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1 **Fibronectin Extra Domains tune cellular responses and confer**
2 **topographically distinct features to fibril networks**

3 Georgios Efthymiou^{1‡}, Agata Radwanska^{1‡}, Anca-Ioana Grapa^{1,2}, Stéphanie Beghelli-de la Forest
4 Divonne^{1,3}, Dominique Grall¹, Sébastien Schaub¹, Maurice Hattab¹, Sabrina Pisano⁴, Mallorie Poet⁴,
5 Didier F. Pisani⁵, Laurent Counillon⁵, Xavier Descombes², Laure Blanc-Féraud², and Ellen Van Obberghen-
6 Schilling^{1*}

7 ¹Université Côte d’Azur, CNRS, INSERM, iBV, Nice, France

8 ²Université Côte d’Azur, Inria, CNRS, i3S, Nice, France

9 ³Centre Antoine Lacassagne, Nice, France

10 ⁴Université Côte d’Azur, Inserm, CNRS, IRCAN, Nice, France

11 ⁵Université Côte d’Azur, CNRS, LP2M, Nice, France

12
13 ‡ These authors contributed equally to this work

14 * Corresponding author:

15 Ellen Van Obberghen-Schilling

16 Institut de Biologie Valrose (iBV)

17 CNRS – INSERM – UCA

18 Université Côte d’Azur

19 Parc Valrose

20 06108 Nice cedex 2, FRANCE

21 Tel +33 (0)4 89 15 0790

22 E-mail: vanobber@unice.fr

23
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28 **Summary statement**

29 Fibronectin, a major building block of tissues and organs, tunes fibroblast behavior and matrix architecture
30 through the presence of alternatively spliced extra domains.

31 **Abstract**

32 Cellular fibronectin (FN; also known as FN1) variants harboring one or two alternatively spliced so-called
33 extra domains (EDB and EDA) play a central bioregulatory role during development, repair processes and
34 fibrosis. Yet, how the extra domains impact fibrillar assembly and function of the molecule remains
35 unclear. Leveraging a unique biological toolset and image analysis pipeline for direct comparison of the
36 variants, we demonstrate that the presence of one or both extra domains impacts FN assembly, function
37 and physical properties of the matrix. When presented to FN-null fibroblasts, extra domain-containing
38 variants differentially regulate pH homeostasis, survival, and TGF- β signaling by tuning the magnitude of
39 cellular responses, rather than triggering independent molecular switches. Numerical analyses of fiber
40 topologies highlight significant differences in variant-specific structural features and provide a first step
41 for the development of a generative model of FN networks to unravel assembly mechanisms and
42 investigate the physical and functional versatility of extracellular matrix landscapes.

43

44 **Introduction**

45 The extracellular matrix (ECM) is a cell-derived bioscaffold composed of more than 100 proteins in a given
46 tissue (Hynes and Naba, 2012) that provides mechanical support to the underlying cells and promotes
47 tissue integrity. In addition, the composition and the physical state of the ECM affect cell physiology by
48 acting as a functionalized platform for the transmission of biochemical and biomechanical cues [reviewed
49 in (Frantz et al., 2010; Hynes, 2009; Theocharis et al., 2016)]. Fibronectin (FN; also known as FN1), a major
50 ECM component of developing and diseased tissues, is at the epicenter of this platform.

51 FN is a large glycoprotein composed of two similar, but not identical, subunits, encoded by a single gene
52 and linked by two disulfide bonds at their C-terminal ends. Each subunit (240-270 kDa) has a repetitive
53 modular structure composed of Type I, Type II, and highly extensible Type III repeats that allow the
54 molecule to acquire compact and stretched conformations and to partner with a large variety of ECM
55 components, cell receptors and soluble factors in a mechanically-tuned fashion (Vogel, 2006). FN is
56 assembled into a fibrillar array, its active form, on the surface of assembly-competent cells. Fibrillogenesis

57 is initiated by the binding of FN to its principal cellular receptor, integrin $\alpha 5\beta 1$. Integrin activation triggers
58 the recruitment of cytoplasmic binding partners and reorganization of the actin cytoskeleton. Intracellular
59 acto-myosin-generated forces applied to FN lead to partial unfolding of the molecule and exposure of
60 cryptic self-association sites that support polymerization and higher-order fiber assembly on the cell
61 surface, as reviewed in (Singh et al., 2010). Importantly, FN networks provide templates for the deposition
62 of other matrix proteins (Sottile and Hocking, 2002; Velling et al., 2002).

63 FN can be found in two distinct forms known as plasma FN (pFN) and cellular FN (cFN). pFN is produced
64 by the liver and circulates in the bloodstream, while cellular FN (cFN) is expressed and assembled in an
65 insoluble matrix by mesenchymal cells such as fibroblasts, endothelial cells, chondrocytes and
66 macrophages (Hynes, 1990). cFN differs from pFN by the presence of highly conserved [reviewed in (White
67 et al., 2008)] alternatively spliced FN Type III repeats termed extra domain B (EDB or EIIIB) and extra
68 domain A (EDA or EIIA), which flank the cell binding (RGD) and synergy sites (PHSRN) located in FNIII10
69 and FNIII9, respectively. Each alternatively spliced extra domain is generated from a single exon and gives
70 rise to a FNIII repeat, the expression of which is tightly regulated and limited to embryonic development,
71 wound healing (Ffrench-Constant, 1989), and tumor progression (Castellani, 1986). Thus, the term
72 “oncofetal” is often used to describe FN isoforms harboring either or both of the extra domains, that are
73 scarce in adult tissues.

74 Early knockout studies in the mouse highlighted the importance of FN in the developing organism, since
75 FN-null mice die during gestation [before embryonic day (E)10.5] due to severe defects in neural tube
76 formation and vascular development (George et al., 1993). Intriguingly, combined deletion of both extra
77 domains also leads to embryonic lethality (Astrof et al., 2007), whereas mice lacking either extra domain
78 are viable and fertile (Fukuda et al., 2002; Muro et al., 2003). EDA-targeted deletion and EDA-constitutive
79 inclusion studies have revealed roles for this FNIII repeat in lymphatic valve morphogenesis,
80 atherosclerosis, tissue injury, inflammation, and lifespan regulation. While several receptors have been
81 shown to bind EDA, such as integrins $\alpha 9\beta 1$, $\alpha 4\beta 1$, $\alpha 7\beta 1$, and TLR4 [reviewed in (White et al., 2008)], much
82 less is known about EDB, for which no receptor has so far been identified. Rather, its presence has been
83 demonstrated to induce conformational changes regulating the accessibility to adjacent cryptic sites
84 (Balza et al., 2009; Carnemolla et al., 1992; Ventura et al., 2010), and facilitating RGD presentation to $\alpha 5\beta 1$
85 integrins, thereby favoring integrin clustering and downstream signaling (Schiefner et al., 2012).

86 Regarding matrix assembly, FNs containing EDB or EDA have been found to be most efficiently integrated
87 by cells into pre-existing ECMs (Guan et al., 1990), consistent with subsequent loss-of-function studies

88 showing that EDB deficiency reduced FN deposition in cultured fibroblasts and endothelial cells (Cseh et
89 al., 2010; Fukuda et al., 2002). These data suggest, that although the extra domains are not essential for
90 FN fibrillogenesis per se, they may regulate the dynamics of polymerization by affecting FN-cell and/or
91 FN-FN interactions.

92 Despite decades of research on fibronectin, the role of extra domains in FN assembly and functions has
93 not been directly assessed using full-length variants in an identical cellular context, devoid of autocrine
94 FN production. To this end, we generated constructs harboring human FN variants containing one, both,
95 or none of the alternatively spliced extra domains for expression in FN-null cells, and production of
96 recombinant proteins in assembly-incompetent cells. As the biological activity of FN is dependent on the
97 conformation of the fibers that constitute its functional form, we enriched our biological toolset with in-
98 depth computational analyses of FN network topology. Here, we report that the presence of EDB and/or
99 EDA facilitates FN fibrillogenesis and regulates cell survival, pH control, and TGF- β signaling in fibroblasts.
100 Quantitative characterization of FN networks has uncovered significant variant-specific differences in
101 structural features and provides a promising framework to enrich the development of imaging-based
102 machine learning methodologies for automatic classification of matrix attributes associated with disease
103 states.

104

105 **Results**

106 **Recombinant human FN isoforms**

107 To explore the functions of alternatively spliced cFN variants and the impact of the extra domains EDB
108 and EDA on FN matrix assembly, we constructed a set of lentiviral expression vectors harboring the full-
109 length coding sequence of the human *FN1* gene containing one, both or none of these alternatively spliced
110 FN Type III repeats (**Fig. 1A**). All constructs contain the alternatively spliced variable region V-89
111 (Schwarzbauer, 1991). We chose not to insert additional sequences encoding cumbersome fluorescent
112 proteins or molecular tags, to avoid modifying the conformation of the protein and disrupting inter- and
113 intra-molecular interactions.

114 **Re-expression of FN variants in FN-null MFs**

115 To eliminate confounding effects of endogenous cFN for re-expression studies, a Cre recombinase vector
116 was introduced in *Fn1 fl/fl* mouse fibroblasts (MFs), which express FN EDB- and EDA-containing transcripts
117 at a ratio of roughly 1:1 (**Fig. S1A-C**). One *Fn1* $-/-$ clone (clone D) was selected for transduction with the

118 human FN expression vectors based on its fibroblast morphology, its ability to readily assemble
119 exogenously presented FN and the absence of multinucleated and/or senescent cells (**Fig. S1D,E**).
120 Following transduction, variant-expressing clones were isolated for analysis, as working with
121 homogeneous populations of FN-re-expressing cells, rather than heterogeneous populations with highly
122 variable FN expression levels, is essential for comparative studies (**Fig. 1A**). Furthermore, for comparison,
123 equivalent amounts of FN must be expressed by the selected clones.

124 A representative clone of each variant is shown in **Fig. 1B-D**. The presence of the expected extra domains
125 in the pericellular FN matrix was confirmed by immunofluorescence staining with extra domain-specific
126 antibodies (**Fig. S2A**). Despite relatively similar FN transcript levels (**Fig. 1B**), more cell-associated FN was
127 observed in lysates of MF clones expressing the extra domain-containing variants than the plasma-like FN
128 B-A-. Conversely, the ratio of soluble FN in the conditioned medium to cell-associated FN was higher in
129 MFs expressing plasma-like FN B-A- (**Fig. 1C**). The facilitating effect of the extra domains on FN assembly
130 was further evidenced by the augmented pericellular deposition of fibers by clones expressing extra
131 domain-containing FN (**Fig. 1D**).

132 We have previously demonstrated that autocrine FN expression is tightly coupled to its fibrillar assembly
133 and that differences in FN expression levels could have a considerable effect on assembly of the protein
134 (Cseh et al., 2010). Indeed, examination of several variant-expressing clones (**Fig. S2B-D**) confirmed that
135 increased expression of the same mRNA generally resulted in more efficient assembly of the molecule.
136 Nonetheless, some clones expressing equivalent amounts of the same transcript displayed marked
137 differences in their ability to deposit a FN matrix, indicating that not only expression levels, but clonal
138 heterogeneity can also impact fibrillogenesis.

139 These re-expression studies indicate that cells assemble FN containing either one or both of the extra
140 domains more efficiently than FN lacking extra domains. However, our findings that FN assembly is
141 particularly sensitive to autocrine expression levels and to clonal variability indicate that direct
142 comparison of FN variant assembly and function cannot be accurately achieved using clones that re-
143 express FN.

144 **Presentation of recombinant variants to FN-deficient MFs**

145 To circumvent the problems associated with the inherent phenotypic variability of recipient MF clones,
146 subsequent studies were performed in an identical cellular context. Hence, as schematized in **Fig. 2A**, FN

147 variants were purified and provided in soluble form to the same *Fn1* ^{-/-} MF clone (clone D) that was used
148 for FN re-expression studies.

149 For purification, the recombinant isoforms were expressed in assembly-incompetent HEK293 cells in
150 which endogenous FN expression is very low (**Fig. S3A,B**). Following transduction, cells secreted high levels
151 of the soluble variants into their culture medium. The purified variants displayed appropriate molecular
152 mass and they dimerized (**Fig. S3C**), as required for fibrillogenesis (Schwarzbauer, 1991). Specific inclusion
153 of each extra domain was confirmed by western blot analysis using isoform-specific anti-FN antibodies
154 (**Fig. S3D**). Cellular FN is considerably less soluble than pFN under physiological buffer conditions (Yamada
155 and Olden 1978). Consequently, it was essential to assure concentration uniformity of the soluble proteins
156 in each experiment and verify the absence of FN aggregates, visualized by immunofluorescence staining
157 of FN variant-coated coverslips (**Fig. S3E** arrowheads).

158 For analysis of FN assembly, subconfluent *Fn1* ^{-/-} MFs were allowed to adhere overnight on non-coated
159 glass coverslips before adding the variants. A concentration of 15 µg/ml was chosen for experiments,
160 following dose-response analyses of exogenous FN assembly by these cells (**Fig. S3F**). First, we compared
161 the quantity of the different recombinant FNs in cell lysates by western blotting. As shown in **Fig. 2B**, all
162 of the FN variants were readily detectable as early as 2 h after addition of the soluble proteins to the cells.
163 Variants containing one or both extra domains were 1.69- to 3.18-fold more abundant in cell lysates than
164 plasma-like FN. The cell-associated protein corresponded exclusively to assembled FN, as determined by
165 immunofluorescence staining (**Fig. 2C**).

166 Similar to what was observed in FN re-expressing cells (**Fig. 1D**), the presence of extra domains enhanced
167 fibrillar assembly of exogenous FN (**Fig. 2C**). Whereas only short thin fibers were detected at 2 h on cells
168 presented with plasma-like FN, a more elaborate pattern of long, coarse fibers was seen in cells treated
169 with the cFN variants. At 2 h we did not observe marked variant-specific patterns of cFN assembly (**Fig.**
170 **2C**), or extra domain-dependent differences in integrin distribution (**Fig. S4**). Assembly of the exogenously
171 provided cFN was predominantly observed at sites of cell-cell adhesion (**Fig. 2C**, thick arrow), or along the
172 lateral and retracting edges of polarized cells (**Fig. 2C**, thin arrow). FN fibrils were not present in the
173 peripheral adhesions at the front of polarized cells (**Fig. 2C**, arrowhead). By 48 h all four recombinant FN
174 variants were incorporated into dense meshworks by cells (**Fig. 2C**).

175 **Effect of FN variants on cell growth and survival**

176 The presence of EDB and EDA in cellular FN have been reported to confer distinct growth promoting
177 properties upon the molecule (Fukuda et al., 2002; Manabe et al., 1999). Therefore, we examined the
178 proliferative effects of the recombinant full-length cFN variants on *Fn1* ^{-/-} MFs. As shown in **Fig. 3A**,
179 addition of FN had no significant effect on early growth of cells. All four FN variants significantly increased
180 exponential growth rates (decreased cell doubling times) compared to that of vehicle-treated control
181 (CTRL) cells (**Fig. 3B**). Interestingly, whereas the number of viable cells began to plateau after 3 days in
182 vehicle-treated cultures and after 4 days in presence of plasma-like FN B-A-, the numbers of cells in
183 cultures with FN containing one or both extra domains continued to increase for at least 5 days. At 5 days,
184 CTRL- and FN B-A- -treated cultures displayed an increased number of round, refractory (dying) cells,
185 compared to cells treated with extra domain-containing FN variants, as depicted in the phase contrast
186 images of FN B-A- and FN B+A+-treated monolayers (**Fig. 3C**). This finding suggests that the cFN variants
187 confer a survival advantage to densely packed cells.

188 **Metabolic effects of FN**

189 While performing growth curve experiments, we observed an increase in acidification of the culture
190 medium of cells treated with the extra domain-containing FN variants, as compared to CTRL (non-treated)
191 cells or cells exposed to plasma-like FN B-A- (**Fig. 3D**). The effect became visible after 2 days and the
192 increment was most pronounced in cells presented with FN B+A-, despite the similar growth rate of these
193 cells (**Fig. 3A,B**). Extracellular acidification is a proxy for lactate production suggesting that FN-induced
194 acidification of the intra- and extra-cellular space could be due to a metabolic switch in the cells from
195 oxidative phosphorylation to anaerobic glycolysis. To assess this, we measured glucose uptake, lactate
196 production, the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) in CTRL and
197 FN variant-treated cells.

198 Glucose transport and lactate extrusion measurements after 48 h showed a tendency for FN-induced
199 stimulation, although statistical significance was not reached (**Fig. S5A,B**). Moreover, expression of
200 glycolytic enzymes was significantly increased by FN (**Fig. S5C,D**), yet no variant-dependent differences
201 were detected. Next, we determined glycolysis and mitochondrial respiration simultaneously after 72 h
202 of FN treatment. Compared to CTRL cells, FN B+A- and FN B-A+ -treated cells displayed a higher basal
203 extracellular acidification rate (ECAR) (**Fig. 3E**), and this is in line with medium color changes. ECAR in
204 response to glucose addition, reflecting anaerobic glycolytic response, remained unchanged across
205 conditions, and even slightly decreased in cells treated with FN B-A+ and B+A+ (**Fig. S5E**). Cells in presence
206 of FN B+A+ displayed lower mitochondrial activity (lower basal OCR and lower maximal OCR) although no

207 differences in OCR were observed in cells treated with the other variants (**Fig. S5F,G**). Respiration coupled
208 to ATP synthase was similar for all conditions and mitochondrial capacity was maximal, even without
209 glucose, as evidenced by the same values in basal and maximal OCR, suggesting the use of another
210 substrate for oxidative phosphorylation in these cells (**Fig. 3F** and **Fig. S5H,I**). As H^+ extrusion can be a
211 consequence of cell acidification, we set out to detect changes in intracellular pH in cells treated with the
212 FN variants (**Fig. 3G**). Indeed, when intracellular pH drops, several pumps extrude H^+ to bring the pH back
213 to normal levels. While intracellular pH decreased significantly in the presence of FN B+A-, only a tendency
214 towards a decrease was observed for B-A+ -treated cells (**Fig. 3G**) which displayed increased ECAR (**Fig.**
215 **3E**). From these results, we conclude that addition of exogenous FN to these fibroblasts does not induce
216 a readily apparent metabolic reprogramming but does induce H^+ extrusion partly independent of
217 anaerobic glycolysis, and this effect is influenced by the presence of extra domains.

218 **FN-induced TGF- β activation and signaling**

219 It has been previously proposed that ECM formation and turnover depend on a feed forward loop
220 involving FN and TGF- β (Fontana et al., 2005). TGF- β regulates FN production (Ignotz and Massagué,
221 1986), alternative splicing (Balza et al., 1988; Borsi et al., 1990; Castellani, 1986), and the expression of
222 the major FN receptor, integrin $\alpha 5\beta 1$ (Ignotz and Massagué, 1987). Moreover, FN was shown to mediate
223 latent TGF- β activation through a mechanism involving αv -containing integrins (Fontana et al. 2005;
224 Henderson et al. 2013). To assess whether the extra domains play a role in FN-induced activation of latent
225 TGF- β and TGF- β signaling in *Fn1* $-/-$ MFs, we monitored expression of the TGF- β response genes *PAI1*
226 (also known as *SERPINE1*), *CTGF* (*CCN2*) and *TGFBI* (Kutz et al., 2001; Leask and Abraham, 2003; Ween et
227 al., 2012), and SMAD2 phosphorylation (**Fig. 4**). The absence of TGF- β in our FN preparations was
228 confirmed by ELISA (**Fig. S5J**). Time courses of *PAI1*, *CTGF* and *TGFBI* mRNA expression following the
229 addition of 15 $\mu\text{g}/\text{ml}$ of FN to cells are shown in **Fig. 4A**. FN stimulated a rapid increase in PAI1 and CTGF
230 mRNA expression which peaked at 2 h and returned to near-basal levels by 6 h, indicating that FN triggers
231 TGF- β signaling in *Fn1* $-/-$ MFs. TGFBI (TGF- β -induced) mRNA expression was unaffected at short times
232 after FN addition (2 h). However, significant downregulation was observed after 48 h (**Fig. 4B**), reflecting
233 a possible FN-dependent retro-control of TGF- β pathway activation. Consistent with this being a TGF- β -
234 mediated effect on gene expression, FN also stimulated SMAD2 phosphorylation in these cells, and the
235 presence of one or both FN extra domains had a similar impact on the magnitude of the response (**Fig.**
236 **4C**). FN B+A+ was found to be the most potent agonist of TGF- β signaling in MFs (**Fig. 4A-C**) whereas FN
237 B-A+ induced the weakest response. The observed differences were not due to differential expression

238 and/or secretion of TGF- β , since total TGF- β levels in acid-treated conditioned medium were equivalent
239 across conditions (**Fig. S5K**). As shown in **Fig. 4D**, a 30-minute pretreatment of cells with the TGF β RI
240 antagonist SB-431542 (10 μ M) precluded FN-induced SMAD2 phosphorylation, indicating that this effect
241 is mediated by TGF β RI. These results suggest that the extent of TGF- β induction most likely involves
242 variant-specific activation of latent TGF- β . In light of the proposed role of mechanical force and ECM
243 stiffness in activation of latent TGF- β (Hinz, 2015; Sarrazy et al., 2014; Wipff et al., 2007), we compared
244 the ability of the FN variants to induce collagen lattice contraction by embedding *Fn1*^{-/-} MFs into collagen
245 lattices containing 15 μ g/ml of the FN variants. The extent of contraction was determined after 24 h.
246 Whereas the presence of FN resulted in pronounced reduction in the lattice surface, only slight differences
247 were observed among the four variants, indicating that presence of the extra domains had no impact on
248 FN-induced collagen gel contraction (**Fig. S5L**).

249 **The effect of the FN extra domains on physical properties of the ECM**

250 As the presence of the extra domains enhanced FN fibrillogenesis and differentially affected cellular
251 responses to FN, we examined whether variant-specific differences could be observed in the physical
252 properties of the matrix assembled by FN-deficient MFs presented with the purified FN proteins. For
253 optimal comparison of the matrix networks, MF cultures plated at 80% confluence were denuded of cells
254 after 7 days and MF-derived matrices (MDMs) were fixed and stained for FN, or other matrix proteins.
255 Interestingly, the organization of FN in the MDMs appeared to be different, depending upon the FN
256 variant presented to cells (**Fig. 5A**). In line with its role as an obligate template for assembly of other matrix
257 components, the architecture of FN fibrils impacted higher order matrix organization. For example,
258 patterns of collagen VI and tenascin C staining appeared similar in mature matrices of cells presented with
259 the same FN variant, whereas they appeared clearly dissimilar in matrices of cells presented with different
260 variants. Differences in the mechanical properties of the four matrices were also noted. The average
261 thickness of the MDMs ranged between 6.3 μ m and 9.0 μ m, with the matrix containing FN B+A- being
262 significantly thicker than the others (**Fig. 5B**). Interestingly, the same matrix was also the most compliant,
263 as determined by indentation using atomic force microscopy (AFM) (**Fig. 5C**). By contrast, the median
264 Young's (elastic) modulus was highest for the MDM containing plasma-like FN, devoid of extra domains,
265 suggesting that their presence in FN can alter the stiffness of the FN-rich ECM. Additionally, the elastic
266 modulus of the FN B-A- MDM displayed the largest range of values suggesting a greater heterogeneity in
267 the composition and/or crosslinking of this matrix.

268 **Structural features of the FN variant matrices**

269 Structural features of the ECM reflect the physiological state of tissues and have a profound impact on
270 cell behavior. To delve deeper into the impact of FN extra domains on fiber network architecture, we
271 employed a computational image analysis approach. Advanced computer vision and machine learning
272 tools were developed to interrogate whether the geometric features of the variant-specific matrices
273 (assembled by the same cells) are distinguishable, and if so, whether they can be described, quantified,
274 modelled, or attributed to extra domain-specific biological functions.

275 *Automatic classification of images of extra-domain-specific FN networks*

276 To optimize the quality of the images for processing, high-resolution images of the decellularized matrices
277 were acquired by confocal microscopy (**Fig. 6A**). Images were subsequently classified using a pre-trained
278 convolutional neural network (CNN), as described in Materials and Methods. The results of this
279 classification are presented in **Fig. 6B** in terms of a confusion matrix, which allows the visualization (on
280 the diagonal) of the percentage of images that were correctly classified as belonging to the expected FN
281 variant class. High intra-variant accuracy scores were observed (general accuracy, 83.3%), indicating that
282 the information contained in the images is sufficient for the algorithm to recognize topological and
283 geometrical differences inherent to the four matrix types. Additionally, machine-based classification
284 outperformed the ability of a biologist who was blind to the experimental conditions to correctly classify
285 the same image set (biologist accuracy, 61.4%). Topological and geometrical properties of fiber
286 arrangements containing only EDA (B-A+) are the most distinguishable (90.5%), followed by the B-A- fibers
287 (85.7%), devoid of extra domains. FN networks containing only EDB (B+A-) are less distinguishable, while
288 B+A+ fibers are the least distinguishable in automatic classification.

289 *Network properties and feature extraction*

290 As a first step in the characterization of the properties of FN fiber networks, fibrillar structures were
291 detected using the workflow shown in **Fig. 6C**. After generating a high-quality dataset of images (1), we
292 defined a set of Gabor kernels, or "dictionary of elementary fibers" (2) that incorporates a set of
293 parameters (described in Materials and methods) that capture the entire range of different fibers. After
294 fiber detection (3), the morphological skeleton, or median axis, of the detected fibers was computed (4)
295 for feature extraction and analysis (5). **Fig. 6D** displays the FN variant-specific graphs, or sets of edges
296 connected by nodes, associated to the morphological skeletons of the fibers. The advantage of using a
297 graph-based representation is that apart from Gabor-specific descriptive parameters, we are able to
298 compute additional features that, in turn, can help characterize the properties of the FN fibers including
299 network connectivity and mesh shape.

300 *PCA analysis of the fiber features*

301 After establishing a faithful representation of the fibers, a set of features was selected to characterize
302 fiber networks and to perform principal component analysis (PCA). The PCA method was used to explore
303 the relatedness between the different FN variants with respect to various physical attributes, or features,
304 including i) connectivity, ii) fiber thickness, iii) fiber heterogeneity, iv) pore shape and v) pore size
305 distribution. To describe the connectivity of the fibers, we computed the relative abundance of nodes of
306 different degrees. The degree of every node is defined by the number of edges connected to it. The
307 variant-specific connectivity distributions are shown in **Fig. S6A**. Interestingly, B-A- fibers are characterized
308 by a higher abundance of fiber ends, delineating a low level of connectivity, compared to the other
309 variants, especially to B-A+. These results reveal that the absence of extra domains leads to a less
310 branched FN fiber arrangement.

311 Next, we considered fiber thickness by computing the proportion of thin to thick fibers. As shown in **Fig.**
312 **S6B**, B-A- fibers display low proportion of thin fibers, and, hence, are characterized by the presence of
313 medium and thick fibers, while the opposite is observed for B-A+. To analyze fiber thickness
314 heterogeneity, or fiber diversity, we implemented the fiber thickness kurtosis, a parameter that indicates
315 how outlier-prone the fiber thickness distribution is relative to a normal distribution with identical
316 variance. In terms of fiber thickness, B-A+ values are distributed around the mean, suggesting a high
317 homogeneity in fiber thickness, compared to B-A- fibers, the thickness of which is more heterogeneous
318 (**Fig. S6C**).

319 Pore shape was measured through a circularity parameter and the average pore size. Circularity reflects
320 pore anisotropy allowing us to distinguish circular and oval-like pores. **Fig. S6D** shows that FN B-A-
321 arrangements are characterized by a high number of oval pores, while pores in FN B-A+ networks are
322 predominantly circular. The same pattern is observed in terms of pore size. Large pore sizes are found
323 within FN B-A- networks, while smaller pore size is observed in FN B-A+ networks (**Fig. S6E**).

324 The PCA (**Fig. 6E**) was performed with the aforementioned features, by adopting the representation
325 provided by the first two principal components. The plot illustrates both the samples (images) projected
326 in a bi-dimensional space, and the five features represented by vectors, the direction and length of which
327 indicate the contribution of each feature to the two principal components. Generally, the samples
328 belonging to FN B-A- and FN B-A+ are concentrated in non-overlapping areas, displaying the
329 distinguishability of these two variant-specific FN networks through the chosen features.

330 Altogether, these analyses demonstrate that FN B-A+ matrices display highly branched, homogeneous,
331 thin fibers that form small pores. In contrast, cells presented with FN B-A- assemble thicker, less branched
332 fibrillar networks with larger more elongated pores. Interestingly, the presence of EDB results in matrices
333 (either B+A-, or B+A+) characterized by a mixture of the attributes seen in B-A- and B-A+ networks.

334

335 **Discussion**

336 In this study we set out to determine how the presence of EDB and EDA in the FN molecule influences
337 cellular functions and impacts ECM architecture. To do so we implemented an in vitro approach consisting
338 of FN-null MFs either re-expressing or presented with extra-domain-specific FN. Several important
339 considerations were factored into the design of our experiments: First, we used FN knock-out cells to
340 preclude interference from endogenous cFN. Second, we generated full-length FN constructs (instead of
341 FN fragments) in light of the involvement of multiple domains in matrix assembly [(Ohashi and Erickson,
342 2011) and references therein]. Furthermore, we did not include additional sequences in our constructs
343 [e.g. GFP or YFP (Ohashi et al., 1999; Ohashi et al., 2002)] to preserve variant-specific spatial
344 conformations and avoid disrupting relevant intra-/inter-molecular interactions, thus improving the
345 biological relevance of our results. Finally, we learned from close examination of multiple clones re-
346 expressing FN variants that defining the precise mechanisms by which EDB and EDA selectively impact
347 fiber assembly and network topology is not possible in a re-expression context. Indeed, assembly of the
348 same variant by different clones was extremely sensitive to FN expression levels and to clonal variability.
349 This is not surprising as FN expression in vivo is exquisitely regulated, both positively and negatively and
350 at multiple levels (transcription and mRNA processing, trafficking, secretion and assembly). Such dynamic
351 regulation is lost upon re-expression of cDNA sequences under an exogenous promoter and lacking
352 untranslated regions of the mRNA. Thus, dose-dependence and cell heterogeneity must be considered
353 when investigating FN assembly in genetically-engineered cell clones or in cells with different genetic
354 backgrounds, such as MFs isolated from different mice [e.g. control and knockout genotypes (Bass et al.,
355 2007; Fukuda et al., 2002; Goetz et al., 2011)]. Although our variant re-expressing clones are of limited
356 use for comparative mechanistic studies, they will be valuable resources for analyzing extra domain-
357 specific paracrine effects of cFN-rich matrices. In addition, the availability of the expression vectors and
358 recombinant variants described here provides not only a means of interrogating specific variant functions,
359 but also a stepping stone for the production and investigation of matrices containing different ratios of
360 FN variants and thus mimicking the heterogeneous FN networks observed in vivo.

361 A key point of our study is the enhanced fibrillar assembly of FN that contains either one or both extra
362 domains by fibroblasts. This finding is highly relevant as cFN is a key provisional matrix component and
363 fibroblasts are the major provisional matrix-producing cells in both physiological and pathological states
364 such as during wound healing, inflammation, fibrosis [reviewed in (Yusuf et al., 2013)], and cancer
365 [reviewed in (Alkasalias et al., 2018)]. Our data are consistent with data from a previous study
366 demonstrating that recombinant rat FNs containing EDB, or EDB together with EDA, were three times
367 more readily incorporated in pre-existing matrices of NIH 3T3 cells than FN lacking extra domains (Guan
368 et al., 1990). More recently, antibody-based approaches (Ventura et al., 2016) and structural analysis of
369 recombinant FN fragments (Schiefner et al., 2012) have shed light on EDB-dependent conformational
370 changes in FN that could impact macromolecular fibril formation. In view of their crystallographic study
371 (Schiefner et al., 2012) and other published data [reviewed in (Mezzenga and Mitsi, 2019)], Schiefner and
372 colleagues proposed that conformational changes resulting from insertion of EDB stabilize head-to-tail
373 homo-dimerization of FN molecules leading to the formation of a quaternary structure that enhances
374 $\alpha 5 \beta 1$ integrin clustering and downstream signaling. Our data would support such a mechanism and
375 suggest that in addition to EDB, the presence of EDA may enhance FN fibrillogenesis through
376 conformational changes that affect self-association of the molecule and how it interacts with the cellular
377 receptors. Indeed, FN conformation affects its interactions with not only receptors, but also with
378 proteoglycan co-receptors in the ECM and at the cell surface, which can, in turn, impact FN assembly.
379 Conformational differences in the FN variants that affect their intermolecular interactions with other
380 matrix proteins (collagens, tenascin C, periostin, etc.) or matrix-associated proteins (LoxL2,
381 transglutaminase or BMP-1) could also impact the growth of FN fibrils on cell surfaces and the higher
382 order assembly of the ECM. Finally, extra domain-specific effects on intramolecular interactions that
383 affect the solubility of the protein are likely to affect its deposition (Castelletti et al., 2008).

384 Our findings that FN variants harboring the extra domains induced a decrease in intra- and extra-cellular
385 pH in *Fn1* $-/-$ MFs and sustained cell growth in dense conditions, as compared to plasma-like FN, was
386 intriguing. The generation of an acidic environment is a common feature of rapidly proliferating cells
387 during several physiological processes (wound healing) and in fibrotic states. Extracellular acidification,
388 best studied in tumor tissue, occurs through the reprogramming of energy metabolism in tumor cells.
389 Moreover, increased glycolysis in the cancer-associated stroma has been proposed to provide high energy
390 metabolites to neighboring cancer cells via what was termed the reverse Warburg effect (Pavlidis et al.,
391 2009; Yoshida, 2015). As fibroblasts are the major matrix-producing cells of epithelial tumors, and cellular
392 FN is a major matrix constituent of the carcinoma-associated fibroblast matrisome (Gopal et al., 2017), it

393 was tempting to speculate that FN could actively contribute to acidification of the tumor
394 microenvironment by stimulating Warburg-like glycolytic rates in stromal cells. Although FN-treatment of
395 MFs led to increased mRNA expression of glycolytic enzymes and a tendency for increased glucose uptake
396 and lactate extrusion, we failed to detect significant increases in anaerobic glycolysis (ECAR) or
397 downregulation of mitochondrial respiration (OCR) in MFs under standard culture conditions. It will be
398 interesting to investigate the role of cFN-regulated metabolic reprogramming of stromal fibroblasts in the
399 tumor setting.

400 It is well documented that cFN expression and ECM deposition are intimately linked to TGF- β signaling in
401 fibroblasts. In an in vivo context, FN containing the EDA domain has been identified as an important
402 protagonist in the TGF- β -mediated conversion of precursor cells into myofibroblasts in several fibrotic
403 disorders (Arslan et al., 2011; Booth et al., 2012; Hirshoren et al., 2013; Kohan et al., 2011; Muro et al.,
404 2003; Serini et al., 1998; White et al., 2008). Here we confirmed that FN B-A+ was able to induce the
405 expression of TGF- β response genes. However, using our complete toolset of purified full-length extra-
406 domain-containing FN variants and FN-deficient fibroblasts we found that effect was most pronounced
407 for the other variants. We further showed that this effect could be attributed to FN-induced activation of
408 latent TGF- β . A series of elegant studies have demonstrated that latent TGF- β can be activated by strain
409 generated within the actin-integrin-ECM axis [reviewed in (Kim et al., 2018)] and involving FN-bound
410 latent TGF- β binding proteins (LTBPs, namely LTBP1 and 3). In our experimental system, TGF- β signaling
411 peaked at 2 h after presenting the variants to the *Fn1* $-/-$ MFs. At this early time point LTBP1 is not likely
412 to be incorporated into nascent FN fibrils (Dallas et al., 2005), suggesting that FN-induced activation of
413 latent TGF- β /TGF β receptors by fibroblasts could involve a mechanism that operates in absence of a
414 mature matrix.

415 The computational tools described here allow robust classification of variant-specific fibrillar FN networks.
416 To analyze scale and fiber orientation, we chose to use the Gabor filters over the curvelet transforms
417 (Grapa et al., 2018; Liu et al., 2017) to eliminate complications related to translation and rotation. The
418 fiber centers were identified after establishing the skeletons, and a reconnection or "gap filling" procedure
419 was implemented to generate a more faithful representation of the fiber networks. Remarkably, our
420 findings unequivocally demonstrate that the presence of the extra domains confers quantitatively
421 tractable topological differences to each of the four FN variant networks. These results, together with
422 those of our functional studies, allow us to propose a scenario in which the presence of one or both extra
423 domains in FN, through distinct conformational changes, alters intra- and inter-molecular interactions

424 that differentially initiate and sustain integrin-mediated signaling events that drive FN fibrillogenesis and
425 a host of cellular responses. Although the cellular responses observed in MFs were modest, availability of
426 extra domain-containing FN should allow further investigations of the specific effects in other cell types.

427 With the ultimate goal of developing a generative model of cFN networks and tools for image-based tumor
428 biomarker analysis, we chose a scale-space strategy for analysis of fiber geometry. Our graph-based
429 description of the fibers based on Gabor filters was designed to compute and extract statistics about
430 network features. The selected features (fiber orientation, connectivity, thickness, anisotropy, and
431 fiber/pore density) are known to play determinant roles in defining cell migration modes and invasion
432 strategies during tumor progression (Kurniawan et al., 2016; Walker et al., 2018; Wolf and Friedl, 2011),
433 and orienting cell division during tissue expansion and development (Dekoninck et al., 2020). Alignment
434 of collagen fibers is a well-established hallmark of tumor-associated stromal matrix and indicator of poor
435 prognosis (Egeblad et al., 2010; Malik et al., 2015; Provenzano et al., 2006). We and others have shown
436 that alignment of FN fibers by cancer-associated fibroblasts (CAFs) promotes directional migration of
437 cancer cells (Attieh et al., 2017; Erdogan and Webb, 2017; Gopal et al., 2017). In the present study, no
438 significant differences in global FN fiber orientation or anisotropy were detected. This is likely due to the
439 fact that the fibers are deposited by normal, and not activated fibroblasts (myofibroblasts). Networks
440 deposited by MFs presented with plasma-like FN B-A- and B-A+ variants were the most distinguishable.
441 Plasma-like FN networks displayed the greatest fiber heterogeneity, with a highest proportion of
442 unbranched fibers and larger and more elongated pores than the extra domain-containing FN networks.
443 High structural heterogeneity could explain why plasma-like FN matrices displayed the widest distribution
444 of AFM measurements. In contrast, networks of FN containing extra domains, especially FN B-A+, were
445 more branched and displayed smaller pores. Interestingly, the presence of EDB resulted in a mixed set of
446 attributes for which further molecular insights into EDB-specific interactions would be necessary to
447 explain how this occurs. Future work will extend these computational analyses of FN networks assembled
448 by fibroblasts in vitro to the characterization of FN network features in the stroma of human tumors in
449 which cFN overexpression is an independent unfavorable prognostic indicator (Gopal et al., 2017). Given
450 the fundamental importance of cFN-rich matrices in orchestrating matrix organization and regulating the
451 behavior of numerous cellular effectors in the tumor microenvironment [reviewed in (Efthymiou et al.,
452 2020)], the experimental tools and computational resources described here should prove valuable for
453 accelerating biologically- and clinically-relevant discoveries in this field.

454 **Materials and methods**

455 **Materials**

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

458 **Cells and culture conditions**

459 Human umbilical vein endothelial cells (HUVECs) were prepared from fresh human umbilical veins and
460 maintained as previously described (Radwanska et al., 2017). *Fn1 fl/fl* mouse kidney fibroblasts (MFs)
461 were kindly provided by Dr. Reinhard Fässler [Max Planck Institute, Martinstread, Germany (Sakai et al.,
462 2001)]. The HEK293FT cell line was from Life Technologies (Saint Aubin, France). All cells were cultured in
463 Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine (Thermo Fisher Scientific, Waltham,
464 MA, USA) and 10% fetal calf serum (FCS; 5% for signaling experiments) (Biowest, Nuaille, France) in a
465 humidified incubator at 37°C with 5% CO₂. For experiments, FN was depleted from FCS using gelatin
466 sepharose-4B columns (GE Healthcare, Uppsala, Sweden), and the culture medium was supplemented
467 with Penicillin-Streptomycin 100 U/ml. No coating was performed on culture dishes and coverslips unless
468 otherwise stated. Absence of Mycoplasma sp. contamination was routinely verified by PCR as described
469 elsewhere (Kong et al., 2001).

470 **Antibodies**

471 Primary antibodies are indicated in Table S1. Secondary antibodies coupled to horseradish peroxidase
472 were from Jackson Immunoresearch Labs (West Grove, PA, USA). Fluorescently labeled (Alexa Fluor 488,
473 564, and 647-conjugated) secondary antibodies were purchased from Thermo Fisher Scientific.

474 **Lentiviral vector construction and production of lentiviral particles**

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

478 Full-length cellular *FN1* cDNA was amplified using primers: 5'-CACCTCTCTCCCCACCGTCTCAACA-3'
479 (forward) and 5'-GATCTTGGCAGAGAGACATGC-3' (reverse) in a two-step RT-PCR with total RNA isolated
480 from HUVECs. Next, *FN1* cDNA was cloned with the pENTR™/D-TOPO® Cloning Kit (Applied Biosystems,
481 Foster City, CA, USA). EDB or EDA domains were inserted or deleted using site-directed mutagenesis to
482 obtain four different constructs (pENTR-FN) corresponding to the following FN variants: FN/EDB-EDA- (FN
483 B-A-), FN/EDB+EDA- (FN B+A-), FN/EDB-EDA+ (FN B-A+), and FN/EDB+EDA+ (FN B+A+). When verified by
484 sequencing, cDNA of each variant was inserted in the 2K7 lentiviral expression vector by recombination

485 between appropriate sites in pENTR-EF-1 α (L4-R1), pENTR-FN variant (L1-L2) and 2K7-BSD vector with a
486 blasticidine resistance gene. Recombination reaction was performed with LR plus clonase (Applied
487 Biosystems, Foster City, CA, USA).

488 Lentiviral particles were produced by transfecting HEK293FT cells with a mixture composed of a 2K7-FN
489 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
490 (Gag, Pol)] using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's
491 instructions. Virus-containing supernatants were collected 48 h after transfection.

492 **Purification of FN variants**

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

499 FN variant purification was performed as described previously with modifications (Akiyama, 2013). Briefly,
500 HEK293 cells expressing FN variants were cultured at confluence in basal culture medium for 2-3 days.
501 Conditioned medium was subsequently collected and centrifuged in the presence of 0.2 mM PMSF. Ten
502 ml of Gelatin-Sepharose[®] 4B (GE Healthcare) were added per 500 ml of supernatant prior to overnight
503 incubation at 4°C with slight agitation. Eluted FN fractions corresponding to the highest absorbance (A280)
504 were pooled, and FN was subsequently dialyzed against CAPS-saline buffer pH 11.0. FN aliquots were flash
505 frozen and stored at -80°C. The purity and concentration of FN variants were verified using Coomassie
506 Blue gel staining, silver stain, and the Pierce[™] BCA Protein Assay Kit (Thermo Fisher Scientific), according
507 to manufacturer's instructions. To assess the presence of insoluble aggregates, glass coverslips were
508 coated with 3 μ g/ml of FN variants at 37°C for 1 h then fixed, blocked, and stained for immunofluorescence
509 analysis with an anti-FN antibody. Detection of TGF- β 1 in the purified FN preparations was performed
510 with the Quantikine[®] ELISA Human TGF- β 1 Immunoassay Kit (R&D Systems, Minneapolis, MN, USA),
511 according to the manufacturer's instructions. Low or no expression of other potentially contaminating
512 factors of the PDGF, FGF, VEGF and TGF- β families (Martino and Hubbell, 2010) by these cells was verified
513 by using the Human Protein Atlas database (<https://www.proteinatlas.org/humanproteome/cell>,
514 retrieved on November 10, 2020).

515 **Knockout of FN in *Fn1 fl/fl* mouse fibroblasts and re-expression of FN variants**

516 Cre recombination was performed by infecting *Fn1 fl/fl* MFs with a self-excising Cre retrovirus [pMMP-RV-
517 Cre-GFP (Silver and Livingston, 2001)] and transduced cells were selected with 6.5 µg/ml puromycin.
518 Clones were obtained by limiting dilution with a flow cytometer and analyzed for FN expression and gene
519 deletion by immunofluorescence staining and PCR analysis, respectively.

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

524 **RNA isolation, reverse transcription, and qRT-PCR**

525 Cells were lysed with Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's
526 instructions. RNA samples were quantified using a small-volume spectrophotometer (DeNovix DS-11
527 series, Wilmington, DE, USA) and 1 µg of RNA was reverse-transcribed into cDNA using the High-Capacity
528 cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The resulting cDNA was diluted tenfold and
529 amplified in a StepOne Plus thermocycler (Thermo Fisher Scientific) using gene-specific primers and
530 PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific). Standard cycling conditions were used.
531 For FN re-expression, results are represented by the $-\Delta\text{Ct}$ values ($\text{Ct}_{\text{gene of interest}} - \text{Ct}_{\text{normalizing gene}}$) as arbitrary
532 units (AU). For FN stimulation experiments, results were analyzed according to the $2^{-\Delta\Delta\text{Ct}}$ method and are
533 expressed as fold change compared to vehicle control (CTRL). *Tbp* was used as the normalizing gene.
534 Primer sequences are indicated in Table S2.

535 **Western blotting**

536 Whole cell lysates and conditioned media were collected with Laemmli buffer 3x (6% SDS, 30% glycerol,
537 187.5 mM Tris HCl pH 6.8) under non-reducing or reducing conditions (5% β-mercaptoethanol), separated
538 by gel electrophoresis and transferred onto Immobilon P membranes (Millipore, Bedford, MA, USA).
539 Membranes were blocked with 3% skim milk in TBS (50 mM Tris-HCl pH 7.4; 150 mM NaCl) before protein
540 immunodetection with the indicated antibodies (Supplemental Table 1). Protein bands were visualized
541 with horseradish peroxidase (HRP)-conjugated secondary antibodies followed by enhanced
542 chemiluminescence (Bio-Rad, Hercules, CA, USA) in a Fusion Fx7 Advanced system (Vilber Lourmat,
543 Eberhardzell, Germany).

544 **Immunofluorescence staining and microscopy**

545 For immunofluorescence analyses, cells or decellularized matrices were fixed in 4% paraformaldehyde
546 with 3% sucrose and permeabilized with 0.2% Triton X-100. The dilutions of the primary and secondary
547 antibodies are shown in Table S1. Nuclei were detected with Hoechst 33342 (Thermo Fisher Scientific).
548 After staining, the coverslips were mounted in ProLong[®] Gold antifade reagent (Thermo Fischer Scientific).
549 Widefield fluorescence was observed through 40X/1.3 NA or 63X/1.4 NA oil objectives on a Zeiss Axiovert
550 200M microscope equipped with a CoolSnap HQ CCD. Image acquisition was performed using the
551 MetaMorph Imaging System. Confocal imaging was performed on a Zeiss LSM710 confocal system using
552 10X/0.45 NA and 63X/1.4 NA objectives. Image analysis was performed using Fiji (Schindelin et al., 2012).

553 **Proliferation rate and cell doubling time**

554 *Fn1* ^{-/-} MFs were plated in triplicate at a density of 2.5X10⁴ cells/well in 24-well plates. Cells were
555 stimulated after 3-4 h with 15 µg/ml of recombinant FN variants, or vehicle buffer (CAPS-saline). At the
556 indicated times, cells were trypsinized, resuspended in culture medium and counted with a Malassez
557 hemocytometer (Thermo Fisher Scientific). Cell doubling time was determined during the exponential
558 phase of growth (between days 2 and 3) in control and variant-treated cells by using the reverse equation
559 and the “Goal-Seek” function in MS Excel.

560 **Internal pH measurement**

561 *Fn1* ^{-/-} MFs were plated in duplicates in 24-well plates (5X10⁴ cells/well). Cells were stimulated with 15
562 µg/ml of recombinant FN variants, or vehicle buffer (CAPS-saline) for 48 h. Internal pH (pHi) measurement
563 was performed as described (Cophignon et al., 2017). Briefly, cells were incubated with 1 µM BCECF-AM
564 (Thermo Fisher Scientific) for 1 h, rinsed with recovery solution (120 mM sodium chloride, 5 mM KCl, 1
565 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 15 mM Hepes), and fluorescence intensity was measured using a
566 multi-well plate spectrophotometer (BioTek Synergy 4, Winooski, VT, USA). Data were collected with the
567 integrated software. Recovery solution was removed, and nigericin solutions were added (140 mM KCl, 1
568 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, 5 µM nigericin at pH values ranging from 6.5 to 8.0 in the presence
569 of 20 mM Hepes).

570 **Mitochondrial metabolism and glycolytic measurements**

571 For metabolic analysis, cells were seeded in 24-well plates (Seahorse Bioscience, Billerica, MA) and
572 stimulated with 15 µg/ml of recombinant FN variants or vehicle buffer for 48 h. The oxygen consumption
573 rate (OCR) and extracellular acidification rate (ECAR) were determined using an XF24 Extracellular Flux
574 Analyzer (Seahorse Bioscience) and Seahorse basal medium supplemented with 2 mM glutamine.
575 Maximal OCR was determined by using FCCP (1 µM), and rotenone and antimycin-A (2 µM each) were

576 used to inhibit Complex I- and Complex III-dependent respiration, respectively. All parameters were
577 calculated as described previously (Brand and Nicholls, 2011).

578 For glucose uptake comparison, *Fn1* ^{-/-} MFs were plated in triplicates in 96-well plates (4X10³ cells/well)
579 and stimulated with 15 µg/ml of FN variants or vehicle buffer for 48 h. Cells were washed with PBS, starved
580 with glucose-depleted medium for 30 min before adding 200 µM of 2-NBDG (Thermo Fisher Scientific) for
581 40 min. Fluorescence intensity was measured in a microplate reader (BioTek Synergy 4). Results are
582 represented as fold difference relative to CTRL. Lactate extrusion in the culture medium of *Fn1* ^{-/-} MFs
583 treated with FN variants for 48 h (same conditions as in proliferation rate experiments) was analyzed using
584 a Dionex ion chromatography column (Thermo Fisher Scientific).

585 **Generation of MF-derived matrices**

586 MF-derived matrices were generated as described previously (Kaukonen et al., 2016). Briefly, coverslips
587 were coated with 0.2% gelatin for 60 min at 37°C, followed by crosslinking with 1% glutaraldehyde for 30
588 min at room temperature. Crosslinker was quenched with 1 M glycine for 20 min and gelatin-coated
589 coverslips were incubated with medium before seeding 2X10⁵ cells. Ascorbic acid (50 µg/ml) and FN
590 variants (15 µg/ml) were added the next day. On day 3, medium was changed, and on day 7, matrices
591 were decellularized in 20 mM NH₄OH, 0.5% Triton X-100 in PBS followed by 10 µM DNase I treatment
592 (Roche Diagnostics GmbH, Penzberg, Germany).

593 **Atomic Force microscopy**

594 Decellularized ECM was prepared on 35mm dishes. Samples were rinsed and immersed in 3 ml of PBS.
595 The mechanical properties of the samples were studied as described previously (Andersen et al., 2019)
596 with slight modifications. Briefly, four areas per sample were analyzed, and 140 force-distance curves
597 were collected. Deflection sensitivity and spring constant of the cantilever were determined using a clean
598 glass dish with PBS. Velocity and trigger threshold were set to 1 µm/s and 2 nN respectively. The elastic
599 (Young's) modulus was calculated as described previously. To eliminate any tilt effect due to the base
600 correction step in the analysis, only force curves with maximum value equal to 2 nN were used to perform
601 the fit.

602 **Collagen gel contraction assay**

603 *Fn1* ^{-/-} MFs were embedded (20X10⁴ cells/lattice) in collagen lattices (1 mg/ml) containing 15 µg/ml of FN
604 variants in duplicates in 12-well plates. A corresponding volume of NaOH was added to bring the pH of
605 the mixture back to 7.4 and lattices were left at room temperature for 30 min to solidify. Medium

606 containing 15 µg/ml of FN variants was added on top of the lattices which were subsequently detached
607 from the well walls and bottom. Gel surface reduction was measured with ImageJ after a 24-hour
608 incubation at 37°C in the presence of 5% CO₂.

609 **Computational analysis of matrix topology**

610 *Image Acquisition for numerical characterization of FN variant fibers and classification*

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

620 *Definition of Gabor kernels*

621 Fibrillar structures were detected and enhanced with Gabor filters (Petkov, 1995), commonly employed
622 in image processing for the detection of structures with different frequencies, and certain directionalities.
623 A set of Gabor kernels was defined, characterized by the formula

$$624 \quad G_{\theta_i, \lambda_j, \varphi, \Sigma_{\theta_i}}(x, y) = \exp\left(-\frac{1}{2} v^t \Sigma_{\theta_i}^{-1} v\right) \cos\left(\frac{2\pi x \theta_i}{\lambda_j} + \varphi\right) (1),$$

625 where $v = (x, y)^t$. The exponential term provides the shape of a bivariate Gaussian kernel, and the cosine
626 function describes its oscillations in space, while v is the 2D coordinate vector, indicating pixel localization
627 in a bi-dimensional Cartesian coordinate system.

628 *Fiber detection computation and morphological skeletonization*

629 Fiber orientation was represented by θ_i with values within the interval $[0, \pi]$, with a stepsize of $\frac{\pi}{20}$. The
630 covariance matrix of the bivariate Gaussian function rotated with θ_i is related to the anisotropy of the
631 kernel spatial support and is designated by Σ_{θ_i} . Fiber thickness is represented by λ_j that corresponds to
632 the wavelength (in pixels) of the cosine term, the values of which are equal to $\frac{\lambda_j}{2}$ and vary between 3 and
633 5 pixels. The thinnest fibers are detected when $\lambda_j = 6$ pixels, medium thickness fibers correspond to $\lambda_j =$

634 8 pixels, while the thickest are characterized by $\lambda_j = 10$ pixels. For accurate localization of fibers we used
635 phase, φ , set to 0. The pixel intensity of a detected fiber at a specific location corresponded to the Gabor
636 filter with the highest coefficient within the Gabor kernel set. The specific parameters of the best
637 corresponding Gabor kernel for a detected fiber could subsequently be linked to physical properties, such
638 as fiber thickness and local fiber orientation.

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

645 *Feature extraction and Principal Component Analysis (PCA)*

646 Features related to fiber thickness and connectivity were directly computed using Gabor kernels and
647 graph-specific parameters. More specifically, connectivity was defined as the proportion of degree 1
648 nodes (those corresponding to fiber ends) relative to the nodes with a degree higher than 2
649 (corresponding to branching and intersecting points). Fiber thickness kurtosis, was determined by the
650 formula

$$651 \quad k = E[(x - \mu)^4]/(\sigma^4) \quad (2),$$

652 where μ is the mean value of x , σ is the standard deviation of x , and $E[s]$ is the expected value of the
653 quantity s . Attributes describing pore regions were computed using the MATLAB tool Regionprops. Pore
654 dimension was measured as the total number of pixels within the area delimited by the skeleton, while
655 pore circularity was determined by the formula

$$656 \quad (4 * Area * \pi)/(Perimeter^2) \quad (3),$$

657 the values of which vary between 0 (line-like) and 1 (perfect circle). For pore size, only pores distributed
658 above the 90th percentile were taken into account, and the mean was calculated. For PCA visualization,
659 each FN network sample (image) was represented in a 5-dimensional space defined by the previously
660 selected features.

661 **Statistical Analysis**

662 Values are represented as the mean \pm s.e.m. when $n \geq 3$ and as mean \pm s.d. when $n=2$, where n is the number
663 of independent experiments (specified in Fig. legends) unless otherwise stated. Statistical significance was
664 assessed by two-tailed Student's *t test* using Microsoft Excel for Windows (function *t test*). The Kruskal-
665 Wallis test for non-parametric distributions, followed by a Dunn's multiple comparison test was applied
666 for statistical analysis of AFM using GraphPad Prism v5.03 for Windows. *P* value is represented by
667 asterisks: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$. Asterisks above bars represent
668 significance between the respective condition and the CTRL. Asterisks above horizontal brackets represent
669 the significance between the conditions at each end of the bracket.

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676 **Competing interests**

677 The authors declare that there are no competing interests.

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683 **Author contributions:** EVOS conceived the project, established collaborations, analyzed the data, and
684 acquired funding for the work. The manuscript was written by EVOS and GE, reviewed and edited by all
685 authors. AR designed and generated the molecular tools. GE, AR, SBFD and DG performed experiments
686 involving molecular, cellular and biochemical analyses, contributed to the experimental design and data
687 analysis. MH, GE, AR and DG purified the recombinant proteins. AG, XD and LBF developed the
688 computational tools and implemented numerical analyses of images. SS developed software and
689 contributed to image analysis. MP, DP and LC provided expertise, performed experiments and analyzed
690 data involving pH regulation and metabolism. SP performed AFM experiments and data analysis.

691 **Materials and data sharing:** All data needed to evaluate the conclusions in the paper are present in the
692 paper and/or the Supplementary Information. All reagents, data, and computer code for this study are
693 available upon request from the authors.

694

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912 **Figure Legends**

913 **Fig. 1. FN variant-expressing MFs.** (A) Schematic representation of the four human FN variants harboring
914 the EDB and/or EDA (or none), including the variable region V-89, and their expression in assembly
915 competent *Fn1* $-/-$ MF clones. (B) Mean \pm s.e.m. FN mRNA levels across selected clones [indicated in (D)]
916 after 7 days in culture, expressed in arbitrary units (AU, n = 3). (C) Western analysis of FN expression in
917 four selected clones [same as in (B)] after 7 days in culture (n = 2 independent experiments performed
918 under identical conditions). Numbers beneath the conditioned medium (CM) and cell lysate blots
919 represent the mean \pm s.d. fold FN levels relative to MF B-A- in the conditioned medium and cell lysates,
920 respectively. ERK1/2 was used as loading control. The mean \pm s.d. ratios of FN in CM to the respective cell
921 associated FN is shown below (n = 2 independent experiments performed under identical conditions). (D)
922 Immunofluorescence staining to assess FN re-expression and assembly in the indicated set of clones after
923 7 days in culture (representative images of n=5 independent experiments are displayed). Nuclei staining
924 is shown in the inserts. Scale bar: 50 μ m.

925 **Fig. 2. Presentation of extra-domain-specific FN variants to *Fn1* $-/-$ MFs.** (A) Schematic representation of
926 the expression of FN variants in assembly-incompetent HEK293 cells for purification from conditioned
927 medium (CM). After purification, *Fn1* $-/-$ MFs are presented with the purified variants for functional
928 analyses. (B) Western blot of whole cell lysates and conditioned medium of *Fn1* $-/-$ MFs presented with
929 FN variants for 2 h. Quantification of FN bands in the conditioned medium verified the addition of equal
930 amounts of FN to the cells, presented here as mean \pm s.e.m. relative to the amount of FN B-A- (n = 9). Cell
931 associated FN represents harvesting and deposition of FN variants by *Fn1* $-/-$ MFs shown as mean \pm s.e.m
932 relative to FN B-A-. ERK1/2 was used as loading control (n = 2 independent experiments performed at this

933 time point). (C) Immunofluorescence staining of FN and nuclei of *Fn1* ^{-/-} MFs presented with FN variants
934 for the indicated times (2 h, n = 2; 48 h, n = 3 independent experiments). Polymerized FN is located at
935 areas of cell-cell contact (thick arrow) and retracting edges of polarized cells (thin arrow). The absence of
936 FN polymerization can be noted at the leading edge of spread cells (arrowhead). In the 48-hour images,
937 nuclei have been added as inserts. Scale bars: 50 μ m.

938 **Fig. 3. The effects of FN extra domains on cell growth and metabolism.** (A) Growth curves of *Fn1* ^{-/-} MFs
939 stimulated with FN variants or vehicle buffer for 5 days (mean \pm s.e.m.; n = 7). (B) Doubling time of cells
940 treated with FN variants relative to cells treated with vehicle CTRL determined from the linear part of the
941 exponential phase of growth (mean \pm s.e.m.; n = 7). (C) Representative phase contrast images of *Fn1* ^{-/-}
942 MFs 5 days after stimulation with FN variants. Scale bar: 100 μ m. (D) Medium acidification observed 4
943 days post treatment. (E) Extracellular acidification rate (ECAR) of *Fn1* ^{-/-} MFs after a 72-hour treatment
944 with FN variants or vehicle buffer measured with the Seahorse analyzer (mean \pm s.d.; from 2 independent
945 experiments; points represent the mean of technical triplicates). (F) Oxygen consumption rate (OCR)
946 measured with the Seahorse analyzer in *Fn1* ^{-/-} cells treated for 72 h with FN variants or vehicle buffer
947 (mean \pm s.d.; 3 technical repeats from 2 independent experiments). The color code is the same as in (A).
948 (G) Intracellular pH measurements in cells treated with FN variants or vehicle CTRL after 48 h (n = 6).
949 Results are represented as mean \pm s.e.m Δ pH_i (difference in internal pH) relative to CTRL. (**p* \leq 0.05; ***p*
950 \leq 0.01; Student's *t* test).

951 **Fig. 4. Regulation of TGF- β signaling by FN extra domain-containing variants.** (A) Time course of TGF- β
952 response gene expression. *Fn1* ^{-/-} MFs were stimulated with FN variants for the indicated time points.
953 Results are represented as mean \pm s.e.m. fold change relative to vehicle control (CTRL) for each time point.
954 Time points later than 8 h have been omitted for clarity (n = 4). (B) Time points at which changes in
955 expression levels are the highest (2 h for *PAI1* and *CTGF*; 48 h for *TGFBI*). (C) Western blot displaying the
956 phosphorylation of SMAD2 (p-SMAD2) after a 2-h stimulation of *Fn1* ^{-/-} MFs with FN variants. Total
957 SMAD2 used as loading CTRL. Numbers beneath the blots represent mean \pm s.e.m. fold phosphorylation
958 relative to CTRL (n = 3). (D) Western blot showing the decrease in SMAD2 phosphorylation following
959 TGFBR1 inhibition. *Fn1* ^{-/-} MFs were treated with FN B+A+ and SB-431542, a TGFBR1 inhibitor, and their
960 respective vehicle solutions for control. Total SMAD2 was used as loading control. Numbers beneath the
961 blots represent mean \pm s.d. fold phosphorylation relative to respective CTRL (n = 2 independent
962 experiments). (**p* \leq 0.05; ***p* \leq 0.01; ****p* \leq 0.001; Student's *t* test).

963 **Fig. 5. The impact of the FN extra domains on FN assembly and ECM physical properties.** (A) Variant-
964 specific decellularized matrices. *Fn1* ^{-/-} MFs were presented with FN variants for 7 days. Matrices were
965 generated, decellularized, fixed and immunofluorescently stained for FN, type VI collagen, and TNC (n = 2
966 technical repeats from 2 independent experiments). Scale bar: 100 μ m. (B) Matrix thickness measured
967 with confocal microscopy (mean \pm s.e.m.; n = 5). (C) FN variant-specific ECM stiffness was measured with
968 atomic force microscopy (n = 3). Data from a representative experiment presented as box-and-whisker
969 plots, with box borders representing the 25% and 75% of the interquartile range, and whiskers
970 representing the 10% and 90% of the data points; median values are shown. ** $p \leq 0.01$; *** $p \leq 0.001$;
971 **** $p \leq 0.0001$ (B, Student's t test; C, Kruskal-Wallis test for non-parametric distributions).

972 **Fig. 6. Computational analyses of FN variant-specific matrices.** (A) Matrices assembled by *Fn1* ^{-/-} MFs
973 presented with FN variants were decellularized after 7 days, stained for FN and visualized by confocal
974 microscopy. Scale bar: 50 μ m. (B) Confusion matrix of FN variant network classification using
975 Convolutional Neural Networks. An algorithm was trained to recognize and classify FN-variant-specific
976 matrices. The diagonal (in blue) displays the classification success rate (correctly classified images). (C)
977 Flow chart of fiber analysis. **1:** confocal images were acquired and cropped to a final size of 512 \times 512
978 pixels; **2:** a set of Gabor kernels representing an elemental-fiber dictionary was established; **3:** fiber
979 detection was performed using Gabor kernels on acquired images; **4:** detected fibers were used to
980 generate variant-specific morphological skeletons; **5:** feature extraction. (D) Graph-based representation
981 of the FN variant fibers detected by Gabor kernels. Edges (black segments) connect nodes (red dots). (E)
982 Principal component analysis (PCA) of the indicated features related to the graphs or derived from
983 characteristic Gabor kernel parameters.

Figure 1

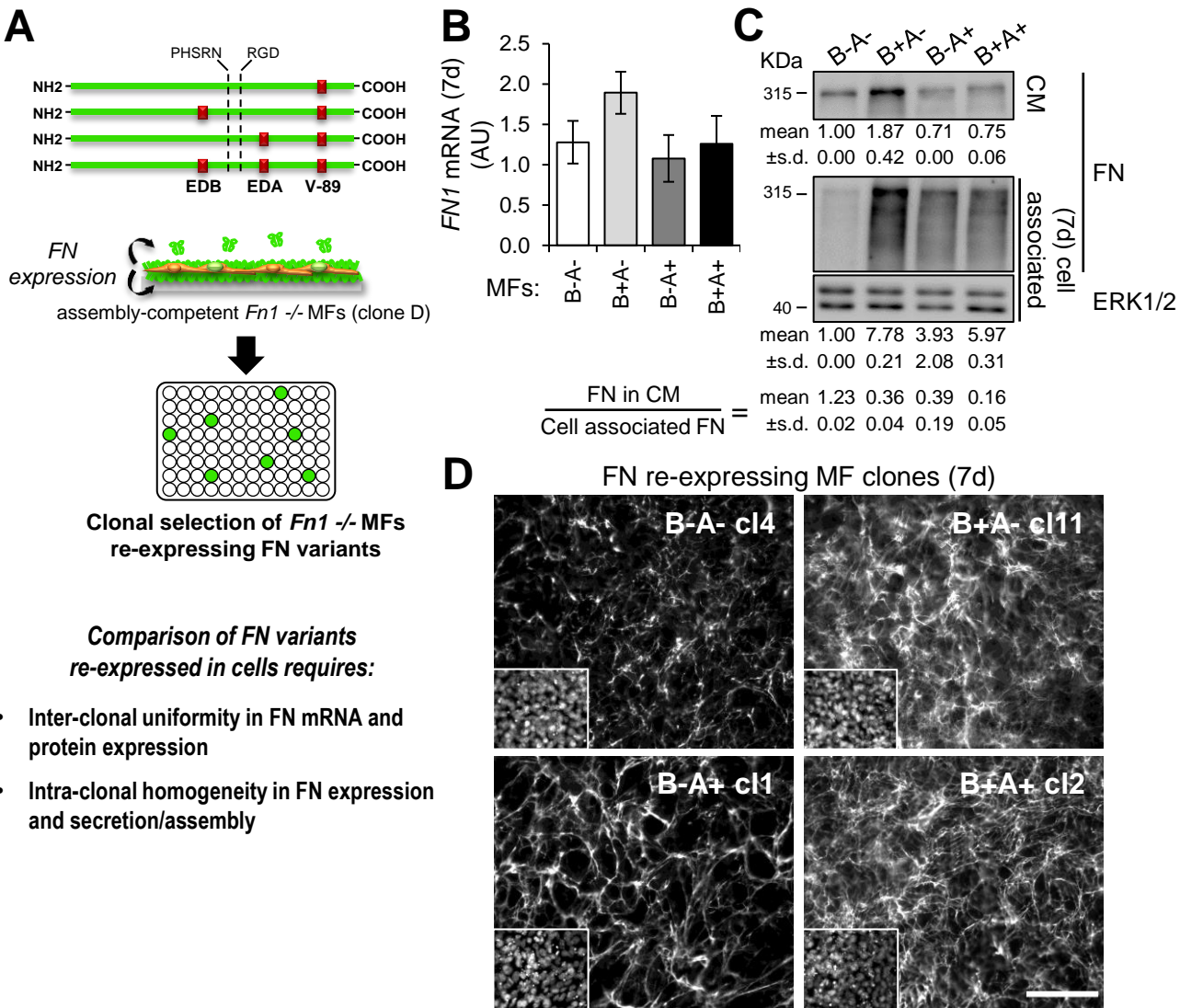
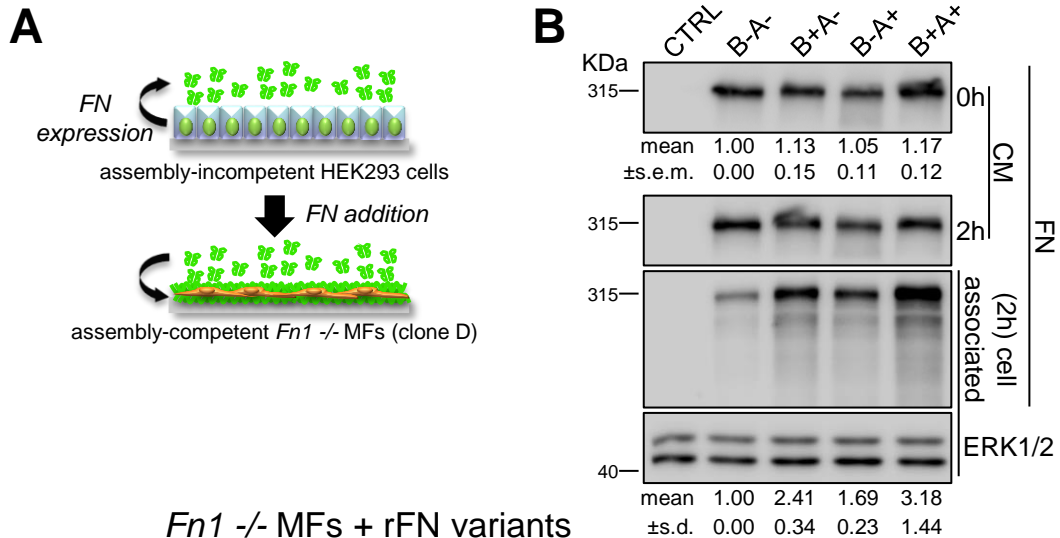


Figure 2



Fn1^{-/-} MFs + rFN variants

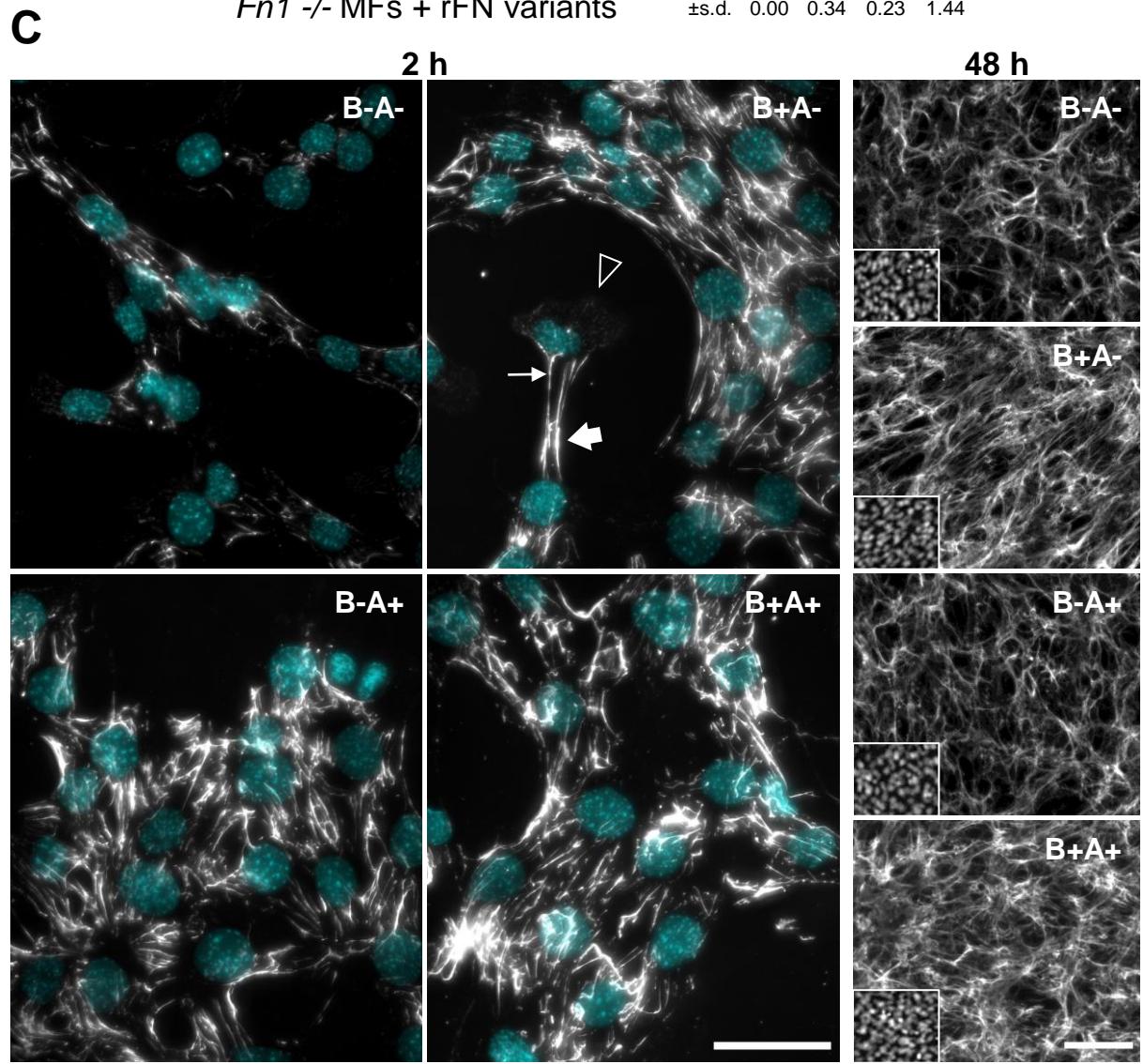


Figure 3

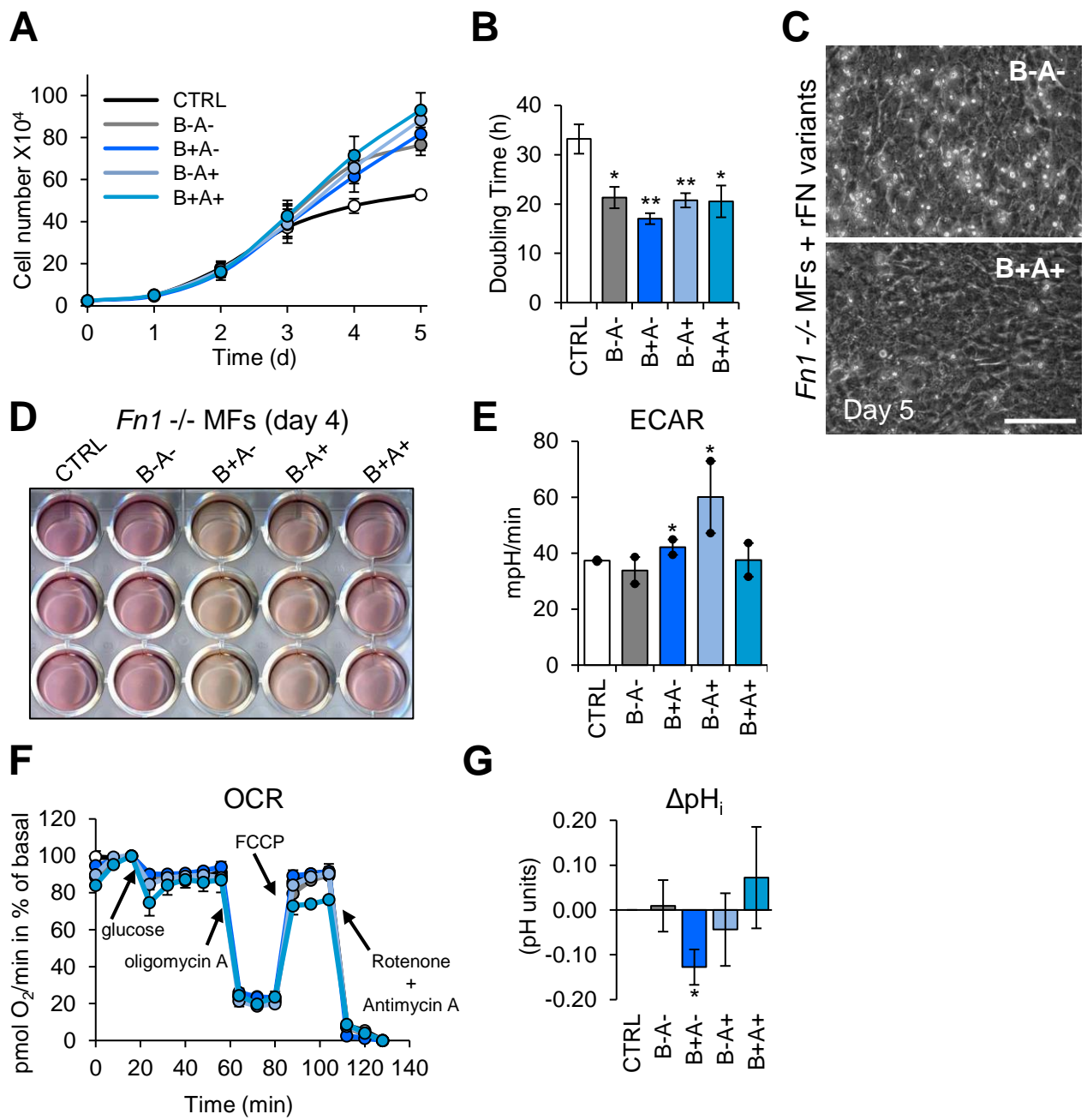


Figure 4

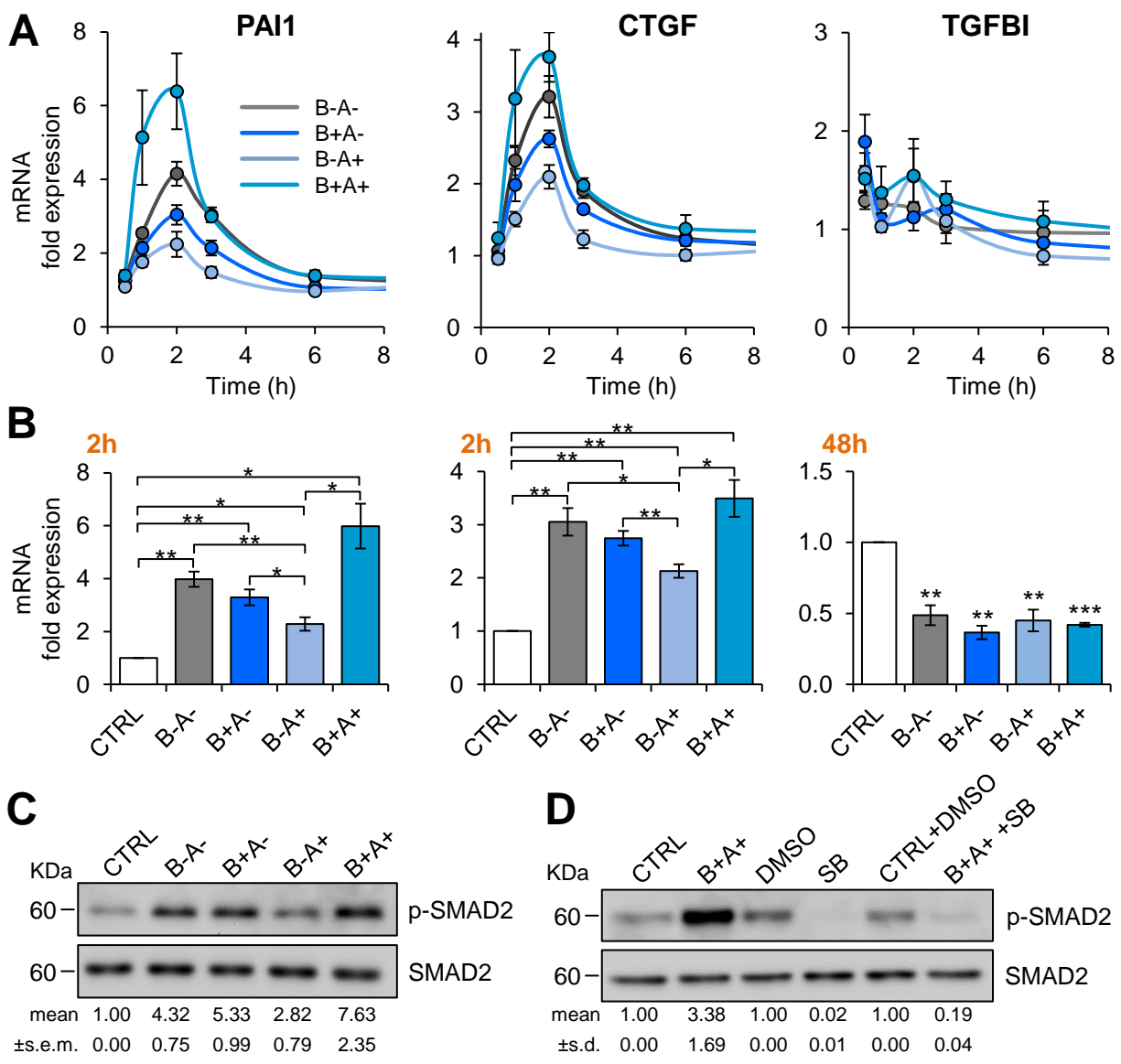


Figure 5

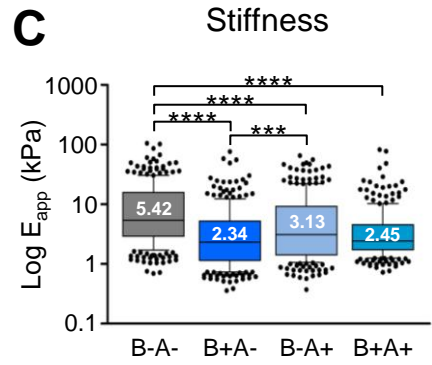
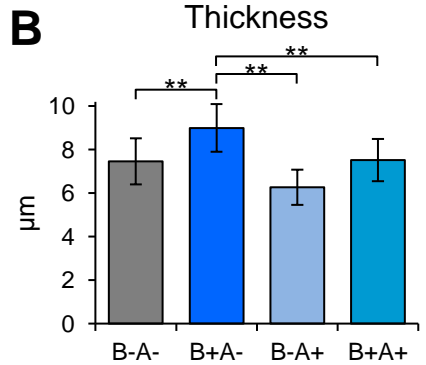
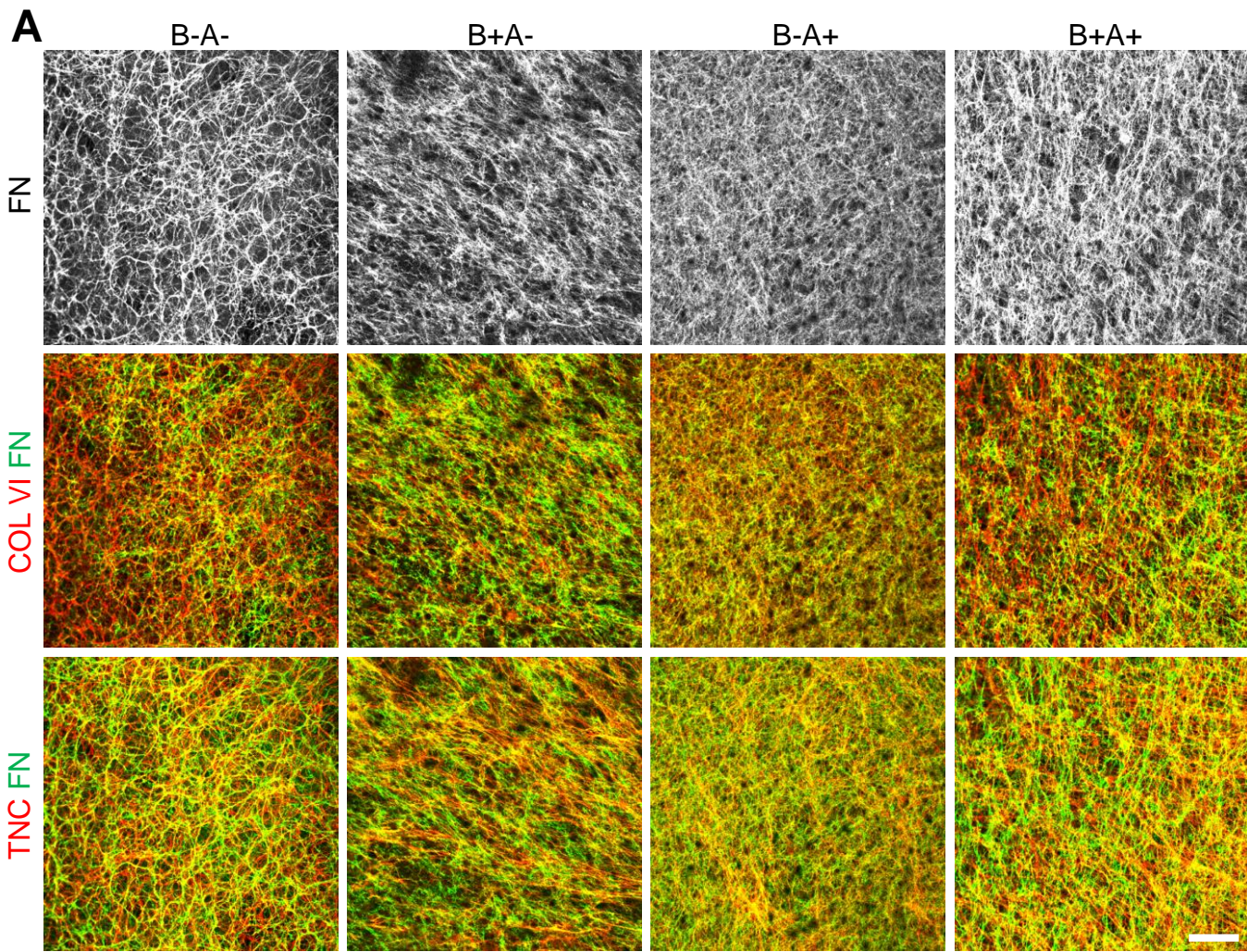
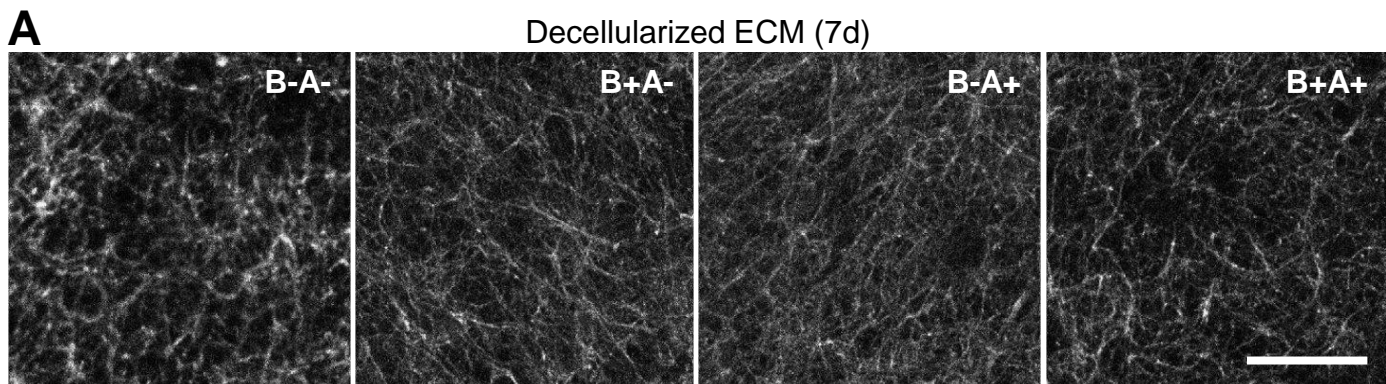


Figure 6

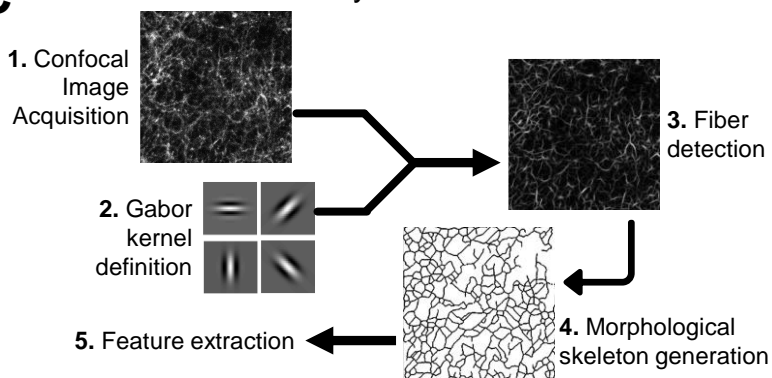


B Image classification with Convolutional Neural Networks

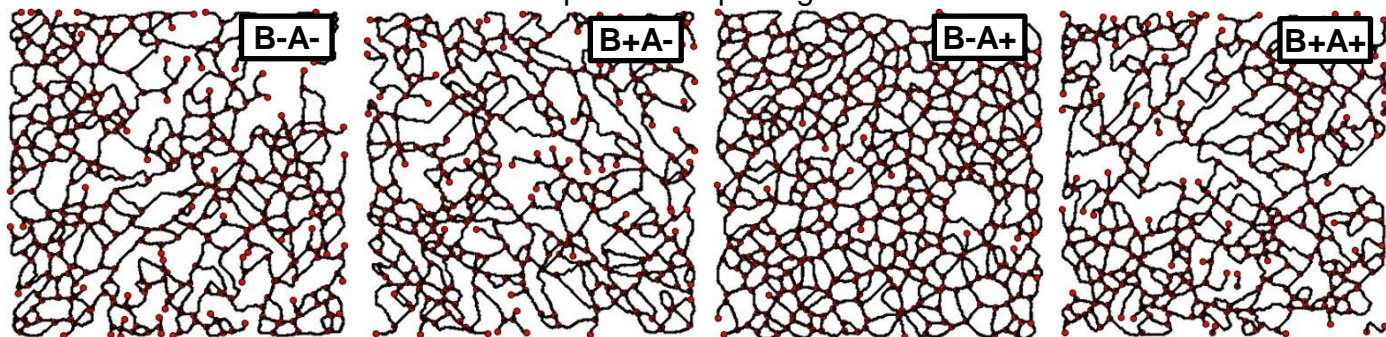
Predicted \ Actual	B-A-	B+A-	B-A+	B+A+
B-A-	85.7	0	0	14.3
B+A-	0	80.9	14.3	4.8
B-A+	0	9.5	90.5	0
B+A+	9.5	14.3	0	76.2

General accuracy: **83.3%**

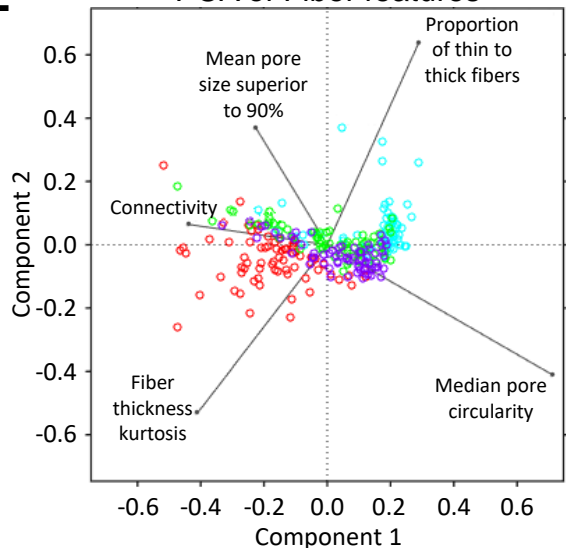
C Fiber analysis workflow



D FN variant-specific morphological skeletons



E PCA of Fiber features



Features

- Proportion of degree 1 nodes relative to the nodes of superior degree (connectivity)
- Proportion of thin fibers to thick fibers (fiber thickness)
- Fiber thickness kurtosis (distribution of fiber thickness values with respect to the mean)
- Pore circularity (median value)
- Pore size (mean derived from values superior to the 90th percentile)

1 **Supplementary Information**

2 **Supplementary Figures**

3 **Fig. S1. Generation of *Fn1* ^{-/-} MF clones.** (A) Relative amounts of FN mRNA containing EDB and/or EDA
4 assessed by qRT-PCR (n = 4). (B) Schematic representation of the relevant position of the two LoxP sites
5 flanking the first exon of the *Fn1* gene, and the positions of the primer set used to confirm the deletion.
6 (C) Agarose gel electrophoresis of the PCR products generated from four separate *Fn1* *fl/fl* populations
7 infected with a lentiviral vector bearing the coding sequence of Cre recombinase. (D) Western analysis
8 showing the elimination of FN protein in whole cell lysates and conditioned medium from *Fn1* ^{-/-} MF Clone
9 D. ERK was used as loading control (n = 4). (E) Phase contrast (top row) and immunofluorescent staining
10 (middle and bottom rows) of FN displaying the absence of FN deposition in *Fn1* ^{-/-} MF Clone D after 2 days
11 in culture (n = 4). Scale bars: Phase, 200 μm; IF, 50 μm.

12 **Fig. S2. Variability in FN variant expressing MFs.** (A) Immunofluorescent staining of FN in the four selected
13 clones. An anti-totalFN antibody was used in combination with an anti-EDB or anti-EDA to verify the
14 expression of the FN variants by the different clones (B-A- cl4, B+A- cl11, B-A+ cl1, B+A+ cl2). Scale bar, 50
15 μm (n = 2). (B) FN mRNA and protein levels in three individual clones expressing FN B-A-. Numbers beneath
16 the blot represent fold expression of FN relative to B-A- cl4 normalized to ERK. (C) FN mRNA and protein
17 levels in three individual clones expressing FN B+A-. Numbers beneath the blot represent FN fold
18 expression relative to B+A- cl11 normalized to ERK. (D) Immunofluorescent staining of three individual
19 clones expressing FN B-A- [same as in (B)] and three individual clones expressing FN B+A- [same as in (C)]
20 to compare FN expression and assembly across clones for 2 different FN variants after 7 days in culture.
21 Nuclei are shown in the inserts. Scale bar: 50 μm (n = 2).

22 **Fig. S3. Recombinant FN variant production in HEK293 cells.** (A) Phase contrast (top row) and
23 immunofluorescent staining of FN and nuclei (bottom row) of HEK293 cells stably expressing the FN
24 variants or the empty vector. Scale bars: Phase, 50 μm; IF, 20 μm. FN is only observed in the cytoplasm
25 and the plasma membrane before being secreted by the assembly-incompetent HEK293 cells. (B)
26 Recombinant FN variant production by HEK293 cells determined by ELISA. (C) Silver staining of FN variants
27 in polyacrylamide gels run under reducing and non-reducing conditions to verify the integrity of the
28 proteins, as well as their ability to dimerize. (D) Western blot of FN variants run in polyacrylamide gels and
29 stained with different antibodies against total FN, Extra Domain B, and Extra Domain A verifying the all-
30 or-nothing inclusion of the Extra Domains in each variant. (E) Immunofluorescent staining of FN-coated (3

31 $\mu\text{g/ml}$) glass coverslips to assess the existence of aggregates, that would greatly affect solubility and
32 bioavailability. Arrowheads indicate examples of isolated FN aggregates. Scale bar: 50 μm . (F)
33 Immunofluorescent staining of FN (left column) and phase contrast (right column) of *Fn1* $-/-$ MFs 5 days
34 after having been presented with increasing concentrations of pFN to find the optimal concentration for
35 our studies. We determined that 15 $\mu\text{g/ml}$ is a good compromise between efficient matrix generation and
36 moderation in the use of the purified variants.

37 **Fig. S4. Integrin $\alpha 5$ and $\beta 3$ distribution in MFs treated with FN variants.** Immunofluorescent staining of
38 FN assembly and integrin $\alpha 5$ and $\beta 3$ localization in MFs stimulated with recombinant FN variants for 2 h.
39 Scale bar, 50 μm . Thin arrows show the FN polymerization along the rear edge of polarized cells. Thick
40 arrows indicate sites of readily polymerized cFN at areas of cell-cell contact. Arrowheads point the
41 absence of FN polymerization at the leading edge of spread cells where $\alpha v\beta 3$ focal adhesions are more
42 prominent (n = 2).

43 **Fig. S5. Metabolic effects of FN variants in *Fn1* $-/-$ MFs and TGF- β detection in FN preparations and**
44 **conditioned media of *Fn1* $-/-$ MFs.** (A) Glucose uptake in *Fn1* $-/-$ MFs stimulated with FN variants for 48
45 h. Results are displayed as mean \pm s.e.m. fold uptake relative to CTRL (n = 9). (B) Extrusion of lactate in the
46 culture medium of *Fn1* $-/-$ MFs treated with FN variants for 48 h, represented as mean \pm s.e.m. fold
47 difference relative to day 0 CTRL (n = 2). (C) and (D) Expression of mRNA encoding glyceraldehyde
48 phosphate dehydrogenase (GAPDH) mRNA and phosphoglycerate kinase 1 (PGK1), enzymes that catalyze
49 the 6th and 7th steps of glycolysis respectively, was determined in *Fn1* $-/-$ MFs after 48 h of stimulation
50 with FN variants (n = 4). (E) Extracellular acidification rate in response to glucose measured in *Fn1* $-/-$ MFs
51 stimulated for 72 h with FN variants with the Seahorse analyzer. (F) Basal mitochondrial activity of *Fn1* $-/-$
52 MFs stimulated with FN variants for 72 h, determined with the Seahorse analyzer by measuring oxygen
53 consumption rate (OCR). (G) Maximal mitochondrial activity of *Fn1* $-/-$ MFs stimulated with FN variants
54 for 72 h, measured as in (E). (H) *Fn1* $-/-$ MFs were stimulated with FN variants for 72 h and OCR was
55 determined in response to glucose with the Seahorse analyzer. (I) Dependence of respiration of *Fn1* $-/-$
56 MFs stimulated with FN variants on ATP synthase demonstrated with the Seahorse analyzer (n = 2). (J)
57 TGF- $\beta 1$ detection in recombinant FN preparations. FN preparations were added in DMEM (15 $\mu\text{g/ml}$) to
58 assess the presence of TGF- $\beta 1$. Standard culture medium (DMEM +5% FCS) was used as reference. (K)
59 Total TGF- $\beta 1$ concentration in the conditioned medium of *Fn1* $-/-$ MFs stimulated with 15 $\mu\text{g/ml}$ of
60 recombinant FN variants for 2 h (n = 2). (L) Collagen based gel contraction assay. *Fn1* $-/-$ MFs were

61 embedded in collagen lattices containing FN variants. Cell surface reduction was measured after a 24-
 62 hour incubation following lattice solidification (n = 4).

63 **Fig. S6. Feature distribution across FN variant matrices.** (A) Connectivity graph displaying the ratio of
 64 degree-1 nodes relative to higher-degree nodes. (B) Thickness graph showing the distribution of thin fibers
 65 relative to thick fibers as described in materials and methods. (C) Graph representing the heterogeneity
 66 in fiber thickness across the different matrices, as determined by the fiber thickness kurtosis distribution.
 67 (D) Median pore circularity distribution defined by pore area and perimeter. (E) Average pore size
 68 distribution across FN matrices after filtering out pores below the 90th percentile within each distribution.

69 **Supplementary Tables**

70 **Table S1. Primary antibodies list 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: 1/200
anti-FN-EDB	Sirius Biotech	C6	S-FN12	Mouse	WB: 1/200 IF: 1/200
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
anti- α 5	Chemicon	Polyclonal	AB1928	Rabbit	IF: 1/1000
anti- β 3	Emfret	Luc.A5	M030-0	Rat	IF: 1/500
anti-COL VI	Millipore	VI-26	MAB3303	Mouse	IF: 1/200
anti-TNC	Sigma-Aldrich	BC-24	T2551	Mouse	IF: 1/200

71

72 **Table S2. Primers sequences used for qPCR studies.**

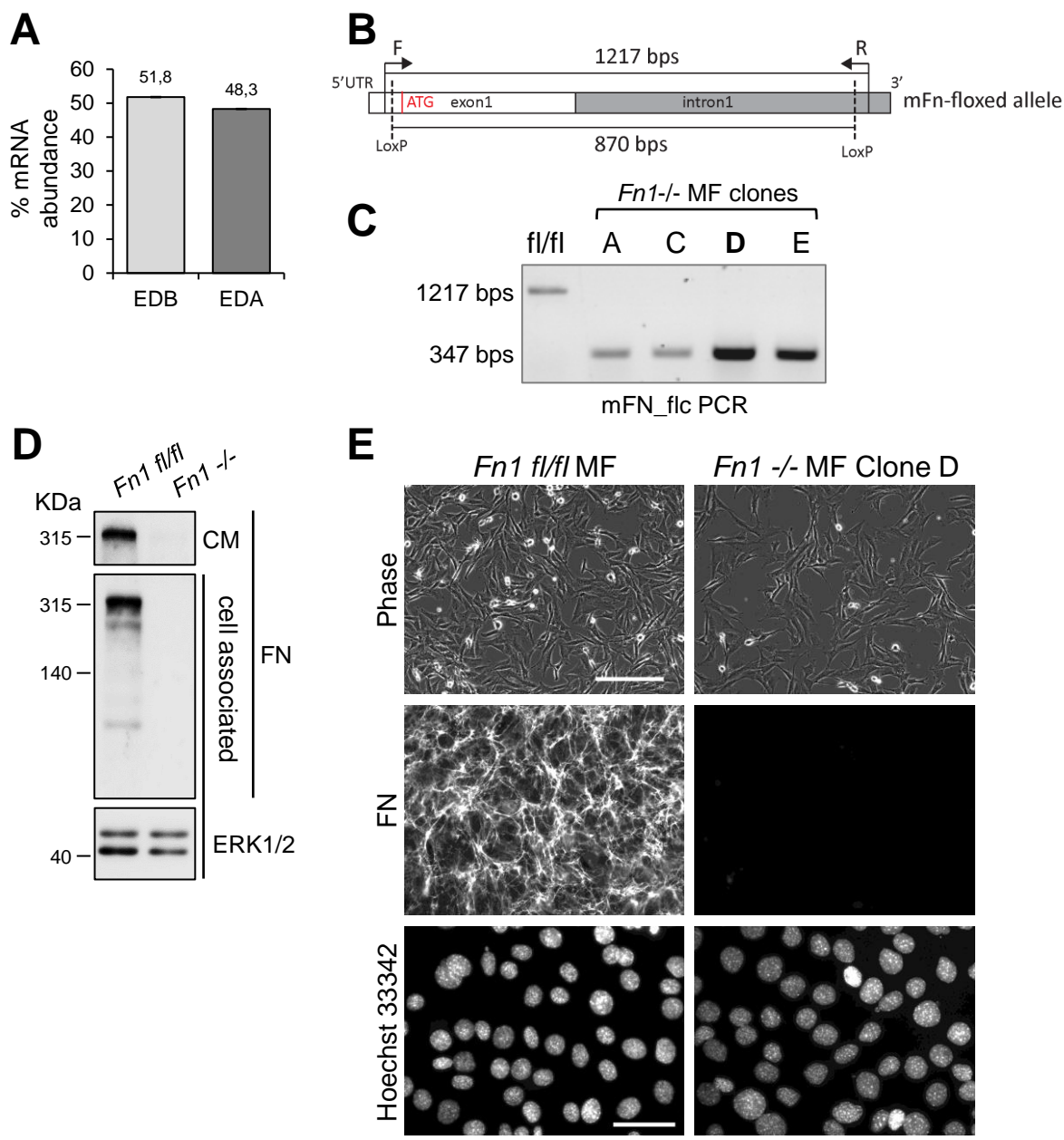
Primer Name	Sequence	
	Forward	Reverse
mTbp	GAA-GAA-CAA-TCC-AGA-CTA-GCA-GCA	CCT-TAT-AGG-GAA-CTT-CAC-ATC-ACA-G

h1_FN_2K7	CCC-AAT-TGA-GTG-CTT-CAT-GCC	TGT-ACA-AGA-AAG-CTG-GGT-CG
mFn1	CTG-TAG-CAC-AAC-TTC-CAA-TTA-CGA-A	GGA-ATT-TCC-GCC-TCG-AGT-CT
mFN-EDB	TGG-TCC-ATG-CTG-ATC-AGA-GT	CCT-CAG-GCC-GAT-GCT-TGA-AT
mFN-EDA	GGA-GCT-TTT-CCC-TGC-ACC-TG	GTG-GGT-GTC-ACC-TGA-CTG-AA
mTgfb1	AAG-GTC-ATT-GGC-ACC-AAC-AAG	AAC-CTT-TCT-CTC-CTG-GGA-CCT-T
mCtgf	CAG-CTG-GGA-GAA-CTG-TGT-ACG	GTA-CAC-CGA-CCC-ACC-GAA-GA
mPai1	TGA-GCT-GTG-CCC-TTC-TCA-TT	GCC-ACC-GAC-TTC-GGA-GTA-AA
Mycoplasma sp.	GGG-AGC-AAA-CAG-GAT-TAG-ATA-CCC-T	TGC-ACC-ATC-TGT-CAC-TCT-GTT-AAC-CTC

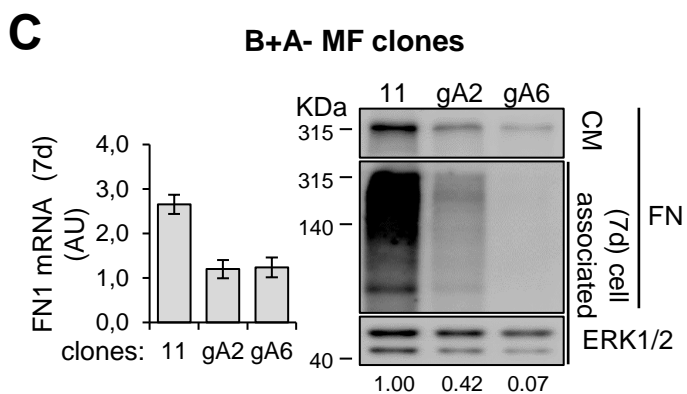
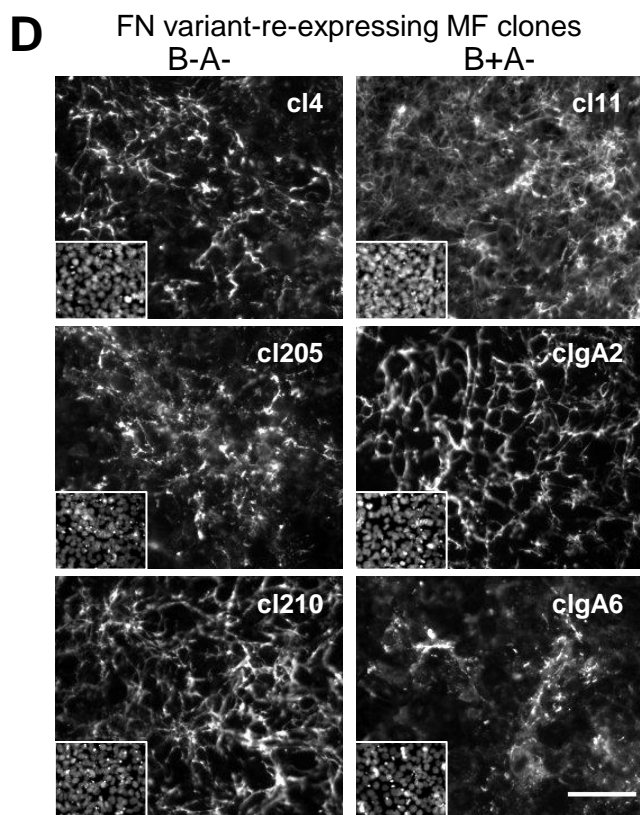
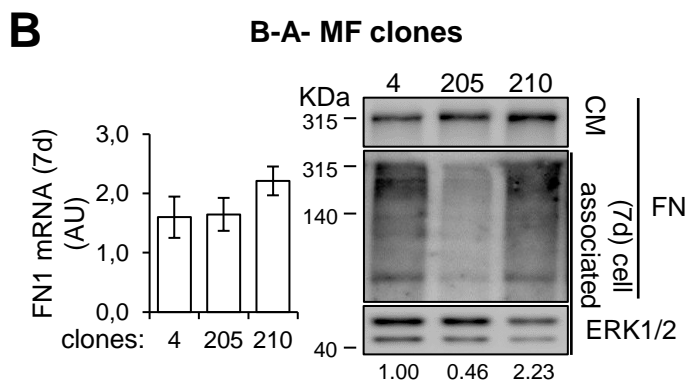
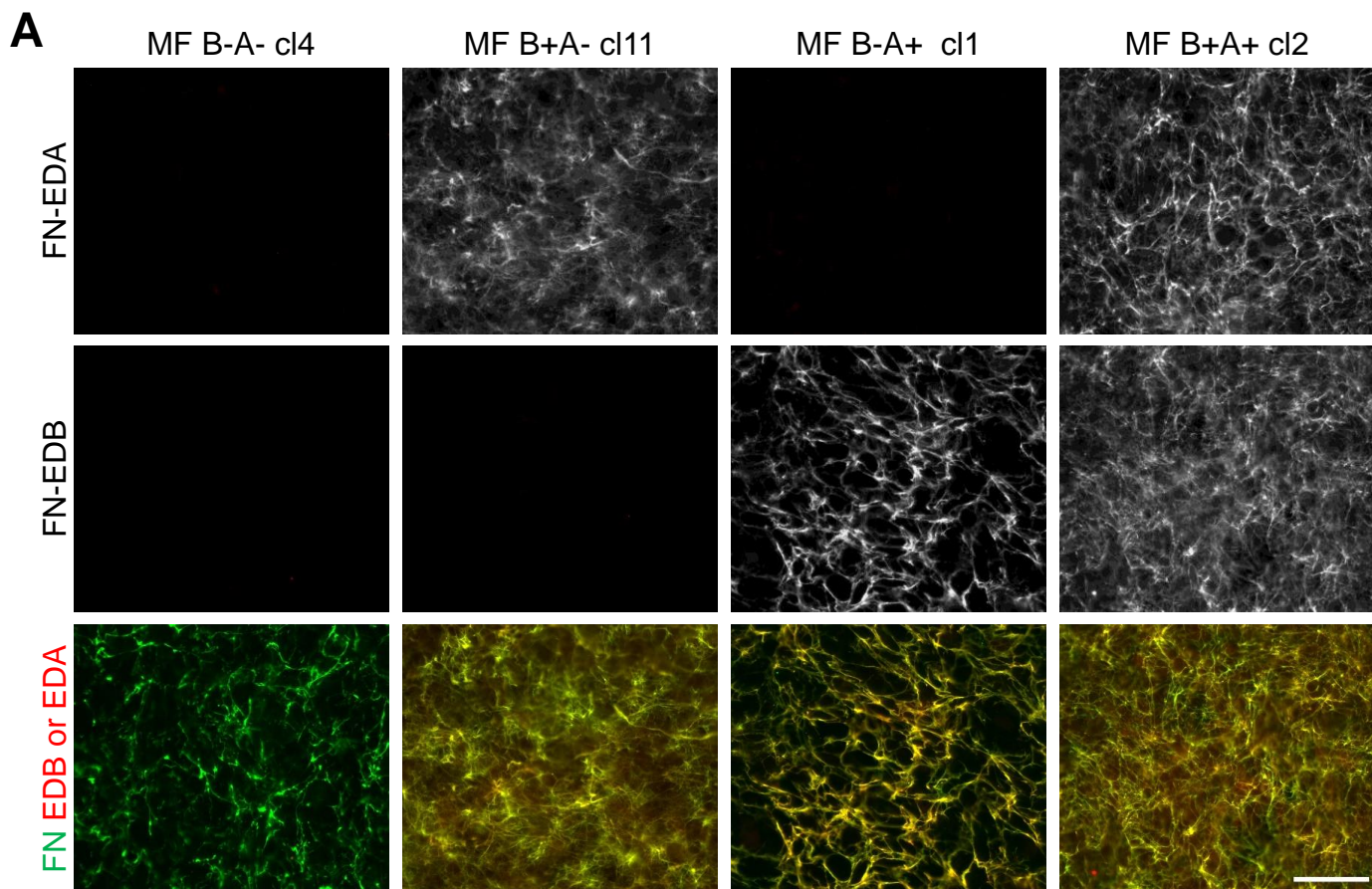
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74

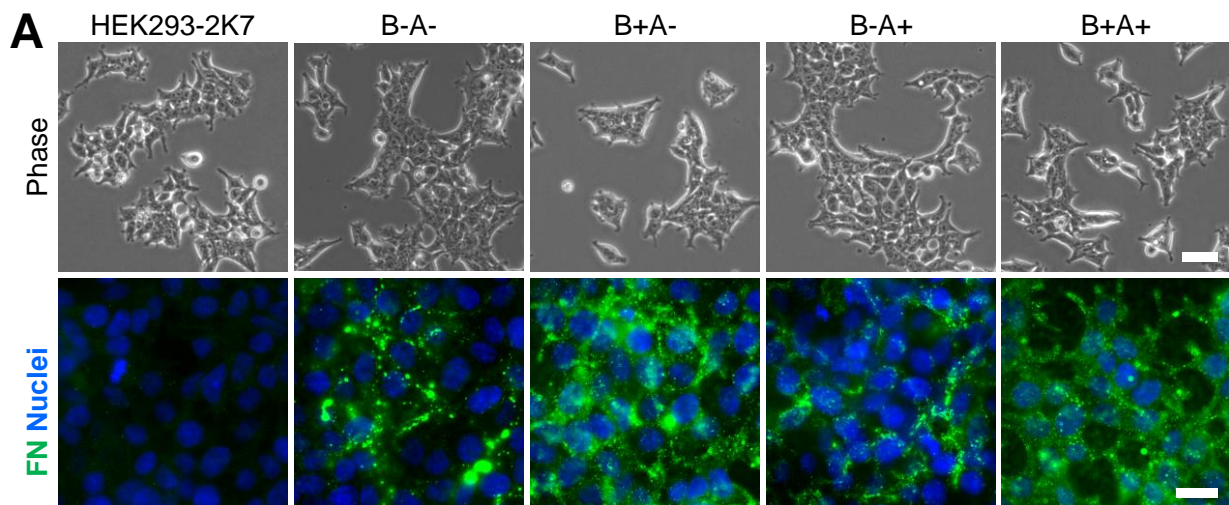
Supplementary Figure 1



Supplementary Figure 2

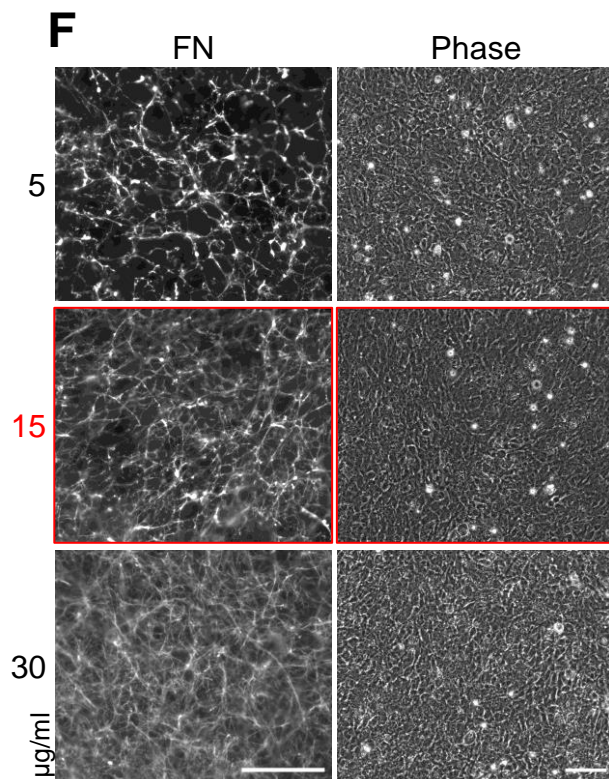
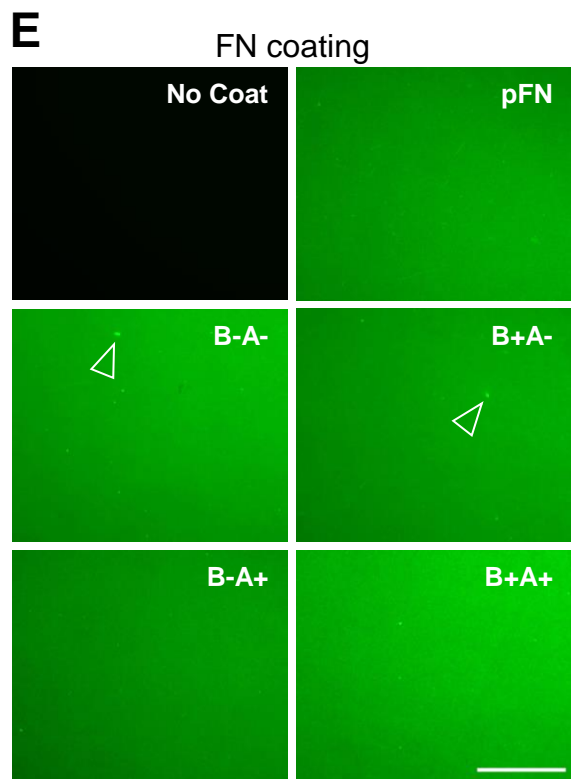
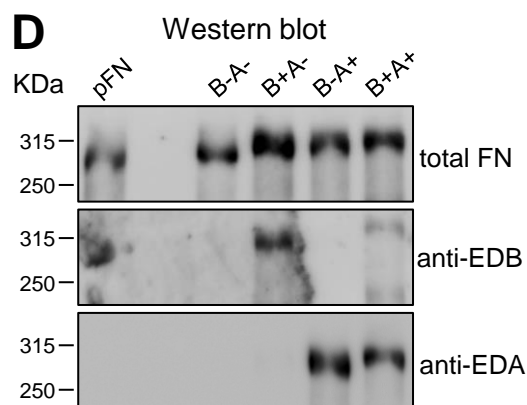
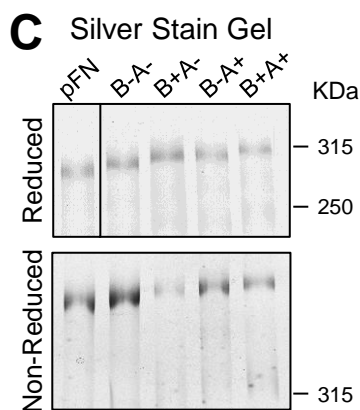


Supplementary Figure 3

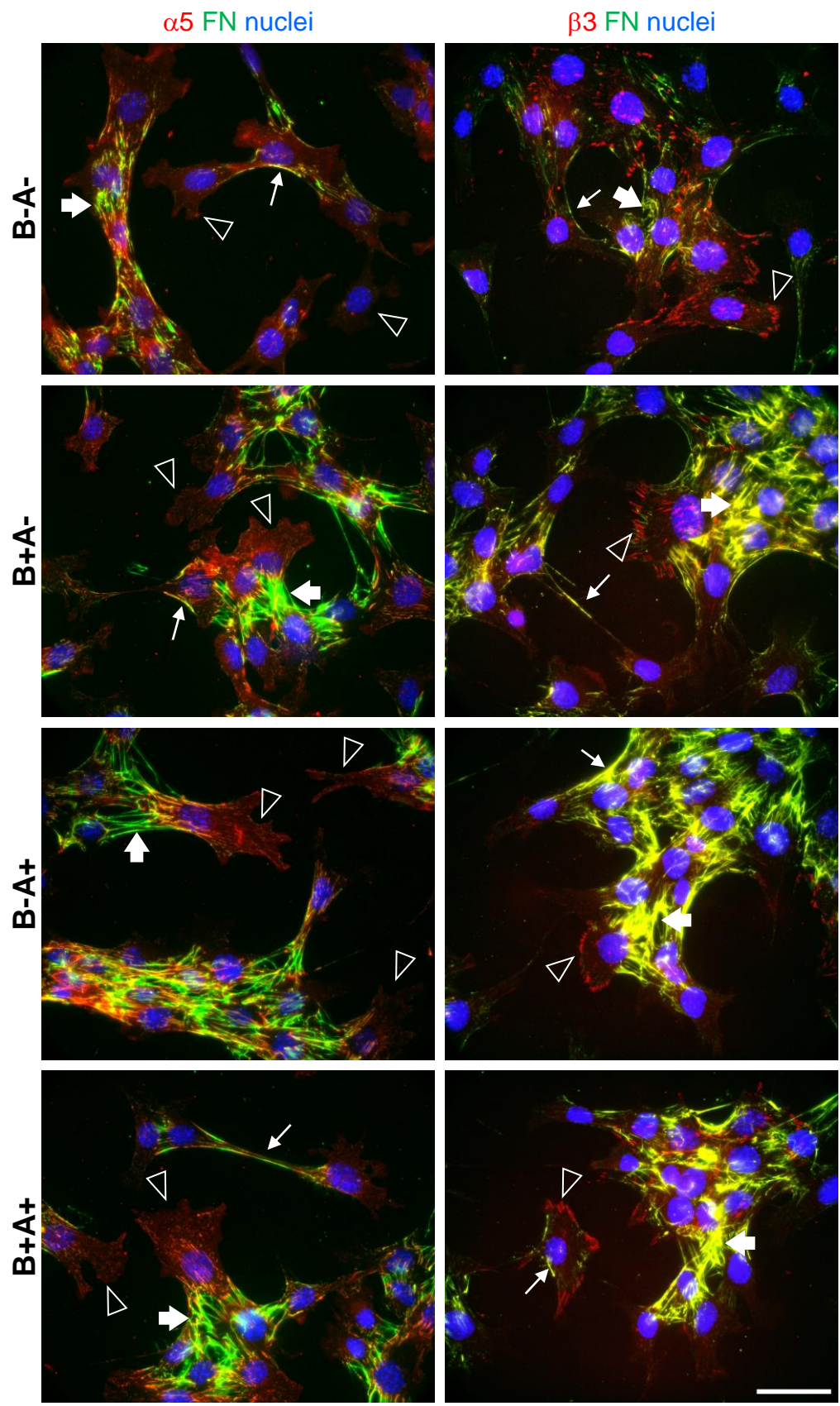


B Elisa

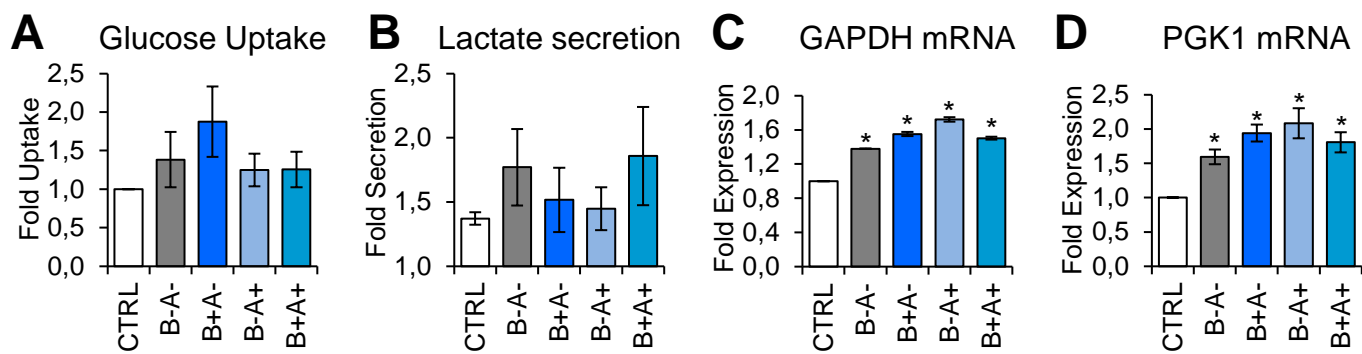
	FN1 ($\mu\text{g/ml}$)
HEK293	0.53
vector cl4	0.33
B-A- cl16	13.5
B+A- cl4	6.83
B-A+ cl4	30.2
B+A+ cl25	16.7



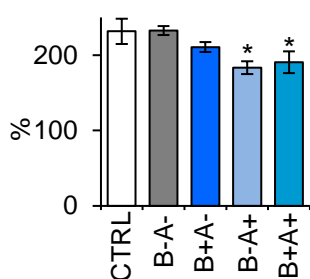
Supplementary Figure 4



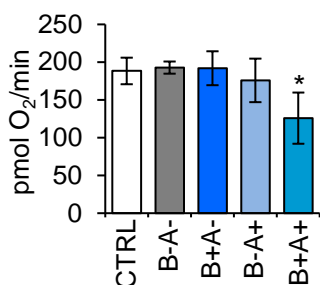
Supplementary Figure 5



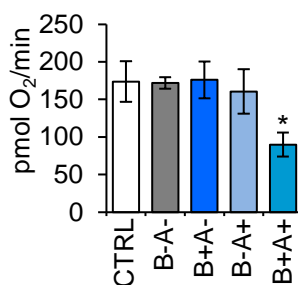
E ECAR in response to glucose



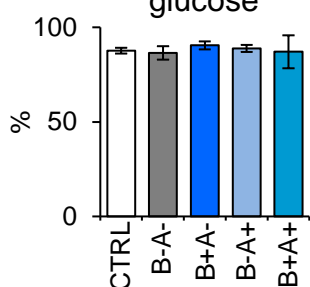
F Basal Mitochondrial OCR



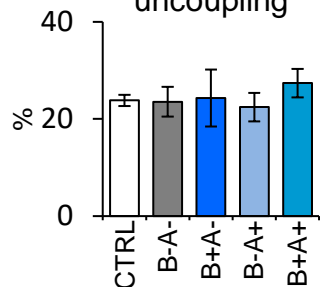
G Maximal OCR



H OCR in response to glucose



I ATP synthase uncoupling

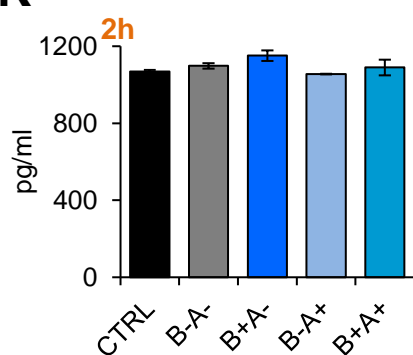


J

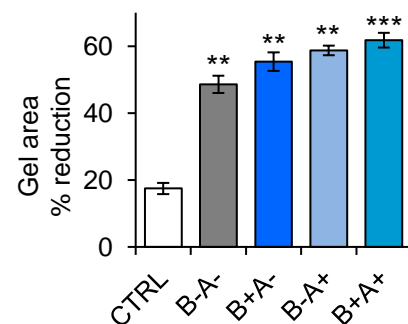
DMEM +	TGF-β1 (pg/ml)
5% FCS	897.5 (±17.1)
CTRL	undetected
B-A-	
B+A-	
B-A+	
B+A+	

FN variants (15 μg/ml)

K TGF-β1 concentration



L



Supplementary Figure 6

