ (Bloom, Ingham, and Hynes 1999), and they are capable of activating signaling pathways (Keum et al. 2004).

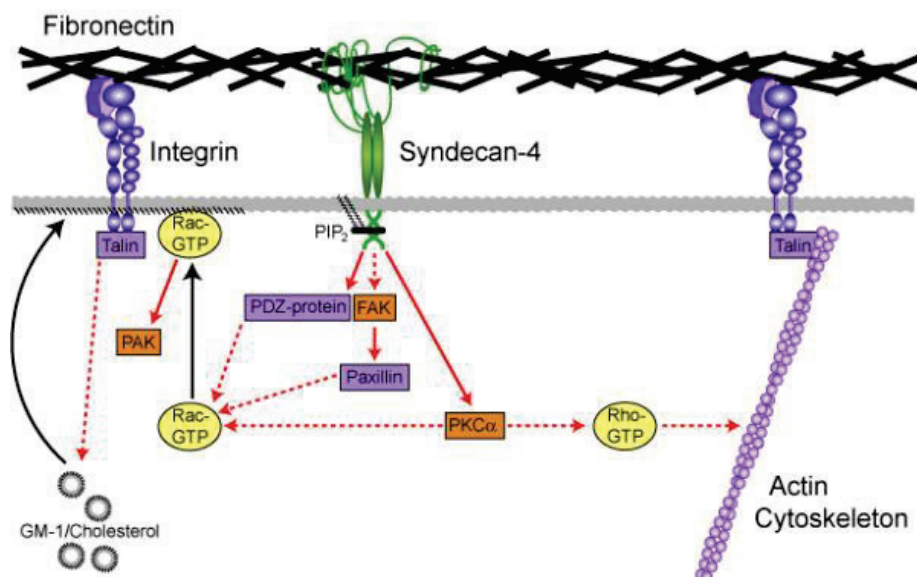


Figure 9 Activation of the cytoskeletal regulators Rac and Rho, upon integrin binding to FN, facilitated by syndecan-4 engagement. The cytoplasmic domain of syndecan-4 mediates GTP loading of Rac and Rho, the localized activation of which initiates downstream signaling leading to actin cytoskeleton re-organization. Acquired from *Morgan et al 2007*.

In Section 1.1.2.2 laminins were referred to as ligands for integrins. Laminins, however, are ligands for additional cell receptors. The dystrophin glycoprotein complex (DGC) is a multimeric apparatus that spans the cell membrane linking the actin cytoskeleton with laminin in the ECM through α -dystroglycan (Ibraghimov-

Beskrovnaya et al. 1992; Haenggi and Fritschy 2006), mediating cell adhesion on BMs (Montanaro, Lindenbaum, and Carbonetto 1999). Other, less well-studied laminin receptors are the Lutheran (Lu) and the basal cell adhesion molecule (B-CAM) that derive from the same gene, and were identified in blood samples of the Lutheran blood group (Udani et al. 1998; I. G. Campbell et al. 1994). Finally, the 67 kDa laminin receptor (67LR) has been identified in tumors and has a high affinity for laminin (Lesot, Köhl, and von der Mark 1983; Malinoff 1983; Terranova et al. 1983).

Similar to laminins, collagens are also recognized by integrins and other receptors. Discoidin domain receptors (DDR) are receptor tyrosine kinases activated upon collagen binding (Shrivastava et al. 1997; W. Vogel et al. 1997), and their distribution is cell type specific (W. F. Vogel, Abdulhusein, and Ford 2006). Additionally, glycoprotein IV (GPIV) is involved in platelet activation by binding collagen together with $\alpha 2\beta 1$ (Auger et al. 2005); leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) regulates collagen-mediated immunosuppression (Lebbink et al. 2006; Meyaard 2008); and urokinase-type plasminogen activator associated protein (uPARAP), also known as Endo180, is involved in collagen phagocytosis after collagen binding (Wienke, MacFadyen, and Isacke 2003; Curino et al. 2005).

Closing this section, it is worth mentioning CD168 and CD44, two cell receptors that recognize and bind HA, resulting in signaling pathway activation by regulation of protein tyrosine kinases, such as Src, and small GTPases such as Rho, while modulating growth factor receptor signaling (Turley, Noble, and Bourguignon 2002).

1.2.1 The Integrins

A major set of proteins that mediate cell-cell and cell-matrix interactions is the integrin family. Integrins are transmembrane complexes involved in a wide range of physiological processes. With their extracellular head regions, integrins bind extracellular matrix glycoproteins such as laminins and collagens in basement membranes, or connective tissue components like fibronectin. Through such interactions, integrins facilitate numerous functions including but not limited to stable adhesion to basement membranes, the formation of extracellular matrices and migration thereon, the formation of platelet aggregates, the establishment of

intercellular junctions in the immune system, as well as bacterial and viral entry in infectious diseases. The aforementioned adhesion-related processes modulate signaling pathways that control cell motility and migration, cell cycle regulation and differentiation, immune response, and mechanotransduction. Thus, integrins link the cell interior with the microenvironment, and are capable of transmitting signals and forces both ways across the plasma membrane depending on the state of the underlying tissue.

1.2.1.1 Integrin Structure

Integrins are heterodimeric type I transmembrane complexes composed of an α and a β subunit (Giancotti and Ruoslahti 1999). In mammals, there are 18 α and 8 β subunits with a differential expression pattern across tissues (Richard O Hynes 2002). These subunits are noncovalently associated in order to form 24 known integrin complexes characterized by distinct function, and ligand specificity (Richard O Hynes 2002; Giancotti and Ruoslahti 1999; M. J. Humphries 2000; Kim, Ye, and Ginsberg 2011). Both α and β subunits are composed of a large extracellular domain [approximately 1000 amino acids for α subunits, and approximately 700 amino acids for β subunits (I. D. Campbell and Humphries 2011)], a single transmembrane domain, and a short cytoplasmic tail. Exception to this rule is integrin $\beta 4$ that possesses an intracellular domain that is significantly larger than the extracellular domain [about 1000 amino acids long (Takada, Ye, and Simon 2007)]. A few integrin subunits can pair with multiple subunits of the opposite set, while others can form functional complexes only with a specific subunit of the opposite set (**Figure 10**). However, all integrin subunits are believed to interact with their respective partners and to exert their functional roles in a similar way.

An $\alpha\beta$ integrin dimer is composed of three distinct regions (**Figure 11**): the extracellular region, the transmembrane domain (TMD), and the cytoplasmic tail. The extracellular region can be further divided in two parts: the head of the complex, which is responsible for ligand binding, and the leg of the complex, that is involved in integrin activity.

The extracellular part that comprises the head of the α subunit is composed of a large domain named β -propeller which contains seven 60-amino-acid repeats that fold into a seven-bladed structure (propeller) (Takada, Ye, and Simon 2007; Barczyk,

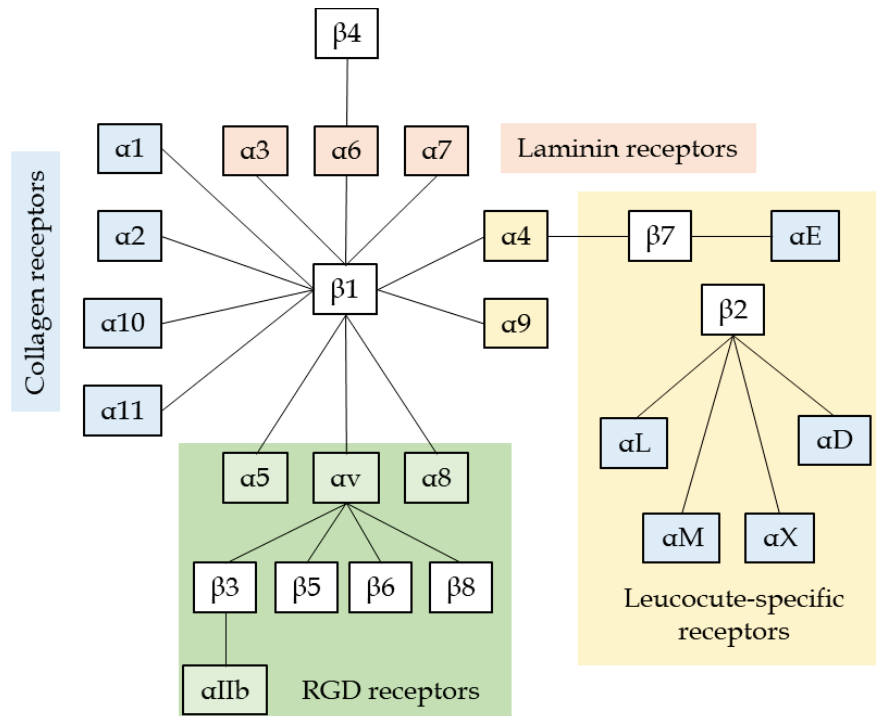


Figure 10 Eight β subunits pair with 18 α subunits and form 24 different combinations that differ in expression across tissues and in binding specificity. Adapted from Hynes 2002.

Carracedo, and Gullberg 2010). Within the β -propeller, between blades 2 and 3, resides the I domain, a domain of approximately 200 amino acids also known as A domain [Figure 11, (Barczyk, Carracedo, and Gullberg 2010)].

The I domain is characterized by the presence of five β -sheets surrounded by seven α -helices and it is only present in half of the α integrin subunits, [$\alpha 1, \alpha 2, \alpha 10, \alpha 11, \alpha L, \alpha M, \alpha X, \alpha D$, and αE (Takada, Ye, and Simon 2007; Barczyk, Carracedo, and Gullberg 2010)]. The I domain is responsible for ligand binding through a motif named metal-ion-dependent adhesion site (MIDAS). Mg^{2+} ions bind within the MIDAS motif facilitating a conformational change that results in ligand binding in a cleft formed between the αI domain and the βI domain (described below). The α integrins that lack an I domain bind their ligands through a ligand binding site formed between the β -propeller and the βI domain (Richard O Hynes 2002; M. J. Humphries 2000; I. D. Campbell and Humphries 2011; Barczyk, Carracedo, and Gullberg 2010).

The α head (β -propeller plus I domain, if present) is connected with a *thigh* region, a calf-1, and a calf-2 domain, which collectively comprise the leg of the α subunit (Figure 11). This region, in concert with the β subunit, orchestrates the activation status of the integrin dimer. Downstream from the leg domain there is the TMD usually spanning around 20 amino acids (both for α and β subunits), and finally lies the cytoplasmic tail of the subunit (M. J. Humphries 2000).

The β subunit is generally shorter than the α subunit. It contains a plexin-semaphorin-integrin (PSI) domain, followed by a hybrid domain within which lies the β I domain (Barczyk, Carracedo, and Gullberg 2010). The β I domain also contains a MIDAS motif that binds a Mg^{2+} ion, as well as an adjacent to MIDAS (ADMIDAS) motif, that either binds an inhibitory Ca^{2+} ion,

or a Mn^{2+} ion resulting in an active form of the integrin through a conformational change (Barczyk, Carracedo, and Gullberg 2010; M. Humphries 2003). As mentioned above, the β I domain is responsible for the formation of the ligand-binding cleft.

The PSI, hybrid, and β I domains, constituting the head of the β subunit, is connected to the leg, which is composed of four cysteine-rich epidermal growth factor (EGF) repeats, as well as a membrane-proximal extracellular domain (Figure 11). The latter is connected to the β TMD region, and the β cytoplasmic tail, which is mainly

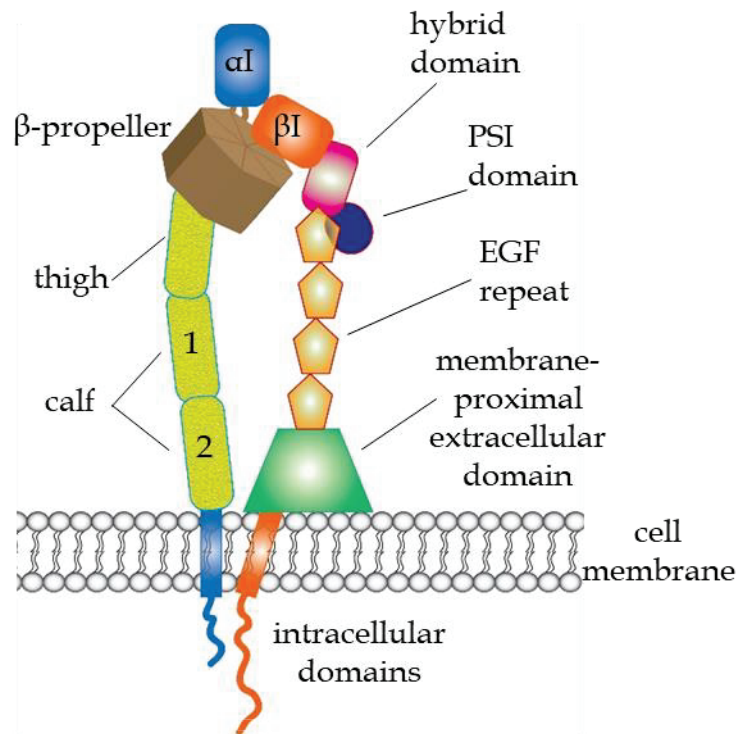


Figure 11 Integrin dimer scheme displaying the active conformation of the dimer. The different subunits, α and β , and the distinct regions of each subunit are shown (see text for details). In the inactive conformation, the extracellular part is in a folded conformation and the transmembrane domains are found in an X-like conformation. Activation drives the extracellular part to adopt an uptight conformation, and the transmembrane and cytoplasmic domains are separated. See also Figure 12. Adapted from *Campbell and Humphries 2011*.

responsible for integrin activity/activation (Kim, Ye, and Ginsberg 2011; Barczyk, Carracedo, and Gullberg 2010). While integrins have no enzymatic activity themselves, the β tails have NPxY motifs that are recognized by proteins that contain phospho-tyrosine binding (PTB) domains. Several proteins have been identified to directly interact with the β subunit PTB domains (Barczyk, Carracedo, and Gullberg 2010).

1.2.1.2 Integrin Activation

As mentioned above, an α subunit will non-covalently bind a β subunit to form a functional integrin, and their head regions will generate a ligand binding site. However, ligand binding is an operation that can be regulated both by the presence of the ligand, and cytoplasm related processes.

Since their identification, integrins have been subjected to extensive studies in order to identify the overall structure and the various conformations, as well as the way they contribute to integrin function. Crystallization and electron microscopy approaches of entire integrins or fragments, with or without ligand, and in presence or absence of bivalent ions led to the establishment of a model in which integrins can be found in three distinct states (Richard O Hynes 2002; M. J. Humphries 2000; Kim, Ye, and Ginsberg 2011). A recent microscopy-based approach (J. Li et al. 2017) confirmed these findings and demonstrated that integrin conformation can be i) bent and inactive, ii) stretched and primed, iii) stretched and active (**Figure 12**). Furthermore, through site-directed mutagenesis, it has been shown that the regions contributing to the different forms of the complex lie mostly within the TMDs and the cytoplasmic tails (I. D. Campbell and Humphries 2011; Takada, Ye, and Simon 2007; Calderwood 2004).

In the proposed model, both cytoplasmic tails and transmembrane domains are found in close proximity through electrostatic interactions, and the hydrophobic properties of the underlying amino acids, respectively. In this state, the extracellular part of the complex adopts a bent, V-shaped conformation that is considered to be inactive and of low affinity for ligand. Activation can occur either by weak ligand binding or by effects that occur on the cytoplasmic side of the complex, leading to

straightening and separation of the legs, the transmembrane domains and the cytoplasmic tails. At this stage, the complex is considered as “primed” and affinity for ligand is increased. Additionally, the straightening of the complex results in the exposure of previously cryptic sites that function as binding/docking sites for cytoplasmic proteins and signaling.

Finally, when the complex is fully active, the cytoplasmic tail binding sites are occupied by a vast set of proteins (adhesome) that exert a wide range of processes such as mechanosensing and signaling (Figure 12 and

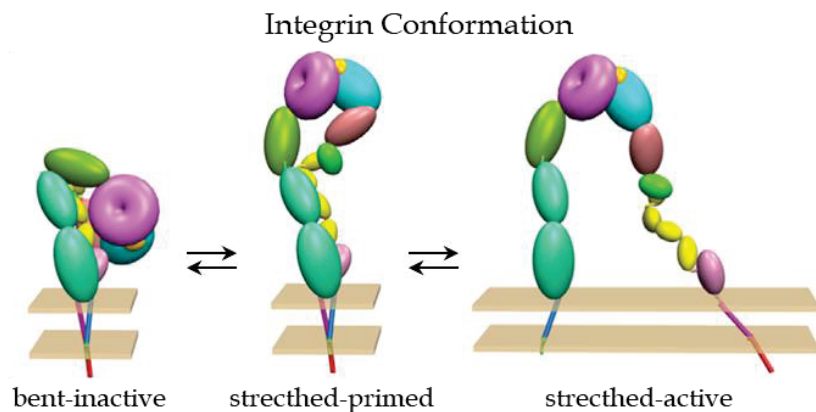


Figure 12 Depending on the presence of ligand and/or intracellular proteins, integrins can be found in three distinct activation states: i) inactive, ii) primed, and iii) active. See text for details. Image adapted from *Li et al. 2017*.

Figure 13). At this state, ligand binding is at its peak forming a catch bond, which gets stronger upon application of higher force (Barczyk, Carracedo, and Gullberg 2010; Ginsberg 2014; Changede and Sheetz 2017; Klapholz and Brown 2017).

The cytoplasmic tails of the integrins are short, but they are also extended and flexible (I. D. Campbell and Humphries 2011). The β tail, more specifically, is able to act as a docking site for multiple interactions with many different proteins, as previously mentioned. More particularly, proteins containing the PTB domain bind directly to one of the two conserved NPxY motifs in β tails, resulting in partial activation of the complex. The most important among these is the protein talin, a large protein of approximately 2500 amino acids that has a head-and-rod structure. Talin has two integrin binding sites, three actin binding sites and can dimerize (Klapholz and Brown 2017). Talin molecules in a closed conformation are recruited to ligand-bound integrins, through their FERM (protein 4.1, ezrin, radixin, moesin) domain and more specifically through the F3 subdomain that binds directly to the β tail. Their orientation depends on which integrin binding site is used, and this can greatly affect

the spacing between integrin subunits, resulting in a stretched integrin configuration. Additionally, talin associated with one ECM-bound integrin can expand the adhesion site by binding to an adjacent non-ligand-bound integrin and stimulating its ECM binding (Klapholz and Brown 2017).

The second step involves integrin-bound talin binding to the actin cytoskeleton. This event is followed by stretching of the molecule by mechanical forces either in the plane of the membrane and the actin cortex, or perpendicular to it. This stretching results in the unfolding of distinct domains and the exposure of additional protein interacting sites, notably vinculin binding sites [VBSs, (Gingras et al. 2005)]. Consequently, talin translates actin-mediated forces on integrins into recruitment of integrin-associated proteins (IAPs), which strengthen and/or modify the adhesion.

Talin also recruits additional proteins independently of force, directly or indirectly, thus acting as a major docking site or hub, initiating numerous cell processes. For instance, the talin head binds Rap1 proteins through the F0 subdomain of the FERM domain (Goult et al. 2010), as well as focal adhesion kinase [FAK, (Lawson et al. 2012)], layilin (Wegener et al. 2008), T-cell lymphoma invasion and metastasis 1 [TIAM1, (Shujie Wang et al. 2012)], and phosphatidylinositol phosphate kinase type I γ [PIPKI γ , (de Pereda et al. 2005)], and the Rap1-GTP-interacting adaptor molecule (RIAM) via the F3 subdomain (H.-S. Lee et al. 2009). Additional regulating proteins that are found docked in the intracellular part of integrin complexes are filamins and migfilin (Ithychanda et al. 2009), ILK (Honda et al. 2009), the family of PINCH proteins (C. Wu 2004), Parvins (Brakebusch 2003), paxillin (Liu et al. 1999), and tensin (Danen 2013).

1.2.1.3 Biological Processes Involving Integrins

Integrin engagement and adhesion establishment can regulate intracellular signal transduction cascades that eventually control differentiation, proliferation, survival, actin dynamics, and metabolism (Figure 13). For most cell types, activation of the Raf – MEK – ERK signaling pathway is weak and transient in the absence of adhesion, but it is accentuated and sustained in the presence of integrin-mediated adhesions (Danen 2013; Renshaw 1997; Q. Chen et al. 1996).

Receptor tyrosine kinase (RTK) signaling is also regulated by integrin clustering and transactivation of PDGFR, EGFR (Miyamoto 1996; Moro 1998), VEGFR (J. F. Wang, Zhang, and Groopman 2001), and other RTKs, while growth factors concentrated

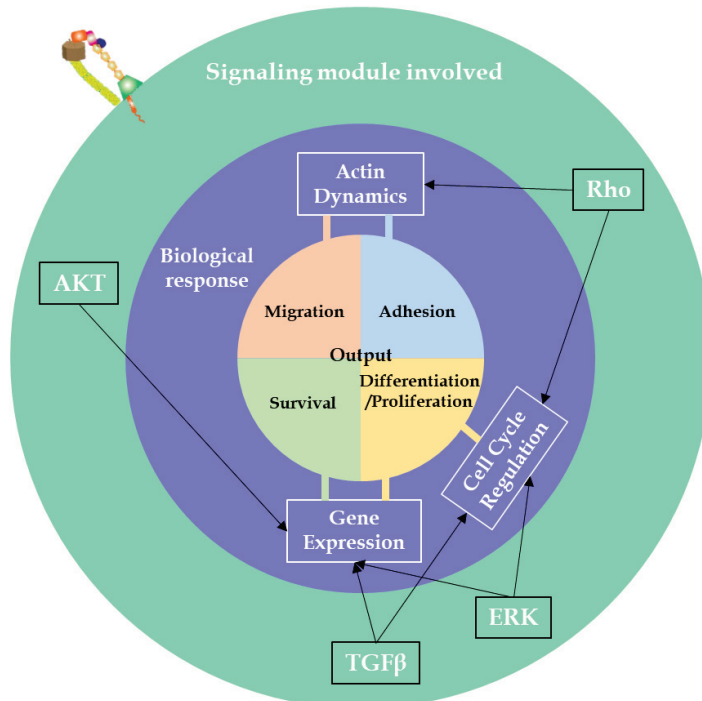


Figure 13 Integrin activation results in the activation of different pathways that in turn initiate biological responses responsible for cell adhesion and motility, or proliferation, differentiation, and survival. See text for details.

by/within the ECM can be activated by integrins and presented to their respective growth factor receptors, as is the case for TGF- β 1 (John S Munger et al. 1999).

Through its effect on multiple signal transduction cascades, integrin-mediated cell adhesion regulates also the cell cycle, and the differentiation state. Integrins cooperate with RTKs to stimulate the cyclin E-cdk2 activity that drives the entry to the S-phase, through organization of the actin cytoskeleton by the integrins (Danen 2013; Assoian and Schwartz 2001; Danen and Yamada 2001). Integrin-mediated adhesion also regulates the expression of genes related to differentiation, such as the synthesis of milk proteins by mammary epithelial cells (M. L. Li et al. 1987; Edwards et al. 1998), the formation of contracting myotubes and expression of meromyosin by embryonic fibroblasts (Menko and Boettiger 1987), the production of inflammatory cytokines by monocytes (Haskill et al. 1988), and the expression of terminally differentiated keratinocyte markers (Adams and Watt 1989).

More recently accumulating evidence suggests that integrin-mediated signaling is tightly linked to metabolic procedures. Metabolic flux, through specific pathways (AMPK, mTOR, HIF1), controls integrin expression, trafficking and degradation, thus affecting cell adhesion and integrin-mediated cell migration (Koike et al. 2004; Keely et al. 2008; Ryu et al. 2010; T. Kong et al. 2004; Tang and Lu 2009; Chiu et al. 2009;

Rainero et al. 2015). Moreover, integrins themselves control various metabolic signals and pathways, establishing a reciprocity of this regulation (Ata and Antonescu 2017).

A major function of integrins and the downstream signaling discussed above is cellular motility. Cell migration is crucial for embryonic development, immune response, tissue repair, while deregulated migration is a primary cause of metastatic cancers (Danen 2013; Ridley et al. 2003). Integrin-mediated adhesion to the ECM or to other cells is required for the establishment of cell polarity during migration. The lamellipodium in the front is characterized by an extended network of stationary focal complexes resulting in the generation of propulsive forces, while the rear edge is rich in F-actin stress fibers generating forces that produce inward sliding focal contacts (Ballestrem et al. 2001). The integrin expression profile, as well as the cell adhesion machinery within each cell type can promote distinct modes of motility, and cells can alter their motile behavior by expressing different integrins and/or adhesion proteins (Danen 2013; Danen et al. 2005).

1.2.1.4 Fibronectin Fibrillogenesis

Given the direct link between integrins and fibronectin/ECM, it only seems fit to close this chapter with ECM assembly. Integrins are found in the core of the assembly process of various types of ECM. In the case of basement membranes, $\beta 1$ integrins cooperate with the dystroglycan receptor to promote synthesis and polymerization of laminin chains into a multivalent network in which eventually, collagens and other components are incorporated. In the case of mesenchymal stroma and other fibronectin-rich matrices, $\alpha 5 \beta 1$ is the typical integrin that, upon interaction with syndecans, drives fibronectin matrix assembly, a crucial step for cell migration during embryonic development, wound healing, and various pathological conditions (George et al. 1993; Wennerberg et al. 1996). Binding of fibronectin to $\alpha 5 \beta 1$ stimulates the activity of RhoA, that in turn stimulates actomyosin-derived contractility, which is required for fibronectin fibrillogenesis (Mao and Schwarzbauer 2005).

Fibronectin fibrillogenesis is a multistep process (Figure 9 and **Figure 14**) that involves inherent physical properties of FN, interactions with other ECM molecules (FN included), binding to cell receptors, and cytoskeletal rearrangements. Functional

studies involving the use of FN or FN fragments revealed that FN assembly is based on self-association, and on FN binding to integrin $\alpha 5 \beta 1$ through the RGD sequence.

FN can be presented on the cell surface in a soluble form, or it can be produced by cells and secreted in close proximity to the plasma membrane. In this state, FN is most probably found in a compact conformation. Several *in vitro* studies have shown that this compact conformation is controlled by the ability of FN to self-associate through several regions (See also section 1.1.3.3). Additionally, the FNIII repeats are characterized by the presence of two β -sheets enclosing a hydrophobic core (Dickinson, Veerapandian, et al. 1994; Dickinson, Gay, et al. 1994), without any covalent interactions, suggesting that each FNIII repeat can acquire an open or stretched conformation if strain is applied. Indeed, *in silico* and *in vitro* studies have demonstrated the extendibility of FNIII domains (Krammer et al. 1999; Baneyx, Baugh, and Vogel 2002).

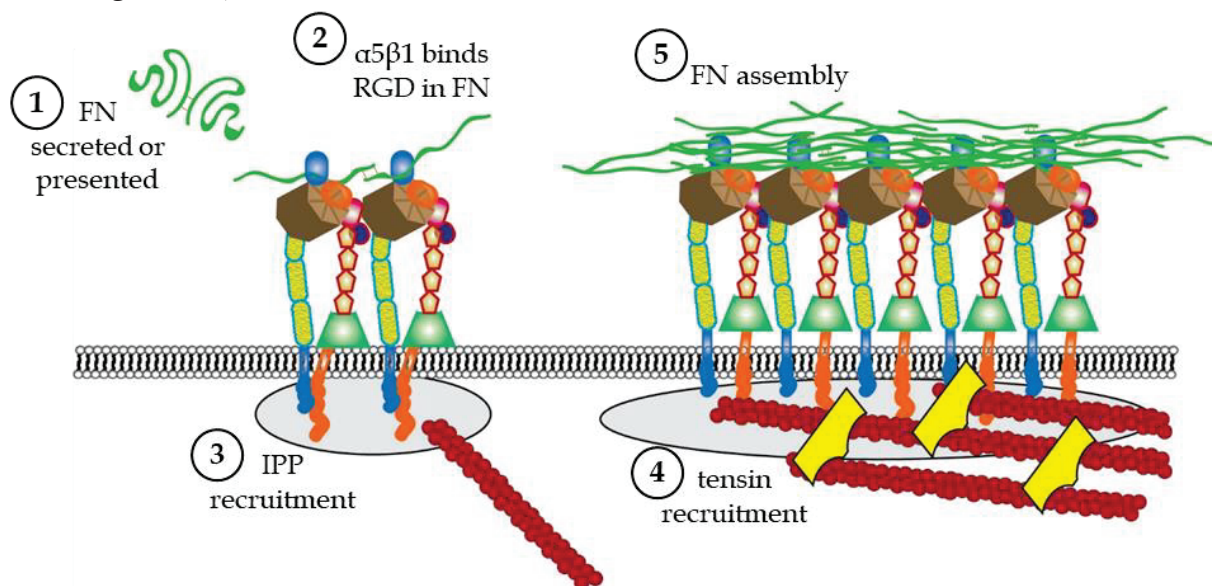


Figure 14 Stages of FN fibrillogenesis. 1) FN is secreted or is exogenously presented to cells. 2) Integrin $\alpha 5 \beta 1$ binds the RGD sequence of FN and is activated resulting in receptor clustering and 3) recruitment of integrin partners in the cytoplasmic site of the plasma membrane (ILK, PINCH, parvin - IPP, depicted by the gray oval). 4) Tensin is recruited in the cytoplasmic side of the plasma membrane, and at the same time FN acquires an extended conformation resulting in 5) binding to additional ECM components and cell receptors subsequently enhancing actin polymerization and further FN fibrillogenesis. Adapted from *Van Obberghen-Schilling et al. 2011*.

Once recognized by $\alpha 5 \beta 1$ (and $\alpha \nu \beta 3$) integrin, FN-integrin interaction takes place through the RGD domain in FNIII₁₀ (E Ruoslahti and Pierschbacher 1987), and this association is facilitated by the synergy sequence (PHSRN) in FNIII₉. Elimination of the regulatory activity of either sequence abolishes early FN fibril formation (Fogerty

et al. 1990; Jean E Schwarzbauer 1991; McDonald et al. 1987), highlighting that FN-integrin interaction is a key step initializing FN fibrillogenesis. FN binding to integrins induces an increase of the local concentration (clustering) of the receptors that are now forming focal complexes by recruiting ILK, PINCH, parvin and tensin, activating Rho-mediated actin polymerization, resulting in cell spreading and motility thus “laying” FN fibrils along the way [Figure 15, and reviewed in (Van Obberghen-Schilling et al. 2011)].

At the same time, integrin clustering and binding of additional cell receptors like syndecan-4 [reviewed in (Morgan, Humphries, and Bass 2007)] or urokinase plasminogen activator receptor [uPAR (Monaghan et al. 2004)] enhances FN assembly by stimulating Rho GTPase and FAK activities (Saoncella et al. 1999; Wilcox-Adelman,

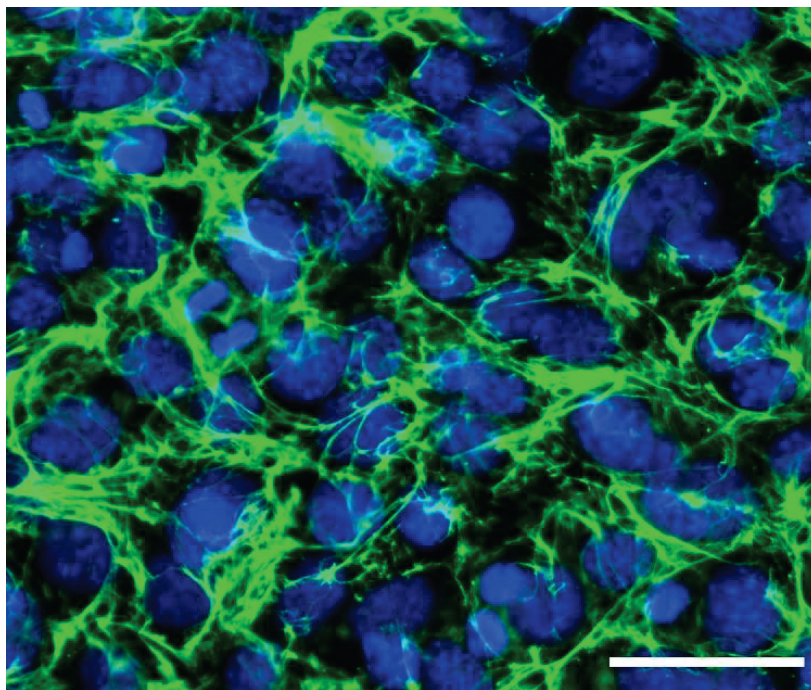


Figure 15 Pericellular deposition of FN fibrils in passively immortalized Mouse Embryo Fibroblasts after 2 days in culture. Scale bar: 50µm.

Denhez, and Goetinck 2002) or by strengthening FN-integrin binding respectively (Monaghan et al. 2004). Finally, longitudinal and lateral association of FN molecules to existing fibrils facilitated by their stretched conformation, results in FN polymerization and promotion of assembly, probably mediated by the protein-disulfide isomerase activity of FN, located in the FNI₁₂ (Langenbach and Sottile 1999).

FN fibrillogenesis is a key step in ECM deposition and maturation since loss of FN has deleterious effects in matrix generation (Cseh et al. 2010), abolishing the deposition and processing of other ECM components such as collagen I (Saunders and

Schwarzbauer 2019) and thrombospondin-1 (Sottile and Hocking 2002), as well as fibrillin-1 (Kinsey et al. 2008; Sabatier et al. 2009).

This introduction has only been a mere scratch on the surface of the integrin information pool, but I think that it contains the necessary elements in order for the reader to understand the complexity and the charm that lies within the cell-ECM interplay.

1.3 The TGF- β Pathway

The TGF- β family of proteins is composed of a large set of secreted peptides that mediate a vast range of cellular processes. Thirty-three genes have been identified in mammals so far (Derynck and Budi 2019), encoding highly specialized secreted proteins that homodimerize or heterodimerize, and bind to specific cell receptors in order to mediate their roles. TGF- β family proteins control cell proliferation and differentiation, they induce or protect from cell death depending on the cell type and the biological context, they control the expression of ECM components, they promote cell motility and invasion, and they are involved in the regulation of cell energetics [reviewed in (Derynck and Budi 2019)].

This work mainly focuses on the most well studied member of the TGF- β family, the TGF- β 1. TGF- β 1 is seen as the archetype TGF- β family member, as it was the first one to be purified, cloned and characterized, and the recombinant protein was subsequently produced for laboratory studies (Moses, Roberts, and Derynck 2016; Derynck et al. 1985). The first documented role of TGF- β 1, together with EGF, was its effect on colony cell proliferation in soft agar. Many cell types stimulated with TGF- β 1 alone displayed decreased proliferation rates including epithelial, endothelial, mesenchymal, hematopoietic, and cells of the immune system (Y. Zhang, Alexander, and Wang 2017). However, the cytostatic effect of TGF- β 1 is not universal. It is now known that there is an extensive crosstalk between TGF- β signaling and other pathways determining cell fate. Such pathways include the ERK-MAPK, JNK, p38-MAPK, PI3K-PKB and mTOR, JAK-STAT (Roberts et al. 1985). This crosstalk can result in either reduced or increased cell proliferation, both in physiological and pathological

conditions, such as in tumor progression where TGF- β 1 may act either as a tumor suppressor or as an oncogene (Roberts et al. 1985; Inman 2011).

1.3.1 TGF- β Signaling, Receptors and Effectors

From a historical perspective, TGF- β 1 was initially characterized in platelets (Moses, Roberts, and Derynck 2016) as a secreted pair of identical subunits linked through disulfide bonding (Assoian et al. 1983). The *TGFB1* gene encodes a pre-pro-peptide composed of an N-terminal signaling sequence responsible for secretion, and a pro-domain termed LAP (latency-associated peptide) adjacent to the mature peptide (**Figure 16**). This pre-pro-peptide is subjected to dimerization and folding in the endoplasmic reticulum (ER). Dimerization takes place through cysteine residues lying within the mature peptide sequences forming a single disulfide bond covalently linking the pro-peptides.

The dimer LAP-TGF- β is called small latent complex (SLC). During its presence in the ER, the SLC is covalently bonded to other proteins, targeting TGF- β to different compartments. (Gleizes et al. 1996; Nunes et al. 1997; Isogai et al. 2003). The glycoprotein-A repetitions predominant (GARP), also known as leucine-rich repeat-containing protein 32 (LRRC32), a cell membrane docked protein, is linked with one disulfide bond per LAP within the dimer complex, thus retaining the inactive complex in close proximity to the extracellular side of plasma membrane. Similarly, disulfide bonding of LAP to a latent TGF- β binding protein (LTBP) results in localization of the resulting large latent complex (LLC) in the ECM (Rifkin 2005).

Proteolytic processing in the trans-Golgi by furin enzymes (Robertson and Rifkin 2016) results in removal of the signaling sequence, and cleavage of LAP from the mature peptide. The mature peptide and the LAP remain associated through non-covalent interactions.

The first step for pathway activation is the release of the mature peptide (the TGF- β 1 dimer) from the complexes that confer latency, LAP and LTBP or GARP. The dimer is then free to bind to TGF- β 1 receptors that are found in the vicinity (**Figure 17**). There are two kinds of receptors, TGF β RI and TGF β RII (or T β RI and T β RII), that are only scarcely found at the plasma membrane, generally in a homodimeric form. Despite

their low abundance (less than 1000 to 5000 per cell, depending on the cell type), TGF- β 1 is able to bind the receptors with very high affinity, the dissociation constants of which are approximately 10^{-11} M (Derynck and Budi 2019). Active TGF- β 1 binds with high affinity to TGF β RII homodimer, followed by the tethering of the TGF β RI homodimer, resulting in ligand induced receptor activation.

Activation of the factor-receptor complex induces conformational changes that

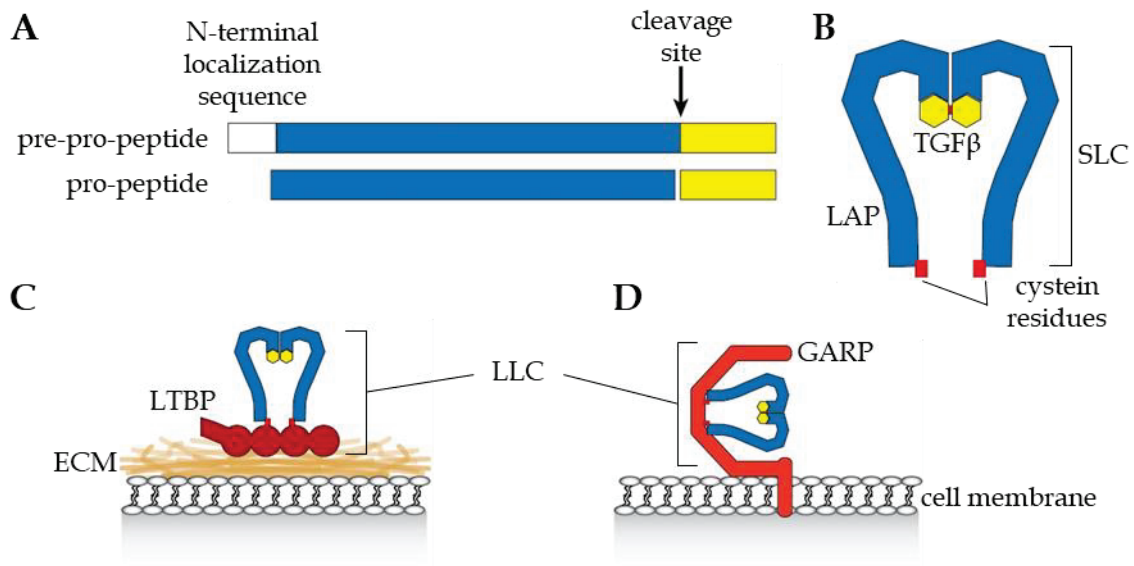


Figure 16 A. TGF- β is produced as a homodimeric pre-pro-peptide in the endoplasmic reticulum. B. Maturation involves cleavage of the LAP from the mature peptide, though they remain associated through non-covalent interactions resulting in TGF- β being in an inactive, or latent, state. TGF- β is finally secreted and docked in the ECM (C), or on the cell membrane (D) through non-covalent association with proteins that confer latency. Image adapted from *Derynck and Budi 2019*.

stabilize the association between the receptor tetramer, which facilitates the cytoplasmic domains of the receptors to be placed in proximity (Chaikuad and Bullock 2016; Hinck, Mueller, and Springer 2016). This interaction results in the phosphorylation of the type II receptor by TGF β RI. More specifically, both receptor types are characterized by the presence of a membrane-proximal domain that is rich in serine and glycine residues, denoted GS domain that has serine kinase activity. The TGF β RI GS domain is phosphorylated at different sites by the TGF β RII kinase upon binding of the ligand (Heldin and Moustakas 2016; Chaikuad and Bullock 2016; Wieser, Wrana, and Massague 1995), namely a threonine residue and several serine residues.

Phosphorylation of the TGF β RI GS domain leads in additional conformational rearrangements that cause FKBP12 to be released from the phosphorylated GS domain. FKBP12 is a cytosolic protein that interacts with the cytoplasmic domain of TGF β RI (T. Wang, Donahoe, and Zervos 1994), preventing its spontaneous phosphorylation by TGF β RII in a ligand-independent manner (Y.-G. Chen, Liu, and Massague 1997). The dissociation of FKBP12 from the type I receptor cytosolic domain results in its kinase activation (Chaikuad and Bullock 2016; Huse et al. 2001).

Upon receptor activation, several intracellular effector proteins transmit the signal

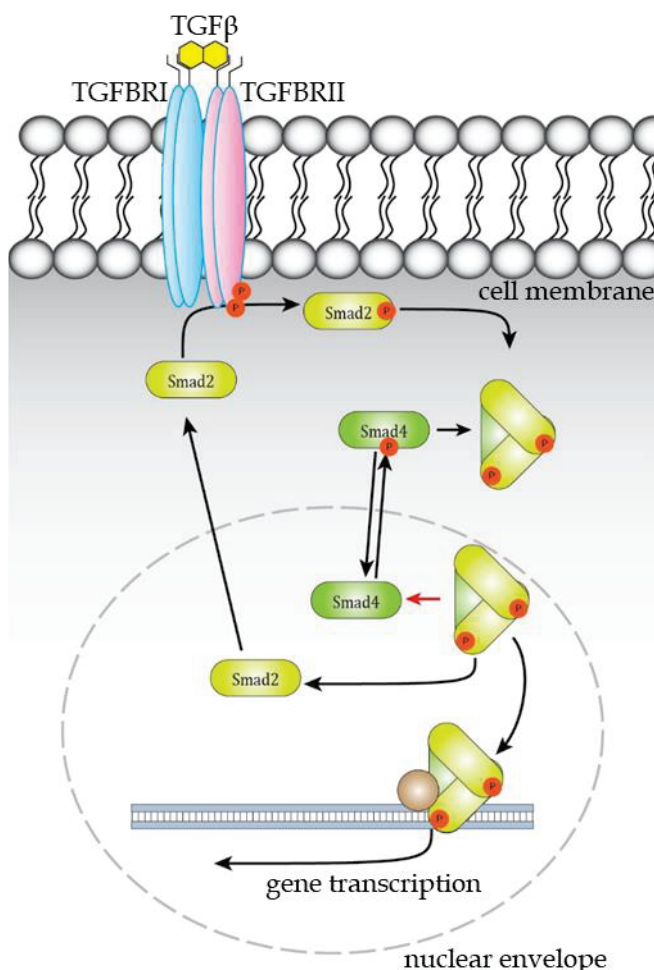


Figure 17 Active TGF- β binds sequentially TGF β RII and TGF β RI, resulting in the activation of the phosphorylation of the cytoplasmic domain of the TGF β RII. This event induces the phosphorylation and activation of the TGF- β signaling effectors SMAD2 and SMAD4 that form a complex that translocates into the nucleus. Interaction with additional transcription factors (brown circle) results in transcriptional regulation of target genes. Adapted from *Schmierer and Hill 2007*.

into the nucleus to guide gene expression. Once TGF β RI kinase is activated, it phosphorylates the SMAD proteins, the principal TGF- β signaling effectors, at two C-terminal serine residues. Typically, these are SMAD2 and SMAD3, which are subsequently released from the receptor complex and partner with SMAD4. The active trimeric SMAD complex then translocates to the nucleus through the combined effect of nuclear localization sequences in the N-terminal domains and the association of SMADs with specific importins or nucleoporins (Hata and Chen 2016; Hill 2009). Inside the nucleus, the SMAD complexes are brought together with highly specific transcription factors and regulatory elements in order to promote activation or repression of target

gene expression, as well as epigenetic modifications and mRNA processing (Derynck and Budi 2019).

The system described so far is a simplified version of the currently known network of TGF- β pathways. There are many different receptors and effectors highly specializing the system in a spatiotemporal and cell-type dependent manner. Additionally, there are proteins that facilitate ligand binding through presentation of the ligand to the receptor, or through regulating the local ligand concentration (Nickel, ten Dijke, and Mueller 2018; Heldin and Moustakas 2016; Hinck, Mueller, and Springer 2016). It is interesting to present two such well-known co-receptors with distinct functions.

Betaglycan, originally named as TGF β RIII (T β RIII), is a transmembrane proteoglycan with CS and Hep side chains attached to its extracellular part, able to bind TGF- β 1 (M. Dong et al. 2007; Bilandzic and Stenvers 2011), and stabilize receptor association. Betaglycan promotes ligand presenting to the receptor resulting in enhanced TGF- β signaling (Bilandzic and Stenvers 2011; Heldin and Moustakas 2016; Hinck, Mueller, and Springer 2016; Nickel, ten Dijke, and Mueller 2018). Conversely, cleavage of the extracellular domain of betaglycan results in decrease of the local concentration of the ligand, finally quenching the signal (M. Dong et al. 2007; Lopez-Casillas et al. 1994).

Endoglin, just like betaglycan, is a single pass transmembrane protein found in a homodimeric form (Heldin and Moustakas 2016; Nickel, ten Dijke, and Mueller 2018; Sugden and Siekmann 2018). Endoglin can act either as a promoter of TGF- β signaling or as a suppressor, depending on the receptor complexes and the effectors implicated in the pathway. More specifically, endoglin binds TGF- β 1 and inhibits TGF β RI mediated signaling (Heldin and Moustakas 2016; Nickel, ten Dijke, and Mueller 2018).

1.3.2 TGF- β Crosstalk with the ECM and the Role of Integrins

It is generally accepted that the ECM serves as a growth factor reservoir, which can locally regulate the bioavailability of active growth factors when they are needed. Many growth factors bind to GAG chains attached to ECM proteins, as well as directly to the proteins themselves (Richard O. Hynes 2009). Fibronectin is a good example of

a matrix protein that has been shown to bind a large variety of growth factors, modulating cell behavior.

As mentioned previously, TGF- β can be found in a latent (inactive) form in the ECM and this localization is facilitated by LTBP. TGF- β 1, as well as other members of the family, are incorporated into the ECM by binding to different ECM components, such as fibrillins, fibronectins, and collagen II (Richard O. Hynes 2009). The apparent question raised by the presence of TGF- β in the matrix was whether the ECM could contribute to the release of the mature peptide and trigger TGF- β signaling. Several lines of evidence have provided insight in TGF- β activation through different ECM mediated routes. Proteolytic degradation of fibrillin and/or LTBP through metalloproteinase activity, MMP2, MMP3, MMP9, MMP13, and MMP14, leads in active TGF- β being released from the LLC [reviewed in (Jenkins 2008)]. Thrombospondin is an ECM protein that can activate metalloproteinases, but it can also bind on LAP resulting in its dissociation from the mature peptide (Crawford et al. 1998). Conformational changes in LAP can also release active TGF- β or result in its exposure towards TGF- β receptors, which are subsequently activated (Rifkin 2005; ten Dijke and Arthur 2007).

The LAP-TGF- β complex is characterized by the ability to bind to integrins. This is mediated through RGD sequences that are found in LAP and are recognized mainly by α v β 6, α v β 8, but also α v β 1, α v β 3, and α v β 5 [see (Robertson and Rifkin 2016) and references therein]. More specifically, α v β 6 integrin binds LAP-TGF- β through the RGD sequence, while being stable in position through its anchoring to the underlying actin cytoskeleton, while LAP-TGF- β is bound to the ECM through LTBP. Generation of traction forces results in the distortion of the steady-state conformation of actin-integrin-LLC-ECM, resulting in the liberation of mature TGF- β which can subsequently bind TGF β RII (Flaumenhaft 1993; John S Munger et al. 1999; Annes et al. 2004). A similar way of TGF- β activation has been described for integrins α v β 3 and α v β 5 (J. S. Munger and Sheppard 2011; Hinz 2013; Worthington, Klementowicz, and Travis 2011; Hinz 2015). Recently, it was shown that the presence of the Extra Domain

A in FN enhances recruitment of LTBP1 to the fibroblast matrix, which in turn confers latency to TGF- β , thus regulating its bioavailability (Klingberg et al. 2018).

Other ways of TGF- β activation have been proposed and are currently under study such as the cooperation of integrins and proteases, deglycosylation of potential glycosylation sites, the presence of mimicking peptides, and other TGF- β interacting proteins. Physicochemical factors such as detergents, ionizing and UV radiation, increased temperature, reactive oxygen species (ROS) and pH extremities also result in TGF- β activation.

ECM mediated regulation of TGF- β activity is not unidirectional. Several studies have shown that TGF- β signaling regulates expression of α v β 3, α v β 5, α v β 6, and several β 1 integrins (Khan and Marshall 2016). It also regulates the expression of ECM components like collagen I and fibronectin (Ignatz and Massague 1986), thus changing the ECM landscape and resulting in a strictly regulated network of interactions, perturbations of which reflect pathological phenotypes like fibrosis, and cancer. In the case of FN, TGF- β expression correlates with inclusion of EDB and EDA (Borsi et al. 1990; Balza et al. 1988; P. Castellani 1986) and involvement of SMAD3 and SMAD4 signaling in different cell lines (Ventura et al. 2018). In line with this is the fact that the SRSF family of splicing factors, that regulate Extra Domain splicing in FN (Du et al. 1997; Lim and Sharp 1998; Caputi et al. 1994), are downstream targets of TGF- β signaling (Hallgren et al. 2012).

Activation of TGF- β signaling results in increased expression of TGF- β -response genes, such as *TGFBI*, *CTGF*, and *PAI1*. TGF- β inducible (TGFBI) is an ECM glycoprotein induced by TGF- β that interacts with FN (Billings et al. 2002) and has a role in normal ECM deposition in several contexts (Ween, Oehler, and Ricciardelli 2012; Ahlfeld et al. 2016). Connective tissue growth factor (CTGF) is a multifunctional matricellular protein (Leask and Abraham 2003) that influences tissue remodeling (Lipson et al. 2012), and it is considered a marker of the fibrotic response (Riser et al. 2000; Gupta et al. 2000). Its expression is positively controlled by TGF- β (Youjun Chen et al. 2002; Riser et al. 2000) and negatively regulated by endoglin (Obreo et al. 2004),

which is also a TGF- β signaling modulator (see Section 1.3.1), while modulating signaling by BMP and TGF- β (Abreu et al. 2002).

Plasminogen activator inhibitor-1 (PAI1) is a matrisome-affiliated protein (Naba et al. 2012) that regulates the plasma fibrinolytic activity. A direct role has been postulated for PAI1 in lung fibrosis, and tissue remodeling in the vasculature [reviewed in (Cesari, Pahor, and Incalzi 2010) and (Yamauchi et al. 2018)]. PAI1 expression is also regulated by TGF- β (Kutz et al. 2001) and it is believed to be involved in the alleviation of elastin network integrity and collagen fiber modifications, thus compromising the structure of BMs (Callaghan and Wilhelm 2008; Calleja-Agius 2007).

Finally, the physical properties of the matrix, reflected for example in tissue stiffness and straining, can directly control and be controlled by the efficacy of TGF- β activation by fibroblasts [reviewed in (Hinz 2015)]. Taken together, these data support the notion of an elaborately regulated crosstalk between cells and ECM, and TGF- β plays a pivotal role in this crosstalk.

2. Aim of the Thesis

Fibronectin is one of the most extensively studied ECM components. Many aspects of the structure and function of FN have been elucidated since its discovery, such as its structural role in matrix deposition and assembly that in turn control normal tissue homeostasis both in developmental processes and in pathophysiological conditions. These effects have been demonstrated through a series of *in vitro* and *in vivo* experiments that have shown that FN is indispensable for normal development by regulating cell adhesion, motility, proliferation, and differentiation (George et al. 1993).

FN is characterized by the presence of repeating modular domains with distinct structure and binding partners, influencing its conformation, which can be compact or stretched [reviewed in (Pankov and Yamada 2002; Zollinger and Smith 2017)]. However, functional studies regarding the two alternatively spliced FN type III repeats (EDB and EDA) have only recently started to provide some insight regarding their function. *In vivo* experiments in the mouse have shown that knockout of either Extra Domain results in viable and fertile mice (Muro et al. 2003), while absence of both leads in embryonic lethality (Astrof, Crowley, and Hynes 2007). For EDA a role has been described in the morphogenesis of the lymphatic valve, atherosclerosis and wound healing, while several cell receptors have been shown to interact with it [reviewed in (White, Baralle, and Muro 2008)]. Conversely, no role or receptor has been described so far for EDB. Furthermore, *in vitro* studies have demonstrated that the Extra Domains influence FN autocrine deposition and remodeling, cell morphology, adhesion and motility, and cell cycle progression (Cseh et al. 2010; Van Obberghen-Schilling et al. 2011). These results alongside the evolutionarily conserved linear and spatial structure of the Extra Domains underline a refined mechanism by which cells deposit FN in a network and respond to it in an autocrine and/or paracrine manner, but this mechanism remains elusive.

In order to understand this mechanism, we implemented a set of biological tools comprised of full-length human recombinant FN proteins containing one, both, or

none of the alternatively spliced domains. So far, most in vitro studies have used gene knock down approaches, and recombinant FN fragments or entire FN constructs labeled with fluorescent proteins or other molecular tags. However, while the generation of recombinant FN fragments has been a valuable method to study FN interactions, they might not reflect the physicochemical properties of the entire native molecule. Furthermore, the presence of extra sequence inserts in the modular structure of FN might result in structural modifications that will interfere with partner binding and assembly. Therefore, we have chosen to analyze full-length FN variants in their native form, rather than introduce voluminous tags or probes that could disrupt their physical properties.

Additionally, we utilized FN-null mouse embryo fibroblasts (MEFs), or MEFs expressing ED-specific FNs. With this toolset, we set out to elucidate how the presence of alternatively spliced (EDA and EDB) domains 1) affects the fibrillar assembly of FN at the surface of assembly-competent cells, and 2) impacts the biochemical, physical, and functional properties of the matrix and affects the behavior of cells growing on it.

3. Results – Article

The presence of alternatively spliced extra domains EDB and or EDA domains of cellular fibronectin confers topographically and functionally distinct features (*in preparation*)

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Running title: Alternatively spliced fibronectin variants

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The authors declare no competing interests.

Financial support for this work was provided by (see acknowledgements)

Abstract

The Extracellular Matrix (ECM) constitutes a structural support and signaling platform for cells to integrate the action of chemical and mechanical cues from the environment. Fibronectin (FN), an early provisional matrix component and obligate partner for the assembly of collagen, lies at the center of this bioregulatory stage during tissue repair and remodeling. FN exists in two forms, plasma FN (pFN) and cellular FN (cFN). cFN differs from pFN by the presence of alternatively spliced FN type III repeats (Extra Domains, EDB and EDA) that flank the cell-binding RGD sequence. Direct functional comparison of full length cFN variants in a defined cellular context has been difficult to achieve. Here we generated a cFN toolset to analyze the impact of the Extra Domains on FN assembly and functions in FN-null fibroblasts expressing the variants, or presented with the purified proteins. Moreover, robust computational tools were developed to classify and characterize cFN network features. Our results show that the presence of Extra Domains differentially affects the fibrillar architecture of the matrix and the induction of both early and late signaling events impacting fibroblast proliferation, energetics and contractility. The different biological readouts were affected to varying extents by the cFN isoforms suggesting that FN Extra Domains are involved in fine-tuning the extent of several processes that enable tissue repair, fibrosis, and tumor progression.

Keywords: fibronectin, oncofetal isoforms, recombinant proteins, fibronectin fibrillogenesis, Extra Domain B, Extra Domain A

Introduction

The extracellular matrix (ECM) is a cell-derived scaffold, composed of more than a thousand macromolecules (R. O. Hynes and Naba 2012), providing mechanical support to the underlying cells and promoting tissue integrity. Furthermore, the composition and the physical state of the ECM affect cell physiology by modulating cell adhesion, motility and morphology, cell proliferation, survival and differentiation [reviewed in (Theocharis et al. 2016; Frantz, Stewart, and Weaver 2010; Richard O. Hynes 2009), thus acting as a multifunctional platform for the transmission of biomechanical and biochemical cues.

Fibronectin (FN), a major component of the ECM, is found in the epicenter of this platform. Abundantly present in almost every tissue in the human body, FN is assembled by cell-driven forces into a fibrillar array that provides an obligate scaffold for the deposition of other matrix proteins, and binding sites for bioavailability regulation of soluble factors in physiological and pathological conditions. FN is a large glycoprotein composed of two similar, but not identical, subunits, encoded by a single gene, and linked by two disulfide bonds at their C-terminal ends. Each subunit (240 – 270 kDa) has a highly repetitive modular structure, composed of FN type I, FN type II and FN type III repeats, giving FN the ability to acquire compact and stretched conformations, and to partner with a large variety of ECM components and cell receptors exerting its various roles in health and disease.

Early knockout studies in the mouse stressed the importance of FN in the developing organism, since FN-null mice die during gestation (before E10.5) due to severe defects in neural tube formation and vascular development (George et al. 1993). Similar results were obtained when a mutant tripeptide sequence (RGE) replaced the wild-type cell binding site (RGD) at the central region of the molecule (Takahashi et al. 2007) which is recognized and bound by integrin $\alpha 5\beta 1$, the principal FN binding integrin.

Binding of $\alpha 5\beta 1$ to FN at the cell membrane leads to the recruitment of cytoplasmic cytoskeleton binding partners. Acto-myosin-generated force applied to FN leads to partial unfolding of the molecule and exposure of cryptic self-association sites that support higher-order assembly (V. Vogel 2006). Thus, modulation of intracellular signaling systems that regulate intrinsic forces, such as the RhoA pathway, modifies matrix assembly. In addition to integrins, members of the syndecan family of FN-binding adhesion co-receptors, have been reported to support FN assembly by mechanisms that are not fully understood (Singh,

Carraher, and Schwarzbauer 2010). Outside cells, matricellular components that interact with FN or integrins, such as decorin or galectins have been reported to enhance FN matrix deposition. Others, including hyaluronan (HA), a multifunctional glycosaminoglycan (GAG) in the pericellular matrix interferes with FN assembly. Interestingly, HA can bind to cFN but not to pFN (Laterra and Culp 1982).

FN is normally found in two distinct forms: plasma FN (pFN) is produced by the liver and circulates in the blood plasma at a high concentration (400 μ g/ml), while cellular FN (cFN) is expressed locally by specialized cells, such as fibroblasts, endothelial cells, chondrocytes, macrophages, and platelets (Richard O Hynes 1990). cFN differs from pFN in the presence of alternatively spliced regions termed Extra Domain B (EDB or EIIIB) and Extra Domain A (EDA or EIIB). Each alternatively spliced Extra Domain is generated from a single exon and gives rise to a FNIII repeat, the expression of which is tightly regulated and limited to embryonic development, wound healing (Ffrench-Constant 1989), and tumor-transformed cells (P. Castellani 1986). Interestingly, EDB and EDA flank the cell binding (RGD) and synergy sites (PHSRN) that reside on FNIII₁₀ and FNIII₉ respectively. The term “oncofetal” was coined in order to describe FN isoforms harboring either of the Extra Domains, that are normally absent in adult tissues.

In vivo studies have demonstrated that mice lacking either Extra Domain are viable and fertile (Muro et al. 2003; Fukuda et al. 2002) but combined deletion of both leads in embryonic lethality (Astrof, Crowley, and Hynes 2007). EDA-targeted deletion and EDA-constitutive inclusion studies have revealed roles for this FNIII repeat in lymphatic valve morphogenesis, atherosclerosis, tissue injury, inflammation, and lifespan, while several receptors have been identified that bind EDA, such as integrins α 9 β 1, α 4 β 1, α 7 β 1, and TLR4 [reviewed in (White, Baralle, and Muro 2008)]. Much less is known about EDB, for which no receptor has so far been identified. Rather, its presence has been demonstrated to induce conformational changes regulating the accessibility to adjacent cryptic sites (Ventura et al. 2010), and facilitating RGD presentation to α 5 β 1 integrins favoring integrin clustering and downstream signaling (Schiefner, Gebauer, and Skerra 2012). Both Extra Domains are considered as markers of angiogenesis (Glukhova et al. 1989; Patrizia Castellani et al. 1994), and are used to evaluate the level of angiogenic process in neoplastic tissue.

In terms of in vitro FN assembly, EDB or EDA containing FNs were shown to be more efficiently integrated in pre-existing extracellular matrices (Guan, Trevithick, and Hynes 1990), concomitant with a more recent study demonstrating that fibroblasts isolated from EDB-lacking mice exhibit reduced FN assembly in vitro (Fukuda et al. 2002). These data suggest that while the Extra Domains are not necessary for FN fibrillogenesis and assembly, they may impact assembly efficiency, through a so far unknown mechanism.

In addition to cellular receptors and key ECM components, FN can specifically interact with matrix cross-linking proteins and remodeling enzymes, and serve as repository for positively charged growth factors [(e.g. FGF, VEGF, TGF- β), reviewed in (Sawicka et al. 2015)] that trigger pro-fibrotic signaling in adjacent cells.

TGF- β , in particular, is a potent cytokine involved in many cellular processes in embryonic and adult tissues, the action and bioavailability of which are regulated, at least in part, by the ECM. Latent TGF- β is bound to the ECM through latent TGF- β binding proteins [LTBPs, (Rifkin 2005)]. Latency is abolished via ECM-integrin-mediated processes [reviewed in (J. S. Munger and Sheppard 2011)] thus releasing the active factor, which is free to bind TGF β Rs and activate signaling.

Several lines of evidence point towards a highly regulated interplay between ECM and TGF- β signaling [reviewed in (Robertson and Rifkin 2016)]. Among others, TGF- β controls FN expression itself (Igotz and Massague 1986), as well as FN splicing, influencing the inclusion of Extra Domains B and A (Balza et al. 1988). Furthermore, TGF- β can be activated by matrix straining and stiffening. Whether or how the presence of the Extra Domains is involved in ECM-mediated TGF- β activation and signaling is currently under investigation.

In order to study the role of the Extra Domains in FN deposition and how cells respond to EDA- and/or EDB-containing FN networks, we implemented the use of a toolset comprised of recombinant FN proteins, as well as assembly-competent and assembly-incompetent cell lines. More specifically, in order to analyze FN variants in their native form, we generated full-length human recombinant FN variants containing one, both, or none of the alternatively spliced Extra Domains. We chose not to utilize FN fragments, as they are unable to form inter- and intra-molecular interactions, thus not reflecting the intrinsic physicochemical and conformational properties of the entire molecule. In addition, given the complexity of the protein, we avoided the inclusion of molecular tags and probes that might disrupt molecule

flexibility and/or naturally occurring interactions that may interfere with assembly and function.

These constructs were used in conjunction with assembly-incompetent cells (HEK293), in which FN expression is not detected, to purify ED-specific FN variants for functional analyses. Furthermore, different assembly-competent cell lines were established from a *Fn1 fl/fl* mouse embryo fibroblast (MEF) population. MEFs represent a model of choice for investigations of FN binding, integrin signaling, adhesion dynamics, and cell-driven mechanisms of FN assembly.

Using this complete toolset, we set out to elucidate how the presence of alternatively spliced (EDA and EDB) domains 1) affects the fibrillar assembly of FN at the surface of assembly-competent cells, and 2) impacts the biochemical, physical, and functional properties of the matrix and affects the behavior of cells growing on it.

Materials and Methods

Materials

Human plasma FN was from BD Biosciences (Bedford, MA, USA). All other chemicals and reagents were purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise stated.

Cell Culture

All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing L-Glutamine (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% Fetal Calf Serum (Biowest, Nuaille, France). Cells were cultured at 37 °C in a humidified incubator with 5% CO₂. For experiments, FN was depleted from FCS using gelatin sepharose-4B columns (GE Healthcare, Uppsala, Sweden), and culture medium was supplemented with Penicillin-Streptomycin 100U/ml. Absence of *Mycoplasma sp.* contamination was routinely verified by PCR as described elsewhere (F. Kong et al. 2001).

Antibodies

Primary antibodies are indicated in **Supplemental Table 1**. Secondary antibodies coupled to horseradish peroxidase were from Jackson ImmunoResearch Labs (West Grove, PA). Fluorescently labeled (Alexa Fluor 488, 564 and 647-conjugated) secondary antibodies were purchased from Thermo Fisher Scientific.

Lentiviral vector construction and production of lentiviral particles

Alternatively spliced FN variants (containing EDA and/or EDB, or no extra domains) with variable region V-89 (also called IIICS) were expressed in the third generation lentiviral vector 2K7 (Suter et al. 2006) under the elongation factor 1 alpha promoter (EF-1 α) in HEK293 cells and *Fn1*^{-/-} MEFs.

Full-length cellular FN1 cDNA was amplified using primers: CACCTCTCT CCCCACCGTCTCAACA (forward) and GATCTTGGCAGAGAGACATGC (reverse) in a two-step RT-PCR with total RNA isolated from primary human endothelial cells (HUVECs). Next, FN1 cDNA was cloned with the pENTR™/D-TOPO® Cloning Kit (Applied Biosystems, Foster City, CA, USA). EDB or EDA domains were inserted or deleted using site-directed mutagenesis to obtain four different constructs

(pENTR-FN) corresponding to the following FN variants: FN/EDB-EDA- (FN B-A-), FN/EDB+EDA- (FN B+A-), FN/EDB-EDA+ (FN B-A+), and FN/EDB+EDA+ (FN B+A+). When verified by sequencing, cDNA of each variant was inserted in the 2K7 lentiviral expression vector by recombination between appropriate sites in pENTR-EF-1 α (L4-R1), pENTR-FN variant (L1-L2) and 2K7-BSD vector with a blasticidine resistance gene. Recombination reaction was performed with LR plus clonase (Applied Biosystems, Foster City, CA, USA).

Lentiviral particles were produced by transfecting the HEK293FT cell line (Life Technologies, Saint Aubin, France) with a mixture composed of a 2K7-FN variant construct (6 μ g) and the virus packaging vectors [6.74 μ g of p8.92 (VSVG), and 6.74 μ g of p8.93 (Gag, Pol)] using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Virus-containing supernatants were collected 48 h after transfection.

Purification of FN variants

To generate FN-expressing clones for purification of recombinant variants from conditioned medium, HEK293 cells were transduced with virus-containing supernatants and selected with 7.5 μ g/ml blasticidine (Invivogen, San Diego, CA). Blasticidine-resistant cells were cloned by limiting dilution with a flow cytometer (FACS Aria, BD Biosciences, San Jose, CA) and FN-expressing clones were selected for variant purification based on the level of FN secretion, as assessed by the Human Fibronectin ELISA kit (Abcam, Cambridge, MA).

FN variant purification was performed according to a modified protocol (Akiyama 2013). Briefly, HEK293 cells expressing FN variants were cultured at confluence in basal culture medium for 2-3 days. Conditioned medium was subsequently collected and centrifuged in the presence of 0.2 mM PMSF. Ten ml of Gelatin-Sepharose[®] 4B (GE Healthcare) were added per 500 ml of supernatant prior to overnight incubation at 4 °C with slight agitation. Eluted FN fractions corresponding to the highest absorbance (A_{280}) were pooled, and FN was subsequently dialyzed against CAPS-saline buffer pH 11.0. FN aliquots were flash frozen and stored at -80 °C. The purity and concentration of FN variants were verified using Coomassie Blue gel staining, silver stain, and the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) according to manufacturer's instructions. To assess the presence of insoluble aggregates, glass coverslips were coated with 10 μ g/ml of FN variants at 37 °C for 1 h then fixed, blocked, and stained for immunofluorescence analysis with an anti-FN antibody.

Knockout of FN in FN floxed mouse embryo fibroblasts and re-expression of FN variants

Fn1 fl/fl MEFs were kindly provided by Dr. Reinhard Fässler [Max Plank Institute, Martinsried, Germany (T. Sakai et al. 2001)]. Cre recombination was performed by infecting cells with a self-excising Cre retrovirus [pMMP-RV-Cre-GFP (Silver and Livingston 2001)] and transduced cells were selected with 6.5 µg/ml puromycin. Clones were obtained by limiting dilution with a flow cytometer and analyzed for FN expression and gene deletion by immunofluorescence staining and PCR analysis, respectively.

MEFs expressing variant-specific FN were generated by infection of *Fn1 -/-* Clone D MEFs with viral supernatants, as described above. Transduced cells were selected in the presence of 7.5 µg/mL blasticidine + 6.5 µg/ml puromycin and cloned by limiting dilution with a flow cytometer. Stable FN variant-expressing clones were selected for analysis based on FN expression levels, as assessed by immunofluorescence staining.

RNA isolation, reverse transcription, and qRT-PCR

Cells were lysed with Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA samples were quantified using a small-volume spectrophotometer (DeNovix DS-11 series, Wilmington, DE) and 1 µg of RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The resulting cDNA was diluted tenfold and amplified in a StepOne Plus thermocycler (Thermo Fisher Scientific) using gene-specific primers and PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific). Standard cycling conditions were used. Results were analyzed according to the $\Delta\Delta C_t$ method, using *Tbp* as normalizing gene, and expressed as fold expression or change compared to respective control. Primer sequences are indicated in **Supplemental Table 2**.

Western blot

Whole cell lysates and conditioned media were collected under non-reducing or reducing conditions (5% β -mercaptoethanol), separated by gel electrophoresis and transferred onto Immobilon P membranes (Millipore, Bedford, MA). Membranes were blocked with 3% skimmed milk in TBS (50 mM Tris-HCl pH 7,4; 150 mM NaCl) before protein immunodetection with the indicated antibodies (**Supplemental Table 1**). Protein bands were visualized with

horseradish peroxidase (HRP)-conjugated secondary antibodies followed by enhanced chemiluminescence (Bio-Rad, Hercules, CA) in a Fusion Fx7 Advanced system (Vilber Lourmat, Eberhardzell, Germany).

Immunofluorescence staining and microscopy

For immunofluorescence analyses, cells or decellularized matrices were fixed in 3% paraformaldehyde/3% sucrose and permeabilised with 0.2% Triton X-100. The dilutions of the primary and secondary antibodies are shown in **Supplemental Table 1**. Nuclei were detected with Hoechst 33342 (Thermo Fisher Scientific). After staining, the coverslips were mounted in ProLong[®] Gold antifade reagent (Thermo Fischer Scientific). Widefield fluorescence was observed through 40X/1.3 oil objectives on a Zeiss Axiovert 200M microscope equipped with a CoolSnap HQ CCD. Image acquisition was performed using the MetaMorph Imaging System. Confocal imaging was performed on a Zeiss LSM710 confocal system using 10X/0.45 and 63X/1.4 objectives. Image analysis was performed using Fiji (Schindelin et al. 2012).

Proliferation rate and cell doubling time

Fn1 ^{-/-} MEFs were plated in triplicate at a density of 2.5X10⁴ cells/well in 24-well plates. Cells were stimulated after 3-4 h with 15 µg/ml of recombinant FN variants, or vehicle buffer (CAPS-saline). At the indicated times, cells were trypsinized, resuspended in culture medium and enumerated with a Malassez hemacytometer (Thermo Fisher Scientific). Cell doubling time was calculated by utilizing the reverse equation and the “Goal-Seek” function in MS Excel.

Internal pH measurement

Fn1 ^{-/-} MEFs were plated in duplicates in 24-well plates (5X10⁴ cells/well). Cells were stimulated with 15 µg/ml of recombinant FN variants, or vehicle buffer (CAPS-saline) for 48 h. Internal pH (pHi) measurement was performed as described (Cophignon et al. 2017). Briefly, cells were incubated with 1 µM BCECF-AM (Thermo Fisher Scientific) for 1 h, rinsed with recovery solution (120 mM choline chloride, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 15 mM Hepes), and fluorescence intensity was measured using a multi-well plate spectrophotometer (BioTek Synergy 4, Winooski, VT). Data was collected with the integrated software. Recovery solution was removed, and nigericin solutions were added (140 mM KCl,

1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 5 μM nigericin at pH values ranging from 6.5 to 8.0 in the presence of 20 mM Hepes).

Mitochondrial metabolism

For metabolic analysis, cells were seeded in 24 multi-well plates (Seahorse Bioscience, Billerica, MA) and stimulated with 15 μg/ml of recombinant FN variants or vehicle buffer for 48 h. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience) and Seahorse basal media supplemented with 2 mM glutamine. Maximal OCR was determined by using FCCP (1 μM), and rotenone and antimycin-A (2 μM each) were used to inhibit Complex I- and Complex III-dependent respiration, respectively. All parameters were calculated as described previously (Brand and Nicholls 2011).

Collagen-based gel contraction assay

Collagen contraction assay was performed as described elsewhere (Su and Chen 2015) with slight modifications. Briefly, *MEF Fn1* ^{-/-} cells were seeded (2.5X10⁵ cells/ml) in a mixture of cell culture medium, FN variant (or vehicle control), 1 mg/ml rat tail collagen I (BD Biosciences, Bedford, MA), and the appropriate volume of NaOH 2.5 N. Mixtures were cast into 12-well plates, and culture medium containing 15 μg/ml of FN variant was added on top of the lattices 30 min later. Lattices were detached from the well walls with a sterile spatula, and were incubated for 24 h prior to scanning the plates and quantifying lattice surface with Fiji.

Detection of TGF-β1 in FN preparations

Detection of TGF-β1 in the purified FN preparations was performed with the Quantikine[®] ELISA Human TGF-β1 Immunoassay Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

Generation of MEF derived matrices

MEF derived matrices were generated as described in (Kaukonen et al. 2016). Briefly, coverslips were coated with 0.2% gelatin for 60 min at 37 °C, followed by crosslinking with 1% glutaraldehyde for 30 min at room temperature. Crosslinker was quenched with 1 M glycine

for 20 min and gelatin-coated coverslips were incubated with medium before seeding 2×10^5 cells. Ascorbic acid (50 $\mu\text{g}/\text{ml}$) and FN variants (15 $\mu\text{g}/\text{ml}$) were added the next day. On day 3 medium was changed and on day 7 matrices were decellularized in 20 mM NH_4OH , 0.5% Triton X-100 in PBS followed by 10 μM DNase I treatment (Roche Diagnostics GmbH, Penzberg, Germany).

Computational analysis of matrix topology

Image acquisition for numerical characterization of FN variant fibers and classification

Confocal images 3128×3128 pixels with a lateral resolution of 0.27 $\mu\text{m}/\text{pixel}$ were acquired with a Zeiss LSM710 confocal system 10X/0.45 with the pinhole diameter set to its maximal value. For each FN variant, 70 images corresponding to a representative region of 512×512 pixels were selected for feature extraction and classification. The set of 280 gray-scale images was classified with the GoogLeNet (Szegedy et al. 2015) pretrained Convolutional Neural Net (CNN) architecture using the MATLAB Deep Learning Toolbox and a 22-layer deep network trained on more than 1 million images for classification into 1000 object categories. A set of 196 images was used for the training of the algorithm, and the remaining 84 for testing it. The training image set was presented to the algorithm 25 times (epochs) as described (Ruder 2017), in order to improve classification accuracy.

Definition of Gabor kernels

Fibrillar structures were detected and enhanced with Gabor filters (Petkov 1995), commonly employed in image processing for the detection of structures with different frequencies, and certain directionalities. A set of Gabor kernels was defined, characterized by the formula $\exp\left(-\frac{1}{2}v^t \Sigma_{\theta_i}^{-1} v\right) \cos\left(\frac{2\pi x \theta_i}{\lambda_j} + \varphi\right)$, where $v = (x, y)^t$. The exponential term provides the shape of a bivariate Gaussian kernel, and the cosine function describes its oscillations in space, while v is the 2D coordinate vector, indicating pixel localization in a bi-dimensional Cartesian coordinate system.

Fiber detection computation and morphological skeletonization

Fiber orientation was represented by θ_i with values within the interval $[0, \pi]$, with a stepsize of $\frac{\pi}{20}$. The covariance matrix of the bivariate Gaussian function rotated with θ_i is related to the

anisotropy of the kernel spatial support and is designated by \sum_{θ_i} . Fiber thickness is represented by λ_j that corresponds to the wavelength (in pixels) of the cosine term, the values of which are equal to $\frac{\lambda_j}{2}$ and vary between 3 and 5 pixels. The thinnest fibers are detected when $\lambda_j = 6$ pixels, medium thickness fibers correspond to $\lambda_j = 8$ pixels, while the thickest are characterized by $\lambda_j = 10$ pixels. For accurate localization of fibers we used phase, φ , set to 0. The pixel intensity of a detected fiber at a specific location corresponded to the Gabor filter with the highest coefficient within the Gabor kernel set. The specific parameters of the best corresponding Gabor kernel for a detected fiber could subsequently be linked to physical properties, such as fiber thickness and local fiber orientation.

Morphological skeletons of the detected fibers were computed using morphological operations provided by the Image Processing Toolbox of MATLAB 2015a. Fiber skeletons were portrayed as graph-based representations in which a set of nodes is linked by edges (connecting segments), using a toolbox (Kollmannsberger et al. 2017) that generates the network graph of a 3D skeleton voxel, that we adapted to the 2D setting. Nodes typically represent intersecting fibers or fiber ends, while edges correspond to the detected fibers connecting the nodes.

Feature extraction and Principal Component Analysis (PCA)

Features related to fiber thickness and connectivity were directly computed using Gabor kernels and graph-specific parameters. More specifically, connectivity was defined as the proportion of degree 1 nodes (those corresponding to fiber ends) relative to the nodes with a degree higher than 2 (corresponding to branching and intersecting points). Fiber thickness kurtosis, was determined by the formula $k = E[(x - \mu)^4]/(\sigma^4)$ where μ is the mean value of x , σ is the standard deviation of x , and $E[s]$ is the expected value of the quantity s . Attributes describing pore regions were computed using the MATLAB tool Regionprops. Pore dimension was measured as the total number of pixels within the area delimited by the skeleton, while pore circularity was determined by the formula $(4 * Area * \pi)/(Perimeter^2)$, the values of which vary between 0 (oval-like) and 1 (perfect circle). For pore size, only pores distributed above the 90th percentile were taken into account, and the mean was calculated. For PCA visualization, each FN network sample (image) was represented in a 5-dimensional space defined by the previously selected features.

Results

Recombinant human FN isoforms

In order to explore the functions of alternatively spliced cellular FN (cFN) variants and the impact of the Extra Domains EDB and EDA on FN matrix assembly, we constructed a set of lentiviral expression vectors harboring the full-length coding sequence of the human *FN1* gene containing one, both or none of these alternatively-spliced FN type III repeats that flank the central cell-binding RGD and synergy sites in the molecule (**Fig. 1A**). All of the constructs contain the alternatively-spliced variable region, more precisely the V89 sequence (Jean E. Schwarzbauer 1991). We chose not to insert additional sequences encoding fluorescent proteins or molecular tags, to avoid modifying the structural conformation of the protein and disrupting inter- and intra-molecular interactions.

Re-expression of FN variants in FN-null MEFs

As recipient cells for FN re-expression studies, *Fn1*^{-/-} MEF clones were generated (**Supplemental Fig. 1**). One clone was selected for transduction with the human FN expression vectors based on its fibroblast morphology, its ability to readily assemble exogenously presented FN and the absence of multinucleated and/or senescent cells. Following transduction, variant-expressing clones were isolated for analysis of FN expression and assembly. A representative clone of each variant is shown in **Fig. 1B-D**. The presence of the expected Extra Domains in the pericellular FN matrix was confirmed by immunofluorescent staining with Extra Domain-specific antibodies (**Supplemental Fig. 2**). Despite similar FN transcript levels (**Fig. 1B**), differences were observed in the amount and organization of the pericellular FN matrix of the variant-expressing clones, at 2 days (**Fig. 1C**) and 7 days of culture (**Supplemental Fig. 3**). At both times assembly of FN containing one or both of the Extra Domains was more efficient than assembly of plasma-like FN, lacking the alternatively-spliced sequences. Western analysis confirmed the increased amounts of cell-associated FN in lysates from MEFs expressing the Extra Domain-containing variants, compared to MEFs expressing plasma-like, B-A- FN. Conversely, more soluble FN was present in cell conditioned medium from FN B-A- MEFs (**Fig. 1D**). The levels of human FN produced and deposited by the MEF

clones 11, 1 and 2 (B+A-,B-A+ and B+A+, respectively) were similar to that of the endogenous protein in control MEFs (*Fn1 fl/fl* MEFs) containing one or both Extra Domains (**Fig. 1D**).

We have previously demonstrated that autocrine FN expression is tightly coupled to its fibrillar assembly whereby differences in FN expression levels could have a considerable effect on assembly of the protein (Cseh et al. 2010). Therefore, it was essential to determine whether the observed increase in assembly of FN containing the Extra Domains, was due to the presence of the alternatively spliced sequences, or whether it simply reflected quantitative differences in FN production by cells. To this end, additional FN variant-expressing clones were examined for FN expression and assembly. Comparison of three clones each expressing either FN B-A- or B+A- variants are depicted in **Fig. 1E-G**. Indeed, assembled FN is more abundant in clones with higher mRNA expression levels (B-A- clone 210 and B+A- clone 11) indicating that FN assembly is extremely sensitive to autocrine expression levels. Further, as exemplified by B+A- clones gA2 and gA6 that express equivalent amounts of the same mRNA, marked differences are observed in their ability to deposit a FN matrix.

We conclude from the above re-expression studies that FN containing either one or both of the Extra Domains is more efficiently assembled by cells than FN lacking Extra Domains. Importantly, although a homogeneous population of cells is required for functional analysis of FN and comparison of the FN variants (i.e. cell clones *versus* a heterogeneous population of transduced cells), varying FN expression levels and clonal differences across MEFs expressing a single variant seriously impair mechanistic studies of FN assembly and the functions of Extra Domain in FN re-expressing MEFs.

FN matrix generated by presentation of recombinant variants to FN-deficient MEFs

To circumvent the problems associated with dissimilar FN expression levels by recipient MEFs and the inherent phenotypic variability of different clones, subsequent studies were performed in an identical cellular context. Hence, as schematized in **Fig. 2A**, FN variants were purified and provided in soluble form to a single clone of *Fn1*^{-/-} MEFs that readily assemble soluble FN into an insoluble extracellular matrix.

For purification, the recombinant isoforms were expressed in HEK293 cells. These cells are well suited for this purpose as expression of endogenous FN is undetectable, transduced cells are assembly-*incompetent* and they secrete high levels of the exogenous variants into their

culture medium. FN expression had little or no effect on the epithelial-like morphology of cells (**Fig. 2B**). The purified variants (B-A-, B+A-, B-A+, B+A+) displayed appropriate molecular mass and the ability to form naturally occurring dimers, absolutely required for fibrillogenesis (Jean E Schwarzbauer 1991), as determined by SDS-PAGE under reducing and non-reducing conditions (**Fig. 2C**). Specific inclusion of each Extra Domain was confirmed by western analysis using isoform-specific anti-FN antibodies (**Fig. 2D**). Cellular FN is much less soluble than plasma FN under physiological buffer conditions (Yamada and Olden 1978). Thus the absence of aggregates in the purified FN preparations was routinely verified by immunofluorescent staining of FN variant-coated coverslips (**Supplemental Fig. 4A**), and concentrations of the soluble proteins were determined in each experiment, prior to their addition to cells.

For comparative studies, FN variants were added to the same *Fn1* ^{-/-} MEF clone that was used for FN re-expression studies. Cells were plated at near confluence and allowed to adhere prior to addition of the variants. A concentration of 15 µg/ml was chosen for experiments, following dose response analyses of plasma FN assembly by cells (**Supplemental Fig. 4B**). First, we examined the ability of cells to assemble the recombinant proteins by western blotting of cell-associated FN and immunofluorescence microscopy. Assembly could be detected as early as 2 h after addition to the culture medium (**Fig. 2E,F**). Similar to the above re-expression studies, FN containing the Extra Domains was more readily assembled at early times of exposure to cells. After 2 days, all of the recombinant FN variants were present in a dense meshwork around cells.

Interestingly, variant-specific differences were observed in the organization of the matrix assembled by FN-deficient MEFs presented with the purified FN proteins. For optimal comparison of the matrices, MEF cultures were denuded of cells after 7 days and MEF-derived matrices (MDM) were stained for FN, or other matrix proteins. Consistent with the role of FN as an essential template for other matrix components, the distinct architecture of FN fibrils impacted higher order matrix organization. The distinct patterning of collagen VI and Tenascin C can be seen in the mature FN variant-specific matrices depicted in **Fig. 3**. Differences in the thickness of the matrices containing the four FN variants were also noted. The average thickness of the MDMs ranged between 6.3 µm (for B-A+) and 9.0 µm (for B+A-), with MDMs of variants containing no, or both Extra Domains of an intermediate thickness of 7.5 µm.

Computational analysis of the FN variant matrices

To explore the specific physical attributes of FN matrices containing the Extra Domains, that appear topographically different by eye, a computational image analysis approach was employed. Mathematical “computer vision” tools were developed to interrogate whether the architectures of the variant-specific matrices (assembled by the same cells) are distinguishable, and if so, whether they can be described, quantified, modelled, or attributed to Extra Domain-specific biological functions.

Automatic classification of Images of Extra-Domain-specific FN Networks

To optimize the quality of the images for processing, high-resolution images of the decellularized matrices were acquired with a confocal microscope (**Fig. 4A**). Images were subsequently classified using a pre-trained algorithm, as described in Methods. The results of this classification are presented in **Fig. 4B** as a confusion matrix, which allows the visualization (on the diagonal) of the percentage of images that were correctly classified as belonging to the expected FN variant class. High intra-variant accuracy scores were observed (general accuracy, 83.3%), indicating that the information contained in the images is sufficient for the algorithm to recognize topological differences inherent to the four matrix types. Additionally, machine based classification outperformed the ability of a blinded biologist to correctly classify the same image set (biologist accuracy, 61.42%). Topological properties of fiber arrangements containing only EDA (B-A+) are the most distinguishable (90.5 %), followed by the B-A- fibers (85.7%), devoid of Extra Domains. FN networks containing only EDB (B+A-) are less distinguishable, while B+A+ fibers are the least distinguishable in automatic classification.

Geometrical Properties and Feature Extraction

As a first step in the characterization of the geometrical properties of FN fiber networks, fibrillar structures were detected using the workflow shown in **Fig. 4C**. After generating a high-quality dataset of images (1), we defined a set of Gabor kernels, or "dictionary of elementary fibers" (2) that incorporates a set of parameters (described in Methods) that capture the entire range of different fibers. After fiber detection (3), the morphological skeleton, or shape descriptor, of the detected fibers was computed (4) for feature extraction and analysis (5). **Fig. 4D** displays the FN variant-specific graphs, or sets of edges connected by nodes, associated to the morphological skeletons of the fibers. The advantage of using a graph-based representation is that apart from Gabor-specific descriptive parameters, we are able to

compute additional features that, in turn, can help us characterize the geometrical properties of the FN fibers.

PCA analysis of the fiber features on the first two components

After establishing a faithful representation of the fibers, a set of features was selected to characterize fiber geometry and to perform principal component analysis (PCA). The PCA method was used to explore the relatedness between the different FN variants with respect to various physical attributes, or features, including i) connectivity, ii) fiber thickness, iii) fiber heterogeneity, iv) pore shape v) and pore size distribution. In order to describe the connectivity of the fibers, we computed the relative abundance of nodes of different degrees. The degree of every node was determined by the number of edges connected to the node. The variant-specific connectivity distributions are shown in **Supplemental Fig. 5A**. Interestingly, B-A- fibers are characterized by a higher abundance of fiber ends, delineating a low level of connectivity, compared to the other variants, especially to B-A+. These results reveal that the absence of Extra Domains leads to a less branched FN fiber arrangement.

Next, we considered fiber thickness by computing the proportion of thin to thick fibers. As shown in **Supplemental Fig. 5B**, B-A- fibers display low proportion of thin fibers, hence characterized by the presence of medium and thick fibers, while the opposite is observed for B-A+. In order to analyze fiber thickness heterogeneity, or fiber diversity, we implemented the fiber thickness kurtosis, a parameter that indicates how outlier-prone the fiber thickness distribution is relative to a normal distribution with identical variance. In terms of fiber thickness, B-A+ values are distributed around the mean, suggesting a high homogeneity in fiber thickness, compared to B-A- fibers which are highly heterogeneous (**Supplemental Fig. 5C**).

Pore shape was measured through a circularity parameter and the average pore size. Circularity measures pore anisotropy allowing us to distinguish circular and oval-like pores. **Supplemental Fig. 5D** shows that B-A- FN arrangements are characterized by a high number of oval pores, while pores in B-A+ FN networks are predominantly circular. The same pattern is observed in terms of pore size. Large pore sizes are found within B-A- FN networks, while smaller pore size is observed in B-A+ FN networks (**Supplemental Fig. 5E**).

The PCA (**Fig. 4E**) was performed with the aforementioned features, by adopting the representation provided by the first two principal components. The plot illustrates both the samples (images) projected in a bi-dimensional space, and the five features represented by vectors, the direction and length of which indicate the contribution of each feature to the two principal components. Generally, the samples belonging to FN B-A- and FN B-A+ are concentrated in non-overlapping areas, displaying the distinguishability of these two variant-specific FN networks through the chosen features.

Altogether, these analyses demonstrate that B-A+ FN matrices feature highly branched, homogeneous, thin fibers that form small pores. In contrast, B-A- FN forms thicker, unbranched networks with larger more elongated pores. Interestingly, the presence of EDB results in matrices (either B+A-, or B+A+) characterized by a mixture of the attributes seen in B-A- and B-A+.

FN-induced collagen remodeling and TGF- β signaling

FN assembly is intimately related to the organization of the cellular actomyosin contractile apparatus (Q. Zhang et al. 1994; Zhong et al. 1998; Fernandez-Sauze et al. 2009). When fibroblasts are embedded into hydrated collagen lattices, the lattices contract (Bell, Ivarsson, and Merrill 1979). This phenomenon, extensively used to study how distinct factors can contribute to matrix contraction, reflects cellular processes that take place during physiological procedures such as tissue remodeling and repair (Ngo et al. 2006). It was previously shown that in the absence of serum FN, fibroblasts can readily interact with collagen lattices but they are unable to cause contraction, and this phenotype can be rescued with exogenously added FN (Gillery, Maquart, and Borel 1986). In order to uncover a potential role of the Extra Domains in FN-induced collagen lattice contraction, we embedded *Fn1* $-/-$ MEFs in collagen lattices containing 15 $\mu\text{g}/\text{ml}$ of the FN variants and determined the extent of contraction after 24 h. Whereas the presence of FN resulted in pronounced reduction in the lattice surface, only slight differences were observed among the four variants, suggesting that presence of the Extra Domains had little impact on FN-induced collagen gel contraction (**Fig. 5A**).

The action of TGF- β , a major fibrinogenic cytokine, has been implicated in cell-mediated collagen lattice contraction since anti-TGF- β neutralizing antibodies were found to suppress

this effect (Levi-Schaffer et al. 1999). As the addition of FN alone was able to induce lattice contraction by *Fn1* ^{-/-} MEFs, we hypothesized that this effect might be due to activation of latent TGF- β in the local milieu. To test this, we analyzed the ability of FN variants to stimulate TGF- β signaling in *Fn1* ^{-/-} MEFs by monitoring the regulation of TGF- β response genes, PAI1, CTGF and TGFBI (Leask and Abraham 2003; Kutz et al. 2001; Ween, Oehler, and Ricciardelli 2012), and SMAD phosphorylation (**Fig 5**). The absence of TGF- β in the FN preparations was confirmed by ELISA (**Fig 5B**). The time course of PAI1, CTGF and TGFBI mRNA expression following the addition of 15 μ g/ml FN to cells is shown in **Fig. 5C**. Indeed, FN stimulated a rapid increase in PAI1 and CTGF mRNA expression, which peaked at 2 h and returned to near-basal levels by 6 h, strongly suggesting that FN triggers TGF- β signaling in *Fn1* ^{-/-} MEFs. Accordingly, FN stimulated SMAD2 phosphorylation in these cells (**Fig. 5D**). For these effects, the presence of FN Extra Domains had a significant impact on the magnitude of stimulation. Thus, FN B+A+ was found to be the most potent agonist of TGF- β signaling in MEFs (**Fig. 5B, C**) whereas FN B-A+ induced the weakest response. Pretreatment of cells with a TGF β RI antagonist precluded FN-induced SMAD2 phosphorylation, indicating that it is mediated by TGF β RI (**Fig. 5E**) and most likely involves FN-dependent activation of latent TGF- β .

TGFBI (TGF- β -induced) mRNA expression was unaffected at early times after FN addition. Rather, significant downregulation was observed after 48h (**Fig. 5C**), reflecting a possible FN-dependent retro-control of TGF- β pathway activation.

Effect of FN variants on cell growth and survival

Extra Domains EDB and EDA in cellular FN have been reported to confer distinct growth promoting properties on the molecule, recombinant FN (Manabe, Oh-e, and Sekiguchi 1999; Fukuda et al. 2002). Therefore, we examined the proliferative effects of the recombinant full-length cellular FN variants on *Fn1* ^{-/-} MEFs. Differences in both growth rates and saturation densities were observed in cells presented with the four variants (**Fig. 6A**). Surprisingly, FN addition had little effect on early exponential growth, with the exception of FN B+A- which increased the doubling time of cells, compared to control (no FN) or other conditions (**Fig. 6B**). Interestingly, cell numbers plateaued by 4 days of culture in absence of FN, or in presence of FN lacking the Extra Domains (B-A-), whereas the numbers of harvested cells continued to increase in presence of FN containing one or both Extra Domains. This variant-dependent

difference in saturation density of confluent monolayers at day 5 is apparent in the phase contrast images of **Fig. 6C**, which depict the densely-packed and aligned B+A+ treated cells to which post-mitotic cells could adhere and survive (**Fig. 6C**, right), and the less-elongated arrangement of FN B-A- treated cells covered with rounded cells unable to integrate into the monolayer (**Fig. 6C**, left). Thus, the presence of a pericellular FN matrix containing the Extra Domains can provide a survival advantage for fibroblasts in crowded conditions (i.e. fibrotic state).

In sparse cultures, we observed variant-specific differences in the cohesive properties of cells (**Fig. 6D**). One day after plating, when intercellular adhesion is virtually absent in control *Fn1* -/- MEFs, the addition of FN promoted cell-cell adhesion and the formation of multicellular arrangements. Intercellular cohesion was highest in cultures treated with the B+A+ variant and lowest in B-A- treated cells, consistent with the least efficient deposit of this variant in the FN matrix (**Fig. 2E, F**).

Metabolic effects of FN

While performing growth curve experiments, we observed that the color of the culture medium became more acidic in cells exposed to FN, as compared to control cells (**Fig. 6E**). The effect became visible after 2 days and the increment was most pronounced in cells presented with FN B+A-. Cell proliferation was slowest in this condition (**Fig. 6B**), thus excluding the possibility that the lower pH resulted from more rapid cell proliferation. Moreover, addition of the FN B+A- variant significantly decreased internal pH (**Fig. 6F**). Extracellular acidification is a proxy for lactate production suggesting that FN-induced acidification of the intra- and extra-cellular space could be due to a shift in the metabolic state of the cells from an oxidative phosphorylation to glycolysis. To assess this, we measured glucose uptake, lactate production extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) in control and FN variant-treated cells. Glucose transport and lactate extrusion measurements after 48 h showed a tendency for FN-induced stimulation although statistical significance was not reached (**Supplemental Fig. 6A, B**). Expression of glycolytic enzymes were significantly increased (**Supplemental Fig. 6C, D**), yet no variant-dependent differences were detected.

Next, we determined glycolysis and mitochondrial respiration simultaneously after 72 h of FN treatment (**Fig. 6G**). Compared to control cells, FN B+A- and FN B-A+-treated cells displayed a higher ECAR both in absence (not shown) and presence of glucose (**Fig. 6H**), and this in line

with media color changes. Surprisingly, ECAR in response to glucose, expressed as % of basal ECAR and reflecting glycolytic response, remained unchanged across conditions, and even slightly decreased in cells treated with B-A+ and B+A+ FN (**Supplemental Fig. 7A**). Cells in presence of B+A+ displayed lower mitochondrial activity (lower basal OCR and lower maximal OCR) although no differences in OCR were observed in cells treated with the other variants (**Supplemental Fig. 7B, C**). Respiration coupled to ATP synthase was similar for all conditions and mitochondria capacity was maximal, even without glucose, evidenced by the same values in basal and maximal OCR, suggesting the use of another substrate for oxidative phosphorylation in these cells (**Supplemental Fig. 7D, E**). From these results, we conclude that exogenous FN does not induce a strong metabolic reprogramming in these cells.

Discussion

We previously performed isoform-specific RNA interference to interrogate the role and functional redundancy of cFN variants in primary endothelial cells (Cseh et al. 2010). The most striking effects were observed following knock down of FN containing the EDB domain, which profoundly impacted cell morphology and migration. Here we have taken a gain-of-function approach to perform a comprehensive study of cellular FN isoforms to determine how the presence of alternatively spliced Extra Domains of FN affects the architecture and function of the ECM. Fibroblasts were selected for our studies because of their advantages as an in vitro model to study cell adhesion, cell cycle regulation, and signal transduction. Additionally, they are ideal for their mesenchymal characteristics, namely their capacity to synthesize and remodel ECM, their involvement in wound healing, inflammation and fibrosis [reviewed in (Yusuf et al. 2013)], and their role in tumor progression [reviewed in (Alkasalias et al. 2018)]. *Fn1*^{-/-} MEFs were used, as it was crucial to perform these analyses in FN-null cells to avoid interference with endogenous FN isoforms of recipient cells. Moreover, these studies required the isolation of clonal populations in which FN variants were homogeneously re-expressed, as FN-deficient fibroblasts can assemble FN produced by neighboring cells. After isolation and extensive characterization of multiple clones for each variant, we concluded that comparison across the variants was more complex than we had imagined. We did observe that the cellular FN variants were assembled by cells more efficiently than their plasma-like FN counterpart

lacking the Extra Domains. However, differences in FN assembly were observed between clones re-expressing the same variant and these differences were linked in part to FN expression levels. Identifying clones that produce the same amounts of FN was complicated by that fact that for a given mRNA level, protein levels were not always the same. Even when levels of the protein were similar by western analysis, cellular localization could vary (cytoplasmic versus matrix-bound) for the same isoform.

These findings demonstrate that caution should be taken when using independent FN-expressing clones for phenotypic comparisons, even when the clones are isolated from the same preparation of mouse embryo fibroblasts (not to mention MEFs from different mice with distinct genetic backgrounds). FN is an exquisitely regulated protein. Both positive and negative controls operate at the levels of transcription, mRNA processing, translation, intracellular trafficking and secretion of the protein (polarized/non-polarized), which is coupled to its assembly. In heterologous expression systems these regulatory mechanisms are circumvented by forced expression, under the control of an exogenous promoter (loss of transcriptional regulation), of the FN cDNA (no splicing) with no mRNA regulatory (e.g. 3' non-coding) sequences for modulation by multiple signaling pathways. Therefore, we turned to analysis of the purified FN variants produced by HEK-293 cells using a single assembly-competent *Fn1*^{-/-} MEF clone for functional readouts.

Considering the modular structure of FN, we chose to generate full-length FN constructs in order to study the protein in its native form, consisting of all the possible binding sites, without disrupting potential interactions or its spatial conformation in solution and in matrix. All constructs were characterized by the presence of the variable region V-89. This IIICS variant has been shown to display a relatively stable expression across different tissues and developmental stages (Oyama et al. 1989), and it has been suggested to facilitate intramolecular interactions to promote fibrillogenesis and cell spreading by utilizing the Hep II domain (Santas et al. 2002). The V-89 sequence also includes the CS1 region which contains the LDV cell binding site (Pagani et al. 1993), recognized by the $\alpha 4\beta 1$ integrin (Mould et al. 1991), but lacks the CS5 region which bears another $\alpha 4\beta 1$ binding site (REDV). This site lies within a highly hydrophobic region, is structurally related to the RGDS cell binding site, and

adds an extra level of complexity in cell binding regulation, adhesion, and migration (M. J. Humphries 1986; M. J. Humphries et al. 1987). All our FN constructs were efficiently secreted by assembly-incompetent cells. However, considerable differences in solubility between our recombinant plasma-like and cellular FN were observed, as previously reported by Yamada using a heterogeneous mixture of cellular FN purified from cultured cells (Olden, Pratt, and Yamada 1979). Given the reduced solubility of cellular FN variants, it was necessary to measure the concentration of each variant prior to every assay to assure experimental reproducibility. The purified isoforms were dimerized as expected, lacked noticeable degradation, and were free of TGF- β , a known contaminant of purified protein preparations (Kaur and Reinhardt 2012; Olsen et al. 2017). Additionally, they were readily harvested by assembly-competent cells and deposited in a pericellular network that gave rise to a mature FN matrix.

cFN re-expression in MEFs

Re-expression of FN variants in a *Fn1* $-/-$ MEF cell line resulted in pronounced differences in FN deposition and FN network organization. More specifically, FN containing either one or both Extra Domains was more efficiently assembled into fibrils, which is consonant with a previous study which demonstrated that Extra Domain-containing FNs were more readily incorporated in pre-existing matrices in vitro (Guan, Trevithick, and Hynes 1990). In this study, however, FN variants differed not only in the inclusion of the Extra Domains, but also in the presence of the Variable region, which is known to confer additional binding sites to FN and play a role in dimerization and secretion (J. E. Schwarzbauer 1989; J. E. Schwarzbauer et al. 1987). It is noteworthy that the most efficient clone in FN assembly was MEF B+A- 11, which also displayed the highest mRNA level, suggesting that transcription levels regulate FN deposition. This is in line with previous results showing the coupling between autocrine FN secretion and matrix assembly (Cseh et al. 2010).

When we established additional clones expressing either FN B-A- or FN B+A, our results were highly variable across clones expressing the same variant. While increased transcription resulted in enhanced FN deposition in the MEF B+A- expressing clone 11, clones gA2 and gA6 secreted variable amounts of FN despite equal transcript levels. Similarly, B-A- expressing MEF

clones 4 and 206 were characterized by similar messenger quantities, yet FN secretion in the medium or assembled into fibrils was considerably different. These results point towards a clonal variability induced by the overexpression approach.

It has previously been reported that overexpression experiments can result in cellular defects triggered by an overload of protein localization processes (Kintaka, Makanae, and Moriya 2016). Furthermore, a series of primary mechanism of cellular defects caused by protein overexpression has been described (Moriya 2015). In the case of a large, dimeric protein like FN, overexpression might require large amounts of cellular resources for translation, folding, localization, or degradation, leading to overloading of these processes. In addition, FN has numerous binding sites, interacting with many partners, and its overexpression might cause promiscuous interactions and aggregation, that might subsequently perturb pathway modulation. These data clearly underlined the need to turn our attention towards a model system that abolishes bias inherent to this approach for direct quantitative comparisons between EDB and EDA functions in FN-re-expressing clones.

ED-dependent FN assembly

By presenting FN variants to the same *Fn1* ^{-/-} MEF clone, we were able to eliminate clonal variations and attribute the various phenotypic differences observed to the FN Extra Domains. Consistent with previous studies, our results show that cFN was more readily harvested by cells compared to the plasma-like FN (B-A-). More specifically, the presence of EDB resulted in enhanced binding of FN by cells and subsequent fibril formation as early as 2 hours after variant addition. A previous study with Extra-Domain-containing FN fragments showed that fragments containing EDB alone or both EDB and EDA enhanced adhesion and spreading in cultured fibroblasts, while sole inclusion of EDA resulted in lower staining of focal adhesions and actin stress fibers (Hashimoto-Uoshima et al. 1997). This study is in agreement with our results, suggesting that EDB-mediated conformational changes enhance presentation of the RGD sequence to $\alpha 5\beta 1$. Xia and Culp reported a complementary role of EDA in RGD-mediated binding of FN to cells, demonstrating that a FN-EDA fragment increased adhesion, stress fiber formation and spreading of cells on a mixture of EDA/pFN substrate compared to pFN alone (Xia and Culp 1995), though they did not include EDB in their comparative analysis.

In a study similar to ours, Manabe and colleagues reported that EDA-containing FN was significantly more potent than FN lacking EDA in promoting cell spreading and motility, irrespective of the presence or absence of EDB (Manabe et al. 1997). They showed that the observed effects were mediated by $\alpha 5\beta 1$ and they proposed that an EDA-modulated conformational modification could result in increased exposure of the RGD site. Although we also observed an increased harvesting of soluble FN B-A+ by cells compared to FN B-A-, the contribution of EDB was considerably greater. Synovial cells were reported to adhere more strongly on FN-EDA coated surfaces compared to pFN, and this effect was facilitated via the Hep II domain, since antibody targeting of the Hep II domain or the EDA repeat, and heparitinase treatment reduced adhesion of synovial cells to levels equivalent to pFN (Hino et al. 1996).

Furthermore, it was recently shown that $\alpha v\beta 3$ integrin, which also binds RGD, altered the deposition and structure of FN fibrils via a $\beta 1$ integrin/ROCK-independent mechanism, and that $\beta 3$ -dependent fibronectin assembly facilitates tumor invasion (Attieh et al. 2017) suggesting that an alternative way of matrix assembly might influence matrix-modulated functions (Filla et al. 2019).

Metabolic reprogramming

We observed here that the addition of cellular FN variants to *Fn1* ^{-/-} MEFs led to a decrease in their intra- and extra-cellular pH, as compared to non-treated control cells. The most significant acidification was observed with FN B+A-, whereas cells treated with FN B+A- displayed the slowest exponential growth rate. This result was unexpected since the generation of an acidic environment is a common feature of rapidly proliferating cells during several physiological processes (wound healing) and disease states. Moreover, it is in contrast to reported results that FN containing EDB might provide a proliferative advantage in cells in vitro, since fibroblasts isolated from EDB-null mice exhibited reduced cell growth in culture (Fukuda et al. 2002). Acidification of the extracellular milieu has been reported to alter expression of proteinases (MMPs and cathepsins) impacting matrix architecture, and proangiogenic factors (VEGF-A and IL-8) promoting migratory capacity and metastatic potential [reviewed in (Kato et al. 2013)] and this is in line with the angiogenic role of both EDB- and EDA-FNs (Glukhova et al. 1989; Patrizia Castellani et al. 1994). Those and our data

suggest that autocrine and paracrine EDB-containing FN may be a contributing factor in tumor progression through intertwining paths that involve angiogenesis, matrix remodeling, and cell energy regulation, while EDA takes also part in cell cycle regulation. It is highly plausible that a mixture of EDB- and/or EDA-positive FN fibrils simultaneously induce different cell responses, the net result of which we observe in vitro.

Extracellular acidification, best studied in tumor tissue, occurs through the reprogramming of energy metabolism in tumor cells and has important tumor-promoting consequences and potential for therapeutic targeting [reviewed in (Corbet and Feron 2017)]. More recently, increased glycolysis in the cancer-associated stroma has been proposed to provide high energy metabolites to neighboring cancer cells via the reverse Warburg effect (Pavrides et al. 2009; Yoshida 2015). As fibroblasts are the major matrix-producing cells of epithelial tumors, and cellular FN is a major matrix constituent of the carcinoma-associated fibroblast matrix (Gopal et al. 2017), it is tempting to postulate that FN could actively contribute to acidification of the tumor microenvironment by stimulating Warburg-like glycolytic rates in stromal cells. FN-treatment of MEFs did lead to increased expression of glycolytic enzymes and a tendency for increased glucose uptake and lactate extrusion, under culture conditions in which enhanced acidification was observed. However, we failed to see significant increases in glycolysis (ECAR) or downregulation of mitochondrial respiration (OCR) in these cells, at least when measured in Seahorse XF24 cell culture microplates, suggesting that metabolic reprogramming of MEFs does not occur.

TGF- β signaling modulation by FN variants

Are there links between FN-induced TGF- β signaling and metabolism? Stromal-derived TGF- β has been shown to shift CAFs towards a catabolic metabolism with lactate production, thus linking TGF- β signaling to the Warburg-like metabolism (Guido et al. 2012). Indeed, in our hands, exogenous FN induced both TGF- β signaling and metabolic modifications in our FN-deficient fibroblasts. Nonetheless, variant-specific effects on TGF- β response genes and extracellular acidification were not correlated. Thus, the highest activation of TGF- β signaling was detected in cells treated with FN B+A+ and A-B- whereas extra-/intra-cellular acidification was most pronounced in cells presented with the B+A- variant.

Nevertheless, soluble FN stimulated an increase in SMAD2 phosphorylation and the expression of various TGF β -related genes as early as two hours post-stimulation. These FN-induced effects were inhibited by a TGF β RI inhibitor, suggesting that exogenous FN variants are capable of activating latent TGF- β located in the extracellular milieu, which subsequently binds the TGF- β receptor complex and activates downstream signaling. It has previously been reported that latent TGF- β can be activated by strain generated within the actin-integrin-ECM axis [reviewed in (Hinz 2015; Richard O. Hynes 2009)]. More specifically, matrix-bound LTBP proteins (namely LTBP1 and 3) are covalently attached to the LAP peptide, which is non-covalently attached to the mature TGF- β , but also bound to α v integrins, through their RGD sites. Applying forces to the LLC can induce conformational modifications resulting in the release of active TGF- β .

In our experiments, however, we used MEFs that do not express FN, thus are effectively unable to assemble other matrix proteins, including fibrillin1 and LTBP1 (Klingberg et al. 2018). FN fibrillogenesis was visible as early as 2 hours after FN addition but we did not examine the co-assembly of LTBP. A recent study showed that the presence of EDA in his-tagged FN resulted in increased binding of LTBP1 to FN, and that function blocking of EDA reduced LTBP1 incorporation in fibroblast ECM and TGF- β activation (Klingberg et al. 2018). Our results are partly consonant with this study since TGF- β pathway activation was mild in our cells upon cell stimulation with FN B-A+, but this effect was significantly reinforced when EDB was also present in the FN molecule. The discrepancies might be due to the use of cell populations stably expressing the FN variants, which as we have shown in our study is not optimal for comparing cFN variant-specific events.

Apart from being docked in the ECM via LTBPs, TGF- β can also be found in a latent form on the outer side of the plasma membrane bound through the LAP peptide to GARP (also known as LRRC32). GARP-bound TGF- β can also be activated by integrins α v β 6 (X. Dong et al. 2017) and α v β 8 (Liénart et al. 2018) by exerting traction forces on the LLC, modulating inflammatory and immunoregulatory functions in the vicinity. We have not analyzed the expression of GARP, but it has recently been reported that GARP, as well as β 8 integrin subunit are present in human and mouse mesenchymal cells (fibroblasts) and they play a pivotal role in TGF- β activation and regulation of T-cell and platelet functions, cell proliferation (Carrillo-Galvez et al. 2015), dendritic cell trafficking, inflammation, and fibrosis (Kitamura et al. 2011).

The presence and expression levels of GARP and $\beta 6$ or $\beta 8$ integrin subunits in the MEFs used in our experiments remain to be determined. However, we can suggest a mechanism by which FN Extra Domains might tune the extent of integrin-mediated TGF- β activation whether it be bound to GARP or LTBP. We propose that addition of exogenous FN variants results in $\alpha 5\beta 1$ integrin binding, the affinity of which may depend on the presence of the Extra Domains. At the same time, $\alpha v\beta 3$ can compete with $\alpha 5\beta 1$ for RGD binding. FN-integrin binding induces outside-in signaling, resulting in acto-myosin cytoskeleton contractility that, in turn, induces 1) fibronectin assembly, and 2) inside-out αv integrin traction forces activating TGF- β . The released factor mediates the generation of a pro-fibrotic stroma characterized by sustained cell proliferation and differential expression of matrix modulators (TGFB1, PAI1), reflected by differential ECM composition and physical properties (Billings et al. 2002; Ahlfeld et al. 2016; Cesari, Pahor, and Incalzi 2010). Finally, the intrinsic properties of this matrix result in a positive feedback loop that maintains the pro-fibrotic phenotype (Parker et al. 2014).

Acknowledgements

We gratefully acknowledge, the PRISM imaging facility of iBV and the PICMI imaging facility of IRCAN. We thank K.-H. Kraus (University of Geneva) and T. Virolle (Université Côte d'Azur) for lentiviral vectors and R. Fässler (Max Planck Institute of Biochemistry) for floxed MEFs. Financial support for this work was provided by the National Agency for Research (ANR10-02, ANR-16-CE93-0005-01), the Fondation ARC (PJA20151203207, fellowship GE), the LabEx SIGNALIFE program (ANR-11-LABX-0028-01) and the Fédération Hospitalo-Universitaire OncoAge.

Figure 1

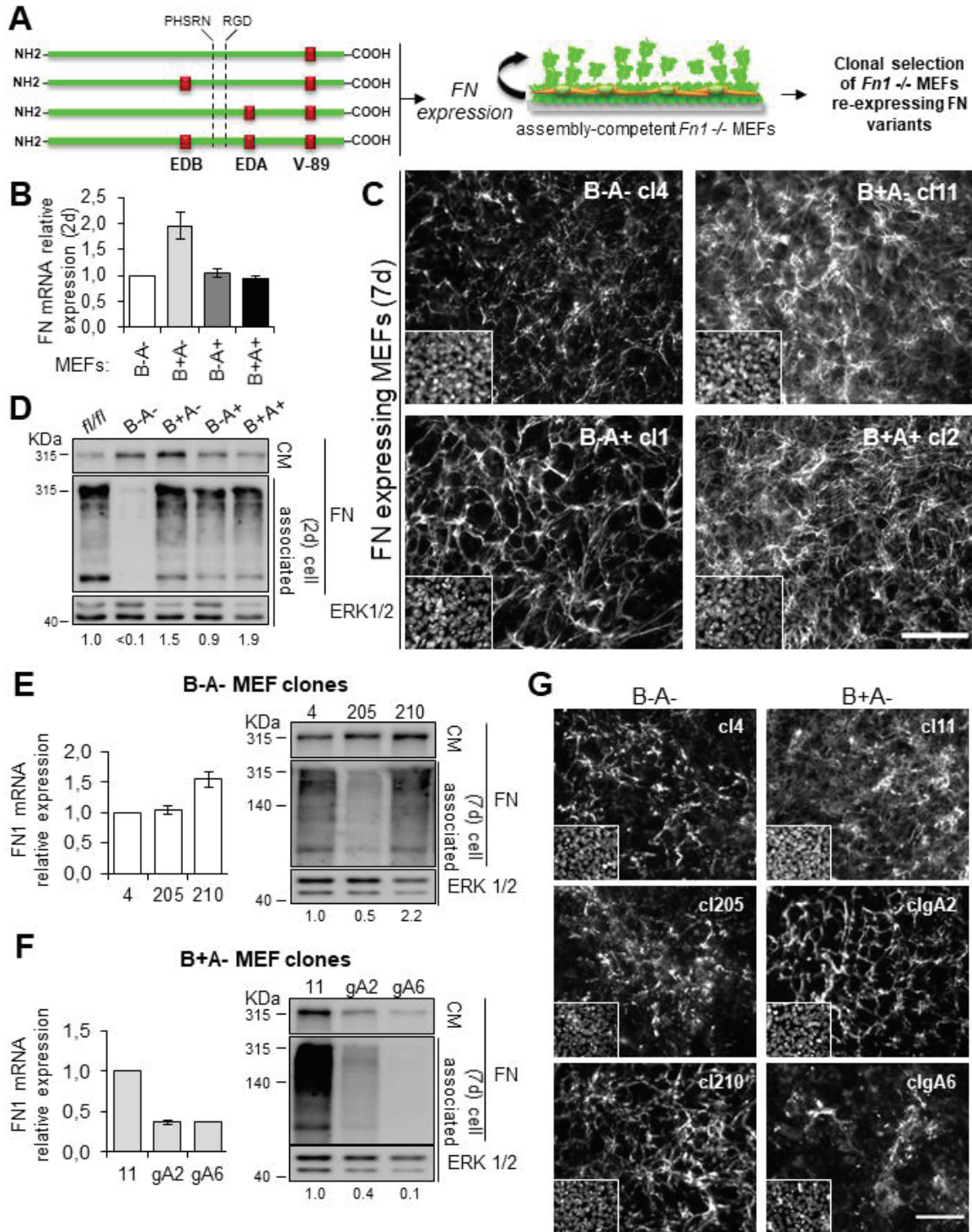


Figure 1. FN variant-expressing MEFs. (A) Schematic representation of the four human FN variants harboring the B and/or A Extra Domains (or none), including the variable region V-89, and their expression in assembly competent *Fn1*^{-/-} MEF clones. (B) Relative FN mRNA levels

across selected clones [indicated in (C)] after two days in culture. (C) Immunofluorescent staining to assess FN re-expression and assembly in the indicated clones, after 7 days in culture. Nuclei staining is shown in the inserts. Scale bar: 50 μ m. (D) Western blot of whole cell lysates and conditioned media (CM) of *Fn1 fl/fl* MEFs and four selected clones re-expressing the FN variants after 2 days in culture. ERK1/2 was used as loading control. Numbers at the bottom of the blots represent FN fold expression relative to *Fn1 fl/fl* normalized to ERK. (E) FN mRNA and protein levels were assessed in three individual clones expressing FN B-A-. Despite similar expression, FN association to cells differs among the clones. Numbers at the bottom of the blot represent fold expression of FN relative to B-A-Clone 4 normalized to ERK. (F) FN mRNA and protein levels were assessed in three individual clones expressing FN B+A-. Despite similar expression, FN association to cells differs among the clones. Numbers at the bottom of the blot represent FN fold expression relative to B+A-Clone 11 normalized to ERK. (G) Immunofluorescent staining of three individual clones expressing FN B-A- [(same as in (E))] and three individual clones expressing FN B+A- [(same as in (F))] to compare FN expression and assembly across clones for two different FN variants after 7 days in culture. Nuclei are shown in the inserts. Scale bar: 50 μ m.

Figure 2

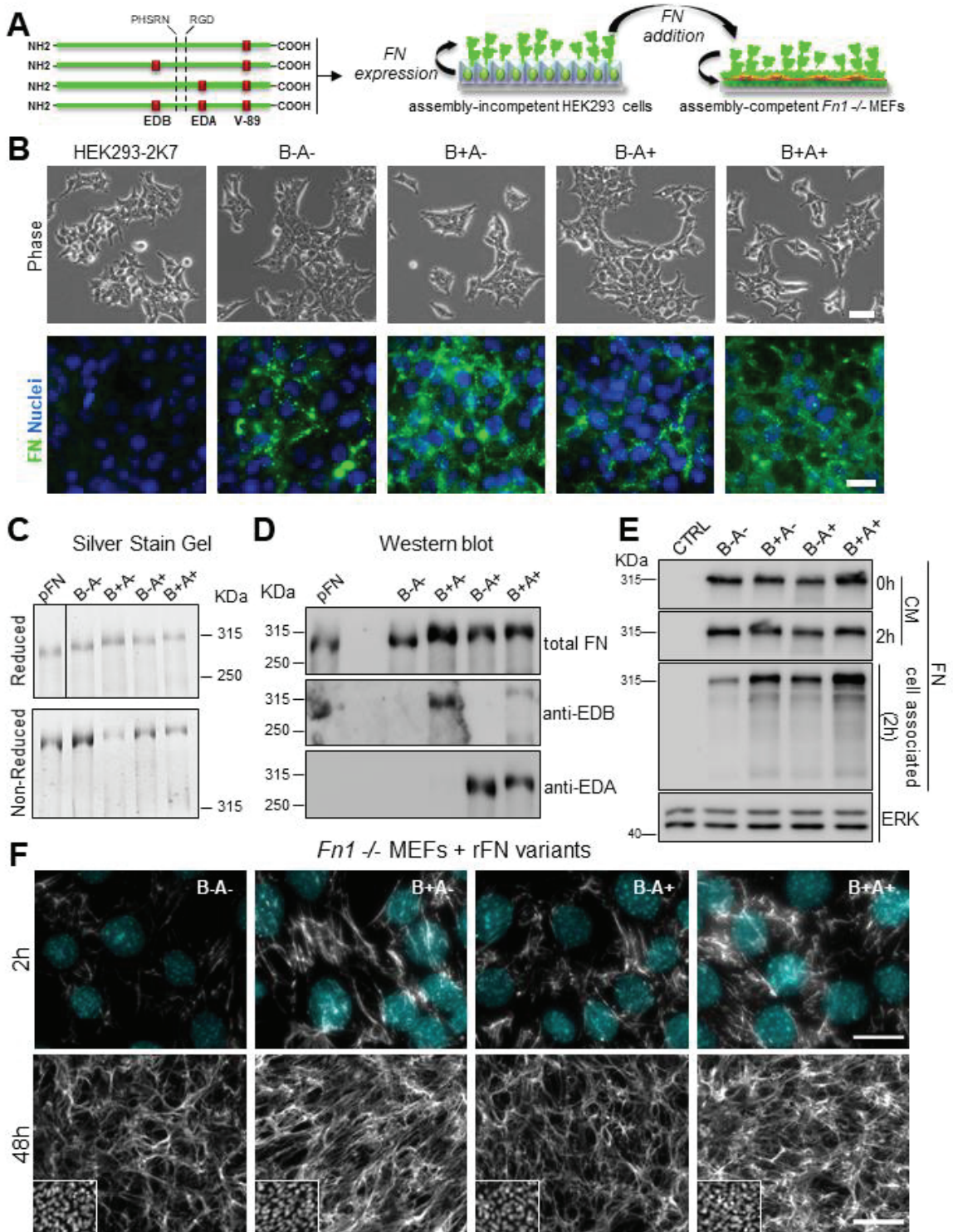


Figure 2. Purification of fully functional Extra-Domain-specific FN variants. (A) Schematic representation of the cDNA constructs encoding the four human FN variants harboring the B and/or A Extra Domains (variable region V-89) and their expression in the assembly-

incompetent HEK293 cells for subsequent FN purification. After purification, *Fn1*^{-/-} MEFs will be presented with the purified variants in order to perform functional analyses. **(B)** Phase contrast (upper row) and immunofluorescent staining of FN and nuclei (lower row) of HEK293 cells stably expressing the FN variants or the empty vector. Scale bars: Phase, 50 μm ; IF, 20 μm . FN is only observed in the cytoplasm and the plasma membrane before being secreted by the assembly-incompetent HEK293 cells. **(C)** Silver staining of FN variants in polyacrylamide gels run under reducing and non-reducing conditions to verify the integrity of the proteins, as well as their ability to dimerize. **(D)** Western blot of FN variants run in polyacrylamide gels and stained with different antibodies against total FN, Extra Domain B, and Extra Domain A verifying the all-or-nothing inclusion of the Extra Domains in each variant. **(E)** Western blot of whole cell lysates and conditioned media of *Fn1*^{-/-} MEFs presented with FN variants for 2 h. ERK1/2 was used as loading control. **(F)** Immunofluorescent staining of FN and nuclei of *Fn1*^{-/-} MEFs presented with FN variants for 2 h (upper row) and 48 h (lower row). In the 48-hour images, nuclei have been added as inserts for clarity. Scale bar: 50 μm .

Figure 3

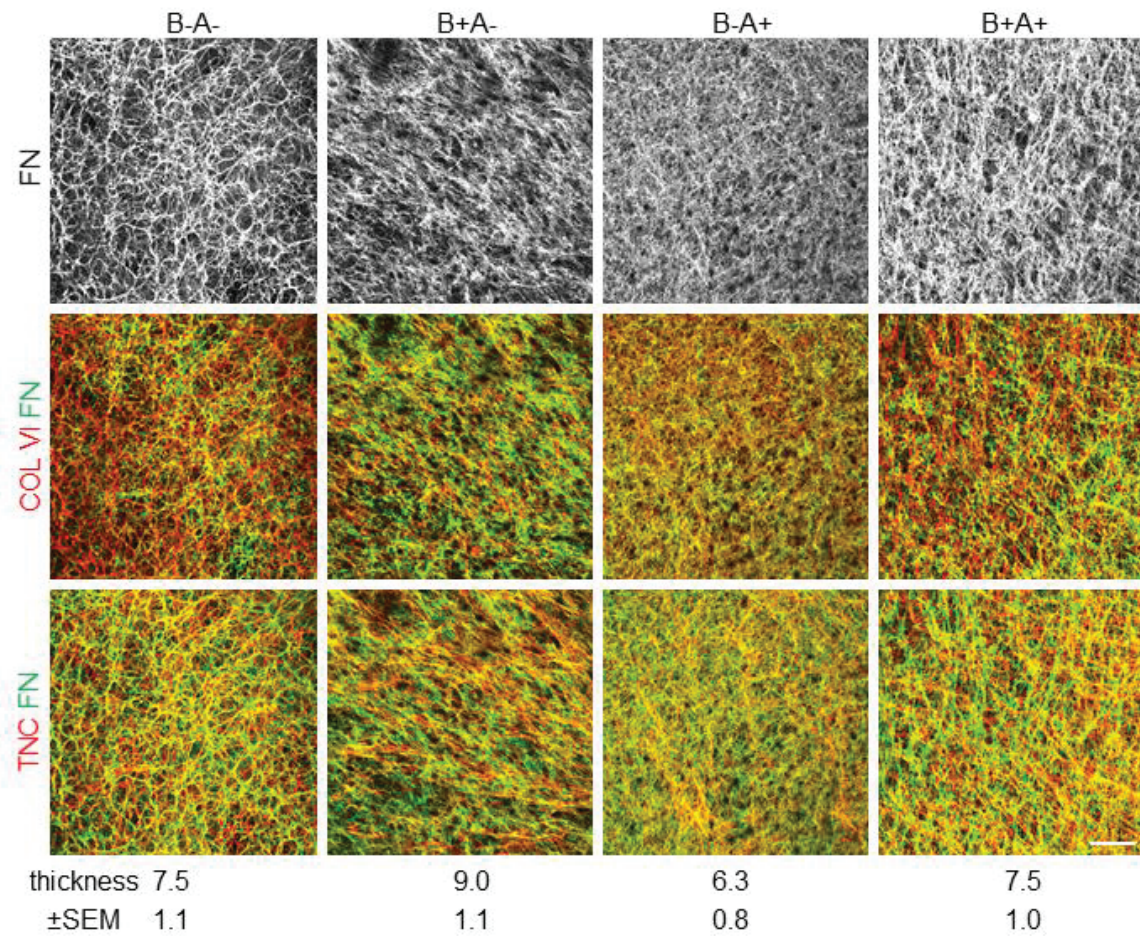


Figure 3. The impact of the FN Extra Domains in FN assembly and ECM physical properties. Variant-specific decellularized matrices. *Fn1*^{-/-} MEFs were presented with FN variants for 7 days. Matrices were generated, decellularized and immunofluorescently stained for FN, type IV collagen, and TNC. Images are representative of at least ten independent experiments. Scale bar: 100 μ m. Thickness was measured with confocal microscopy and mean \pm SEM values of five independent experiments are shown.

Figure 4

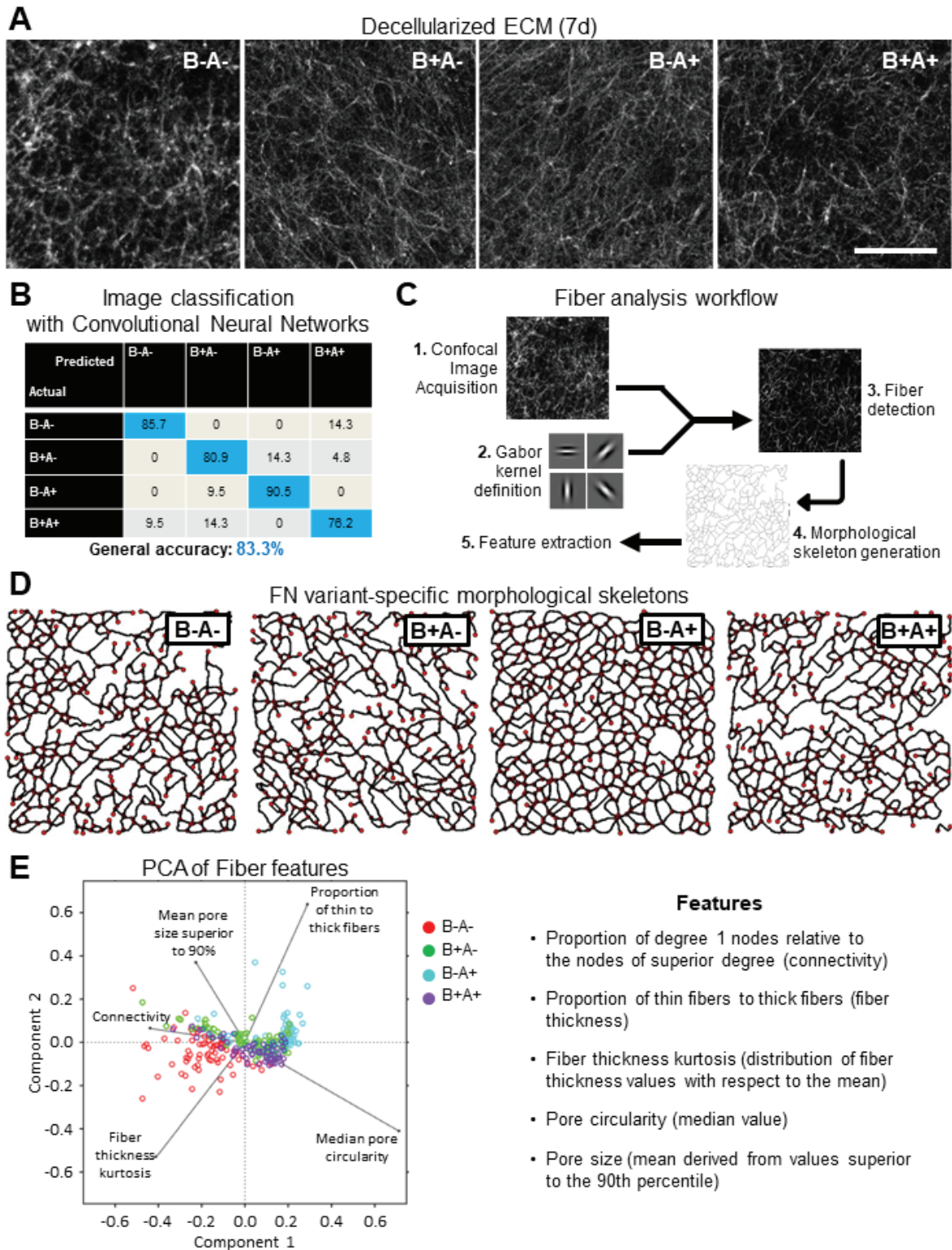


Figure 4. Computational analyses of FN variant-specific matrices. (A) Matrices assembled by Fn1 $-/-$ MEFs presented with FN variants were decellularized after 7 days, stained for FN and visualized by confocal microscopy. Representative images of at least 10 independent

experiments are shown. Scale bar: 50 μm . **(B)** Confusion matrix of FN variant network classification using Convolutional Neural Networks. An algorithm was trained to recognize and classify FN-variant-specific matrices. The diagonal (in blue) displays the classification success rate (correctly classified images). **(C)** Flow chart of fiber analysis. 1: confocal images were acquired and cropped to a final size 512×512 pixels; 2: a set of Gabor kernels representing an elemental-fiber dictionary was established; 3: fiber detection was performed using Gabor kernels on acquired images; 4: detected fibers were used to generate variant-specific morphological skeletons. **(D)** Graph-based representation of the FN variant fibers detected by Gabor kernels. Edges (black segments) connect nodes (red dots). **(E)** Principal component analysis (PCA) of the indicated features related to the graphs or derived from characteristic Gabor kernel parameters.

Figure 5

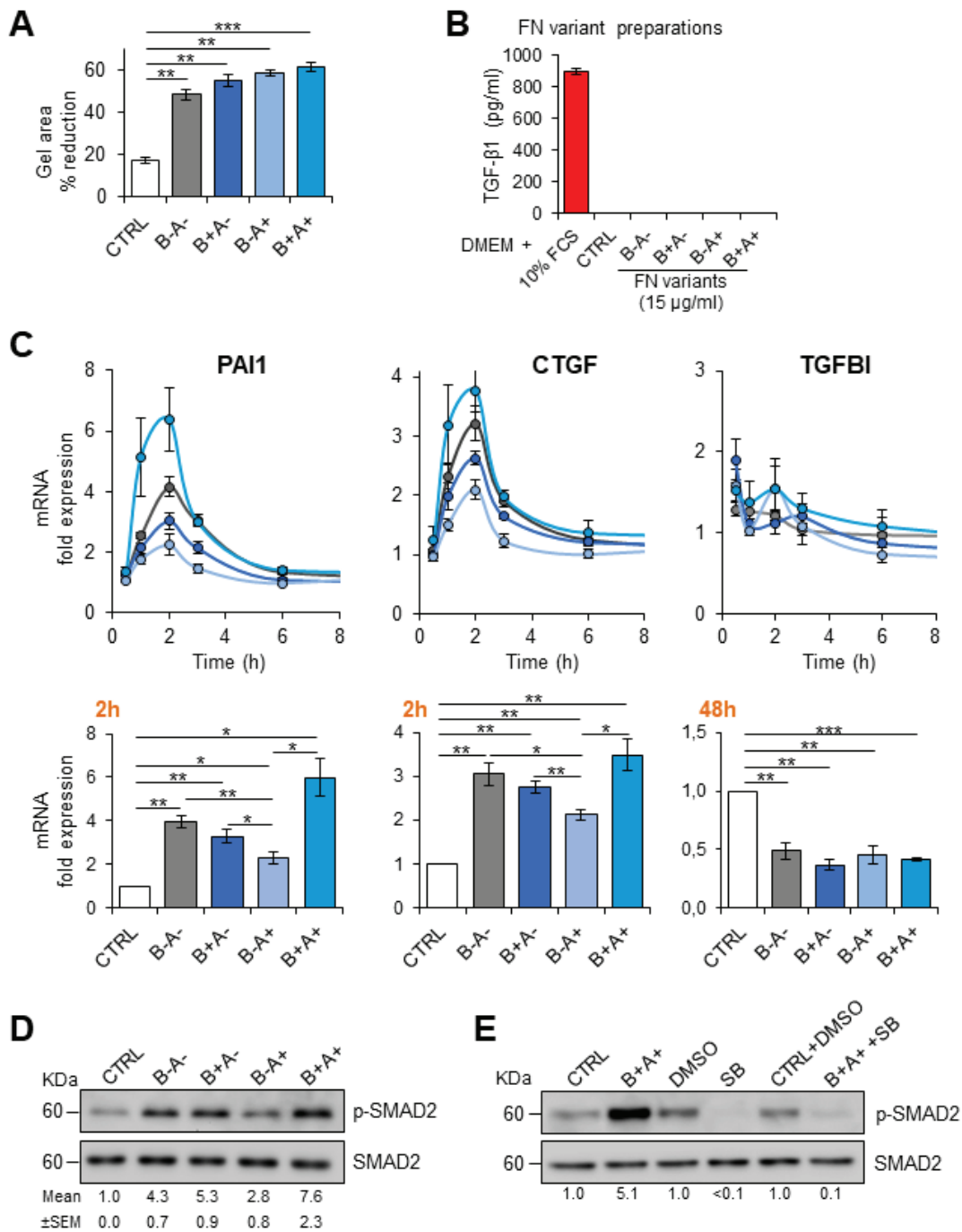


Figure 5. The presence of the Extra Domains in the FN molecule differentially regulates TGF- β signaling through activation of TGF- β 1. (A) Collagen gel contraction assay. FN1 $-/-$ MEFs were embedded in collagen lattices containing FN variants. Cell surface reduction was

measured after a 24-hour incubation following lattice solidification. Results are the mean \pm SEM values from four independent experiments, and are displayed as a % reduction in gel surface measured with ImageJ. $**p \leq 0.01$; $***p \leq 0.001$. **(B)** TGF- β 1 detection in recombinant FN preparations. FN preparations were added in DMEM (15 μ g/ml) to assess the presence of TGF- β 1. Standard culture medium (DMEM +10% FCS) was used as reference. **(C)** Top row: Expression levels of TGF- β response genes between 15 min and 48 h. Fn1 $-/-$ MEFs were stimulated with FN variants and a time course was established. Results are mean \pm SEM values from at least three independent experiments, represented as fold change relative to CTRL for each time point. Time points later than 8 h have been omitted for clarity. Bottom row: Isolated time points for each TGF- β response gene, in which expression changes are significant. PAI1 and CTGF, 2 h; TGFBI, 48 h. Results are mean \pm SEM values from at least three independent experiments. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. **(D)** Western blot displaying the phosphorylation of SMAD2 after a 2-hour stimulation of Fn1 $-/-$ MEFs with FN variants. Total SMAD2 used as loading control. Numbers at the bottom of the blots are mean \pm SEM values of three independent experiments. **(E)** Western blot showing diminishing of SMAD2 phosphorylation after TGFBR1 inhibition. Fn1 $-/-$ MEFs were treated with FN B+A+ and SB-431542, a TGFBR1 inhibitor, and their respective vehicle solutions for control. Total SMAD2 used as loading control. Numbers at the bottom of the blots are SMAD2 phosphorylation fold difference relative to respective controls.

Figure 6

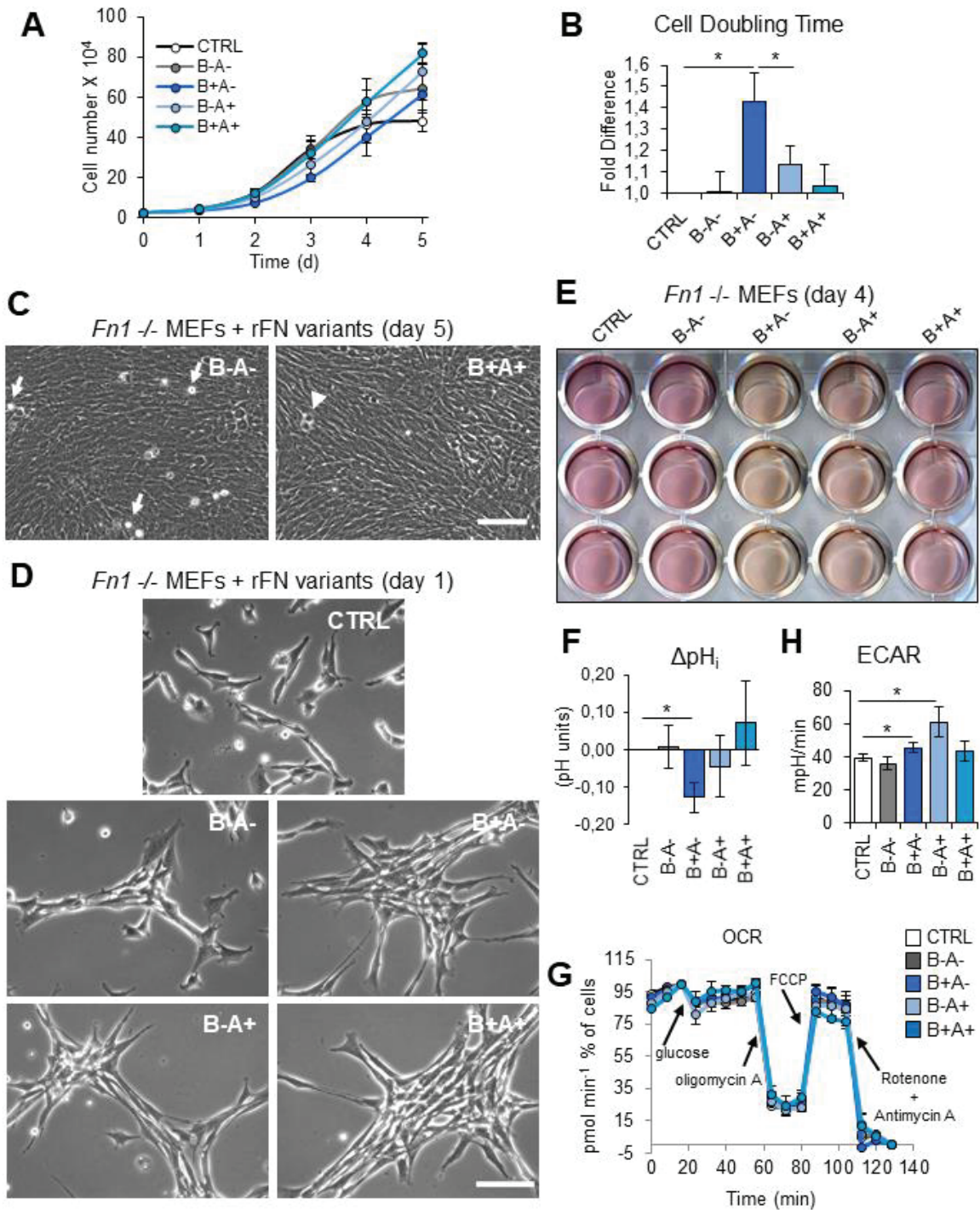


Figure 6. The presence of FN Extra Domains affects cell growth and metabolism (A) Growth curves of *Fn1*^{-/-} MEFs stimulated with FN variants or vehicle buffer. Mean \pm SEM values from 3 independent experiments are shown. **(B)** Doubling time of cells treated with FN variants

relative to cells treated with vehicle CTRL determined from the growth curves shown in (A). Values represent mean \pm SEM from 4 independent experiments. * $p \leq 0.05$. (C) Representative phase contrast images (7 independent experiments) of Fn1 -/- MEFs 5 days after stimulation with FN variants. (D) Representative phase contrast images of Fn1 -/- MEFs (7 independent experiments) after overnight stimulation with FN variants. (E) Medium acidification observed at 4 days post treatment (representative image from 3 independent experiments). (F) Internal pH measurements in cells treated with FN variants or vehicle CTRL after 48 h. Results are represented as Δ pHi (difference in internal pH) relative to CTRL. Mean \pm SEM from 7 independent experiments are shown. * $p \leq 0.05$. (G) Oxygen consumption rate (OCR) measured with the Seahorse analyzer in Fn1 -/- cells treated for 48 h with FN variants or vehicle buffer. (H) Extracellular acidification rate (ECAR) of Fn1 -/- MEFs after treatment with rFN variants or vehicle buffer measured with the Seahorse analyzer after 72 h. Values are mean \pm SEM from 2 independent experiments. * $p \leq 0.05$.

Supplementary Material

Supplemental Table 1

Primary antibodies used, suppliers and dilutions

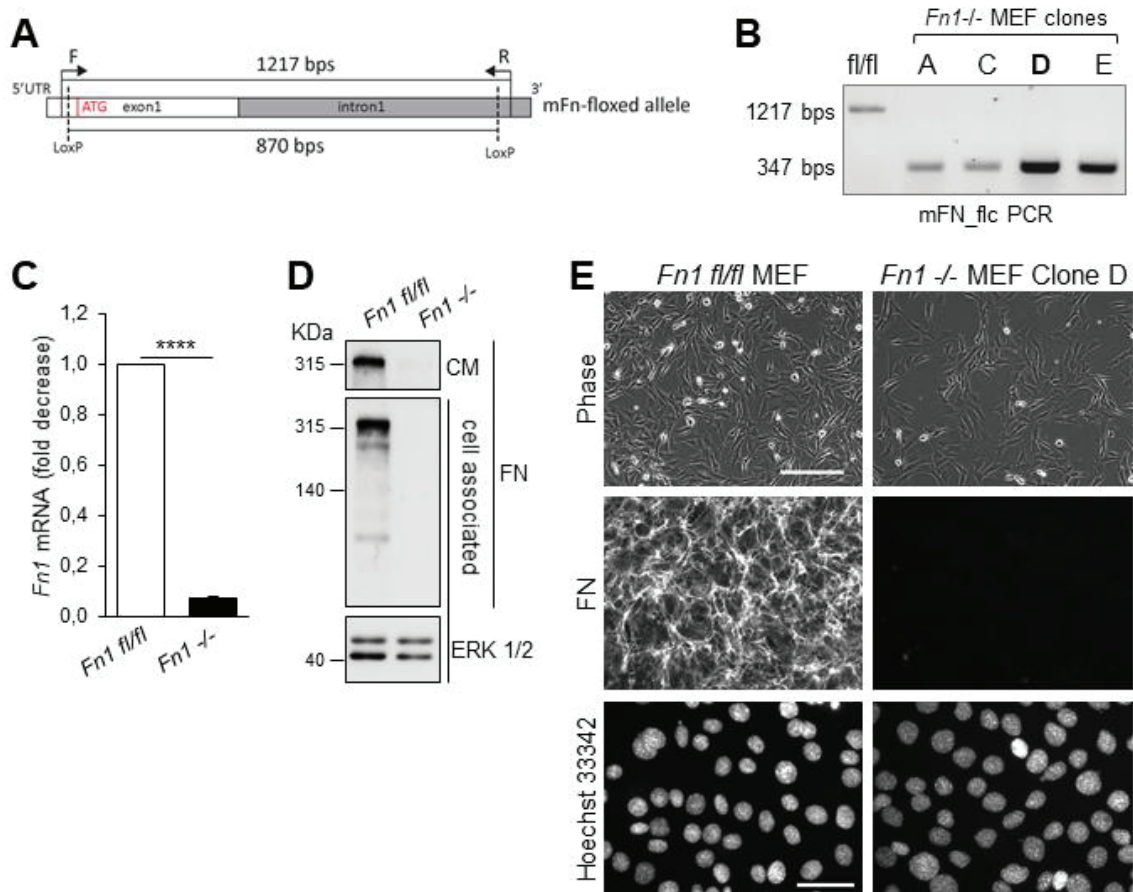
Specificity	Company	Clone	Reference	Raised in	Application
anti-FN-EDA	Sirius Biotech	IST-9	S-FN5	Mouse	WB: 1/200 IF: 1/XXXX
anti-FN-EDB	Sirius Biotech	C6	S-FN12	Mouse	WB: 1/200 IF: 1/XXXX
anti-FN	Millipore	Polyclonal	AB1945	Rabbit	WB: 1/1000 IF: 1/400
anti-FN	BD Biosciences	10/Fibronectin	#610077	Mouse	WB: 1/1000 IF: 1/500
anti-ERK1/2 (diphosphorylated)	Sigma-Aldrich	MAPK-YT	M8159	Mouse	WB: 1/1000
anti-ERK1/2	Santa Cruz Biotechnology	C-16	sc-93	Rabbit	WB: 1/2000
anti-SMAD2 (Ser465/467)	Cell Signaling Technology	138D4	#3108	Rabbit	WB: 1/1000
anti-SMAD2	Cell Signaling Technology	86F7	#3122	Rabbit	WB: 1/2000

Supplemental Table 2

Primer sets used for qPCR analyses

Target	Sequence	
	Forward	Reverse
Tbp	GAAGAACAATCCAGACTAGCAGCA	CCTTATAGGGAACCTCACATCACAG
h1_FN_2K7	CCCAATTGAGTGCTTCATGCC	TGTACAAGAAAGCTGGGTCG
Fn1	CTGTAGCACAACCTTCCAATTACGAA	GGAATTTCCGCCTCGAGTCT
Tgfbi	AAGGTCATTGGCACCAACAAG	AACCTTTCTCTCCTGGGACCTT
Ctgf	CAGCTGGGAGAACTGTGTACG	GTACACCGACCCACCGAAGA
Pai1	TGAGCTGTGCCCTTCTCATT	GCCACCGACTTCGGAGTAAA
<i>Mycoplasma sp.</i>	GGGAGCAAACAGGATTAGATACCCT	TGCACCATCTGTCACTCTGTTAACCTC

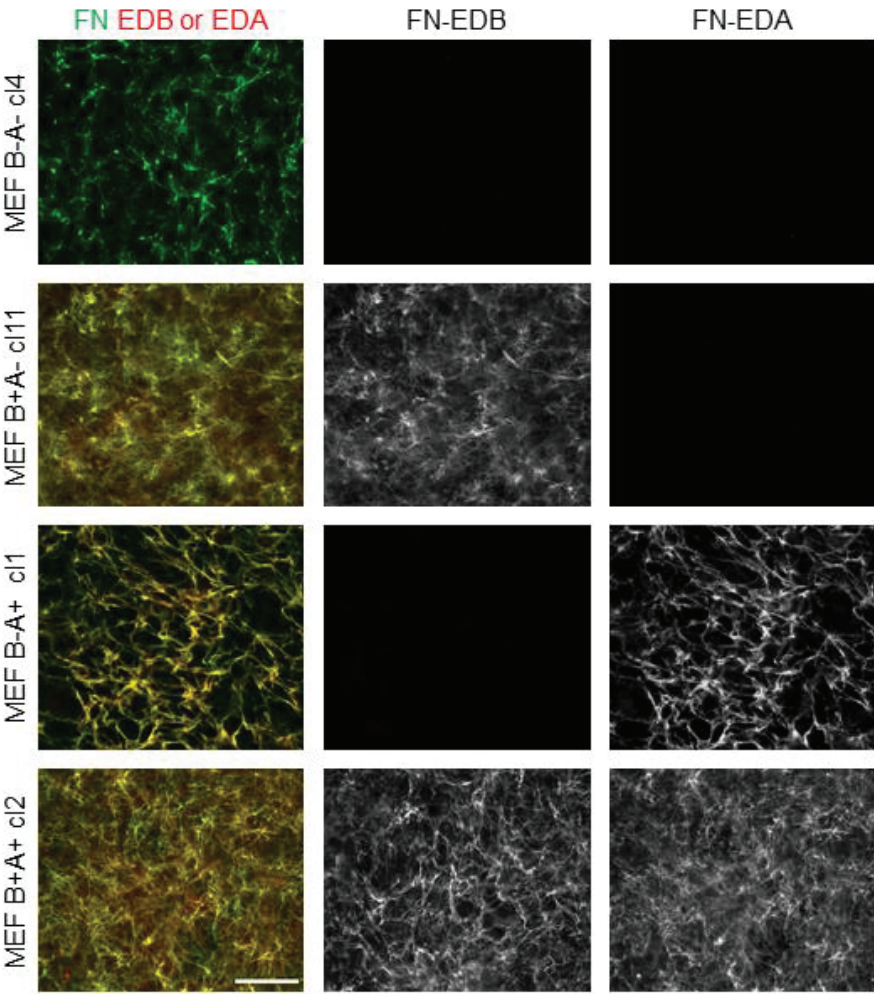
Supplemental Figure 1



Supplemental Figure 1. Generation of *Fn1*^{-/-} MEF clones. (A) Schematic representation of the relevant position of the two LoxP sites flanking the first exon of the *Fn1* gene, as well as the positions of the primer set used to confirm the deletion. (B) Agarose gel electrophoresis of the PCR products generated from four separate *Fn1*^{fl/fl} populations infected with a lentiviral vector bearing the coding sequence of Cre recombinase. (C) qRT-PCR analysis showing the decrease of mRNA levels of the endogenous *Fn1* gene in MEF *Fn1*^{-/-} CloneD. ****p ≤ 0.0001. (D) Western analysis showing the elimination of FN protein in whole cell lysates and conditioned medium from MEF *Fn1*^{-/-} CloneD. ERK was used as loading control. (E) Phase contrast (upper row) and immunofluorescent staining (middle and lower rows) for

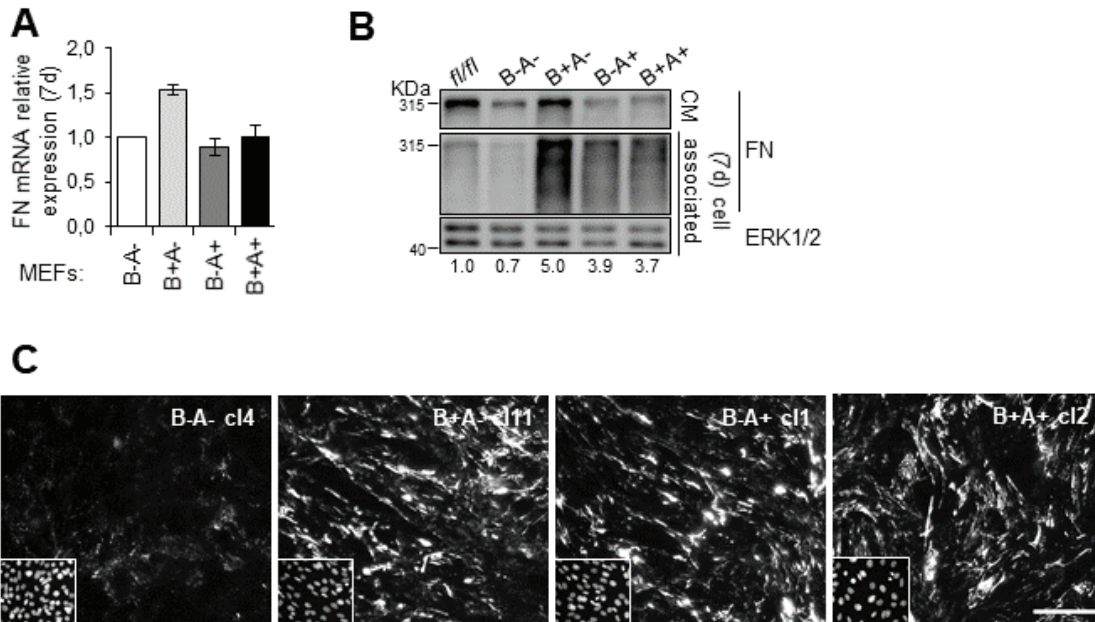
FN displaying the absence of FN deposition in MEF Fn1 -/- CloneD after 2 days in culture. Scale bars: Phase, 200 μm ; IF, 50 μm

Supplemental Figure 2



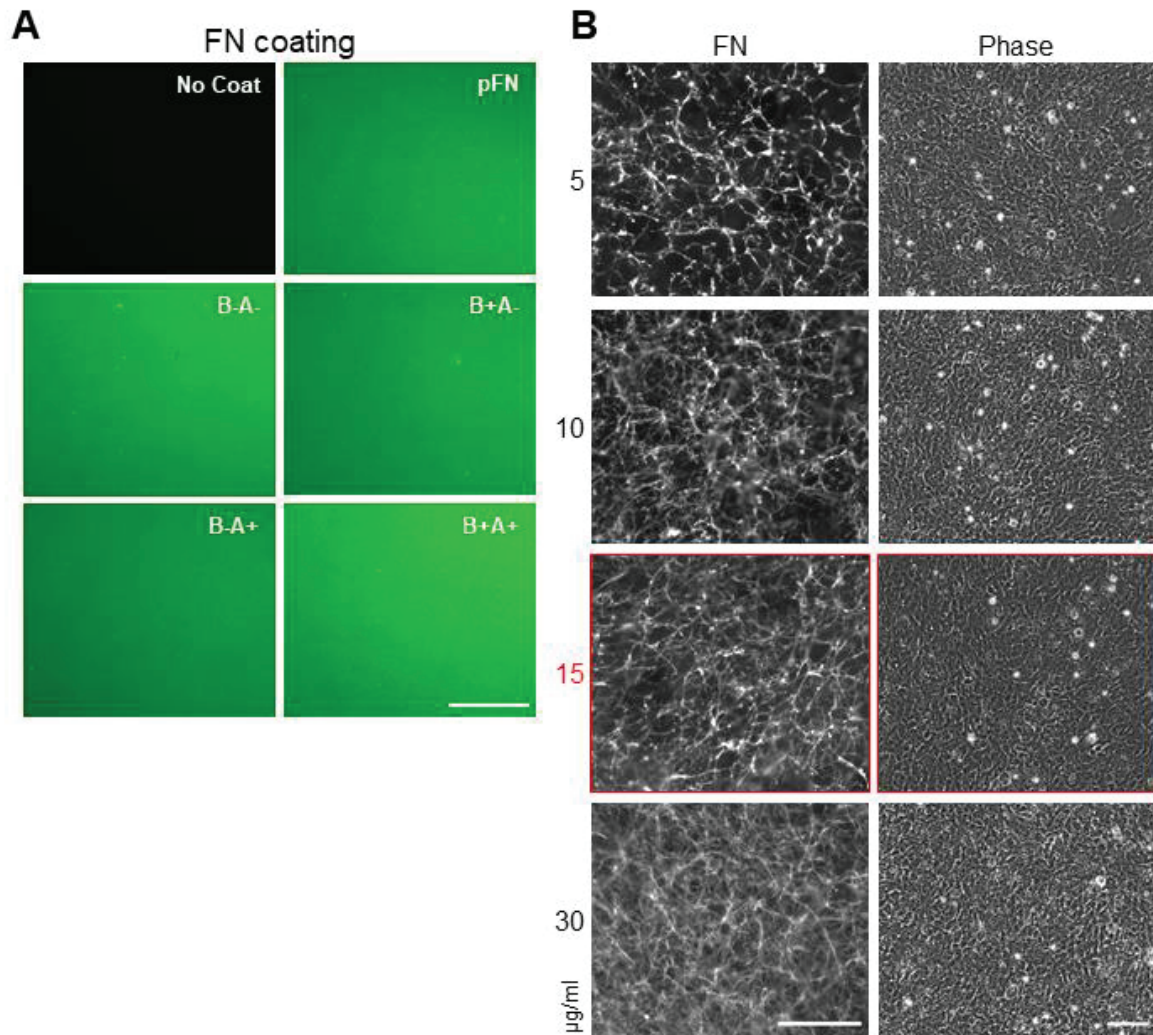
Supplemental Figure 2. Immunofluorescent staining of FN in the four selected clones. (B-A-cl 4, B+A- cl11, B-A+ cl1, B+A+ cl2). An anti-totalFN antibody was used in combination with an anti-EDB or anti-EDA in order to verify the expression of the FN variants by the different clones. Scale bar, 50 μ m

Supplemental Figure 3



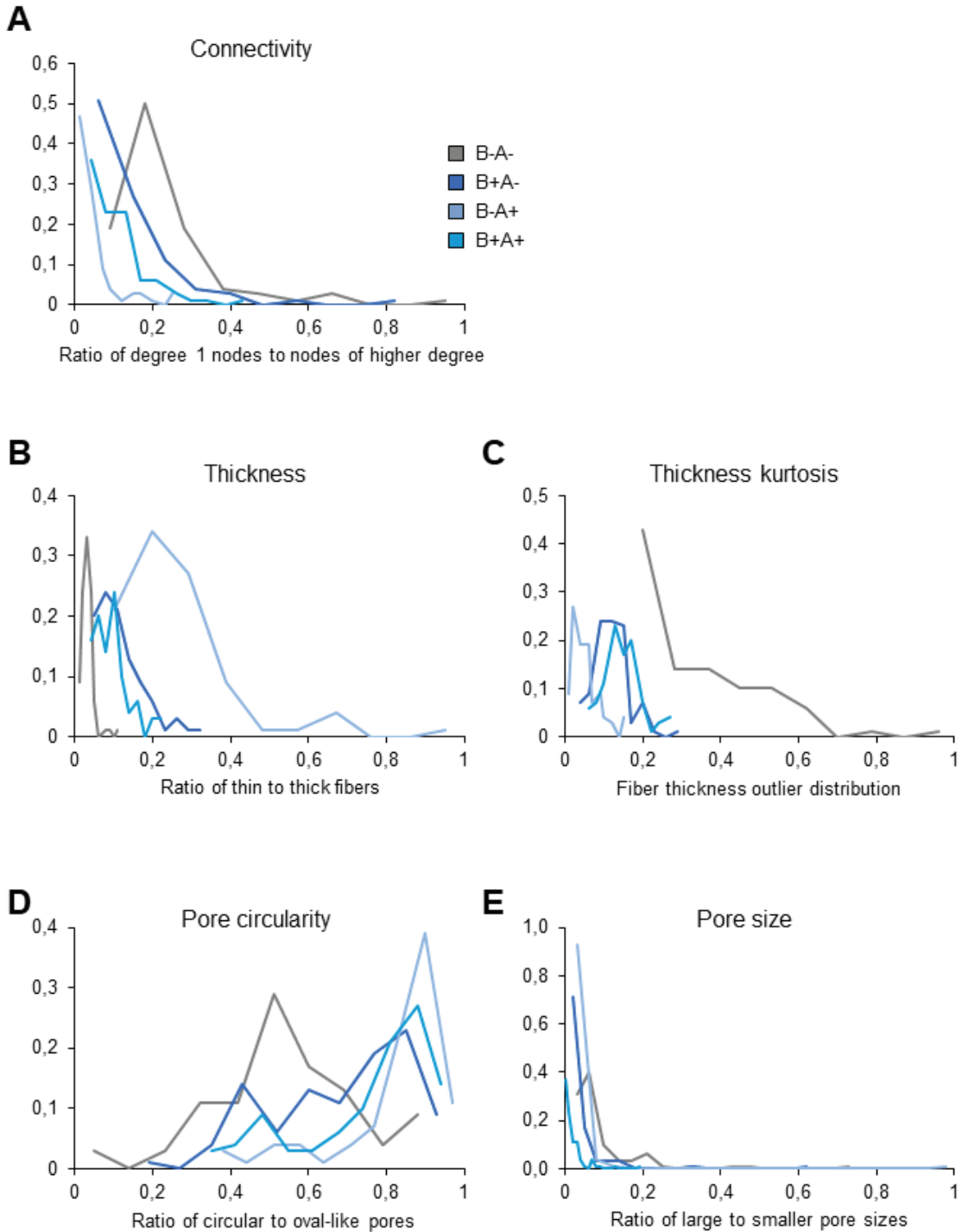
Supplemental Figure 3. FN expression and assembly in MEF clones. (A) Relative FN mRNA expression levels across the selected clones after 7 days in culture. (B) Western blot of whole cell lysates and conditioned media of Fn1 fl/fl and the four selected clones re-expressing the FN variants, to detect FN secreted in the medium and associated to the cells after 7 days in culture. ERK1/2 was used as loading control. Numbers at the bottom of the blots represent FN fold expression relative to Fn1 fl/fl normalized to ERK. (C) Immunofluorescent staining to assess FN re-expression and deposition in the indicated clones, after 2 days in culture. Nuclei staining is shown in the inserts. Scale bar: 50 μ m.

Supplemental Figure 4



Supplemental Figure 4. Dose-dependent assembly of pFN. (A) FN coating of glass coverslips. Immunofluorescent staining of FN-coated (3 $\mu\text{g/ml}$) glass coverslips to assess the existence of aggregates, that would greatly affect solubility and bioavailability. Scale bar: 50 μm . (B) Immunofluorescent staining of FN (left column) and phase contrast (right column) of Fn1 $-/-$ MEFs 5 days after having been presented with increasing concentrations of pFN in order to find the optimal concentration for our studies. We determined that 15 $\mu\text{g/ml}$ is a good compromise between efficient matrix generation and moderation in the use of the purified variants.

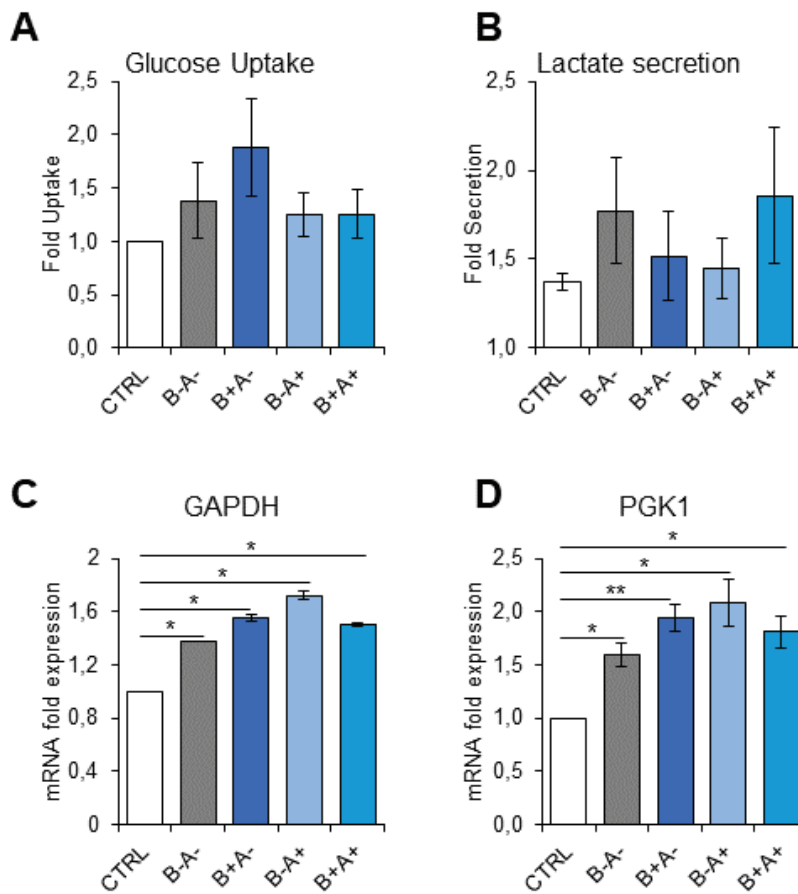
Supplemental Figure 5



Supplemental Figure 5. Feature distribution across FN variant matrices. (A) Connectivity graph displaying the ratio of degree-1 nodes relative to higher-degree nodes. (B) Thickness graph showing the distribution of thin fibers relative to thick fibers as described in material

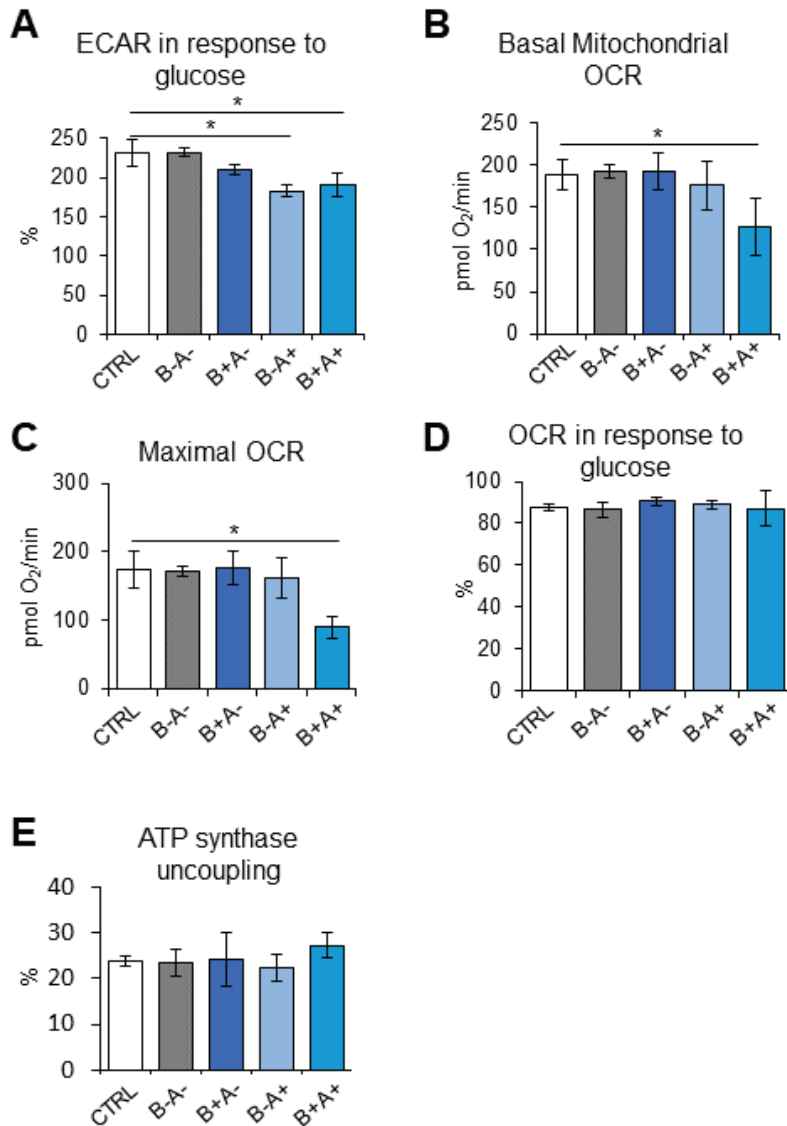
and methods. **(C)** Graph representing the heterogeneity in fiber thickness across the different matrices, as determined by the fiber thickness kurtosis. **(D)** Pore circularity distribution defined by pore area and pore perimeter. **(E)** Pore size graph across FN matrices after filtering out pores below the 90th percentile within each distribution.

Supplemental Figure 6



Supplemental Figure 6 Metabolic effects of FN in Fn1 ^{-/-} MEFs. (A) Glucose uptake in Fn1 ^{-/-} MEFs stimulated with FN variants for 48 h. To do so, Fn1 ^{-/-} MEFs were plated in triplicates in 96-well plates (4X10³ cells/well) and stimulated with 15 μ g/ml FN variants or vehicle buffer for 48 h. Cells were washed with PBS, starved with glucose-depleted medium for 30 min before adding 2-NBDG (Thermo Fisher Scientific) 200 μ M for 40 min. Fluorescence intensity was measured in a microplate reader. Results represent mean fold difference \pm SEM relative to CTRL from 9 independent experiments. (B) Lactate extrusion in the culture medium of Fn1 ^{-/-} MEFs treated with FN variants for 48 h, analyzed using an ion chromatography column. Graph displays the mean \pm SEM fold difference relative to day 0 CTRL from 2 independent experiments. Expression of mRNA encoding (C) glutaraldehyde phosphate dehydrogenase (GAPDH) mRNA and (D) phosphoglycerate kinase 1 (PGK1), enzymes that catalyze the 6th and 7th steps of glycolysis respectively, were determined in Fn1 ^{-/-} MEFs after 48 h of stimulation with FN variants. Results are expressed as mean fold difference \pm SEM relative to CTRL from 4 independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$.

Supplemental Figure 7



Supplemental Figure 7. The presence of FN does not affect mitochondrial activity in Fn1 ^{-/-} MEFs. (A) Extracellular acidification rate in response to glucose measured in Fn1 ^{-/-} MEFs stimulated for 72 h with FN variants with the Seahorse analyzer. Results are mean ±SEM of two independent experiments. *p ≤ 0.05. (B) Basal mitochondrial activity of Fn1 ^{-/-} MEFs stimulated with FN variants for 72 h, determined with the Seahorse analyzer by measuring oxygen consumption rate (OCR). Results are mean ±SEM of two independent experiments. *p ≤ 0.05. (C) Maximal mitochondrial activity of Fn1 ^{-/-} MEFs stimulated with FN variants for 72 h, measured as in (A). Results are mean ±SEM of two independent experiments. *p ≤ 0.05. (D) Fn1 ^{-/-} MEFs were stimulated with FN variants for 72 h and OCR was determined in response

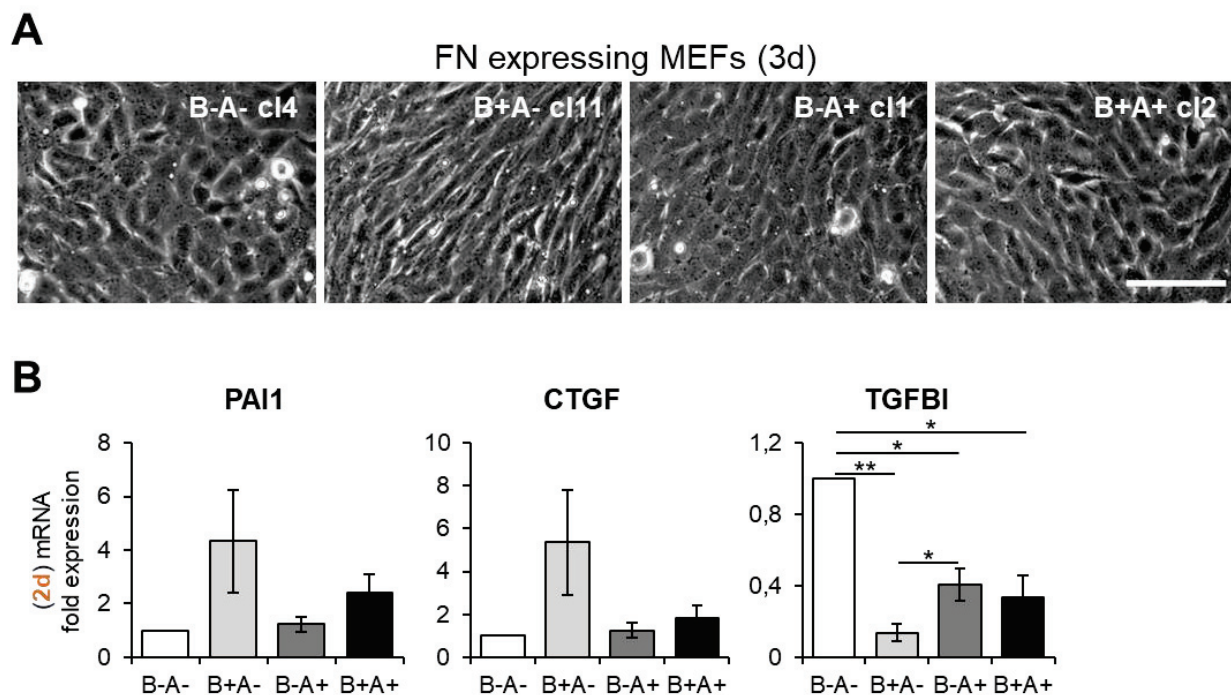
to glucose with the Seahorse analyzer. (E) Dependence of respiration of Fn1 ^{-/-} MEFs stimulated with FN variants on ATP synthase demonstrated with the Seahorse analyzer.

4. Complementary Results

Re-expression of FN variants in FN-null MEFs

We saw previously that addition of cFN in *Fn1*^{-/-} MEFs resulted in cells adopting a spindle-like morphology, resembling activated fibroblasts, or myofibroblasts. Stable re-expression of cFN variants by MEFs resulted in a similar effect that appeared to be dose-dependent. FN B+A- MEFs, which express the highest quantity of FN, were more spindle-like (**Complementary Fig. 1A**), followed by FN B+A+ MEFs.

Furthermore, the expression pattern of TGF- β targets PAI1, CTGF, and TGFBI reflected both the level of FN expression and the efficiency of FN assembly (**Complementary Fig. 1B**).

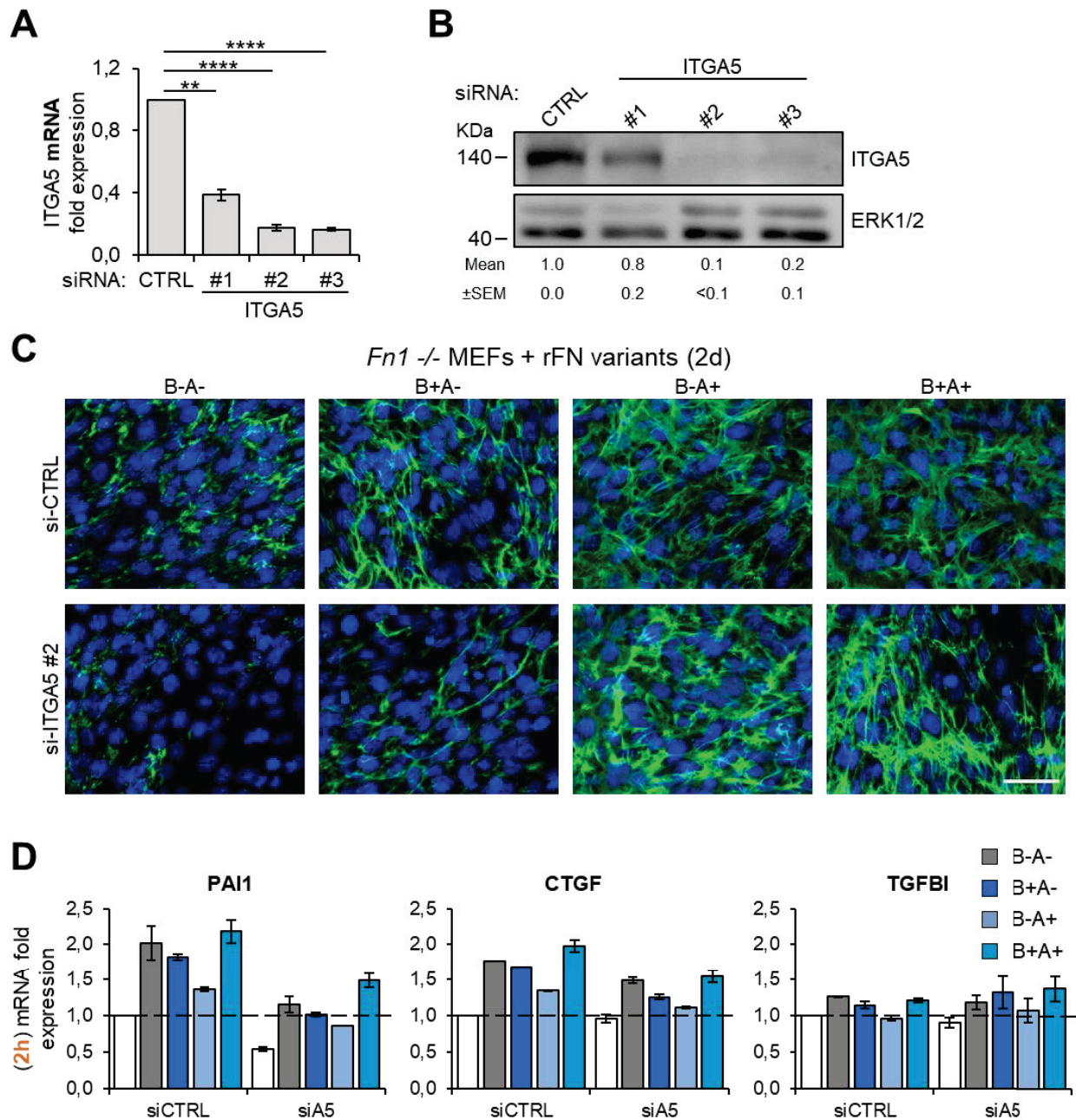


Complementary Fig. 1. Re-expression of FN variants by FN-null MEFs

Re-expression of variant specific FNs by *Fn1*^{-/-} cells. (A) Phase contrast images of *Fn1*^{-/-} MEFs re-expressing FN variants after 3 days in culture. Scale bar: 100 μ m. (B) Relative mRNA levels of three TGF- β response genes (PAI1, CTGF, TGFBI) in the MEF clones after 2 days in culture. * $p \leq 0.05$, ** $p \leq 0.01$.

FN deposition and TGF- β signaling dependence on $\alpha 5\beta 1$

Integrin $\alpha 5\beta 1$ is viewed as the “classic” FN binding integrin, responsible for FN fibrillogenesis and mechanosensing. It binds a tripeptide sequence (RGD) residing on FNIII₁₀ (Main et al. 1992), flanked by the splice sites where the two Extra Domains are



found in cFN. In order to examine whether the effects we previously saw are entirely due to FN binding to integrin $\alpha 5 \beta 1$, or whether other integrins/receptors are involved, we targeted *ITGA5* mRNA with a small interfering RNA (siRNA). Utilizing the algorithm developed by Vert and colleagues (Vert et al. 2006), we generated three independent siRNA sets the efficiency of which we subsequently tested. si-ITGA5 #2 was the most efficient in terms of mRNA (Complementary Fig. 2A) and protein (Complementary Fig. 2B) decrease, and was selected for further experiments.

◀ **Complementary Fig. 2. FN deposition and TGF- β signaling dependence on $\alpha 5\beta 1$ (previous page).**

Fn1 $-/-$ MEFs were plated in 6-well plates (10^5 cells/ml) in DMEM supplemented with 10% FN-depleted FCS. Transfection with three siRNAs targeting $\alpha 5$ subunit (siRNA sequences shown in **Table 3**) or with scramble siRNA (Control siRNA duplex negative control, Eurogentec, Liège, Belgium) was performed the day after with RNAiMAX reagent (Thermo Fisher Scientific) according to manufacturer's instructions. Cells were harvested 3 d later and equally distributed in 6-well plates for qPCR and Western blot. (A) $\alpha 5$ mRNA fold decrease in si-ITGA5-transfected cells compared to CTRL. ** $p \leq 0.01$, **** $p \leq 0.0001$. $\alpha 5$ mRNA was detected by qPCR with the following primers F: 5'-CTCGGCTTCTTCAAACGTTCC-3' and R: 5'-CGGGATCATGAGTCTGAGATCAGG-3'. (B) $\alpha 5$ protein decrease in si-ITGA5-transfected cells was visualized with an anti- $\alpha 5$ antibody (Thermo Fisher Scientific). ERK was used as loading control. Numbers at the bottom of the figure are the mean decrease \pm SEM of $\alpha 5$ levels relative to CTRL. (C) *Fn1* $-/-$ MEFs were transfected with si-ITGA5 #2 as described. Two days later, cells were trypsinized and plated in 12-well plates with coverslips (10^5 cells/ml). Upon attachment, cells were presented with FN variants (15 μ g/ml) or vehicle buffer (CTRL, not shown) for 48 h. Cells were subsequently fixed and stained with an anti-totalFN antibody as described in Methods. Scale bar, 50 μ m. Representative images of at least 2 independent experiments. (D) Relative mRNA levels of PAI1, CTGF, and TGFBI in *Fn1* $-/-$ MEFs stimulated with FN variants for 2 h after $\alpha 5$ knockdown. *Fn1* $-/-$ MEFs were transfected with si-ITGA5 #2 as described. Two days later, cells were trypsinized and plated in 6-well plates (10^5 cells/ml) with DMEM supplemented with 5% FN-depleted FCS. The day after, cells were presented with FN variants (15 μ g/ml) or vehicle buffer (CTRL) for 2 h. RNA extraction and qPCR were performed as described in Methods.

Table 3. siRNA sequences used in $\alpha 5$ -knockdown experiments

	Sense Sequence	Anti-sense Sequence
si-ITGA5-1	GCAGGGAGAUGAAGAUCUACC	UAGAUCUUCAUCUCCUGCAG
si-ITGA5-2	CAUCAAUUUGACAGCAAAGG	UUUGCUGUCAAUUGAAUGGU
si-ITGA5-3	GCAUGUGCUCACUGUAUAGC	UAUACAGUGGAGCACAUGCCA

As expected, knockdown of $\alpha 5$ resulted in disrupted FN variant harvesting and deposition by *Fn1* $-/-$ MEFs and this effect was more striking in the case of plasma-like FN (B-A-) (**Complementary Fig. 2C**), followed by B+A- which was somewhat more efficiently incorporated and some fibrils were formed. Intriguingly, EDA-containing FNs (B-A+, and B+A+) were equally set up into complex network arrangements in spite of the absence of $\alpha 5\beta 1$, though the fine fibrils seen in si-CTRL are replaced by fiber-like aggregates. These results are in accordance with previous works that despite the importance of RGD binding by $\alpha 5\beta 1$ in FN fibrillogenesis and development (J. T. Yang, Rayburn, and Hynes 1993), $\alpha 5\beta 1$ is dispensable for FN fibrillogenesis in culture when EDA is included in the molecule. It has been reported that integrin $\alpha v\beta 3$ might trigger an RGD-independent FN assembly pathway (Takahashi et al. 2007), but further studies are needed to assess a possible interaction between this integrin and the alternatively spliced type III repeats.

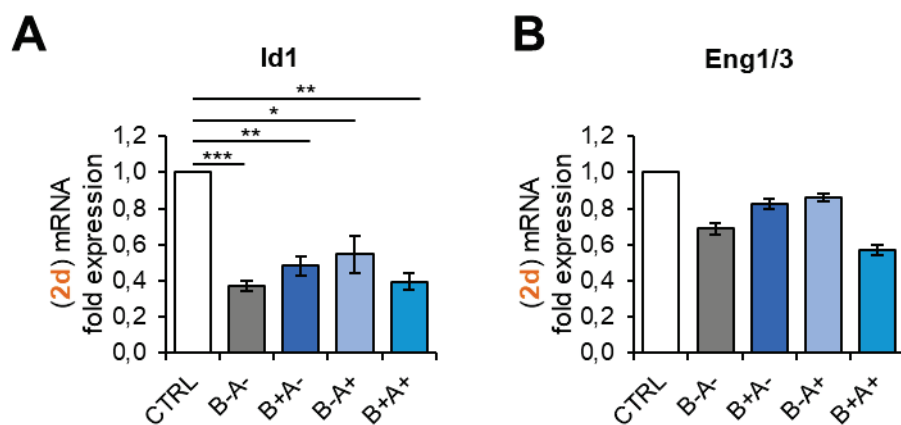
In terms of TGF- β signaling, $\alpha 5$ integrin knockdown resulted in reduced induction of PAI1 and CTGF, while TGFBI levels remained unchanged, as expected, pointing towards a synergistic effect of $\alpha 5\beta 1$ in TGF- β activation (**Complementary Fig. 2D**).

Of note, the extent of the induction was lower (for si-CTRL and si-ITGA5) compared to untransfected cells, suggesting a possible interference of the liposomal nature of the transfection reagent with cell receptor distribution on the cell membrane.

FN-mediated expression regulation of TGF- β modulators

Apart from the three aforementioned canonical TGF- β downstream targets, we assessed the induction of a non-canonical one, namely Id1, the expression of which is involved in cell proliferation and differentiation [reviewed in (Derynck and Budi 2019)]. Interestingly, Id1 mRNA expression was significantly diminished 2 h after FN variant stimulation of *Fn1* $-/-$ MEFs, and the reduction pattern was opposite to that observed for the aforementioned induced genes, with B-A- and B+A+ resulting in the strongest decrease, followed by B+A- and then by B-A+ (**Complementary Fig. 3A**). This drop in Id1 expression might explain, at least in part, the sustained proliferation of cFN-treated MEFs in culture.

Furthermore, mRNA expression of Endoglin1/3, an inhibitor of TGF- β signaling [See (Heldin and Moustakas 2016) and references therein], displayed a similar expression pattern, though weaker, to Id1 (**Complementary Fig. 3B**), suggesting that



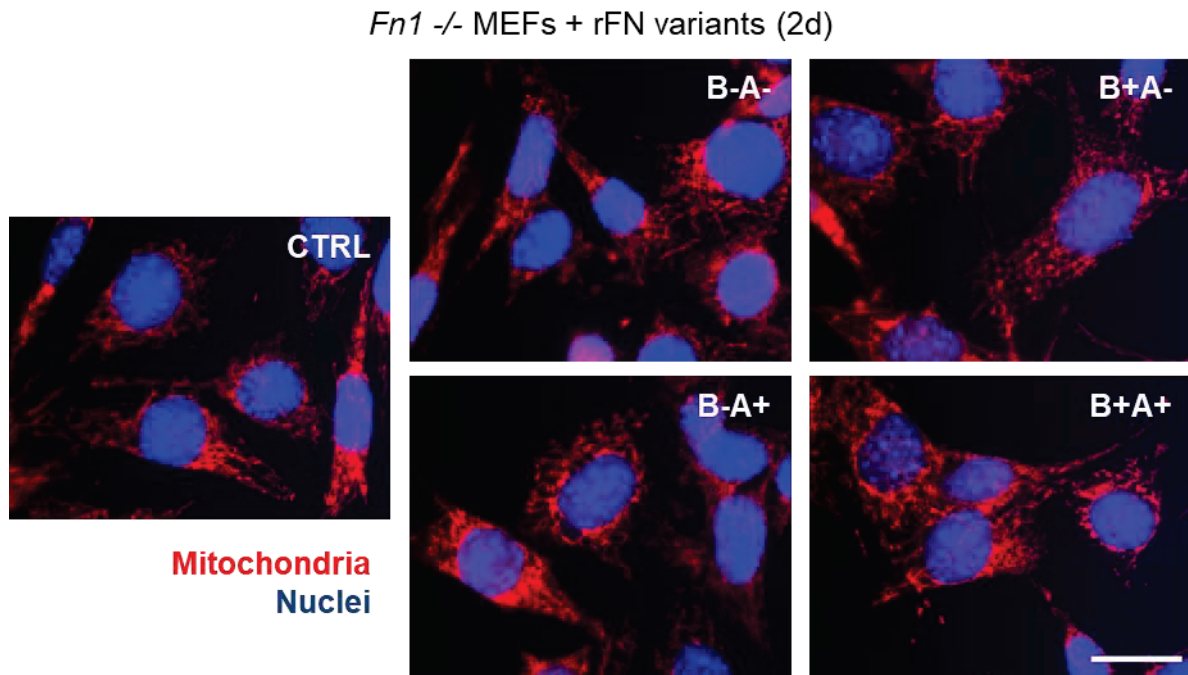
Complementary Fig. 3. FN-mediated expression regulation of TGF- β modulators.

Relative mRNA expression of two known TGF- β signaling regulators, (A) Id1 and (B) Endoglins 1 and 3 after a 2-hour stimulation of *Fn1* $-/-$ MEFs with FN variants. Results are mean \pm SEM values of two or more independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

FN variants modulate TGF- β activation and signaling through multiple branches within the pathway.

Role of FN variants in mitochondrial morphology

In order to examine the possible effect of FN variants in mitochondrial quantity and morphology, we used a mitochondria-specific tracer, in cells stimulated with FN



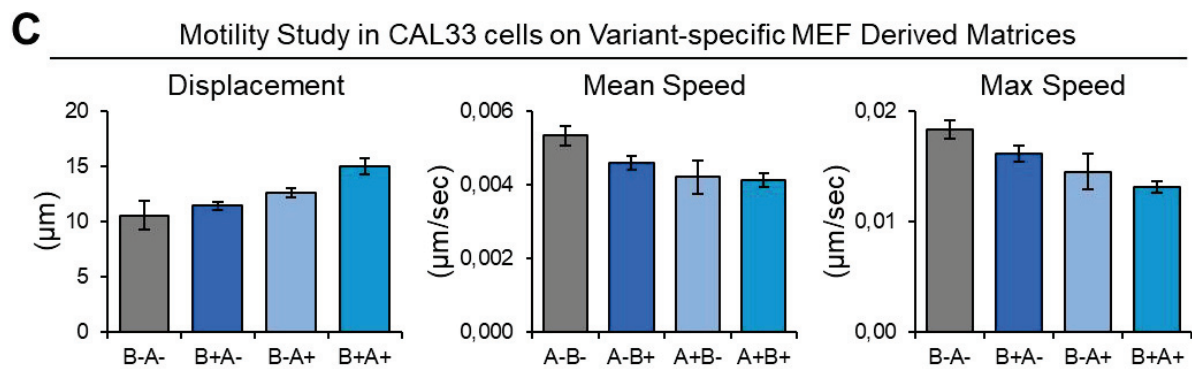
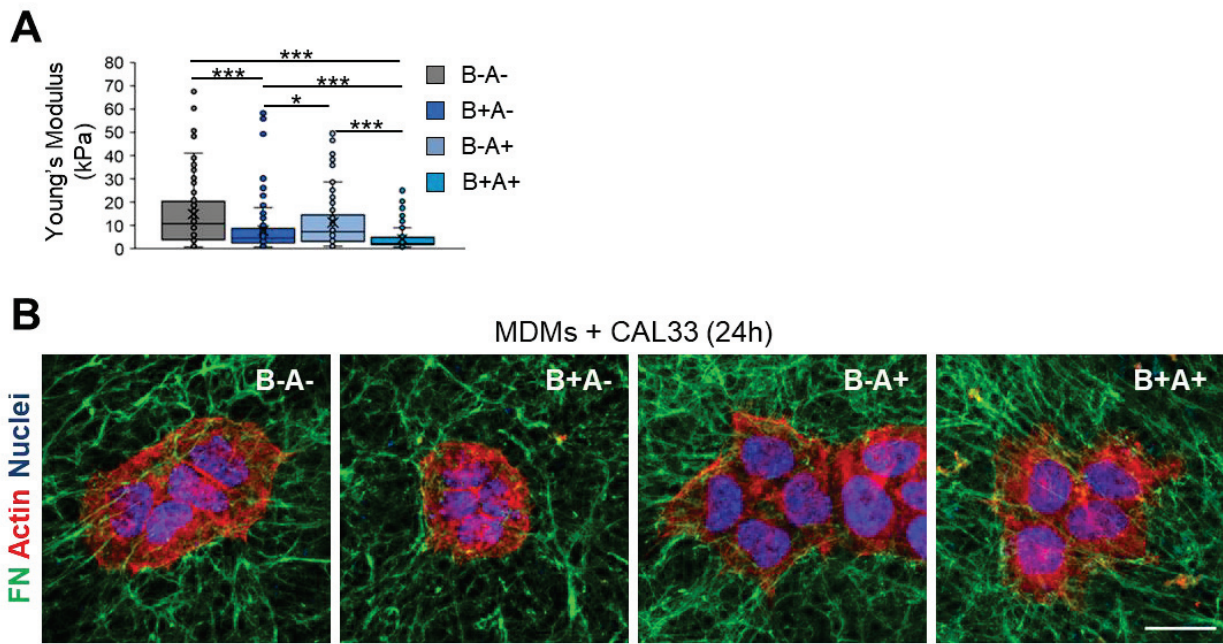
Complementary Fig. 4. FN role in mitochondrial morphology

Treatment of *Fn1*^{-/-} MEFs with FN variants for 48h to study the effect of FN Extra Domains in mitochondrial morphology. Representative immunofluorescence images of two independent experiments. *Fn1*^{-/-} MEFs were plated in 12-well plates with coverslips (5x10⁴ cells/ml) in DMEM supplemented with 10% FN-depleted FCS and incubated at 37°C. Upon attachment, cells were stimulated with FN variants (15 μ g/ml) for 48 h. They were subsequently incubated with 200 nM MitoTracker CMX Ros (Thermo Fisher Scientific) for 40 min in DMEM before fixation and further staining according to standard procedures. Red, mitochondria; blue, nuclei. FN staining has been omitted for clarity. Scale bar: 25 μ m

variants or vehicle buffer for 48 h. In line with the results obtained with the Seahorse analyzer, no differences were observed in mitochondrial morphology and quantity between FN-stimulated and CTRL cells (Complementary Fig. 4).

Physical and biochemical properties of FN variant-specific matrices.

We saw previously that the presence (or absence) of the Extra Domains in the FN molecule results in differences in FN fibrillogenesis and assembly, and in MEF-derived matrices with different physical properties, namely fiber topology, porosity, and



thickness. In order to study whether these properties reflect differences in mechanical resilience of the matrices, we measured the elastic (Young's) modulus by Atomic Force Microscopy (AFM). As expected, the thick B+A- matrices were less stiff compared to the thin B-A+ matrices, though the differences were only slight. Conversely, both B-A- and B+A+ were of equivalent thickness but the former was the matrix most resistant to indentation, while the latter was the softest (**Complementary Fig. 5A**), showing that there is no direct correlation between matrix thickness and stiffness. However, additional elegant techniques are required to measure local mechanical properties of these matrices, since their mean thickness is close to the limit of the AFM movement in the z-axis, which can possibly introduce technical artefacts to the analysis.

◀**Complementary Fig. 5. Physical and biochemical properties of FN variant-specific matrices (previous page).**

(A) AFM microscopy results displaying variant-specific matrix stiffness. Variant-specific MEF-derived matrices were generated in 35 mm dishes as described in Methods. Matrices were rinsed and covered with 3 ml of PBS. The mechanical properties of the samples were studied using a BioScope Catalyst atomic force microscope (Bruker Nano Surfaces, Santa Barbara, CA, USA) equipped with a Nanoscope V controller and coupled with an optical microscope (Leica DMI6000B, Leica Microsystems Ltd., UK). After thermal stabilization for each sample 4 different areas were analyzed using the “Point and Shoot” method, collecting a maximum of 140 force-distance curves at just as many discrete points spaced at least 20 μm from each other. The experiments were performed using a borosilicate glass spherical tip (5 μm of diameter) and a cantilever with a nominal spring constant of 0.06 N/m (Novascan Technologies, Ames, IA USA). After determining both the deflection sensitivity of the system in the PBS using a clean glass dish and the spring constant of the cantilever by the thermal tune method, force-distance curves were collected on samples using a velocity of 1 $\mu\text{m/s}$, in relative trigger mode and by setting the trigger threshold to 2 nN. The apparent Young's (elastic) modulus was calculated using the NanoScope Analysis 1.80 software (Bruker Nano Surfaces, Santa Barbara, CA, USA), fitting the force curves to the Hertz spherical indentation model and using a Poisson's ratio of 0.5. To avoid large indentation, a minimum and a maximum Force Fit Boundary of 5% and 25% respectively of the whole force curve was considered for the fit. To eliminate any tilt effect due to the base correction step in the analysis, only the force curves having their maximum value at 2 nN were taken into account for performing the fit. Data analysis was performed with GraphPad Prism v 5.03. Results are shown as a Tukey boxplot. Statistical significance was determined with the Kruskal-Wallis test for non-parametric distributions, followed by a Dunns multiple comparison test. (B) Representative immunofluorescence images of CAL33 (an oral squamous cell carcinoma cell line) attached on FN variant-specific MEF-derived matrices. MEF-derived matrices were generated as described in Methods. CAL33 cells were plated on top of these matrices (2×10^5 cells/ml) for 24 h and were subsequently fixed and stained according to standard protocol. Scale bar: 25 μm . (C) Motility study in CAL33 plated on top of variant-specific matrices, as in (B). MEF-derived matrices were generated as described in Methods. CAL33 cells were plated on top of these matrices (2×10^5 cells/ml) and were monitored with timelapse microscopy for 24 h. Displacement, mean and maximal speed were calculated. Graphs display mean \pm SEM values from three fields per variant in a representative experiment.

From a different point of view, these matrices are ideal substrates for customized cell culture. In contrast to tissue culture plastic surfaces, these matrices offer a physiologically more relevant environment, containing a wide range of ECM components. ECM properties have been known to modulate the behavior of cells that reside in them [see (Walker, Mojares, and del Río Hernández 2018; Kato et al. 2013) and references therein]. In order to test whether engineered Extra-Domain-specific FN matrices influence cell behavior, we plated squamous cell carcinoma cells (CAL33) on variant-specific matrices and examined their morphology (**Complementary Fig. 5B**) and motility (**Complementary Fig. 5C**). Although CAL33 cells attached and proliferated normally on MDMs (data not shown), their motility was profoundly perturbed. Instead of maintaining a guided migratory pattern (Gopal et al. 2017), they amble around their initial position, probably due to the embryonic nature of these matrices. Though the role of variant-specific matrices in CAL33 motility remains

inconclusive, these results underline the importance of cell-substrate compatibility in cell culture experimentation.

5. Conclusions and Perspectives

In this work, we addressed the role of the Extra Domains B and A of FN in *Fn1* $-/-$ MEFs using native, full-length, human FN constructs containing either, both or none of the Extra Domains. We used MEF clones that stably express ED-specific FN, and we concluded that the abrogation of the multi-level FN regulation as well as the clonal variabilities mandated for an alternative approach, in which purified FN variants were presented to a single assembly-competent FN-null MEF clone.

Using this model system, we observed that FNs containing the Extra Domain B (FN B+A-, and FN B+A+) were more readily harvested by cells and assembled into a fibrillar network, followed by FN B-A+. Plasma-like FN (B-A-) was the least efficient in FN fibrillogenesis. Additionally, the presence of the Extra Domains resulted in ECMs with differential physical properties, such as thickness and fiber topology, and these differences were efficiently recognized by an unbiased numerical method, suggesting a distinctive FN fibrillogenesis and assembly.

An important aspect of this work was the variant-dependent induction extent of the SMAD2/3 branch of TGF- β signaling. FN B+A+ was the most potent agonist of TGF- β activation followed, intriguingly, by FN B-A-, while FN B+A- and FN B-A+ resulted in lower pathway induction. More studies are required in order to understand the mechanism of this FN-mediated TGF- β activation, and to discover which other pathways are involved that result in the distinct patterns of cell proliferation and metabolic phenotypes demonstrated.

As a first step, elegant studies to examine the conformational modifications of FN variants in solution and during early fibrillogenesis is an interesting approach. The combination of our toolset with the use of FRET as an indicator of FN conformation (Baneyx, Baugh, and Vogel 2002) is an attractive perspective. Furthermore, ED-specific integrin-mediated FN fibrillogenesis and the identification of partners that facilitate these interactions are of substantial scientific interest.

In terms of TGF- β signaling, LTBP- and GARP-oriented studies will provide insight in ED-dependent TGF- β activation, while further understanding of TGF- β target gene regulation may elucidate roles of cFN in sustaining proliferative signaling

and in modulation of cell metabolism. To that end, the involvement of known regulators of cell energetics should be assessed upon stimulation of cells with FN variants.

Finally, variant-specific MEF-derived matrices constitute an ideal substrate to study the paracrine effects of the FN variants on integrin signaling, cytoskeletal organization, contractile behavior, and motility of various cell types, such as immune, vascular, and tumor cells. These models may be used as effective tools in novel drug design and screening, and in translational research.

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7. Annex 1: Fibronectin Timeline

1948 - A protein fraction separable from the bulk of the fibrinogen in Cohn fraction I (**Morisson et al**)

1955 - The “Morisson” fraction is a mixture of several components among which is the “cold insoluble globulin” and some fibrinogen (**Edsall et al**)

1957 - A protein in heparin-induced precipitate of plasma has properties similar to cold insoluble globulin (**Smith and van Korff**)

1967 - Partial purification of a plasma fraction that precipitated with gelatin (**Wolff et al**)

1968 - A cryoprecipitate was observed in plasma from a patient with intravascular coagulation syndrome. It contained fibrinogen and another component (**Mosesson et al**)

1970 - Cold insoluble globulin was purified to homogeneity from plasma cryoprecipitate. It is found in fibrin clot during coagulation and in platelet lysates (**Mosesson and Umfleet**)

1973 - A large glycoprotein (galactoprotein a) was identified on the cell surface of fibroblasts that was lost upon oncogenic transformation by tumor viruses (**Hynes et al; Gahmberg and Hakamori**)

1973 - An antiserum was described that recognized a fibroblast surface antigen - SF-A (**Ruoslahti et al**)

1974 - The aforementioned antiserum reacts both with SF-A as well as an antigen in the serum (**Ruoslahti, Vaheri et al**)

1974 - Isolation of the Large Cell Surface Protein (CSP) from fibroblasts, which proved to be the same as galactoprotein a (**Yamada and Weston**)

1974 - CSP depends on the growth state in normal cells (**Hynes and Bye**)

1974 - A serum factor promotes cell adhesion to collagen (**Klebe**)

1974 - SFA localization was studied by immunofluorescence and scanning electron microscopy of chick embryo fibroblasts. The antigen was found to membrane processes and ridges, it was removed upon trypsin treatment, and it almost undetected after transformation by RSV (**Wartiovaara et al**)

1975 - The serum protein is identified as the cold insoluble globulin (**Ruoslahti and Vaheri**)

1975 - The cold insoluble protein is probably very similar to the large cell surface protein

1976 - A serum factor promotes cell adhesion to plastic (**Grinnell**). This factor (and the previously mentioned one -Klebe) were shown to be identical with cold insoluble protein

1976 - An anti-CIG antibody is used to study the localization of the large external transformation sensitive protein in adenovirus-transformed cell lines and in established tumor cell lines. The cold insoluble globulin in blood plasma is the soluble form of the cell surface protein (**Chen et al**)

1977 - Both the plasma and the cellular protein were involved in various aspects of cell adhesion and morphology (**several references**)

1977 - The gelatin affinity chromatography was developed as a rapid and simple method for purifying both forms of the protein (**Engvall and Ruoslahti**)

1977 - The term "fibronectins" was coined, to denote a class of large glycoproteins widely distribution, that interacted with a variety of other molecules, and were involved in cellular adhesion (see reviews of **Vaheri and Mosher 1978; Yamada and Olden 1978**)

1982 - A relatively clear outline of fibronectin structure and function emerged (See **Mosher 1980; Ruoslahti et al 1981; Hynes 1981; Hynes and Yamada 1982**)

1983 - Isolation of cDNA clones for FN from a rat liver library in the expression vector λ gt11. Restriction mapping and sequencing reveal at least three different FN mRNAs in rat mRNA liver which differ in coding potential. The three mRNAs probably arise from a single gene by alternative splicing (**JE Schwarzbauer**)

1984 - Four independent FN cDNA clones were established from a library with a synthetic oligonucleotide primer. Two different FN mRNA species were detected: mRNA I and mRNA II are both found in a human cell line, but mRNA I is undetectable in the liver. This mRNA species harbors an Extra Domain (ED) of 270 nucleotides that corresponds to a 90 amino acids type III repeat near the C-terminus of FN (**Kornblihtt AR et al**)

1984 - With the use of five independent cDNA clones and S1 nuclease mapping, the aforementioned mRNA species were found to be cell type specific with mRNA I being expressed only by normal fibroblasts in culture, hepatoma cells, and breast tumor cells. Furthermore, with sequence alignment a 89nt alternatively spliced region in the C-terminal end of human FN was described (**Kornblihtt A et al**)

1984 - By using a monoclonal antibody which inhibits cell attachment, the domain within the fibronectin molecule which endows it with the ability to interact with the cell surface of fibroblasts was isolated as a peptidic fragment, and the complete amino acid sequence was determined (**Pierschbacher M et al**)

1987 - Through isolation of the human genomic clone containing part of the human FN locus, restriction mapping, and S1 nuclease mapping, a second type III repeat was found to be alternatively spliced through an exon skipping mechanism (**Gutman A et al**)

1988 - In situ hybridization in developing chicken embryos shows the distribution and alternative splicing of FN mRNA (**Ffrench-Constant C et al**)

1988 - Using a monoclonal antibody specific for EDA, inclusion of the extra domain was studied upon treatment of normal human fibroblasts with transforming growth factor β , and dexamethasone. While the latter does not significantly change the ratio between the total FN and the EDA-FN, TGF β preferentially increases the expression of the FN isoform containing the EDA sequence (**Balza E et al**)

1989 - Construction of α -globin - fibronectin minigenes containing the EDA and/or EDB regions and expression of the minigenes in different cell lines shows that all the information required to produce accurate and regulated alternative splicing is contained within the fragments, and cell specific factors are necessary for differential splicing across various cell lines (**Barone MV et al**)

1989 - The distribution of the different spliced forms of FN in developing chicken embryos was determined using probes specific for the spliced regions in ribonuclease protection and in situ hybridization experiments. Spatially distinct splicing differences were described with cell-type-specific splicing excluding EIIIA and/or EIIB in some tissues (**Ffrench-Constant C et al**)

1989 - In situ hybridization with segment-specific probes was performed on healing wounds in adult rat skin. FN expression is increased after wounding and the pattern of FN mRNA splicing was different, resembling that found during early embryogenesis (**Ffrench-Constant C et al**)

1989 - Focus on the V region of the rat fibronectin and its role in FN secretion. Fragments of FN (deminectins) containing the V120 or the V95 segments are readily secreted, while the V0 containing fragments never reach the cell surface and are most probably degraded intracellularly. Co-expression of V0 and V120 fragments leads in the formation of heterodimers among which the ones containing only the V0 are retained intracellularly (**Schwarzbauer J et al**)

1990 - Utilizing the S1 nuclease analysis, the levels of EDA and EDB containing mRNAs of FN in cultured normal human skin fibroblasts before and after TGF β treatment were examined. It was shown that TGF β increases the relative amount of mRNA for EDA and EDB containing FN isoforms, demonstrating that a growth factor may regulate the splicing pattern of a pre-mRNA (**Borsi L et al**)

1990 - Retroviral vectors containing the full-length rat FN with or without the extra domain and/or the variable region were generated (8 variants in total). Constructs were stably expressed in NIH 3T3 cells (*not FN -/-*), and in lymphoid WEHI231 cells for rFN variant isolation. A-B-, B+, A+, V+ promote adhesion and spreading in all cell lines tested. All FN isoforms induced cytoskeletal organization. Cell migration was studied with a modification of the Boyden chamber approach. All variants promoted cell migration to similar extents. Incorporation of variants into matrix was tested. The inclusion of EDA or EDB enhances the ability of FN to incorporate into existing matrix. Observation that the lymphoid cell line that expresses the IIICS+ FN attaches on the

bottom of the culture dish instead of being a suspension culture as the control (**Guan J et al**)

1991 - Inclusion of EDA, EDB and the different regions of the IIIICS was assessed by RT-PCR in cells derived from young and old rats, as well as from different human cell lines. It was observed that aging tissues show a decreased inclusion of the alternatively spliced regions. Additionally, the inclusion of the extra domains was enhanced in permanent cell lines compared to primary cultures, probably due to the presence of growth factors. Indeed, TGF β -1, retinoic acid, and 1.25-dihydroxyvitamin D3 can all change the alternative splicing of FN pre-mRNA in the EDA, EDB, and IIIICS exons (**Magnuson VL et al**)

1992 - Using nuclear magnetic resonance techniques, the solution structure of the tenth FNIII repeat has been determined. The cell binding domain is found in the repeat and is composed of an Arg-Gly-Asp tripeptide that lies on a mobile loop between strands FN and G (**Main AL et al**)

1992 - Generation of an antibody against EDB and subsequent staining of different clones that express different forms of FN or an EDB-containing β -galactosidase-FN fusion protein. The antibody is specific for EDB containing FN but does not bind to EDB itself. The recognized epitope lies within the adjacent FNIII₇ domain and it is masked when EDB is absent, clearly demonstrating that the presence of the EDB generates conformational modification in the central part of the molecule (**Carnemolla B et al**)

1993 - The FN gene was inactivated in a rat model by deleting the translation initiation sequence. Homozygous mutant embryos show severe defects in mesodermally derived tissues related to mesodermal migration, adhesion, proliferation or differentiation. Heterozygous embryos express half the amount of normally produced FN, yet are normal (**George EL et al**)

1994 - A series of mutated fragments harboring the FN-EDA domain were transfected into cell lines to study the differential RNA processing. Two short nucleotide sequences have been described: one positive modulator (element A) and a negative modulator (element B) within the sequence of FN-EDA (**Caputi M et al**)

1994 - The expression of EDA and EDB FN was measured in an experimental hepatic fibrosis model in the rat. EDA-FN was expressed 12h after injury preceding fibrosis, while EDB-FN was expressed at lower level with a 24h lag. EDA expression was confirmed in a cell culture model. Matrices deposited by endothelial cells from injured liver accelerated the conversion (activation) of normal lipocytes to myofibroblast-like cells, while pretreatment with a specific anti-EDA antibody blocked this response. Finally, recombinant 6xHis-tagged FN peptides containing EDA promoted the activation of normal lipocytes in culture (**Jarnagin WR et al**)

1995 - RT-PCR was used to estimate the FN transcription rate and inclusion of EDA and EDB before and after partial hepatectomy in rats. Despite the fact that the liver is the major source of plasma FN, which is characterized by the absence of EDA and EDB, in the rat regenerating liver there is a significant reprogramming of the splicing machinery that results in the synthesis by the liver of up to 17% of EDA-FN linked with all the IIICS forms. On the other hand, EDB is completely absent both in normal and regenerating liver (**Caputi M et al**)

1995 - Human plasma FN and recombinant FN segments (FNIII₁₁, FNIII₁₂, EDA) expressed in bacteria were used to show that EDA has a complementary action to that of plasma FN in promoting adhesions and transmembrane signaling responses, including organizations of stress fibers and assembly of focal contacts (**Xia P et al**)

1997 - Alternative splicing of EDB (in the level of mRNA and protein) was studied in rats during liver growth. HRS expression correlated with expression of FN containing EDB in a temporal manner, particularly in non-parenchymal liver cells. Using a FN-EDB minigene it was shown that HRS has the potential to mediate inclusion of EDB during processing of FN pre-mRNA (**Keyong D et al**)

1997 - Full length human recombinant F variants, MBP-FN fragment fusion proteins, and GST-FN fragment fusion proteins were generated. The full-length FN construct harboring the extra domains include the IIICS₁₂₀ region and were expressed as chimeric proteins with the signal sequence of human protein C inhibitor. HT1080 cells were plated on FN coated substrates. EDA+ FN was significantly more potent than EDA- FN in promoting cell spreading and cell migration, irrespective of the presence or absence of the second alternatively spliced EDB (**Manabe R et al**)

1997 - Full length human FN isoforms differing in the presence or absence of the EDA and/or EDB segments were constructed to test their cell-adhesive activities, promotion of cell spreading, and cell migration. EDA+ FN was more potent in promoting cell spreading, migration, irrespective of the presence or absence of a second alternatively spliced segment (EDB). The cell spreading activity of EDA+ FN was not affected by antibodies recognizing the EDA segment but was abolished by antibodies against $\alpha 5\beta 1$ and by Gly-Arg-Gly-Asp-Ser-Pro peptide, indicating that the EDA segment enhanced the cell-adhesive activity of FN by potentiating the interaction of FN with $\alpha 5\beta 1$. Additionally, purified $\alpha 5\beta 1$ bound more avidly to EDA+ FN than to EDA- FN. These effects were observed only in the context of the intact FN molecule, since the difference in integrin-binding activity between EDA+ FN and EDA- FN was abolished in the case of case of FN fragments (**Manabe R et al**)

1998 - Plasma FN and cellular FN (A+B+) were purified from human plasma and from conditioned medium of the SV-40-transformed embryonic human lung WI-38-VA cell line. 6xHis-tagged EDA was expressed in E. coli and purified in a Ni-NTA resin column. In vivo skin wounds were performed in rats for granulation tissue

sample collection. EDA-FN deposition precedes α SMA expression by fibroblasts during granulation tissue evolution in vivo and after TGF β 1 stimulation in vitro.

1999 - Molecular dynamics simulations for the FNIII₁₀ were carried out. The N-terminal end was fix in position and a force was applied to the C-terminal end. Increasing the applied force, the domain is distorted with hydrogen bonds between the β -sheets being broken apart. Further pulling leads to total disintegration of the modules with all the strands aligned. This suggests that FNIII₁₀ constitutes a mechanosensitive control of ligand recognition (**Krammer A et al**)

1999 - Full length human recombinant FN variants were generated for functional analysis of the extra domains B and A (B-A-, B+A-, B-A+, B+A+). Coating experiments were performed with CHO-K1 cells to study attachment and spreading, integrin binding, induction of DNA synthesis, MAP kinase activation and tyrosine phosphorylation of p130 and FAK. Authors conclude that the inclusion of EDA through alternative splicing promotes FN-induced cell cycle progression through upregulation of integrin-mediated mitogenic signal transduction (**Manabe R et al**)

1999 - His-tagged FNIII repeats, EDA containing fragments, and purified placental FN (all human) were used for functional studies in rabbit cartilage explants, chondrocytes and synovial cells. EDA alone can induce the production of MMPs (-1, -3, -9), while other FNIII domains cannot. This induction depends on IL1 and is abolished when EDA is flanked by FNIII repeats, suggesting that exposure of the NH₃- or the -COOH end of the EDA domain by proteolytic cleavage releases the inducing activity (**Saito S et al**)

2001 - FN Extra Domain fragments (6xHis-tagged EDA and EDB), FNIII₁ C-terminus, human cellular FN (by Sigma) were used. EDA exclusively was able to activate TLR4. This activation persisted in the presence of the LPS-binding antibiotic polymyxin B, and a potent LPS antagonist. EDA-containing FN fragments promote expression of genes involved in the inflammatory response (**Okamura Y et al**)

2001 - Generation of mice harboring a "floxed" FN gene in order to establish mice with an inducible tissue specific deletion of the fibronectin gene. pFN-deficient mice show increased neuronal apoptosis and larger infarction areas after transient focal cerebral ischemia. On the other hand, pFN is dispensable for skin-wound healing and hemostasis. (**Sakai T et al**)

2001 - Study the levels of circulating cellular fibronectin in diabetes patients using a monoclonal antibody against the EDA segment of FN. Circulating cellular fibrinectin was significantly elevated in patients with diabetes compared with patients with ischemic stroke, patients with renovascular hypertension, and with healthy subjects. Cellular fibrinectin may be a marker protein for endothelial cell activation, especially in diabetes (**Kanters SDJM et al**)

2002 - FN in solution was probed with two fluorophores that display FRET when FN is in a compact form. Cell forces can induce conformation changes in the FNIII domains resulting in a stretched conformation that abolishes FRET, supporting a model of FN fibril elasticity based on unraveling and refolding of FNIII modules (**Baneyx G et al**)

2002 - Rat FN-EDA sequence was compared against known proteins that contain peptides bound by $\alpha 9\beta 1$ and $\alpha 4\beta 1$. Wild type and mutant FN-EDA segments were coated in microtiter plates and cell adhesion was measured (**Yung-Feng L et al**)

2002 - Mice lacking the EDB exon were generated using the Cre-Lox system. EDB-deficient mice develop normally and are fertile. No significant phenotypes observed in vivo. Isolated fibroblasts, however, grow more slowly and deposit less FN in the pericellular matrix than wild type fibroblasts underlying a potential role in the growth of connective tissue cells via the FN matrix (**Fukuda T et al**)

2003 - Establishment of a mouse model comprised of animals that constitutively express EDA+ FN, and animals that are homozygous for EDA- FN. EDA is not necessary for FN matrix assembly, neither for embryonic development. On the other hand, EDA lacking mice display abnormal wound healing. Both mutant mice have a significantly shorter lifespan, suggesting that EDA splicing regulation is necessary for efficient long-term maintenance of biological functions (**Muro AF et al**)

2003 - Generation of EDA-/- mice and crossing with ApoE-/- mice to study the effect of EDA in arterial wall lesion development. EDA-FN was increased in the ApoE-/- mice and total plasma cholesterol was reduced in EDA-/-ApoE-/- mice, suggesting a potential role of FN-EDA in plasma lipoprotein metabolism (**Tan MH et al**)

2004 - A series of FN-EDA minigenes was generated with variations in the splicing enhancer and splicing silencing elements. The sequence context as well as the primary sequence identity can contribute to the making of functional units capable of influencing pre-mRNA splicing. (**Buratti E et al**)

2004 - EDB-/- and EDA-/- mice strains were used to examine neovascularization of mouse retinas, pancreatic tumors and transplanted melanomas. The absence of EDA and EDB did not significantly affect physiological or tumor angiogenesis. Tumor growth was not affected neither was the expression level of α SMA. The presence of EDA or EDB splice variants individually is not essential for neovascularization (**Astrof S et al**)

2005 - EDA +/+ and EDA -/- mice were generated by using gene targeting. The absence of regulated splicing of EDA has behavioral consequences. Deletion of EDA results in reduced motor coordination abilities and vertical exploratory capacity, whereas mice constitutively expressing EDA+ FN display a in locomotor activity in the open field test. Results suggest that the EDA exon is necessary for a normal function of the brain. (**Chauhan AK et al**)

2006 - Generation of antibodies and compounds to target EDB FN for visualization of tumors in mice provides a potentially useful clinical tool for imaging of angiogenesis-associated EDB-FN expressing human tumors. (**Berndorff D et al**)

2007 - Blood was collected from affected and non-affected members of 15 pedigrees with incidents of glomerulopathy with FN deposits and linkage analysis was performed on the FN chromosomal region. The mutations found were introduced into human cDNA encoding repeats III12-14 and expressed as His-tagged fusion proteins in Sf9 cells by the Baculovirus system. Mutated proteins resulted in reduced cell spreading and impaired cytoskeletal reorganization (**Castelleti F et al**)

2007 - The RGD motif was replaced with the inactive RGE motif in mice. FN-RGE homozygous mice died at embryonic day 10, though FN matrix assembly was not compromised in mutant embryos or on mutant cells. FN-RGE matrix assembly can be mediated by $\alpha V\beta 3$ integrin instead as shown by $\alpha 5$ knockdown experiments, as well as assembly assays utilizing FN fragments (FNI₁₋₁₀, FNIII₁₋₅, FNIII₇₋₁₀) (**Takahashi et al**)

2007 - Both EDA and EDB exons were simultaneously deleted from the FN gene in mice. Embryonic lethality was observed on day E10.5. FN synthesis and assembly were not affected. Double null embryos displayed extensive cardiovascular defects, including vascular hemorrhage, failure of remodeling embryonic and yolk sac vasculature, defective placental angiogenesis and heart defects. (**Astrof S et al**)

2007 - Patient derived primary lung fibroblasts, mice lacking the EDA domain and their wild type littermates were used as experimental model to investigate the role of EDA-containing fibronectin in lung fibrogenesis. Idiopathic pulmonary fibrosis lung fibroblasts produced markedly more EDA-containing fibronectin and α SMA than control fibroblasts. EDA-null mice failed to develop significant fibrosis 21 days after bleomycin challenge, whereas wild type controls developed the expected increase in total lung collagen. Failure to develop lung fibrosis in EDA-null mice correlated with diminished activation of latent transforming growth factor (TGF)- β and decreased lung fibroblast responsiveness to active TGF- β in vitro. (**Muro AF et al**)

2007 - Mice devoid of regulated splicing at the EDA exon were used to study the role of EDA-FN in thrombosis. EDA+ FN accelerates thrombosis both in vitro and in vivo at arterial shear rates. However, the presence of EDA affected neither single platelet adhesion to subendothelium nor thrombosis in veins. EDA+ FN has prothrombotic activity and its presence in plasma may worsen pathological conditions in which this form is elevated. (**Chauhan AK et al**)

2007 - Generation of mice modified to produce EDA+ FN in the liver showed that these animals have normal extracellular matrix FN levels but secrete less soluble FN. EDA-exon deletion resulted in restoring the FN levels in both plasma and tissues.

Thus, an important fraction of tissue FN is actually plasma derived, suggesting that plasma is an important source of tissue FN (**Moretti FA et al**)

2007 - His-tagged rat FN-EDA segment or FNIII₄-EDA hybrids were used in order to identify the peptide sequence within the EDA domain that mediates $\alpha 9\beta 1$ -dependent cellular responses. The residues responsible for integrin binding were found to be Asp⁴¹ and Arg⁴² that reside within the C-C' loop of EDA. rFN constructs (His-tagged A+RGD+, A+RGD-, A-RGD+, A-RGD-) were also used in coating experiments to assess integrin binding and cell behavior. The presence of EDA stimulates the formation of filopodial protrusions at the cell periphery, as well as Cdc42 activation (**Shinde AV et al**)

2007 - To analyze the role of FN isoforms in atherosclerotic lesion formation, mouse strains devoid of EDA-exon regulated splicing were utilized, that constitutively include (EDA+/+) or exclude (EDA-/-) the exon. Both mutant mice had a 40% reduction in atherosclerotic lesions after the atherogenic diet treatment associated to a lower capacity of macrophages to uptake modified LDL and undergo foam-cell formation. Lesions in control mice were more numerous and bigger with augmented and deeper macrophage infiltration, and increased FN expression in the sub-endothelial area (**Babaev VR et al**)

2009 - Using a mouse tumor model, the impact of different angiogenic microenvironments on FN-EDB expression was investigated. The angiogenic microenvironment, and in particular the VEGF/VEGFR-2 system, plays a key role in modulating EDB-FN expression by tumor cells in vivo. This may have implications for the design of therapeutic strategies targeting EDB-FN in combination with anti-angiogenic and/or cytotoxic drugs (**Coltrini D et al**)

2010 - An siRNA approach targeting specifically EDA- or EDB-containing FN showed that silencing of cFN variants differentially affects integrin usage, cell spreading, motility and capillary morphogenesis in vitro. cFN-deficient cells undergo a switch from $\alpha 5\beta 1$ - to $\alpha v\beta 3$ -based adhesion, accompanied by a Src-regulated disruption of adherens junctions. (**Cseh et al**)

2012 - 6xHis-tagged FN fragments (FNIII₇₋₁₀ including EDB) were subject to x-ray structural analysis. A tendency toward RDB-dependent dimer formation in solution was described. Insertion of EDB appears to stabilize overall head-to-tail dimerization of two separate FN chains which together with alternating homodimer formation via S-S bridges at the C-terminus should lead to the known macromolecular fibril formation (**Schiefner A et al**)

2012 - Using engineered antibody fragments, new EDB-specific anticalins were generated that can provide useful reagents in research and biomedical drug development, both for in vivo imaging and for directed cancer therapy (**Gebauer M et al**)

2016 - The osteoblast-mediated role of FN in hematopoiesis was studied in a mouse model. FN was depleted specifically in osteoblasts in mice affecting myelopoiesis. FN constructs (pFN, EDA, EDB - no mention if they are fragments, full length, nor their source) were transfected into a cancer cell line in which FN had been deleted using shRNA (check the figure!). EDA-FN augments myeloid cells in vivo via $\alpha 5\beta 1$ thereby increasing cancer growth, which is reverted after $\alpha 5\beta 1$ blocking. These results establish an immune regulatory function for EDA-FN originating from the osteoblasts and identify new avenues for enhancing the immune reaction against cancer (**Rosnagl S et al**)

2016 - A mouse model and cell culture was used to determine the role of FN in endocytosis. Fn constructs were the same as those in Rosnagl et al 2016 (same team). Fibronectin containing an extra domain called EDB is released in bacterial meningitis. EDB-containing FN enhances phagocytosis more than plasma FN. The enhancement is mediated by activation of $\alpha v\beta 3$ in the presence of EDB. (**Kraft S et al**)

2017 - Patient derived cells, human cerebral microvascular endothelial cells, and human glioblastoma cell lines were used to study the interplay between TGF β and oncofetal FN. A positive correlation was identified in the expression of EDA+ and EDB+ with molecules associated with TGF β signaling. Using FNIII fragments (single Extra Domains, or Extra Domains flanked by their naturally adjacent sequences), the authors described that FN modulates TGF β superfamily signaling in endothelial cells via the EDA and EDB domains pointing toward a bidirectional influence between oncofetal FN and TGF β superfamily signaling (**Ventura E et al**)

2017 - A human pFN cDNA clone and a human cDNA fibronectin clone containing the EDA and EDB domains were used to generate expression constructs (no A+B+ used). Constructs were used for stable transfections. Fibronectin containing EDA produced by osteoblasts enhances their differentiation via $\alpha 4\beta 1$. On the other hand, EDB enhances FN binding to $\beta 3$ integrin through the RGD sequence, resulting in increased mineralization by and differentiation of osteoblasts. (**Sens C et al**)

2018 - Rat FN domain peptides expressed in *E. coli*, and full length rat FN constructs harboring one, both, or no extra domains (all 6xHis-tagged) were used to study the role of the extra domains in LTBP1 binding and TGF β activation. High ECM stiffness promotes expression and co-localization of LTBP1 and EDA-containing FN. Additionally, LTBP1 bound more effectively to ED+ FN than to EDB+ FN and FN lacking extra domains. Authors suggest that EDA domain enhances association of the latent TGF $\beta 1$ by promoting weak direct binding to LTBP1 and by enhancing heparin-mediated protein interactions through HepII in FN (**Klingberg F et al**)

8. Annex 2: Classification of the fibronectin variants with curvelets

Classification of the fibronectin variants with curvelets

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HAL Id: hal-01868726

<https://hal.archives-ouvertes.fr/hal-01868726>

Submitted on 5 Sep 2018

To cite this work:

Anca-Ioana Grapa, Raphael Meunier, Laure Blanc-Féraud, Georgios Efthymiou, Sébastien Schaub, et al. Classification of the fibronectin variants with curvelets. ISBI 2018 - IEEE 15th International Symposium on Biomedical Imaging, Apr 2018, Washington, DC, United States. pp. 930-933, 10.1109/ISBI.2018.8363723. hal-01868726

CLASSIFICATION OF THE FIBRONECTIN VARIANTS WITH CURVELETS

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ABSTRACT

The role of the extracellular matrix (ECM) in the evolution of certain diseases (e.g. fibrosis, cancer) is generally accepted but yet to be completely understood. A numerical model that captures the physical properties of the ECM, could convey certain connections between the topology of its constituents and their associated biological features. This study addresses the analysis and modeling of fibrillar networks containing Fibronectin (FN) networks, a major ECM molecule, from 2D confocal microscopy images. We leveraged the advantages of the fast discrete curvelet transform (FDCT), in order to obtain a multiscale and multidirectional representation of the FN fibrillar networks. This step was validated by performing a classification among the different variants of FN upregulated in disease states with a multi-class classification algorithm, DAG-SVM. Subsequently, we designed a method to ensure the invariance to rotation of the curvelet features. Our results indicate that the curvelets offer an appropriate discriminative model for the FN networks, that is able to characterize the local fiber geometry.

Index Terms—FN networks, ECM, curvelets, DAG-SVM classification

1. INTRODUCTION

The ECM represents a molecular scaffold used by cells to adhere, migrate and communicate in tissues. One of its major components is the Fibronectin (FN) molecule, a large multi-domain protein which is assembled by cells into fibrillar networks. It is known that the fibrillar organization of the cellular FN is a key feature of the ECM and can take various conformations depending on the presence of alternatively spliced variants [1]. FN variants differ by the inclusion (or exclusion) of two FN Type III Domains (Extra Domains A and B). In this study, we focused on the analysis and modeling with curvelets of four variants: FN A-B-, FN A+, FN B+ and FN A+B+ illustrated in Fig. 1.

A significant number of methodologies have been proposed in the literature to extract highly discriminative features

out of images. We are interested in multiresolution analytical tools that are able to capture the geometrical properties (e.g. scale, orientation) of the anisotropic fibers in a multiscale-based fashion. Several studies on multi-resolution analysis show that curvelet basis is suitable to represent curvilinear features [2]. A fast discrete curvelet transform [3] was used in order to capture and describe the geometry of the FN networks with curvelets. However, the extracted features are not invariant to rotation. For example, two images that have different main orientations but correspond to the same FN variant will be assigned to two different classes.

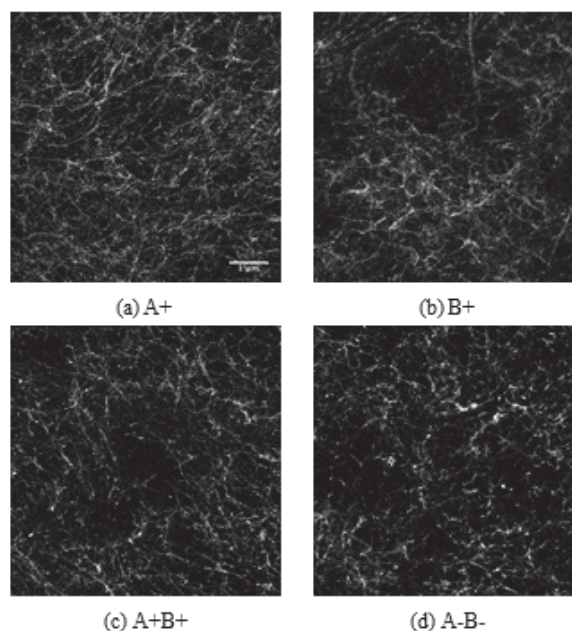


Fig. 1: Different variants of FN. Scale bar = 1 μm

In this paper, we describe a method to align images in a given direction before computing the discrete curvelet coeffi-

cients. Our approach was validated by performing a classification among the four variants, with a DAG-SVM classifier. In order to obtain an alternative representation of the image in the feature space, called image signature, we built a Bag of Features model of the curvelet coefficients. The results of signature classification were compared to those of a specialist. We, therefore, show that our feature extractor model can outperform a trained biologist in terms of classification accuracy.

2. THE CURVELET TRANSFORM AND FEATURE EXTRACTION

In the field of image processing, multiscale/multiresolution tools have been extensively used for anisotropic feature extraction (points, lines, edges) and detection, compression etc. Among existing methods, the wavelets can detect features such as point singularities [4] but do not constitute a well-suited technique to represent curvilinear features, such as those that describe the FN fibers.

The curvelet transform is a family of frames that is proposed as a means to tackle this inconvenience. We chose the second generation of curvelets, which is a multiscale pyramid allowing the representation of a certain number of possible directions at multiple scales [4].

2.1. Fast Discrete Curvelet Transform

The output of the linear transform is a collection of coefficients $c_{j,l,k}$ evaluated in Fourier domain (real-valued), indexed by discrete-valued scale j , orientation l and location k . Usually, a finer scale is associated with a higher number of possible orientations. This property allows for highly anisotropic elements being represented at a fine scale.

Conceptually, the transformation implements a tight frame, meaning that every function $f \in L^2(\mathbb{R}^2)$ can be represented as follows [3]:

$$f = \sum_{j,l,k} c_{j,l,k} \psi_{j,l,k} \quad (1)$$

where

$\psi_{j,l,k}$ is the discrete curvelet waveform

and

$$c_{j,l,k} = \langle f, \psi_{j,l,k} \rangle \quad (2)$$

The Parseval identity then holds:

$$\sum_{j,l,k} |c_{j,l,k}|^2 = \|f\|_{L^2(\mathbb{R}^2)}^2 \quad \forall f \in L^2(\mathbb{R}^2) \quad (3)$$

Regarding the digital implementation of the curvelet transform, there are multiple options for the construction of cartesian arrays, instead of polar tiling, in the frequency

plane. We chose the wrapping method implemented in Curvelet Toolbox [3] for its simplicity in the handling of the discretization grid.

2.2. Curvelet feature extraction

Each image of size $N \times N$ is decomposed into $(\log_2(N) - 3)$ dyadic scales and the number of angular sectors for each scale differs according to the following example: for $N = 512$, the curvelet transform returns 6 scales with 1, 16, 32, 32, 64, 64 possible orientations from coarse to fine scales respectively. Fig. 2 illustrates the curvelet coefficients amplitude matrices for 3 levels of decomposition corresponding to coarsest scale 1, 2, and finest scale 6. The multiple matrices at each level belong to different orientations. A certain fiber will be reconstituted by a linear combination of curvelet coefficients at different scales and orientations.

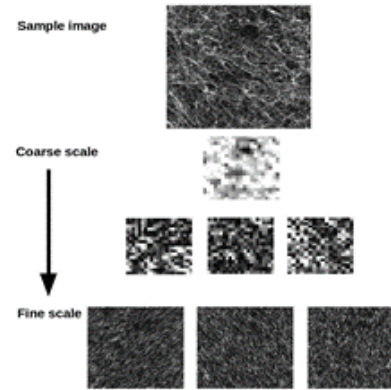


Fig. 2: Curvelet scale decomposition of a sample image at 3 scales, from coarse to fine: 1, 2, 6.

Related studies consider different statistical properties of the curvelet coefficients for texture characterization, such as energy [5], entropy or curvelet subband distribution [6]. As our main interest is to perform geometrical modeling of the fibers rather than a pure assessment of their discriminative power, instead of computing average statistical features to facilitate the classification, we have worked with the coefficients themselves.

However, we chose to reduce the vast number of coefficients taken into account for the classification, and thus keep the most significant ones. To do so, we selected the largest curvelet coefficients that contain a suitable percentage of the total energy. A percentage of 85% seems to be a good compromise between the speed of the training and classification algorithm and the fidelity of image reconstruction, as illustrated in Fig. 3. Finally, the coefficients that belong to the

finest scale, susceptible of capturing the eventual acquisition noise present in the images, were not taken into account.

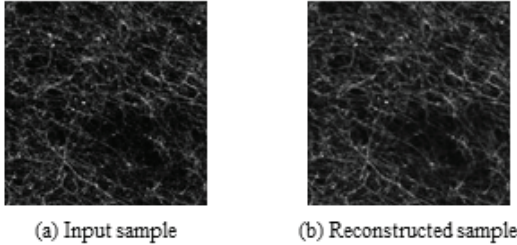


Fig. 3: Reconstruction of a sample image after keeping 85% of total curvelet coefficients energy.

2.3. Invariance to rotation

The curvelets that are described above are not invariant to rotation. In a discriminating context, this aspect can be quite problematic, as it can impact the accuracy of the classification. What is important in the FN images is the presence of multiple dominant orientations, relative to each other. During the acquisition process, the samples of the ECM may also be differently oriented. Hence we needed to ensure that the images follow the same main privileged direction.

To do so, we estimated the dominant orientation of the fibers and rotated every image according to its own dominant orientation. Since this different information is hidden in the energy distribution over the subbands, we opted for an estimation of the dominant orientation using the gradient vector of the images. For a function $f \in \mathbb{R}^2$, we consider its gradient vector $\nabla f = (f_x, f_y)$ with magnitude defined by $|\nabla f| = \sqrt{f_x^2 + f_y^2}$ and orientation $\theta = \arctan(\frac{f_y}{f_x})$. We can now estimate the dominant orientation Θ as:

$$\Theta = \frac{\sum_i |\nabla f_i|^2 \theta_i}{\sum_i |\nabla f_i|^2} \quad (4)$$

where $|\nabla f_i|$ is the magnitude and θ_i is the orientation of the image gradient at pixel i .

Subsequently, the images were aligned to the same direction, after performing a rotation by interpolation with the corresponding Θ .

3. BAG OF FEATURES MODEL

In order to validate the assumption that curvelets can provide a suitable model for the characterization of FN fibers, we first needed to show their ability to describe the fiber geometry in terms of physical characteristics (e.g. scale, orientation, location). In addition to that, we were interested in determining

the discriminating capacity of the curvelet features (i.e. ability to discriminate among the different FN variants). Therefore a bag of features model [7], adapted to our data, was developed in order to analyze the classification results of the four FN variants, as detailed below.

The curvelet features that describe the fibers are the collection of coefficients $C_{j,l,a}$ with scale j , orientation l and magnitude a . We performed a K-means clustering of the curvelet coefficients after the curvelet decomposition of the image database, referred to as the training dataset. In order to determine an appropriate number of clusters, we used a heuristic elbow method [8] and found $K = 400$ number of total clusters. The normalized feature histogram was computed as the rate of the number of its curvelet coefficients assigned to each cluster, as shown in Fig. 4. Also referred as image signature, it is stored as a K-dimension vector of real-positive values. The image signature constitutes the input data for the chosen classifier.

We decided to use a non-exhaustive k -fold cross validation technique with $k = 4$ to evaluate the classification performance and its generalization capabilities. The classification of the feature histograms is performed using a DAG-SVM classifier, using LibSVM [9], as described below.

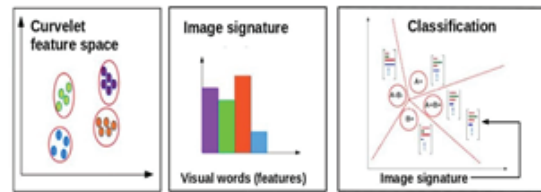


Fig. 4: Bag of features pipeline (from left to right): K-means clustering in curvelet feature space, image signature (feature histogram) and classification of the image signatures.

3.1. DAG-SVM classifier

In terms of classification of the previous curvelet feature histograms, we are faced with a supervised non-linear problem. Support vector machine (SVM) constitutes a standard statistical learning technique to solve binary classification and regression problems. An extension of SVM to multi-class techniques, DAG-SVM [10], was used in this study for the classification of the image signatures corresponding to the FN variants. For a 4-class problem, we built and trained $4(4-1)/2$ binary SVM classifiers, arranged in a tree structure [7] with 4 layers and $4(4-1)/2$ nodes. The classification was hence performed by evaluating a decision function in each node, resulting in $(4-1)$ decisions to be made for a test sample. The classification process with DAG-SVM is therefore relatively fast.

4. SIMULATION RESULTS

This section describes the implementation of the proposed method for the classification of the four FN variants. We deployed a database of 280 images of 3128×3128 pixels with a lateral resolution of $0.27 \mu\text{m}/\text{pixel}$, acquired with a Zeiss 710 confocal system. Each class contains 70 images corresponding to the four FN variants. For speed convenience, we selected a representative region of 512×512 pixels from each image and used those regions for feature extraction and classification. The classification results were compared to those of a trained specialist, in terms of general classification accuracy, as well as confusion matrices.

Table 1 indicates the values of the confusion matrix for the automatic classification, while Table 2 shows the results of the specialist. The confusion matrix indicates that the classifier is highly capable of distinguishing the FN images belonging to variant A-B- from the rest of the others. Additionally, the classifier is presented with a greater challenge when it comes to distinguishing among classes B+ and A+. A similar pattern was noted in the confusion matrix that corresponds to the classification performed by the specialist.

Actual \ Predicted	Predicted			
	A+	A-B-	B+	A+B+
A+	64.3	2.9	25.7	7.1
A-B-	0	90	0	10
B+	25.7	4.3	45.7	24.3
A+B+	0	15.7	8.6	75.7

Table 1: Confusion matrix in percentage form - automatic classification

Actual \ Predicted	Predicted			
	A+	A-B-	B+	A+B+
A+	77.2	0	18.5	4.3
A-B-	0	65.7	5.7	28.6
B+	34.3	0	48.6	17.1
A+B+	2.9	5.7	37.2	54.2

Table 2: Confusion matrix in percentage form - Trained specialist

The fiber geometry associated to the A-B- FN variant, characterized by short filaments without a specific pattern, seems to be represented by a more discriminative geometric model. On the other hand, the topological properties of the fibers corresponding to FN A+, and FN B+ (i.e. fiber length and the presence of an apparent directionality) are quite similar, thus increasing the difficulty in differentiating between them. FN variant that incorporate the B+ domain is the least distinguishable, both in automatic and manual classification.

Regarding the general accuracy of classification, the clas-

sification scheme that is proposed in this paper (68.92%) outperforms the results obtained by a trained specialist (61.42%).

5. CONCLUSION

FN network fibers exhibit local geometric properties that can be captured by curvelet features. We can reconstruct the fibers as a linear combination of curvelet coefficients at multiple scales and orientations. In addition, we are able to classify among the four variants of interest, with a similar performance to that of a trained specialist. Future studies will focus on the development of generative models of the 2D/3D FN variants, as well as the generation of a model based on differences in FN architecture caused by tumor-like cells.

Acknowledgements. This work was supported by the French Government (National Research Agency, ANR) through the "Investments for the Future" LABEX SIGNALIFE: program reference ANR-11-LABX-0028-01.

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