Fibrillin-1 directly regulates osteoclast formation and function by a dual mechanism

Kerstin Tiedemann, Iris Boraschi-Diaz, Irina Rajakumar, Jasvir Kaur, Peter Roughley, Dieter P. Reinhardt and Svetlana V. Komarova

Supplemental Figure S1.

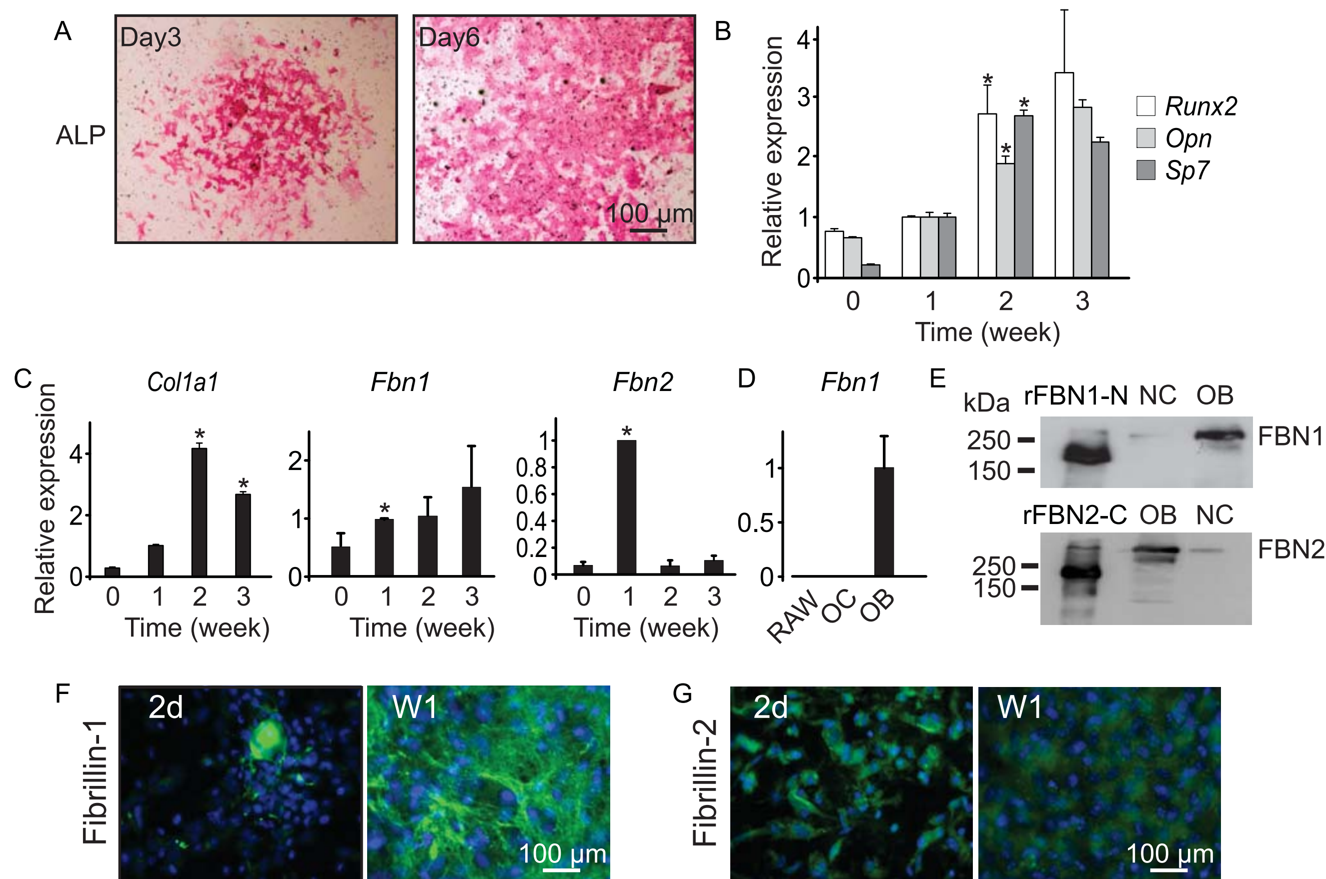

Fig. S1. Osteoblasts produce fibrillin-1 and -2. Mouse bone marrow cells were treated with ascorbic acid (AA; 50 µg/ml. (A) Osteoblast differentiation was monitored by the expression of alkaline phosphatase (ALP). (B) The expression of osteoblast marker genes including Runt-related transcription factor 2 (*Runx2)*, osteopontin (*Opn*), and osterix (*Sp7*) was assessed during the differentiation period. *Runx2, Opn* and *Sp7* reached maximum expression after 2 weeks of culture and remained up-regulated during the third week of differentiation. (C) The expression of the extracellular matrix proteins by osteoblastic cells. Collagen type IA (*Col1a1*) gradually increased over the first two weeks of culture and remained up-regulated thereafter. Both fibrillin-1 (*Fbn1)* and fibrillin-2 (*Fbn2)* mRNA were strongly induced during the first week of osteoblast differentiation. In the subsequent weeks, *Fbn1* mRNA remained up- regulated, but the expression of *Fbn2* mRNA decreased to levels similar to undifferentiated cells. Data are means ± SEM, n = 3-5 experiments, *p < 0.05 indicates significance compared to week 0 assessed by ANOVA. (D) RAW 264.7 cells (RAW) or differentiated osteoclasts (OC) do not express *Fbn1*. Mature osteoblasts (OB) were used as a positive control. (E) Differentiated osteoblasts (OB) secreted fibrillin-1 and -2 into the conditioned medium. In contrast, untreated bone marrow cells (negative control, NC) did not secrete detectable amounts of fibrillin-1 or -2. (F) Development of fibrillin-1-containing microfibrils was apparent by immunofluorescence during the first week (W1) of osteoblast differentiation. (G) Only diffuse staining was observed for fibrillin-2 in osteoblast cultures.

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Supplemental Figure S2.

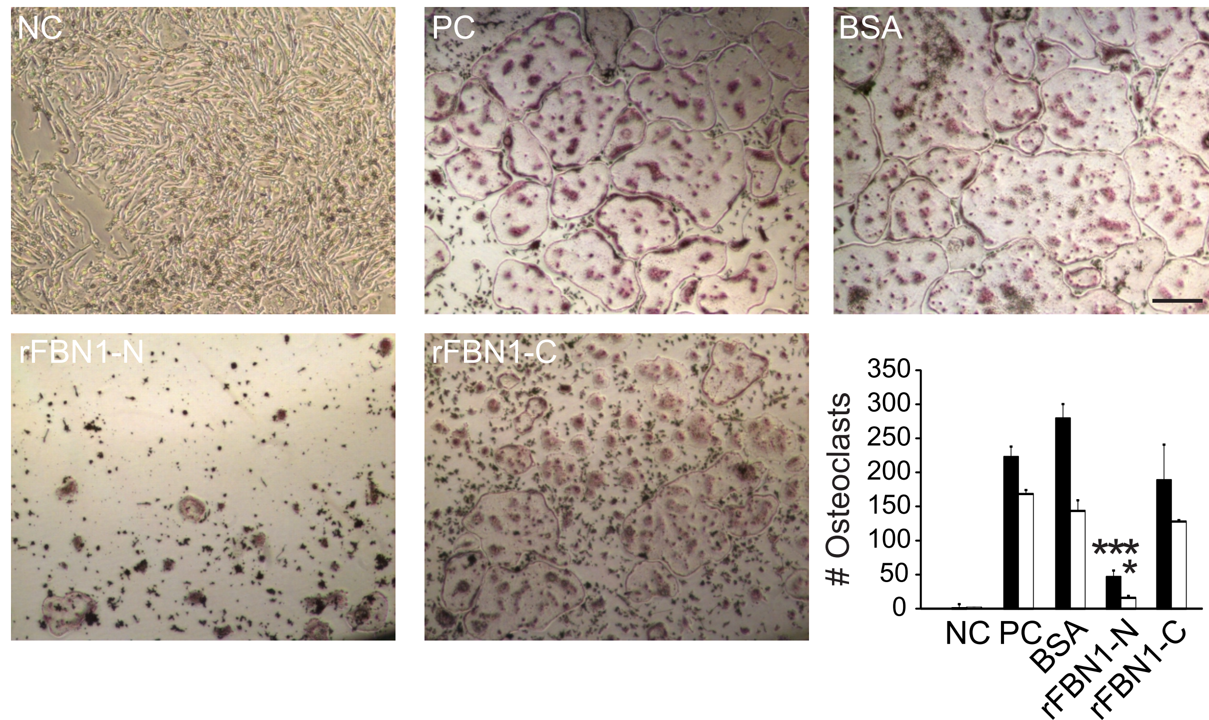

Fig. S2. Bovine serum albumin (BSA) does not affect osteoclast formation. RAW 264.7 cells (black bars) and mouse bone marrow cells (white bars) were cultured for 5 days untreated (negative control, NC), treated with RANKL only (50 ng/ml, positive control, PC), or treated with RANKL and BSA (50 µg/ml), or soluble fibrillin-1 fragments rFBN1-N or rFBN1-C (50 µg/ml). Samples were fixed and stained for TRAP. Shown are representative images of osteoclasts formed in bone marrow cultures under the indicated conditions. Scale bar of 250 µm applies to all images. The addition of BSA did not affect osteoclast formation, only addition of rFBN1-N resulted in significant decrease in osteoclast numbers. Data are means ± SEM, n = 3-5 independent experiments, *p<0.05, ***p<0.001 compared to PC.

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Supplemental Information S3.

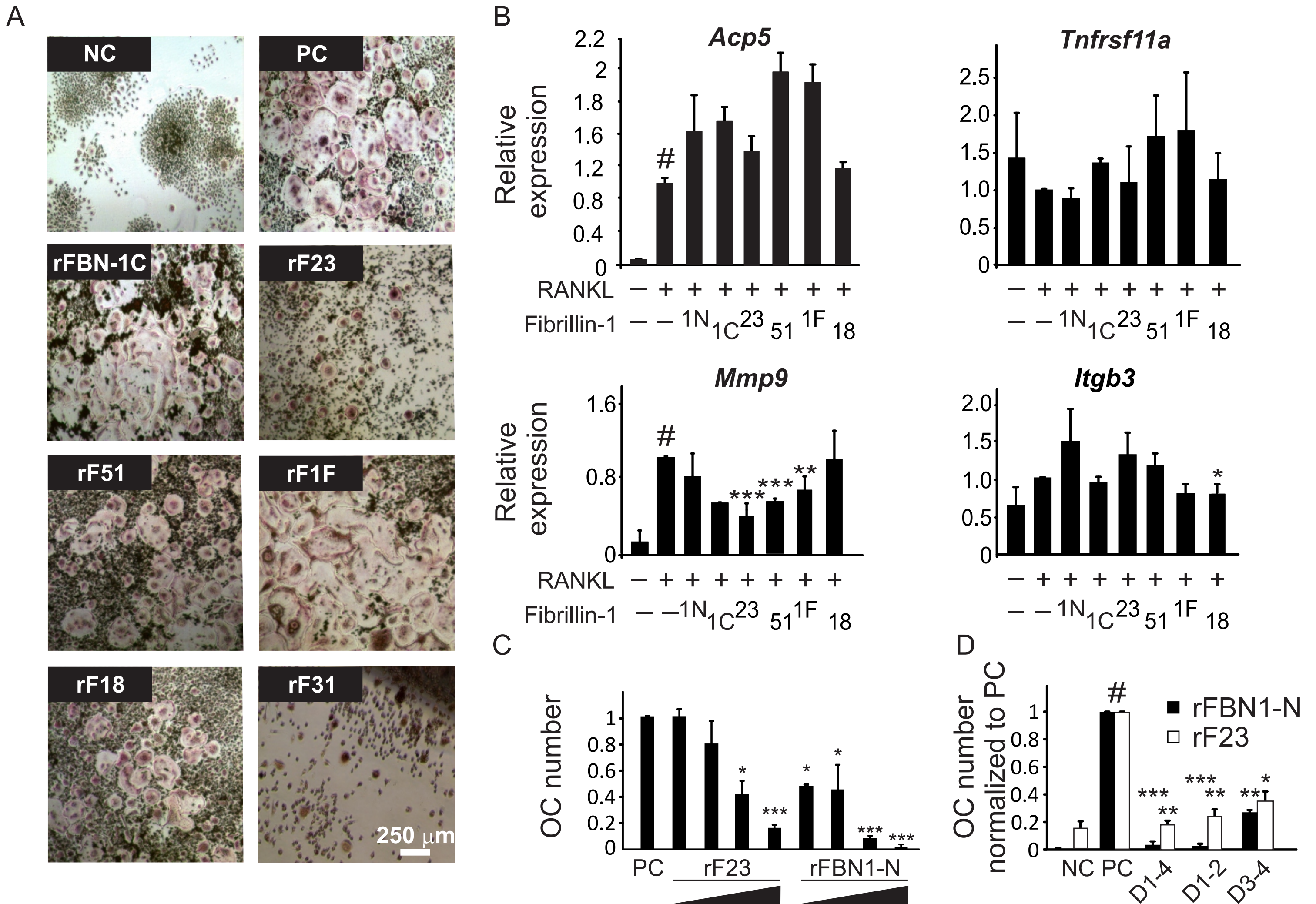

Fig. S3. Effect of fibrillin-1 fragments on osteoclast formation and gene expression. (A) Representative images of osteoclasts formed from RAW 264.7 monocytes after addition of soluble rFBN1-N, rFBN1-C, rF23, rF51, rF1F, or rF18 (50 µg/ml) for the duration of the experiment. Scale bar applies to all images. (B) RAW 264.7 cells were cultured for 5 days untreated, treated with RANKL only (50 ng/ml), or treated with RANKL and soluble rFBN1-N, rFBN1-C, rF23, rF51, rF1F, or rF18 (50 µg/ml), and mRNA expression of tartrate resistant acid phosphatase (*Acp5*), receptor activator of nuclear factor B (*Tnfrsf11a*), metalloproteinase 9 (*Mmp9) and integrin* β*3* (*Itgb3*) wasassessed. RANKL treatment alone significantly increased gene expression of *Acp5 and Mmp9.* rF23, rF51, and rF1F significantly decreased gene expression of *Mmp9.* Data are means ± SEM, n = 3 experiments, ***$p < 0.001$, compared to PC as assessed by t test; # $p < 0.05$, compared to NC as assessed by t-test. (C) Bone marrow cells were cultured with 50 ng/ml MCSF and RANKL for 4 days with or without rF23 and rFBN1-N in concentrations of 6.25, 12.5, 25 and 50 µg/ml. (D) Bone marrow cells were cultured with 50 ng/ml MCSF and RANKL for 4 days with or without rF23 and rFBN1-N added for the duration of the experiment (day 1-4, D1-4), for the first 2 days only (D1-2), or for the last 2 days only (D3-4). Data are means ± SEM, n = 3 experiments normalized to osteoclast numbers observed in positive control (PC), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to positive control as assessed by t-test; # $p < 0.05$, compared to negative control (NC) as determined by t-test.

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Supplemental Figure S4.

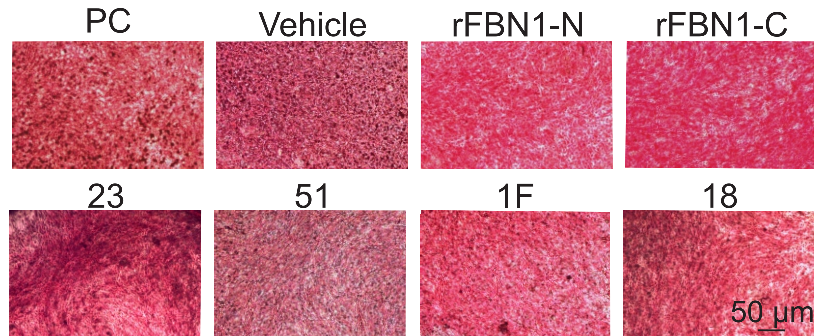

Fig. S4. Fibrillin-1 has no effect on osteoblast differentiation. Osteoblast differentiation of mouse bone marrow cells was induced with ascorbic acid (50 µg/ml) in the absence (positive control, PC) or presence of vehicle (Tris-based buffer) or soluble fibrillin-1 fragments (50 µg/ml), rFBN1-N, rFBN1-C, rF23 (23), rF51 (51), rF1F (1F) and rF18 (18). After 1 week, cells were fixed and stained for ALP (red). Shown are representative images of osteoblastic cells formed under different conditions. Scale bar applies to all images.

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Supplemental Figure S5.

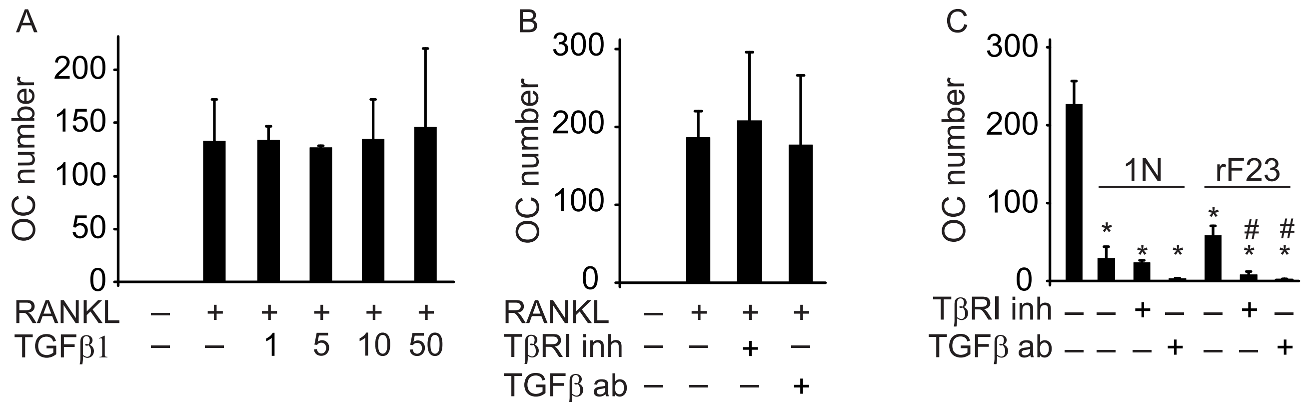

Fig. S5. TGFβ does not affect osteoclastgenesis in the cell culture model used. (A) RAW 264.7 cells were cultured for 5 days untreated or treated with RANKL (50 ng/ml) and recombinant TGFβ1 (1-50 ng/ml) and osteoclast numbers were quantified. (B) Mouse bone marrow cells were treated with MCSF (50 ng/ml) and RANKL (50 ng/ml) in the presence of TGFβ receptor I inhibitor (TβRI inh, 5 μM) or neutralizing antibody against TGFβ1 (TGFβ ab, 15 μg/ml) and osteoclast numbers were determined. For A and B, data are means ± SEM, n=3-5 experiments, no significant differences between RANKL-treated conditions. (C) Inhibition of TGFβ minimally affects anti-osteoclastogenic effect of fibrillin-1 fragments. Bone marrow cells were cultured for 5 days with MCSF and RANKL without (positive control) or with 50 μg/ml of rFBN1-N or rF23 in the absence or presence of TβRI inhibitor (5 μM) or TGFβ neutralizing antibody (15 μg/ml), and osteoclast numbers were counted. Data are means ± SEM, n = 3-5 experiments, *$p < 0.001$ indicates significance compared to positive control and #$p < 0.05$ indicates significance compared to the samples without inhibitors, as determined by t-test.

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Supplemental Figure S6.

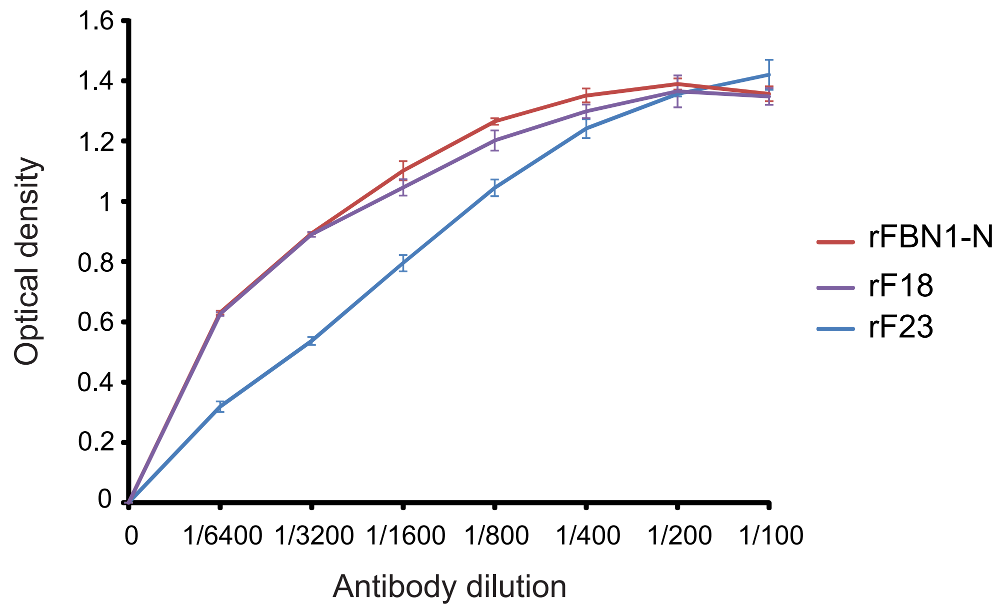

Fig. S6. Efficiency of the detector antibody for rFBN1-N and its fragments rF18 and rF23. ELISA demonstrates similar titers for rFBN1-N and the tested fragments. Background values without coated proteins were ≤ 0.075. Data are means ± SD, n = 3 for rFBN1-N and rF23, and n = 2 for rF18.

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Supplemental Figure S7.

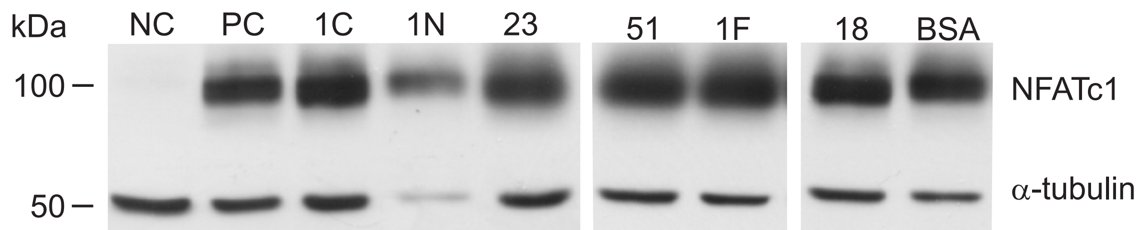

Fig. S7. NFAT protein levels are not affected by fibrillin-1 fragments. RAW 264.7 cells were either untreated (NC) or treated with RANKL (50 ng/ml, PC) alone or RANKL and rFBN1-C (1C), rFBN1-N (1N), rF23 (23), rF51 (51), rF1F (1F), rF18 (18) or BSA (50 μg/ml) for 3 days. Cell lysates were collected and NFATc1 protein levels were assessed using immunoblotting, α-tubulin was used as a loading control. Fibrillin-1 fragments did not affect protein level of NFATc1. Data are representative of three independent experiments.