Figures and figure supplements

The fibronectin synergy site re-enforces cell adhesion and mediates a crosstalk between integrin classes

Maria Benito-Jardón et al
Figure 1. Normal tissue development and prolonged bleeding in $Fn^{1\text{syn/syn}}$ mice. (A) Cartoon of FN and the nucleotide point mutations disrupting the function of the synergy site. (B) Representative images of 3-months-old $Fn^{1\text{+/+}}$ and $Fn^{1\text{syn/syn}}$ heart sections stained with H and E and immunostained for FN. (C) Confocal images of ear whole-mounts from 3 months-old mice immunostained with anti-PECAM-1 and anti-$\alpha$SMA to visualize the dermal endothelial cell tubes and smooth muscle cells. (D) Bleeding time of 3-months-old $Fn^{1\text{+/+}}$ (n = 11) and $Fn^{1\text{syn/syn}}$ (n = 11) mice. (E) Platelet counts in blood samples of $Fn^{1\text{+/+}}$ (n = 18) and $Fn^{1\text{syn/syn}}$ (n = 19) mice. (F) FN content in platelets derived from $Fn^{1\text{+/+}}$ (n = 6) and $Fn^{1\text{syn/syn}}$ (n = 6) mice relative to their vinculin levels. (G) Occlusion time of injured arterioles in the cremaster muscle of 3-months-old $Fn^{1\text{+/+}}$ (n = 11) and $Fn^{1\text{syn/syn}}$ (n = 11) mice. (H) Figure 1 continued on next page
Figure 1 continued

Representative still images of the arteriolar occlusion (white: platelets). Values are shown as mean ± SD; statistical significances were calculated using the Student t-test; **p < 0.01 and ***p < 0.001.

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Figure 1—figure supplement 1. Strategy used to generate the $Fn1^{syn/syn}$ mice and tissue and platelet analysis. (A) Scheme of the FN gene and the targeting vector to generate the mouse with a dysfunctional synergy site. The synergy region is located in the FNIII9 and encoded in exon 28, shown in red. (B) Homologous recombination of the targeting vector was re-tested in ES cell clones 56 and 266 by Southern-blot using probe 1 Sacl digested DNA. (C) Mice were genotyped by PCR using primers shown as arrows in (a). (D) Liver, kidney and lung sections from 3-months-old $Fn1^{+/+}$ and $Fn1^{syn/syn}$ mice stained with H and E (scale bar, 100 μm) and immunostained for FN (scale bar, 50 μm). (E) Ear whole-mount staining of laminin (Lam), FN, collagen IV, and erythrocytes (Ter119) to analyze the sub-endothelial ECM composition and the integrity of blood vessels (scale bars, 50 μm and 25 μm for FN immunostaining).

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Figure 1—figure supplement 2. FN levels in platelets and blood from Fn1<sup>+/+</sup> mice and platelet aggregation assays. (A) Western-blot to estimate FN levels in non-activated, washed platelets from Fn1<sup>+/+</sup> (n = 6) and Fn1<sup><i>syn/syn</i></sup> mice (n = 6). (B) Western-blot to calculate FN and fibrinogen (Fg) levels in blood plasma from Fn1<sup>+/+</sup> (n = 6) and Fn1<sup><i>syn/syn</i></sup> mice (n = 6). The first two lanes are commercial Fg and FN, respectively. (C–E) Representative in vitro aggregation assays using washed platelets from Fn1<sup>+/+</sup> (n = 6) and Fn1<sup><i>syn/syn</i></sup> mice (n = 8). Aggregation curves of platelets activated with 5 μg/ml collagen (C), with 0.5 u/ml thrombin (D) or with 20 μM ADP (E). DOI: 10.7554/eLife.22264.004
Figure 2. The FN synergy site is dispensable for FN fibrillogenesis, cell adhesion and spreading. (A) Fn1-Knock-Out (Fn1-KO) fibroblasts grown in 1% plasma derived from either Fn1+/+ or Fn1m/m mice, fixed at the indicated times and stained for FN (green), F-actin stain (with Phalloidin; red) and nuclei (with DAPI; blue). Scale bar, 10 μm. (B) Fn1-KO cells seeded on pFNwt or pFNsyn, fixed at the indicated times and stained for F-actin (red), paxillin (white) and total β1 integrin (green). Scale bar, 20 μm. (C–E) Cell size (C), number of FAs per cell (D) and percentage coverage by FAs (paxillin-positive) (E) were quantified (n = 25 cells assessed from three independent experiments; mean ± sem). Statistical significances were calculated using the Student t-test; **p < 0.01 and ***p < 0.001.

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Figure 2—figure supplement 1. Integrin surface levels and plasma FN purification and glass coating. (A) Integrin levels on the surface of Fn1-KO fibroblasts analyzed by flow cytometry (graphs show representative results of 3 independent experiments). (B) Coomassie blue staining of purified pFN<sup>wt</sup> and pFN<sup>syn</sup> after PAGE. The second lane shows the starting flow-through, and the 5-11<sup>th</sup> the fractions with FN. (C) Western-blot of the purified pFN<sup>wt</sup> and pFN<sup>syn</sup>. (D) Western-blot to detect fibrinogen in the purified mouse pFN. The fibrinogen content was below the 4% of protein. (E) ELISA of glass-

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coverslips coated with different pFN concentrations. (F) Adhesion of Fn1-KO fibroblasts plated to pFN<sup>wt</sup>, pFN<sup>syn</sup>, polylysine and BSA (n = 3 independent experiments for each ligand). (G) FACS analysis of integrin surface levels on Fn1-KO, pKO-αv/β1, pKO-αv and pKO-β1 fibroblasts. DOI: 10.7554/eLife.22264.006
Figure 2—figure supplement 2. Captures of life-time microscopy videos of Fn1-KO fibroblasts spreading on pFN\textsuperscript{wt} or pFN\textsuperscript{syn}. DOI: 10.7554/eLife.22264.007
**Figure 3.** The FN synergy site is required to establish tensioned FN-α5β1 bonds. (A) Quantification of adhesion strength. $7 \times 10^5$ Fn1-KO cells attached onto purified, full-length (fl) pFN$^{wt}$ or pFN$^{syn}$ or FNIII7-10$^{wt}$ or FNIII7-10$^{syn}$ and spun with a spinning disk device (n = 7 independent experiments with fl-FN; n = 3 independent experiments with FNIII7-10; mean ± sem). (B) Western-blot analysis (left) and quantification (right) of cross-linked α5 integrins to pFN$^{wt}$ or pFN$^{syn}$ before and after applying shear forces (n = 6 independent experiments; mean ± sem). (C) Western-blot analysis (left) and quantification (right) of pY397- and pY861-FAK levels in Fn1-KO cells plated on pFN$^{wt}$ or pFN$^{syn}$ (n = 6 independent experiments; mean ± sem). Statistical significances were calculated using the Student t-test; *p < 0.05, **p < 0.01 and ***p < 0.001.

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Figure 3—figure supplement 1. Representative spinning disk experiment showing the cell distribution profile against the shear force. The shear force was calculated according to the method described by Friedland and Boettiger (Friedland et al., 2009). The force corresponding to the inflexion point of the curve is the $t_{50}$.
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**Figure 4.** α5β1 integrins require the synergy site in FN to induce cell spreading. (A) Adhesion of pKO-β1, pKO-αv and pKO-αv/β1 fibroblasts seeded on pFN wt or pFN syn for indicated times (n = 3 independent experiments; mean ± sem). (B) pKO-β1, pKO-αv and pKO-αv/β1 fibroblasts were seeded on pFN wt or pFN syn, fixed at the indicated times and stained for total β1 integrin (green), paxillin (white) and F-actin (red). Scale bar, 50 μm. (C–E) Quantification of cell area of pKO-β1 (C), pKO-αv (D) and pKO-αv/β1 (E) cells seeded on pFN wt or pFN syn for indicated times. (F–H) Quantification of the number of FAs (F), the percentage of FA coverage measured as paxillin-positive area (G) and the percentage of β1 integrin-positive areas referred to the total cell area (H) in pKO-β1 cells (n = 25 cells for each measurement and three independent experiments; mean ± sem). The binding probability Figure 4 continued on next page
of integrins to FNIII7-10<sup>wt</sup> or FNIII7-10<sup>syn</sup> fragments (I) and to full length (β-FN) pFN<sup>wt</sup> or pFN<sup>syn</sup> (J) determined by single-cell force spectroscopy. Numbers in parentheses indicate events studied for each condition. Statistical significances were calculated using the Student t-test; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

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Figure 4—figure supplement 1. Captures of life-time microscopy videos of pKO-β1 fibroblasts spreading on pFN^wt or pFN^syn.
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Figure 5. Fn1<sup>syn/</sup>;Itgb3<sup>-/-</sup> mice suffer from severe hemorrhages and fail to separate the blood and lymphatic vasculatures. (A) E12.5 Fn1<sup>syn/</sup>;Itgb3<sup>-/-</sup> embryos display hemorrhages in the jugular and axilar areas in the left side (arrowheads). Scale bar, 50 mm. (B) Representative images from E15.5 littermates embryos resulting from Fn1<sup>syn/</sup>;Itgb3<sup>-/-</sup> intercrosses. Compound Fn1<sup>syn/</sup>;Itgb3<sup>-/-</sup> embryos display cutaneous edema (arrowhead) and abundant skin hemorrhages (arrows); scale bars, 50 mm. (C) Skin whole-mount from E15.5 embryos showing Lyve1-positive lymphatic vessels (green), αSMA-positive blood vessels (red) and Terr119-positive erythrocytes (white). The lymphatic vessels of compound Fn1<sup>syn/</sup>;Itgb3<sup>-/-</sup> embryos are dilated, covered by ectopic αSMA-positive cells and filled with erythrocytes. Scale bar, 50 μm. (D) Representative images of skin sections stained with H and E (upper panel) and Lyve1 and Terr119 (lower panel) showing erythrocytes in lymphatic vessels. Scale bar, 50 μm. (E) Quantification of the percentage of lymphatic vessels filled with Terr119-positive erythrocytes (n = 40 vessels counted per embryo, in two embryos per each genotype; mean ± sem). Statistical significances were calculated using the Student t-test: ****p<0.0001.

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**Figure 5—figure supplement 1.** Blood vessel formation in $F_{n1}^{syn/syn}$, $Itgb3^{-/-}$ embryos. (A) E11.5 embryos of indicated genotype. Scale bar, 2 mm (B) E11.5 whole mount embryos of indicated genotype stained with PECAM. High magnifications indicate that $F_{n1}^{syn/syn}$, $Itgb3^{-/-}$ embryos have normal angiogenesis. Scale bar, 1 mm. DOI: 10.7554/eLife.22264.019
Figure 6. Shear flow exposed platelets fail to adhere to pFN<sup>syn</sup>. (A) Cartoon showing the platelet integrins that can be ligated to the different substrates used in the experiments. The color intensity of the integrin denotes whether the integrin is active or inactive. (B) Spreading of Itgb3<sup>+/+</sup> and Itgb3<sup>-/-</sup> platelets after 1 hr on fibrinogen, pFN<sup>wt</sup>, pFN<sup>syn</sup> and type I collagen. Scale bars, 10 μm. (C) Quantification of the platelet area at indicated times (n = 100 platelets per each condition in three independent experiments; mean ± sem). (D) Representative figures of fluorescently labeled Itgb3<sup>+/+</sup> or Itgb3<sup>-/-</sup> platelets seeded on indicated substrates and exposed to shear flow. Scale bar, 40 μm. (E) Platelet coverage after 10 min shear flow of 1000 s<sup>-1</sup>. (n = 10 pictures per experiment, four independent experiments for each condition; mean ± sem). Statistical significances were calculated using the Student t-test; *p<0.05, **p<0.01 and ***p<0.001.
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Figure 7. Malformed blood vessels in \( \text{Fn}^{\text{syn/syn}, \text{Itgb}3^{-/-}} \) embryos. (A) PECAM-positive endothelial cells (red) and \( \alpha \)-SMA-positive smooth muscle cells (green) in dermal whole mounts from E15.5 \( \text{Fn}^{1+/+}, \text{Itgb}3^{+/+} \) and \( \text{Fn}^{\text{syn/syn}, \text{Itgb}3^{-/-}} \) littermate embryos indicate veins (V) and arteries (A). (B) Quantification of the number of branching points (\( n = 10-15 \) images of 2–3 embryos; mean ± sem). (C) Vascular basement membranes in dermal whole mounts from E15.5 \( \text{Fn}^{1+/+}, \text{Itgb}3^{+/+} \), \( \text{Fn}^{1+/+}, \text{Itgb}3^{-/-} \) and \( \text{Fn}^{\text{syn/syn}, \text{Itgb}3^{-/-}} \) littermate embryos stained for type IV collagen (green) and PECAM-positive endothelial cells (red). Arrowheads show small vessels lacking lumen. (D) Quantification of retracted vessels (\( n = 14-23 \) from 4–7 embryos; mean ± sem). (E) PECAM-positive endothelial cells (red) and NG2-positive pericytes (green) in dermal whole-mounts from E15.5 \( \text{Fn}^{1+/+}, \text{Itgb}3^{+/+} \), \( \text{Fn}^{1+/+}, \text{Itgb}3^{-/-} \) and \( \text{Fn}^{\text{syn/syn}, \text{Itgb}3^{-/-}} \) littermate embryos. Note pericytes are sparse, absent or aggregate on mutant vessels (arrowheads). Statistical significances were calculated using the Student t-test; \( *p < 0.05 \), and \( **p < 0.001 \). Scale bars, 50 \( \mu \text{m} \).

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Figure 8. The major role of the FN synergy site is to re-enforce cell adhesion. (A) Hydrodynamic shear force-exposed fibroblasts seeded on a FN\textsuperscript{wt}-coated surface form catch-bonds that strengthen $\alpha5\beta1$ integrin-mediated adhesions to FN and trigger phosphorylation of Y397-FAK (upper image). On FN\textsuperscript{syn}-coated surfaces, the $\alpha\nu\beta3$ integrins compensate for the absent synergy site allowing fibroblast adhesion and the reduced $\alpha5\beta1$ binding strength leads to diminished phosphorylation of pY397-FAK (middle image). The elimination of $\alpha\nu$-class integrins decreases cell adhesion on FN\textsuperscript{syn}-coated surfaces, reduces cell spreading and delays the maturation of FA and fibrillar adhesions (lower image). (B) Platelets in Fn1\textsuperscript{+/+} mice form tight aggregates on injured vessel walls that withstand the shear forces of the blood flow (upper image), while platelets in an injured vessel in Fn1\textsuperscript{syn/syn} mice fail to withstand the blood flow leading to a delayed thrombus formation (lower image). Endothelial cells (EC); vascular smooth muscle cells (VSMC).

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