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New mechanism of fibronectin fibril assembly revealed by live imaging and super-resolution microscopy.

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Abstract

Fibronectin (Fn1) is an essential ECM glycoprotein important for embryonic development and homeostasis. The functions of Fn1 in regulating cell fate decisions, morphogenesis and cellular responses to injury are intimately linked to the process of Fn1 fibrillogenesis. Therefore, understanding the mechanisms by which Fn1 proteins assemble into fibrils is necessary to gain insights into diverse functions of Fn1. Using CRISPR/Cas9 mutagenesis, we generated mice and cell lines wherein a sequence encoding a fluorescent protein (FP) was knocked into the *Fn1* locus replacing the termination codon, resulting in the expression of Fn1-FP proteins subject to endogenous regulation. Live imaging and super-resolution microscopy revealed that Fn1 fibrils are not continuous fibers as was thought before, instead, they are comprised of a discontinuous array of small nanodomains. Live imaging showed that Fn1 nanodomains are mobile and that they become arranged into progressively longer linear arrays as they move toward the nucleus in parallel with the rearward actin flow. The organization of Fn1 nanodomains into linear fibrillar arrays but not the formation of Fn1 nanodomains is regulated by the interactions mediated by the Fn1 N-terminal assembly domain. The nanodomain architecture of Fn1 fibrils is observed in multiple contexts: in three-dimensional ECM *in vivo*, on substrata of different composition and stiffness, and is retained when the linkage of Fn1 fibrils to cells is disrupted. The modular assembly and structure of Fn1 fibrils bears important implications for mechanisms of ECM remodeling and signal transduction.

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Introduction

Fibronectin (Fn1) is a requisite component of extracellular matrix (ECM) necessary for embryogenesis and homeostasis (Schwarzbauer and DeSimone, 2011). It is noteworthy that in the absence of Fn1 fibrillogenesis, the binding of Fn1 to cells is not sufficient to regulate key biological processes including those governing embryonic development, angiogenesis, vascular remodeling, or cartilage condensation (Chiang et al., 2009; Rozario et al., 2009; Singh and Schwarzbauer, 2014; Zhou et al., 2008). Therefore, understanding the mechanisms by which Fn1 proteins assemble into macromolecular fibrils is essential to gain insights into *in vivo* functions of Fn1. Fn1 fibrillogenesis occurs following the binding of secreted Fn1 homodimers to cell-surface integrins. Following integrin binding, intracellular cytoskeletal forces such as actomyosin contractility acting through integrins generate pulling forces on Fn1 dimers, exposing epitopes that promote Fn1 fibrillogenesis (Chernousov et al., 1987; Smith et al., 2007; Zhang et al., 1994; Zhang et al., 1997; Zhong et al., 1998). At the cell biological level, the process of Fn1 fibrillogenesis is correlated with the formation of fibrillar adhesions, whereby mobile adhesions containing Fn1 and integrin $\alpha5\beta1$ translocate toward the nucleus resulting in elongated linear arrays termed focal and fibrillar adhesions, containing both Fn1 and intracellular cytoplasmic effectors linking Fn1 and actin cytoskeleton (Geiger et al., 2001; Geiger and Yamada, 2011; Lu et al., 2020; Pankov et al., 2000; Zamir et al., 1999; Zamir et al., 2000).

It has been thought that Fn1 fibrils arise following partial unfolding and alignment of Fn1 dimers in a periodic end-to-end fashion of alternating N- and C-termini, forming continuous fibers (Chen et al., 1997; Dzamba and Peters, 1991; Fruh et al., 2015). However, this model is based on the analyses of fixed samples and electron microscopy wherein the exact composition of the fibrous material imaged could not be easily determined. To evaluate the process of fibrillogenesis in real time, we adopted a CRISPR/Cas9-mediated mutagenesis

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approach to generate fluorescently-labeled Fn1, subject to physiological regulation of expression and splicing. This approach has enabled us to visualize the initiation and progression of Fn1 fibrillogenesis over an extended period of time. Using live imaging and super resolution microscopy, we uncovered an unexpected mechanism of Fn1 fibrillogenesis. Our data demonstrate that Fn1 fibrils are composed of centripetally-moving Fn1 nanodomains originating at cell periphery. As Fn1 nanodomains move toward the nucleus, they become arranged into progressively longer and longer arrays wherein nanodomains containing a high number of Fn1 dimers alternate with regions containing low or no Fn1. We show that the N-terminal Fn1 assembly region is not required for the formation of Fn1 nanodomains or their centripetal translocation. Instead, the N-terminus of Fn1 regulates the organization of Fn1 nanodomains into linear arrays. This model of fibrillogenesis integrates the process of fibrillogenesis with adhesion maturation and provides significant new insights into the mechanisms of ECM formation, remodeling, and signaling.

Results

While examining Fn1^{+} ECM by confocal immunofluorescence microscopy in mouse embryos, we observed that Fn1 fibrils appeared discontinuous (**Fig. 1a-b**, arrows), containing regularly-spaced regions of high and low fluorescence intensity (**Fig. 1c, Movie 1**; *Movie legends are at the end of the manuscript*). This suggested that Fn1 fibrils consisted of regions with a high number of Fn1 dimers

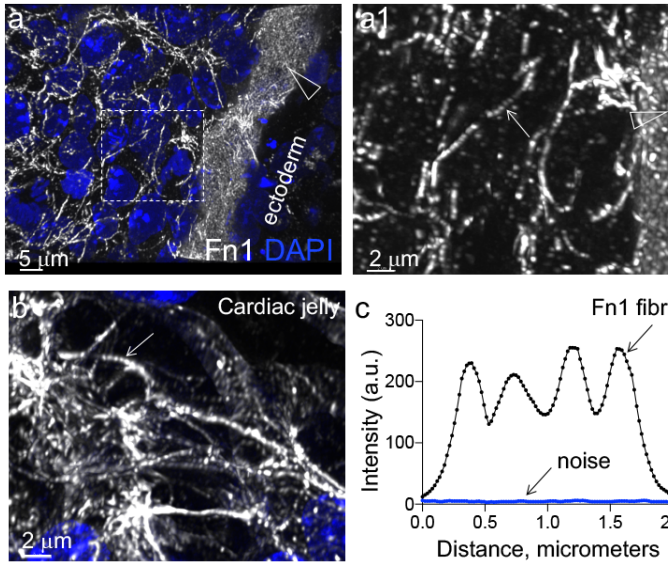

Figure 1. Beaded architecture of Fn1 fibrils in embryonic ECM. Wild-type E9.5 mouse embryos were fixed and stained with the monoclonal antibody to Fn1 (white) and DAPI, and imaged using 100x oil objective, N.A. 1.49, pinhole 0.8, and sampling rate of 40 nm/pixel. **a – a1.** Sagittal optical section through the first pharyngeal arch and **b.** the cardiac jelly, an ECM-rich region between the myocardial and endocardial layers of the outflow tract of the heart. Large arrowheads in **a – a1** point to the ECM at the ectoderm-mesenchyme boundary of the 1st pharyngeal arch. The box in **(a)** is expanded in **a1** to show the beaded Fn1 microarchitecture. Arrow in **a1** points to Fn1 fibril within the arch mesenchyme; **b.** Beaded architecture of Fn1 fibrils in cardiac jelly, e.g., arrow. **c.** Intensity profile plot of a Fn1 fibril shows a regularly-undulating profile, with peaks corresponding with Fn1 "beads"; a.u. arbitrary units.

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separated by regions containing a low number of Fn1 dimers (**Fig. 1c**). To test this hypothesis and to determine mechanisms regulating the formation of Fn1 fibrils, we employed a CRISPR/Cas9 knock-in strategy to modify the endogenous Fn1 locus by replacing the termination codon of Fn1 with a sequence encoding a fluorescent protein. This strategy has allowed to generate fluorescently-labeled Fn1 proteins subject to endogenous regulation (**Sup. Fig. 1a-b**), and enabled extended live imaging of Fn1 fibrillogenesis. Using this strategy, we obtained multiple independent lines of mouse embryo fibroblasts (MEFs) expressing Fn1-mEGFP, Fn1-mScarlet-I, Fn1-Neon Green, or Fn1-tdTomato fusion proteins (FP) generated by CRISPR/Cas9-mediated insertion. Western blots showed that FP fusions to Fn1 were specific: FPs were only fused to Fn1 as no other FP fusions were detected either by western blotting or immunofluorescence (IF) (**Sup. Fig. 2a-b**).

Deoxycholate (DOC) insolubility of Fn1 ECM is a classical biochemical assay for proteins stably incorporated into the assembled ECM (Choi and Hynes, 1979; McKeown-Longo and Mosher, 1983; Singh et al., 2010; Wierzbicka-Patynowski et al., 2004). DOC assays demonstrated that the incorporation of Fn1-FPs into ECM was indistinguishable from wild-type, untagged Fn1 (**Sup. Fig. 2c**). To determine whether Fn1-FP proteins carried out the physiological functions of Fn1, we generated $Fn1^{mEGFP}$ knock-in mice. $Fn1^{mEGFP/mEGFP}$ homozygous knock-in animals are viable and fertile (**Sup. Fig. 1b** panels 4 and 5). Furthermore, Fn1-mEGFP proteins were expressed in the same pattern as total Fn1 protein (**Sup. Fig. 2d**) (Peters and Hynes, 1996). Together, these studies demonstrated that Fn1-FP fusion proteins are suitable reagents for investigating mechanisms of Fn1 fibrillogenesis.

To visualize the process of fibrillogenesis in real time, we plated $Fn1^{mEGFP/+}$ MEFs on gelatin-coated cover glass and imaged cells 16 hours after plating using total internal reflection (TIRF) microscopy at the critical angle of incidence. These experiments showed that Fn1 fibrillogenesis initiated at cell periphery as distinct bright Fn1 densities that moved centripetally in parallel with F-actin and aligned into linear arrays of “beads” (arrows in **Movie 2**). TIRF

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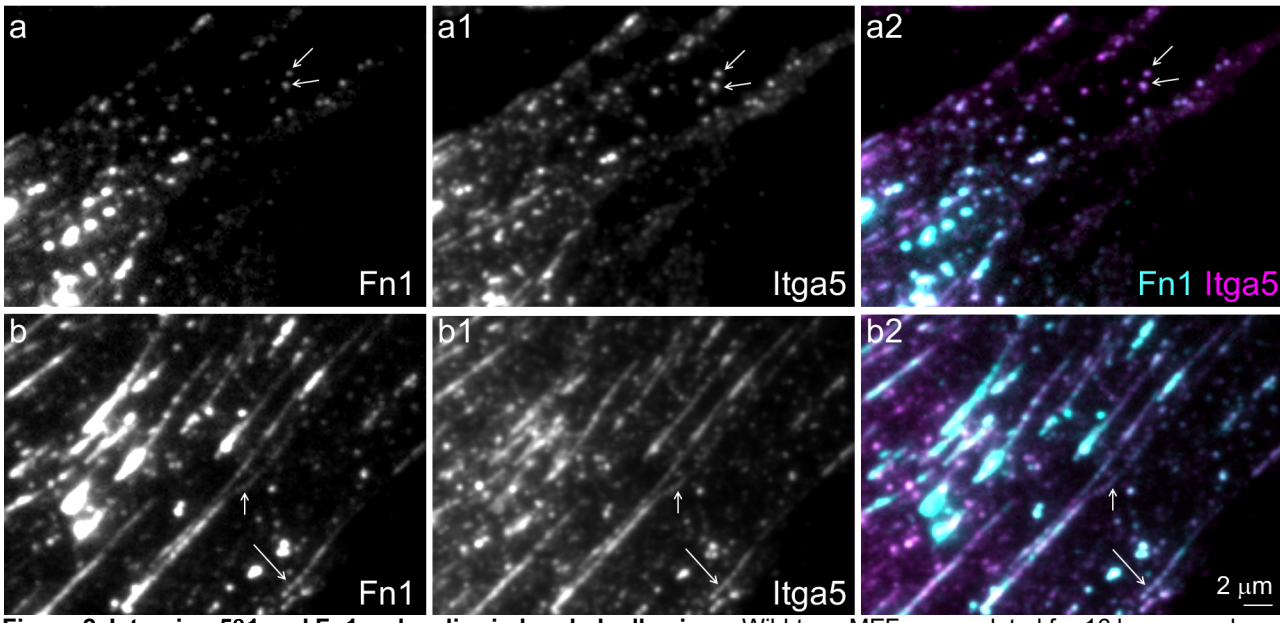

Figure 2. Integrin α5β1 and Fn1 co-localize in beaded adhesions. Wild-type MEFs were plated for 16 hours on glass coverslips, then fixed, stained with antibodies to Fn1 and integrin α5 (Itga5), and imaged at the critical angle of incidence using 100x oil objective, NA 1.49. **a – a2** cell periphery. Arrows in **a-a2** point to examples of non-fibrillar Fn1 adhesions ("beads") at cell periphery. **b - b2** medial portion of a cell containing beaded fibrillar adhesions (arrows). Note that both Itga5 and Fn1 stainings are beaded. Magnifications in all panels are the same.

imaging also showed that the domains of higher fluorescence intensity of Fn1 co-localized with integrin α5β1 both in non-fibrillar adhesions (arrows in **Fig. 2a-a2**) and in fibrillar adhesions (arrows in **Fig. 2b-b2**), and that Fn1 and α5β1 fibrillar adhesions are beaded (**Fig. 2b-b2**, arrows). We also observed the beaded architecture of Fn1 fibrils using an independent imaging method by employing Zeiss Airyscan (**Sup. Fig. 3**). Staining using monoclonal and polyclonal antibodies recognizing distinct and multiple epitopes in Fn1 resulted in the discontinuous appearance of Fn1 fibrils (**Fig. 3a1-c1**). Fn1 fibrils formed by cells plated on glass, gelatin, laminin 111 or vitronectin were beaded Fn1 fibrils (**Fig. 3**, and **Sup. Figs. 3-4**), and so were Fn1 fibrils in cell-free areas (**Fig. 3a, a1**) and between cells (**Fig. 3b-c**). In addition, the beaded appearance of Fn1 fibrils was observed when cells were plated on soft substrata such as hydrogels of variable stiffness (**Sup. Fig. 4c-d**). In the latter experiment, Fn1 was detected by imaging the native fluorescence of Fn1-mEGFP, indicating that the beaded

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appearance of Fn1 fibrils was independent of antibody staining. Taken together, these studies indicated that the beaded appearance of Fn1 fibrils was a general feature of Fn1 ECM seen in 3D ECM *in vivo* and under different conditions *in vitro*.

To test the hypothesis that the "beads" in Fn1 strings were contiguous, cultures were treated with 2% DOC. 2% DOC treatment dissolves cell membranes and cytoplasmic components, leaving insoluble ECM devoid of cell contact (see **Movie 3** for time-laps of dissolution of cellular components, F-actin and DNA). This experiment showed that Fn1 fibrils in assembled ECM retained their beaded architecture in the absence of cell contact (**Fig. 3d**). Together, these data indicated that the beaded topology of Fn1 fibrils is a feature of physiological three-dimensional Fn1 ECM *in vivo* and suggested that the beads are contiguous.

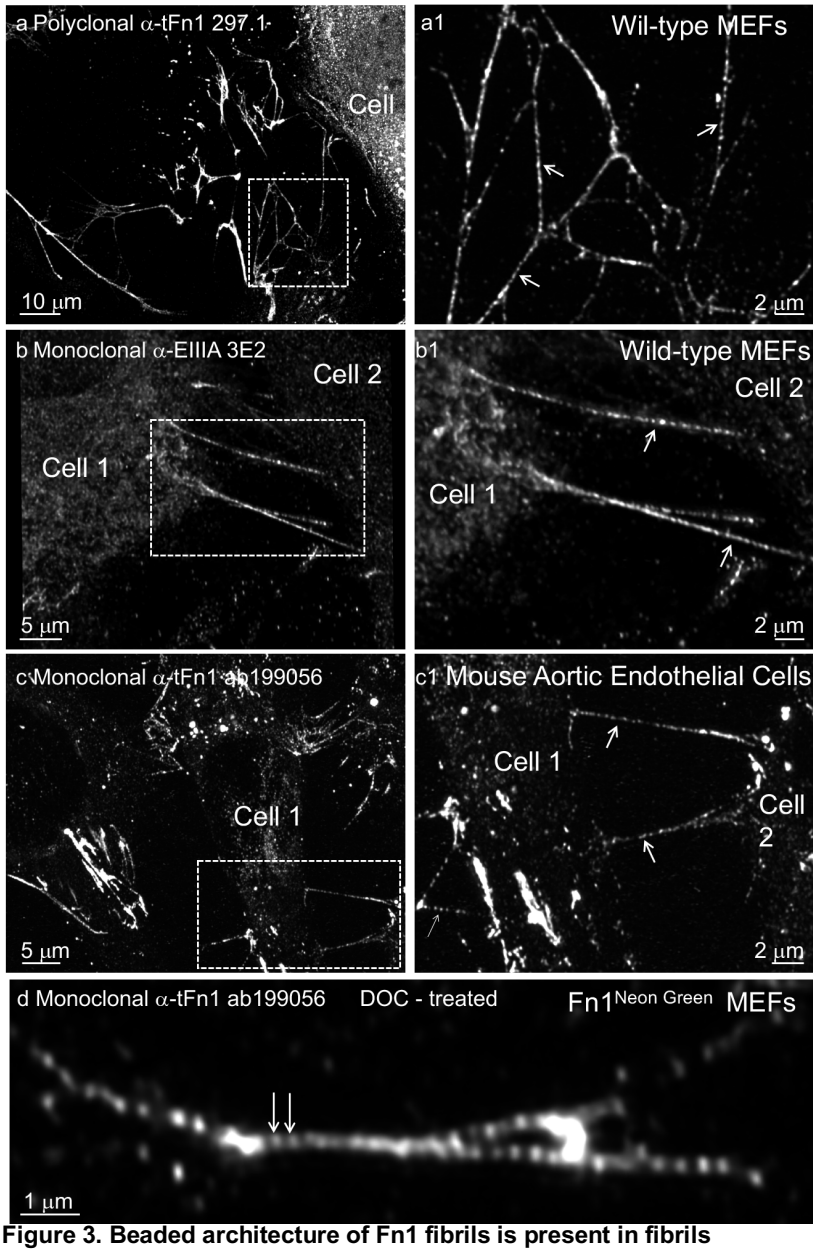

Figure 3. Beaded architecture of Fn1 fibrils is present in fibrils between cells and is retained in the absence of cell contact. a-b. Fn1 secreted by wild-type MEFs and deposited **a)** on glass or **b)** between cells. **c.** Fn1 fibrils between endothelial cells. Boxes in **a – c** are magnified in **a1-c1**. Arrows point to fibrils left behind on substrata (**a1**) or between cells (**b1-c1**). **d.** $Fn1^{Neon\ Green}$ MEFs were treated with 2% DOC pH 8 until cells were dissolved (see Movie 3). Following DOC treatment, Fn1 was imaged using 100x oil objective, NA 1.49, pinhole size 0.8 Airy units, and sampling resolution of 40 nm/pixel in x,y. Note beaded architecture (arrows).

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To determine the relationship between the beaded architecture of Fn1 seen by diffraction-limited microscopy with Fn1 nanoarchitecture, we plated Fn1$^{mEGFP/+}$ MEFs on glass for 16 hours, then fixed and stained cells using a monoclonal antibody to the central region of Fn1. Fn1-mEGFP fluorescence was imaged in the TIRF mode at the critical angle of incidence, while the binding of the monoclonal αFn1 antibody was detected with Alexa Fluor-647-conjugated secondary antibody and imaged by Stochastic Optical Reconstruction Microscopy (STORM). STORM was performed by illuminating samples at the critical angle of incidence, as described in (Jimenez et al., 2020). Thin beaded fibrillar adhesions (arrow in **Fig. 4a**) were resolved by STORM (**Fig. 4b-b2**) to be arrays of regularly-spaced nanodomains that were

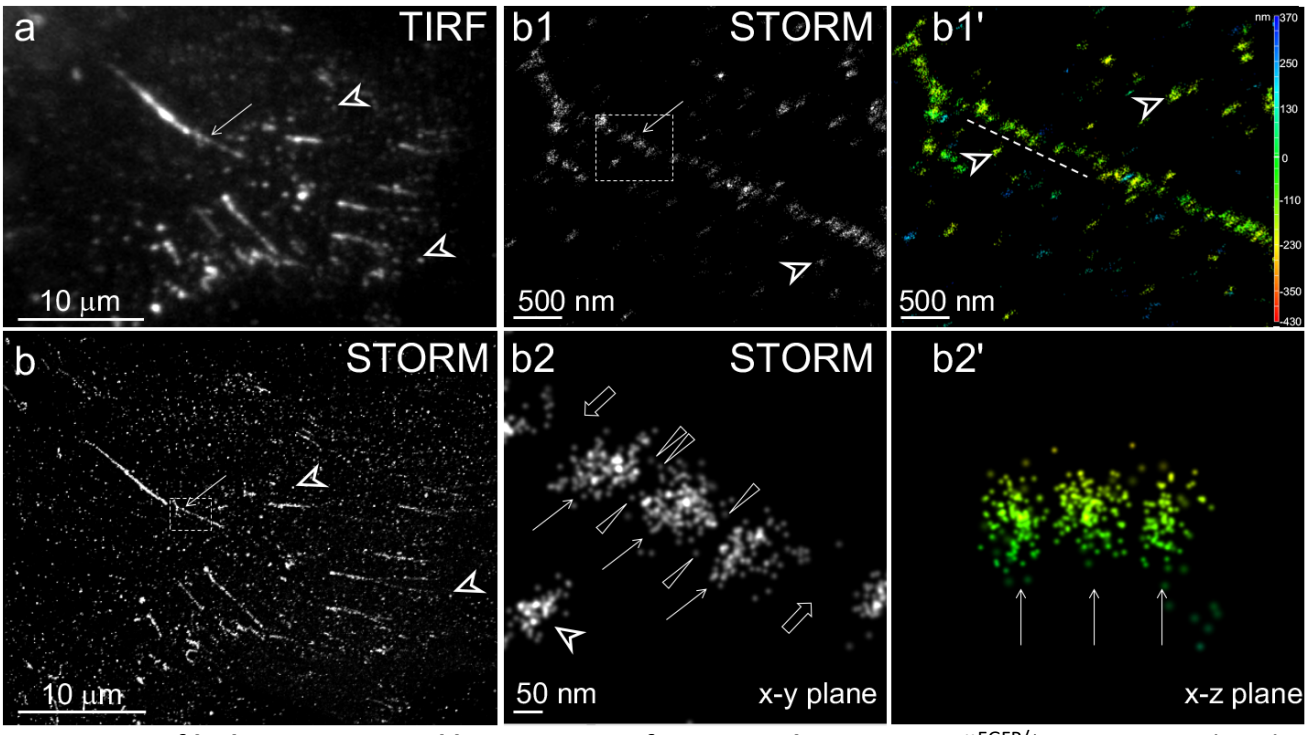

Figure 4. Fn1 fibrils are composed linear arrays of Fn1 nanodomains. Fn1$^{mEGFP/+}$ MEFs were plated on glass for 16 hrs, then fixed and stained with antibodies to GFP followed by secondary antibodies conjugated to Alexa-647. **a.** Native GFP fluorescence imaged by TIRF using 100x objective NA 1.49. Arrow in **a** points to a thin, beaded Fn1 fibril resolved by STORM in **b1, b1'** and **b2. b.** The entire field of view in **a** is imaged by STORM detecting Alexa-Fluor 647-conjugated antibody (see Methods). The box in **b** is expanded in **b1**. The box in **b1** is expanded in **b2**. The arrow in **b1** points to the same region as the arrow in **a**. Arrows in **b2** point to Fn1 nanodomains; arrowheads point to Fn1 localizations between the nanodomains, notched arrowheads point to Fn1 nanodomains that are not in fibrils and wide arrows point to Fn1-free areas between the nanodomains in the fibril marked by the arrow in **a**. **a, b, b1, b1'** and **b2** are in x-y planes; **b2'** shows the nanodomains in **b2** in the x-z plane. Images in **b1'** and **b2'** are depth-coded according to the scale in **b1'**. See Movie 4 for 3D rotation of the fibril underlined in **b1'** around the x-axis.

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symmetrical in x, y, and z dimensions (**Fig. 4b1, b2, b2'** and **Movie 4**). The space between nanodomains contained a few or no Fn1 localizations (**Fig. 4b2** and **Movie 4**).

Fn1 is a large, multi-domain, ~250 kDa glycoprotein secreted as a homodimer, wherein Fn1 subunits are linked in the anti-parallel orientation by two di-sulfide bonds at their C-termini (Schwarzbauer and DeSimone, 2011; Skorstengaard et al., 1986). To investigate the relationship between the domain structure of Fn1 protein and the nanodomain architecture of Fn1 fibrils, we used antibodies to distinct parts of Fn1 protein (depicted in **Model 1** at the bottom of **Fig. 5**,) and STORM. For these experiments, wild-type and $Fn1^{mEGFP/+}$ MEFs were sparsely-plated in Ibidi 8-well glass-bottom chambers overnight, allowing to observe the structure of thin fibrils. Cells were then fixed and stained with antibodies recognizing different Fn1 epitopes: polyclonal rabbit antibodies raised to recognize the first six type III repeats of Fn1 (Fn1 $III_{1\text{-}6}$) (Vega et al., 2020) (**Fig. 5** column 1), a monoclonal antibody recognizing an epitope within the central region of Fn1 (**Fig. 5** columns 2, 4-5), polyclonal anti-serum 297.1 raised against the entire Fn1 protein (Rickelt and Hynes, 2018) (**Fig. 5**, column 3), or polyclonal antibodies to GFP, recognizing the C-terminus of Fn1-mEGFP protein (**Fig. 6**, column 5). To maximize the labeling density, the polyclonal anti-Fn1 III_{1-6} and 297.1 antisera were used at a 40- and 4-fold higher concentrations than for routine immunofluorescence microscopy, respectively. Together with the use of excess secondary antibodies each conjugated to 3 – 6 molecules of Alexa-647, this approach maximizes the chance that all the epitopes recognized by the 1° antibodies will be localized by STORM (Jimenez et al., 2020).

We first focused on the analyses of thin Fn1 fibrils like those marked by the arrows in (**Fig. 4a-b**) to study the initiation of fibril formation rather than fibril bundling or branching. Each antibody resulted in the detection of long characteristic Fn1 fibrils (**Fig. 5a-c**). Successive magnifications show that independent of the antibody used for staining, Fn1 fibrils contained linear arrays of nanodomains (thin arrows in **Fig. 5d, magnified in 5e**) separated by regions containing a small number or no Fn1 localizations (as in **Fig. 4, Movie 4**).

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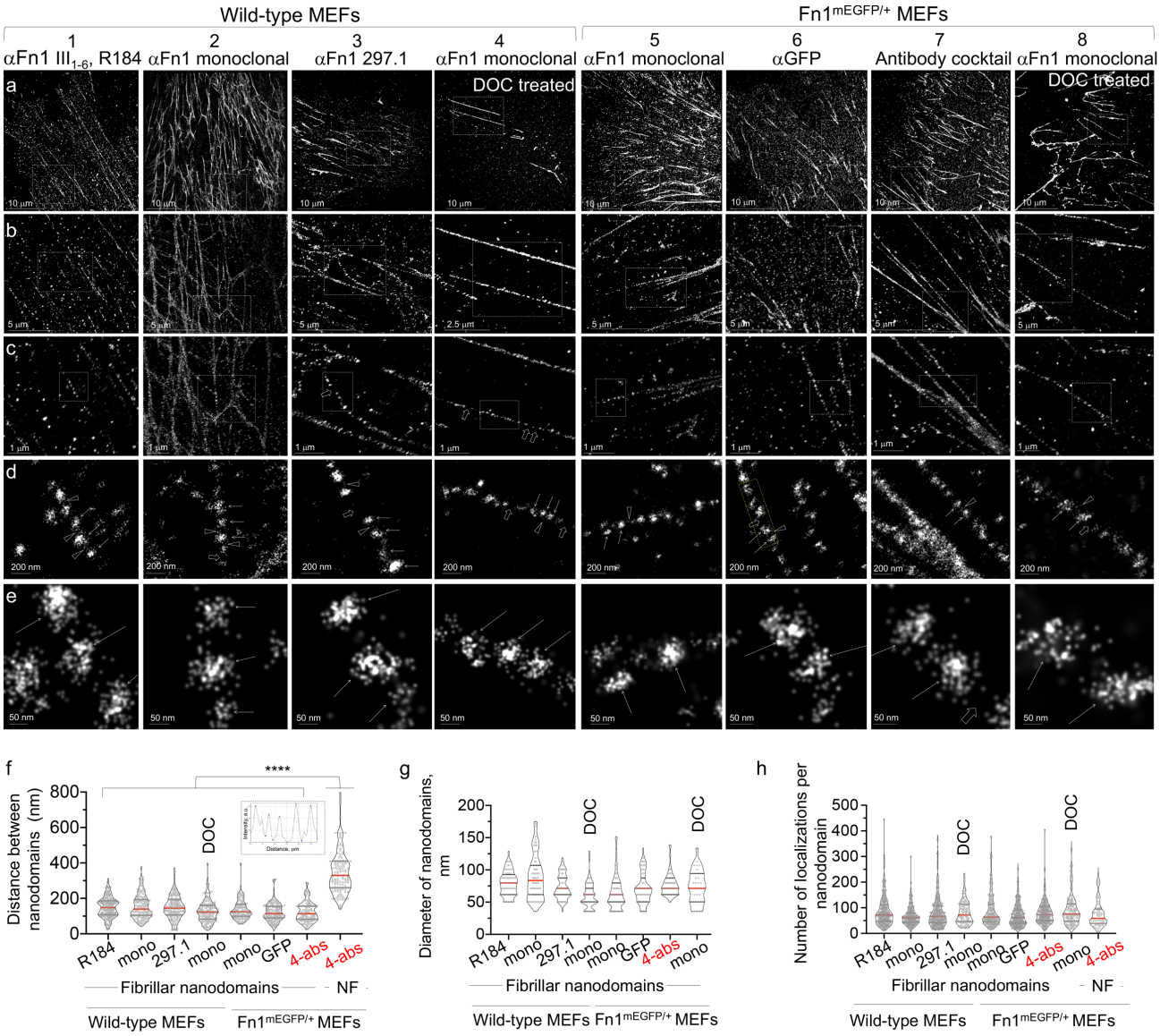
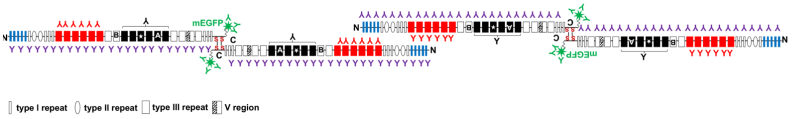


Model 1 Two Fn1 dimers aligned in a periodical end-to-end fashion. If fibrils were composed of periodically aligned dimers, they would be uniformly stained by the antibody cocktail:



Model 2 Fn1 fibrils form from nanodomains containing multiple Fn1 dimers

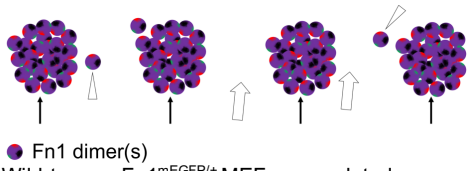


Figure 5. Fn1 fibrils are composed of nanodomains containing multiple Fn1 dimers. Wild-type or Fn1$^{mEGFP/+}$ MEFs were plated on glass for 16 hrs, fixed and stained with different antibodies to Fn1 followed by Alexa 647-conjugated secondary antibodies. Columns showing cells treated with DOC prior to fixation are marked. Cells were imaged using STORM. **a.** zoom-out views to show the overall appearance of Fn1 fibrils. **b-e.** Successive magnifications of fibrils shown in (**a**). Arrows in **d** point to nanodomains magnified in **e;** arrowheads in **d-e** point to Fn1 molecules between nanodomains, wide open arrows point to Fn1-free zones between nanodomains in a fibril. **f.** distances between nanodomains within fibrils or non-fibrillar (NF) nanodomains, **** $p<10^{-4}$, Kruskal-Wallis test, with Dunn's correction for multiple testing. Inset in **f** is a plot profile of the fibril marked by the box in the column **6d** showing regularly-spaced peaks of intensity. DOC-treated samples are marked. 4-antibody cocktail contains four antibodies as in **Model 1**. **g.** Diameter of nanodomains in fibrils. **h.** number of Fn1 localizations per nanodomain in fibrils. **Model 1**. Two Fn1 dimers in a fibril. Depiction of antibody coverage of Fn1 fibrils if dimers were oriented in end-to-end fashion with alternating N- and C-termini. **Model 2.** Our data show that Fn1 fibrils consist of a linear array of nanodomains (black arrows) containing multiple Fn1 dimers (Fn1 dimers are depicted as balls and color-coded according to the antibody scheme in **Model 1**. Arrowheads point to Fn1 and open arrows point to Fn1-free areas between nanodomains.

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Surprisingly, staining with the polyclonal anti-serum 297.1 raised to the entire Fn1 and, presumably, recognizing multiple epitopes along Fn1, resulted in the same pattern of regularly-spaced nanodomains as staining with antibodies recognizing distinct parts of Fn1 molecule (**Fig. 5,** column 3), suggesting that each Fn1 localization contained the entire Fn1 sequence, and that each nanodomain contained multiple Fn1 dimers.

These data are not consistent with previous models suggesting that Fn1 fibrils are composed of periodically aligned Fn1 dimers arranged in an end-to-end fashion of alternating N- and C-termini (Chen et al., 1997; Dzamba and Peters, 1991; Fruh et al., 2015) (**Model 1** at the bottom of **Fig. 5**). Such periodic alignment of Fn1 dimers necessitates that staining using polyclonal antibodies would result in a uniform labeling of thin Fn1 fibrils, as depicted in **Model 1.** The dimers in the **Model 1** are aligned in an end-to-end fashion, according to the current Fn1 fibrillogenesis model, with the predicted overlap between the N-terminal Fn1 assembly domain (blue) and the first six type III repeats of Fn1 (red) (Singh et al., 2010)). If Fn1 fibrils, were indeed composed of continuous, linear arrays of dimers, the use of all four depicted in **Model 1** would be predicted to uniformly label Fn1 fibrils.

To test this prediction, we used $Fn1^{mEGFP/+}$ MEFs and a cocktail of four antibodies recognizing the beginning (αFn1 $III_{1\text{-}6}$), middle- (αFn1 monoclonal), and the end (αGFP) of Fn1-mEGFP protein in addition to all the epitopes recognized by 297.1 polyclonal antibody. The binding of all the antibodies in the cocktail was detected by a cocktail of secondary antibodies that were each conjugated to 3 – 6 molecules of Alexa-647 (**Fig. 5, column 7**). The nanoarchitecture of thin Fn1 fibrils, the nanodomain spacing (124 $\pm$ 25 nm between nanodomains in fibrils, on average), nanodomain size (average diameter 77 $\pm$ 18 nm), and the number of Fn1 localizations per nanodomain (average of 80 $\pm$ 43 localizations) detected by the cocktail of four

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antibodies were indistinguishable from those produced by each type of the antibody individually (**Fig. 5d – e**, quantified in **Fig. 5f-h** and **Tables 1 –3**). These results indicate that Fn1 fibrils are not uniform. Instead, our data show that Fn1 fibrils are arrays of nanodomains containing multiple Fn1 dimers separated by areas containing a few or no Fn1 molecules (**Fig. 5, Model 2**). The nanoarchitecture of Fn1 fibrils following the treatment with 2% DOC which removes cells and cellular components, was similar to untreated fibrils (**Fig. 5,** columns 4 and 8, **Fig. 5f – h**). These data indicate that the nanodomain architecture is a feature of mature Fn1 fibrils.

Fn1 nanodomains were also present outside of fibrils, we term them non-fibrillar nanodomains (e.g. notched arrowheads in **Fig. 4**). About 3 – 5 such nanodomains are seen as a "bead" in conventional diffraction-limited microscopy. Non-fibrillar Fn1 nanodomains contained a similar number of Fn1 localizations per nanodomain and were of similar size compared with Fn1 nanodomains in fibrils (**Fig. 5h, Tables 2 – 3**). Staining using antibodies to endosomal and lysosomal markers (Rab5 and LAMP1) showed no appreciable co-localization with Fn1 nanodomains (data not shown). Together with imaging using TIRF microscopy, these findings indicated that Fn1 nanodomains in fibrils and non-fibrillar nanodomains are on the cell surface. Non-fibrillar Fn1 nanodomains were present at cell periphery and throughout the cell surface, but they were not organized into linear arrays and were spaced at an median distance of 329 nm (**Table 1**).

Live imaging experiments suggested that Fn1 fibrils form from centripetally-translocating Fn1 nanodomains originating at cell periphery (**Movie 2**). To understand the relationship between the observed nanodomain architecture of Fn1 fibrils and the process of fibrillogenesis, we adopted a live imaging approach using $Fn1^{mEGFP/+}$ MEFs and inhibitors of fibrillogenesis. Fn1 fibrillogenesis critically depends on the interactions mediated by the N-terminal assembly domain of Fn1 (domains shaded in blue in **Model 1, Fig. 5**), and inhibitors that interfere with

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these interactions block the formation of Fn1 fibrils (Filla et al., 2017; Morla et al., 1994; Schwarzbauer, 1991; Sechler et al., 2001; Sechler et al., 1996; Tomasini-Johansson et al., 2001). One such inhibitor is a 49-amino acid peptide derived from *Streptococcus pyogenes* adhesin F1, termed the functional upstream domain (FUD) (Tomasini-Johansson et al., 2001). FUD binds the N-terminal assembly domain of Fn1 and functions as a competitive inhibitor of Fn1-Fn1 interactions (Maurer et al., 2010; Tomasini-Johansson et al., 2001). To determine how N-terminal interactions regulate Fn1 fibrillogenesis, $Fn1^{mEGFP/+}$ MEFs were plated on glass for 4 hours, and then imaged for 15 – 18 hours either in the imaging medium alone, or in the medium containing either 225 nM FUD or 274 nM 11-IIIC, a 68 amino-acid control peptide that does not interfere with Fn1 fibrillogenesis (Morla et al., 1994; Sottile and Chandler, 2005). Untreated cells or cells treated with the control peptide developed and accumulated long Fn1 fibrils (**Movie 5**). In contrast, treatment with FUD led to dismantling of the pre-existing Fn1 fibrils and inhibited the formation of new Fn1 fibrils (**Movie 6**). Instead of fibrils, cells cultured in the presence of FUD mainly contained centripetally-moving Fn1-mEGFP fluorescent “beads” that only rarely formed strings (**Movie 6**). These experiments suggested that FUD inhibits fibrillogenesis by interfering with the process by which Fn1 “beads” become arranged or connected into linear arrays. To test this hypothesis, $Fn1^{mEGFP/+}$ MEFs were plated for 16 hours in the continuous presence of either 225 nM FUD or 274 nM III-11C control peptides, or were left untreated. Cell were then fixed and stained without permeabilization using monoclonal anti-Fn1 antibodies which were detected with Alexa Fluor 647-conjugated secondary antibodies, and imaged at the critical angle of incidence by STORM at the excitation wavelength of 640 nm (Jimenez et al., 2020). This approach abolishes the detection of intracellular Fn1-mEGFP and maximizes the detection of cell-surface Fn1 due to the following: a) the absence of a detergent during fixation, staining and washing, b) detecting the emission of Alexa 647-conjugated antibodies at $\geq$ 670 nm, c) the use of oxygen scavengers in the STORM buffer that inhibits GFP fluorescence (Tsien, 1998), and d) imaging at the critical angle of incidence to

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detect fluorescence in close proximity to the plasma membrane. These experiments demonstrated that the organization of nanodomains into linear arrays was lost upon incubation with FUD (compare **Fig. 6a, b, a1, b1** with **Fig. 6c, c1**). Non-fibrillar Fn1 nanodomains in cells treated with FUD had a similar number of Fn1 localizations per nanodomain, and were of similar sizes compared with fibrillar or non-fibrillar Fn1 nanodomains in untreated cells or cells incubated with the control peptide (**Fig. 62-c1-1**, quantified in Fig. **6d** and **Tables 2-3**). Taken together, these data indicate that FUD does not interfere with the formation of Fn1 nanodomains but inhibits the organization of Fn1 nanodomains into linear arrays. Since Fn1 proteins lacking the N-terminal assembly domain do not form fibrils, our experiments suggest

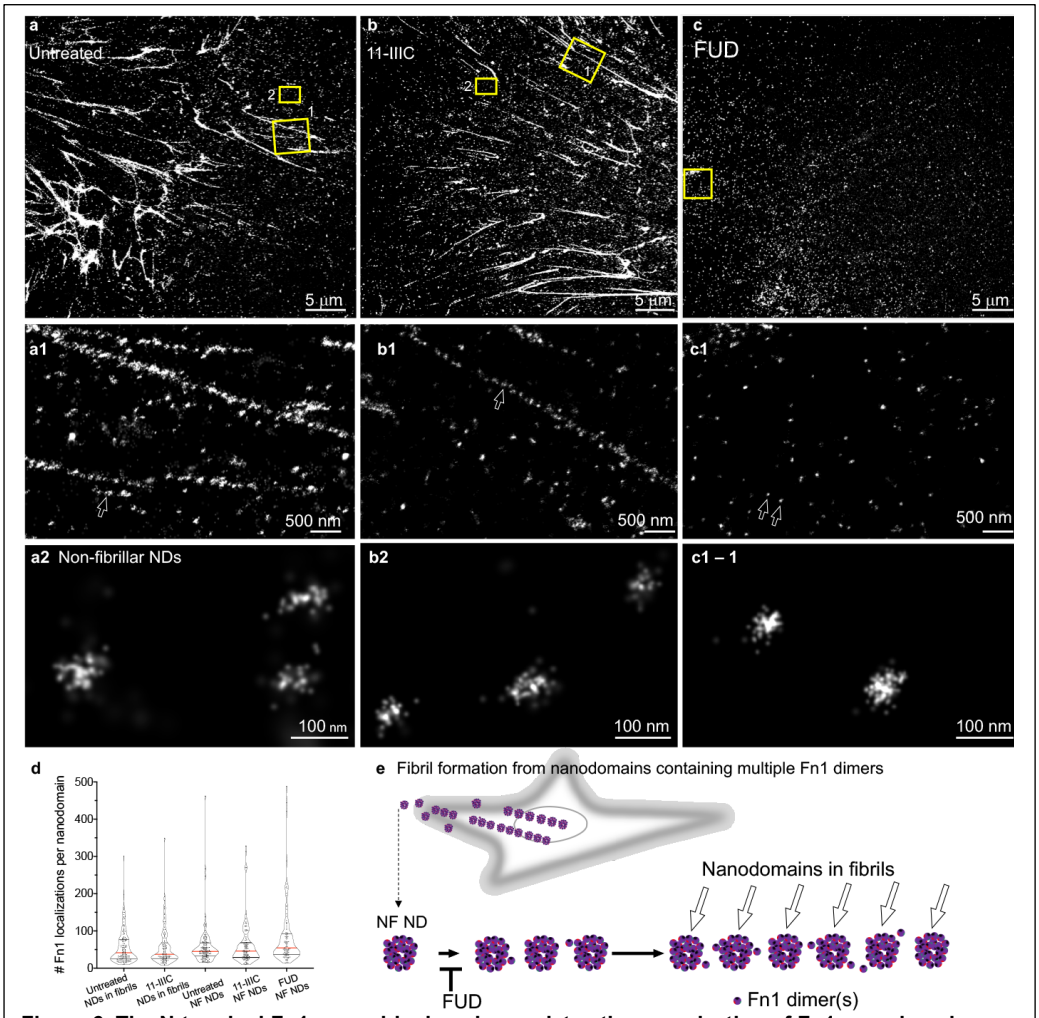

Figure 6. The N-terminal Fn1 assembly domain regulates the organization of Fn1 nanodomains into linear fibrillar arrays. Fn1$^{mEGFP/+}$ MEFs were plated on glass and were either left untreated, or were incubated with the control 11-IIIC peptide, or the FUD peptide for 16 hrs. Cell were then fixed and stained with the monoclonal antibody to Fn1 followed by Alexa 647-conjugated secondary antibodies. Cells were imaged at the critical angle of incidence by STORM. **a – a2**. Untreated, unpermeabilized cells. **b – b2.** Cells incubated with controls 11-IIIC peptide. **c – c1, c1-1** FUD-treated, unpermeabilized cells. Boxes marked 1 in **a-b** were expanded in **a1-b1**. Boxes marked 2 in **a-b**, were expanded in **a2 – b2**. The box in **c** is expanded in **c1** and **c1-1**. Arrows in **a1-b1** point to Fn1 nanodomains (NDs) in fibrils. Arrows in **c1** points to non-fibrillar nanodomains expanded in c1-1; **d.** Quantification of the number of Fn1 localizations in NDs in fibrils and in non-fibrillar NDs after various conditions. Red lines mark medians. Differences are not statistically significant, Kruskal-Wallis test with Dunn's correction for multiple testing. **e.** Model of fibril formation: Fn1 dimers assemble into small nanodomains containing integrin $\alpha5\beta1$ at cell periphery, move rearward with actin flow, and become organized into linear arrays of nanodomains. Joining of the additional Fn1 nanodomains to these arrays leads to the generation of longer fibrils as the assembly moves toward the cell center. FUD does not interfere with the formation of Fn1 nanodomains. But instead, it blocks the organization of Fn1 nanodomains into linear arrays.

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that interactions mediated by the N-terminal assembly domain of Fn1 are critical for the linking of Fn1 nanodomains into strings.

Discussion

In this manuscript, we describe the discovery of a novel mechanism underlying the process of Fn1 fibrillogenesis. We found that the three-dimensional beaded architecture of Fn1 ECM fibrils *in vivo* and in two-dimensional cell culture is due to the presence of Fn1 nanodomains, and showed that each Fn1 nanodomain is composed of multiple Fn1 dimers. Time-laps imaging by confocal and TIRF microscopy showed that Fn1 fibrillogenesis initiated at cell periphery, as bright fluorescent "beads" of Fn1-mEGFP moved centripetally in parallel with F-actin and became organized into linear arrays. Interestingly, the beaded appearance of focal and fibrillar adhesions has been noted before (Zamir et al., 1999; Zamir et al., 2000), and the beaded architecture of cell adhesions can be seen in micrographs from multiple studies (e.g. Fig. 1C in (Zamir et al., 1999), Fig 7a and Sup. Figs 1a and b in (Choi et al., 2008); Fig. 8B in (Damsky et al., 1985); and lower left cell in Fig. 3A (Zhang et al., 2012). However, the significance of these observations has not been investigated to the best of our knowledge. Fn1 domains and beaded strings in our movies resemble mobile, $Fn1^+$ adhesions similar to those described previously (Lu et al., 2020; Pankov et al., 2000; Zamir et al., 1999; Zamir et al., 2000). The centripetal movement of these adhesions was dependent on the rearward actin flow, and the linkage of Fn1 to actin was mediated by integrin $\alpha5\beta1$ and tensin (Pankov et al., 2000; Zamir et al., 1999; Zamir et al., 2000). Our TIRF microscopy experiments are consistent with these studies and show that integrin $\alpha5\beta1$ co-localizes with the regions of higher Fn1 intensity in fibrillar and non-fibrillar adhesions, and that $\alpha5\beta1^+$ focal and fibrillar adhesions are beaded. Taken together, our studies suggest that Fn1 fibrils arise from small mobile nanodomains containing $Fn1^+$ and integrin $\alpha5\beta1^+$ that move toward the cell's center. This

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centripetal translocation of Fn1 nanodomains is coordinated with their organization into linear arrays, which become longer and longer as more nanodomains are added.

The number of Fn1 localizations in nanodomains is independent of the antibody type or amount used for staining, indicating that we are using saturating amounts of antibodies. Together with the preservation of the nanodomain architecture upon the treatment with DOC, our studies indicate that the non-uniform, beaded architecture is a salient feature of mature Fn1 fibrils. Two pieces of evidence suggest that Fn1 nanodomains in fibrils are contiguous: 1) the preservation of the linear organization and the nanoarchitecture of Fn1 fibrils after the treatment of cells with DOC which dissolves cell membranes, and 2) the presence of fibrous material between immunogold densities in electron micrographs (Chen et al., 1997; Dzamba and Peters, 1991). The sparsity of Fn1 localizations between Fn1 nanodomains in fibrils indicates that molecules other than or in addition to Fn1 participate in the linking of Fn1 nanodomains into strings.

FUD peptide specifically binds to the N-terminal assembly domain of Fn1 (Maurer et al., 2015; Maurer et al., 2010) and acts as a competitive inhibitor of Fn1-Fn1 interactions (Ma et al., 2015; Tomasini-Johansson et al., 2001). In the absence of FUD, individual fibrils in an established matrix are stable and can be tracked for over 16 hours (data not shown). When FUD is added to cells, it specifically co-localizes with Fn1 fibrils and dismantles the mature Fn1 ECM (Filla et al., 2017; Tomasini-Johansson et al., 2001), suggesting that the linear arrangement of Fn1 nanodomains in the fibrillar ECM is maintained through dynamic interactions mediated at least in part by the Fn1 N-terminal assembly domain. Our live imaging experiments demonstrated that in addition to dismantling pre-existing fibrils, FUD effectively blocks their de-novo formation. STORM showed that FUD does not affect the formation of Fn1 nanodomains, instead, it blocks the organization of Fn1 nanodomains into linear arrays.

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Together, these data suggest that FUD may block the dynamic interactions between the N-terminal Fn1 assembly domain and the factor(s) linking Fn1 nanodomains into fibrils.

The beaded architecture of Fn1 ECM has important implications for the mechanisms of ECM formation, remodeling and signal transduction. The tensile strength of knotted strings is significantly lower than that of strings with uniformly-aligned fibers (Arai et al., 1999; Saitta et al., 1999), thus the beaded architecture of Fn1 fibrils may facilitate their rupture under strain (Ohashi et al., 1999). The non-uniform, nanodomain architecture of Fn1 may facilitate the accessibility of Fn1 fibrils to matrix metalloproteases. In this model, degradation of Fn1 fibrils by metalloproteases may be accomplished by cleaving between Fn1 nanodomains facilitating ECM remodeling. Finally, Fn1 is known to bind growth factors (Martino and Hubbell, 2010; Saunders and Schwarzbauer, 2019; Wijelath et al., 2002), and cell adhesion to ECM is known to orchestrate growth factor signaling (Hynes, 2009). Thus, Fn1 nanodomains could serve as platforms for the binding and presentation of concentrated packets of growth factors to cells, the organization of Fn1 nanodomains into closely-spaced arrays could further facilitate clustering and signaling by growth factor receptors.

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Materials and Methods

Materials and Methods are in the Supplemental Methods Word File.

Data availability

Reagents generated as a part of this study are available upon request

Legends for Figures are included with Figures

Legends for Movies

Movie 1. Rotational views through the Fn1+ ECM in the cardiac jelly. Whole E9.5 embryo was stained using rabbit monoclonal anti-Fn1 antibody and imaged using 100x objective, N.A. 1.49, with the pinhole set at 0.8 Airy units, and sampling of 40 nm per pixel in x, y. The movie shows 3D reconstruction through 3.4 μm of tissue sampled every 0.121 μm in z. Fn1 is in white, DAPI is in blue. Arrows point to examples of beaded Fn1 fibrils.

Movie 2. Fn1 fibrillogenesis imaged by TIRF microscopy. $Fn1^{mEGFP}$ MEFs were transiently transfected with mCardinal-lifeact, plated on gelatin-coated glass cover slips, and imaged 48 hours later. Filming was done every 2 min for 30 min using TIRF and 100x objective, N.A.

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406 1.49. The first set shows Fn1-mEGFP channel. Yellow arrows point to centripetally-moving
407 Fn1 nanodomains that appear to be organizing into an elongating linear fibril. The second set
408 is an overlay between Fn1-mEGFP and mCardinal-lifeact.
409
410 **Movie 3. 2% DOC dissolved cytoplasm and nucleus in under 13 min leaving Fn1 fibrils.**
411 MEFs expressing Fn1-mEGFP were plated on glass-bottom slides and labeled with SiRActin
412 (magenta) to visualize F-actin and Hoechst (blue) to visualize DNA. Time lapse was recorded
413 every 54 sec immediately following the addition of DOC solution, pH 8.01, to live cells. The
414 addition of 2% DOC dissolves actin cytoskeleton and nuclei, and leaves Fn1 ECM fibrils
415 (green). Fn1 fibrils collapse following the dissolution of the actin cytoskeleton due to the loss of
416 tension.
417
418 **Movie 4. 3D-rendering of STORM data.** Fn1 fibril underlined in Fig. 4b1 is rotated around the
419 x-axis to show the arrangement of Fn1 localizations in 3D. The movie starts in x-y plane.
420 Yellow arrows point at Fn1 nanodomains in the fibrils. Red arrows point to the space between
421 the nanodomains.
422
423 **Movie 5. Cells incubated with 11-IIIC, show robust fibrillogenesis.** $Fn1^{mEGFP}$ MEFs were
424 plated on glass in 8-well Ibidi chambers for 4 hours, Medium containing 11-IIIC control peptide
425 was then added and cells were filmed every 90 sec for about 15 hours, as described in
426 Methods. The movie begins approximately 30 min after the 11-IIIC-containing medium was
427 added, the time it takes to set up time-laps recording. Arrows point to cell periphery and
428 examples of centripetally moving Fn1 fibrils.
429
430 **Movie 6. FUD interferes with linking centripetally moving Fn1+ nanodomains into fibrils.**
431 $Fn1^{mEGFP}$ MEFs were plated on glass in 8-well Ibidi chambers for 4 hours. Medium containing

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FUD peptide was then added and cells were filmed every 3 min for about 15 hours, as described in Methods. The movie begins approximately 30 min after the FUD-containing medium was added, the time it takes to set up time-laps recording. Note the dismantling of pre-existing fibrils at the beginning of the movie. Yellow and red arrows point to cell periphery. Note the presence of centripetally moving Fn1-mEGFP “beads” and the scarcity of Fn1 fibrils for the majority of the duration of the movie.

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