Supporting Information

Figures

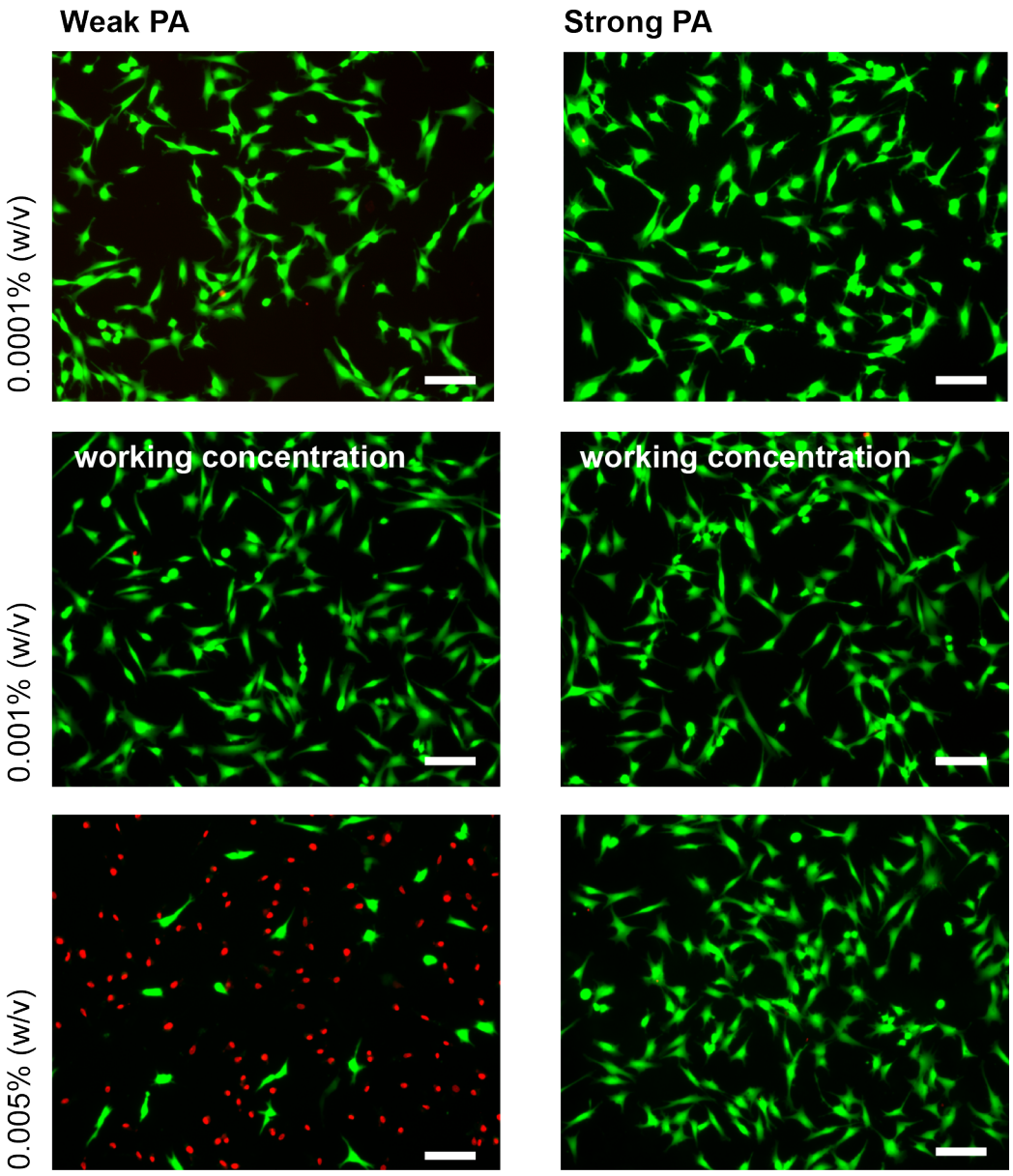

Fig. S1: Viability of C2C12 cells following exposure to Strong or Weak PA with 10 nM BMP-2. Following treatment with either Strong β-sheet or Weak β-sheet forming PA nanofibers and BMP-2 for 24 hr, C212 cells were were stained to evaluate the viable (calcein AM, green) and dead (ethidium homodimer-1, red) cell populations. Scale bar: 100 μm.

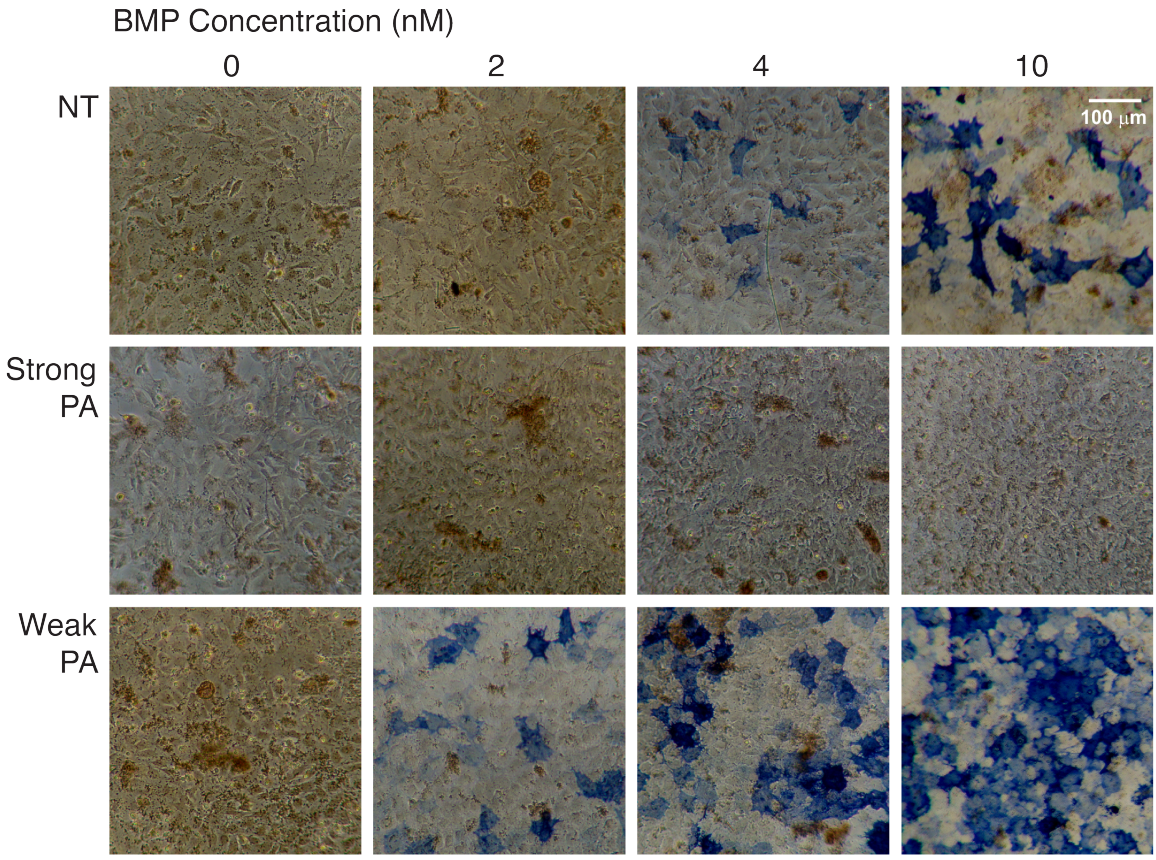

Fig. S2: Differentiation of C2C12 cells with PA and varying BMP-2 concentration. Fast Blue staining of C2C12 cells after a 3 day treatment with increasing concentrations of BMP-2 (left to right) and either Strong β-sheet PA (middle row) or Weak β-sheet PA nanofibers (bottom row). Cells treated with only BMP-2 served as controls (top row). Fast Blue staining for alkaline phosphatase (ALP) expression was used to record ALP expression. Enhanced ALP expression was observed for only those cells treated with Weak β-sheet forming PA materials and BMP-2. Enhanced ALP expression was observed with as low 2 nM BMP-2 concentration in the presence of the Weak β-sheet PA nanofibers. Scale bar: 100 μm.

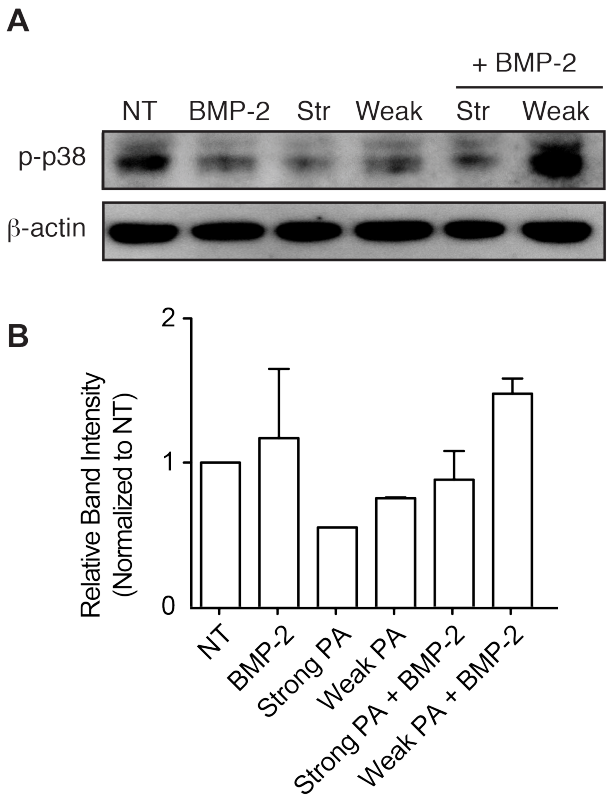

Fig. S3: Non-Smad BMP-2 mediated signaling upon treatment with PA. (A) Whole cell lysates were extracted from C2C12 cells following treatment with PA and/or BMP-2. Western blot analysis was performed for phospho-p38 (p-p38) with β-actin to reference total protein content. (B) Densitometry from Western blot band luminescent signal from the p-Smad experiment that appears in (A).

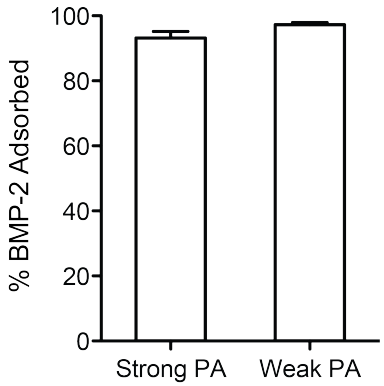

Fig. S4: Evaluation of BMP-2 affinity to Strong and Weak PAs. Coatings of Weak or Strong PA were treated with 10 nM BMP-2 and the percent adsorbed was quantified. BMP-2 adsorbs to both PA coatings with minimal preference for strong or weak PA.

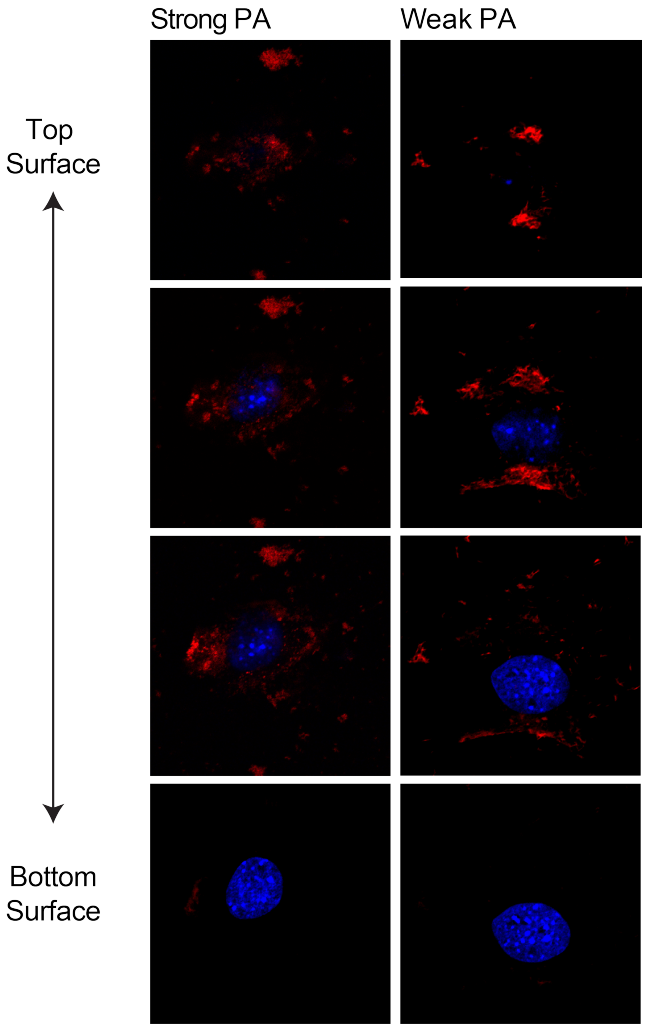

Fig. S5: Cellular distribution of PA following treatment of cells. C2C12 cells were treated with fluorescent-labeled Strong β-sheet or Weak β-sheet forming PA material (red) and nuclei visualized with DAPI (blue). Individual confocal optical slices reveal the distribution of PA from the top surface of the cell to the bottom surface of the cell. Z-stacked images were acquired from the images shown in Fig. 4A.

Materials and Methods

PA Synthesis. Molecules were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc)-solid phase peptide chemistry. The peptides were synthesized on a Rink amide MBHA low loading resin at the Peptide Synthesis Core at the Simpson Querrey Institute for BioNanotechnology. First, Fmoc-Lys(Mtt)-OH was coupled to the resin followed by deprotection of the Mtt group using 3% trifluoroacetic acid (TFA), 5% triisopropylsilane (TIPS) and 93% dichloromethane (DCM). The remainder of the peptide was synthesized using a CEM Liberty microwave-assisted peptide synthesizer. Amino acid couplings were performed using 5 eq. of protected amino acid or palmitic acid, 5 eq. of O-benzotriazole-N, N, N', N'-tetramethyluronium hexafluorophosphate (HBTU), and 10 eq. of N,N-diisopropylethylamine (DIEA) at 75°C for 5-10 minutes. Fmoc deprotection was performed using 20% 4-methyl piperidine and 0.1M hydroxybenzotriazole (HOBt) in dimethylformamide (DMF) at 75°C for 3-4 minutes. Capping steps were achieved using a mixture of 0.5 M acetic anhydride, 0.125 M DIEA, and 0.015 M HOBt in DMF at 65°C for 2 minutes. Default microwave settings were used. To couple the fluorophore, 1.1 eq. of 5(6)-carboxy-tetramethylrhodamine (EMD Chemicals) was coupled with 1.1 eq. HBTU and 2.2 eq. DIEA in 2 mL of DMF overnight without the microwave.

The molecules were cleaved in 95:2.5:2.5 TFA:TIPS:water for 2-3 hours. After removing volatile solvents and precipitating the product with diethyl ether, the product was dried under vacuum and purified using preparative scale reverse phase high performance liquid chromatography (Varian) with a Phenomenex Jupiter Proteo column (C_{12} stationary phase, 10 μm, 90 Å pore size, 150 x 30 mm). All molecules were purified using a mobile phase gradient of acetonitrile and water with 0.1% TFA to aid with solubility. Pure fractions were identified using ESI mass spectrometry and excess acetonitrile was removed with rotary evaporation. The final product was lyophilized and stored at -20 °C until use.

X-ray Scattering. Wide-angle X-ray diffraction was performed using the bending magnet station 14-BMC at Argonne National Laboratory operating at 12.67 keV. Samples were dissolved to 1% (w/v) in a 1:1 mixture of water:MEMAlpha and injected into 1.5-mm diameter quartz capillaries (Charles Supper). Background solvent subtraction and radial integration were performed using FIT2D software.

Cryogenic Transmission Electron Microscopy. PA samples were dissolved to 0.1% (w/v) in a 1:1 mixture of water:MEMAlpha. Using a Vitrobot Mark IV (FEI) vitrification robot, samples were pipeted onto a copper TEM grid with a lacey carbon support in a humidified (95-100% humidity) chamber at ambient temperature. Samples were blotted, plunged into liquid ethane, and transferred to a Gatan 626 cryo-holder where they were imaged using a JEOL 1230 TEM fitted with a LaB_6 filament operating at an accelerating voltage of 100 kV. Images were acquired using a Gatan 831 bottom-mounted CCD camera.

Enzyme Linked Immunosorbent Assay. To quantify the amount of BMP-2 bound to PA nanostructures, PA assemblies were coated on a 48 well tissue culture plate as previously described.[16, 19] Briefly, the culture plate was coated sequentially with poly-D-lysine, sodium alginate, and 0.001% (w/v) PA. The coatings were washed twice with PBS and incubated with 10 nM BMP-2 in DMEM at 37°C, 5% CO_2 for 3 hours. The supernatant was removed from the

coating and frozen at -80°C until the ELISA was performed. The BMP-2 Quantikine ELISA Kit (R&D Systems) was used per manufacturer's instructions. The supernatant was diluted 10-fold and compared to the concentration of the initial BMP-2 10 nM stock to determine the percentage of growth factor bound to the PA ($N = 4$).

Cell Culture. Recombinant human BMP-2 was obtained from Medtronic Sofamor Danek (Minneapolis, MN) and reconstituted and stored according to the manufacturer's instructions. C2C12 mouse myoblasts (ATCC) were maintained in Dulbecco's Modified Eagle's Medium with high glucose and L-glutamine (DMEM, ATCC), supplemented with 10% heat inactivated FBS (Life Technologies), 100 U/mL of penicillin and 100 μg/mL streptomycin (Life Technologies) and passaged at 80-90% confluence. Cells were grown at 37°C, 5% CO_2 in a humidified atmosphere. Cells were detached following a 5 minute incubation at 37°C with 0.05% Trypsin-EDTA (Life Technologies), seeded, and allowed to attach for a minimum of 3 hours prior to experiments.

LRAP was chemically synthesized and HPLC purified. ST2 cells were maintained in α-minimal essential medium containing 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. To induce osteogenesis, over-confluent cells were switched to mineralization media containing 25 μg/ml ascorbic acid and 10 mM β-glycerophosphate.

EdU Staining. 5-ethynyl-2'-deoxyuridine (EdU), a nucleoside analog that incorporates into DNA, was used to identify proliferating cells. To evaluate cell proliferation, C2C12 cells were seeded on 12 mm PDL coated glass coverslips in a 12 well plate (50,000 cells/well) with 10% FBS DMEM. After 3 hours, cells were cultured overnight with 2.5% FBS DMEM containing various treatments. Cells were then treated with 4 μM EdU for 8 hours followed by fixation with 4% paraformaldehyde in PBS (5 min, RT) and permeabilization with 0.4% Triton X-100 in PBS (15 min, RT). A reaction cocktail containing 0.1 M Tris (pH 8.5), 1mM $CuSO_4$, 0.1 M ascorbic acid, and 1μg/mL Alexa Fluor 555 azide (Life Technologies) was prepared and added to the cells (30 min incubation at RT, dark) to allow for fluorescent labeling of the EdU. Cell nuclei were counterstained with 5 μg/mL DAPI (Life Technologies) and imaged using a TissueGnostics High Throughput Imaging System. Each experiment was performed in triplicate (n=2 for each experiment) and for each coverslip, ten fields were imaged at 20X magnification and quantified using NIH ImageJ.

Alkaline Phosphatase Staining. Alkaline phosphatase was stained using Fast Blue. Cells were fixed with 4% paraformaldehyde in PBS for 30 seconds, washed three times with PBS and stained using Fast Blue for 30 minutes at room temperature. Following staining, the samples were washed with PBS until the solution was clear.

For the Fast Blue stain, napthol AS-MX (10 mg/mL, Sigma Aldrich) was dissolved in a glass vial using dimethyl formamide and added to a solution of 0.1M Tris-HCl, pH 8.2 containing Fast Blue BB hemizinc salt (1 mg/mL, Sigma Aldrich). The solution was sonicated and filtered immediately prior to staining.

Alizarin Red Staining. Two weeks following the osteogenic induction of ST2 cells, cells were stained with Alizarin Red. Quantification of calcium concentration was done by measuring

absorbance at 612 nm (QuantiChrom Calcium Assay kit; BioAssay Systems). The total amount of protein in each sample was used as a standard with which to normalize calcium concentration.

Western Blot. C2C12 cells were seeded at 150,000 cells/well in 6 well plates with 10% FBS DMEM for 48 hours. The cells were starved for 24 hours in 0.2% FBS DMEM (PA-treated samples contained 0.001% (w/v) PA during the starvation period). Following starvation, the cells were rinsed once with serum-free DMEM and treated with 0.001% (w/v) PA with or without BMP-2 (10 nM) for 30 minutes. Cells were lysed with M-PER lysis buffer (Thermo Scientific) containing 1X of both Halt protease and Halt phosphatase inhibitors (Thermo Scientific) and horn sonicated prior to determining protein concentration using the Micro BCA Protein Assay (Thermo Scientific). 9 µg of protein was loaded in each well of the NuPAGE 10% Bis-Tris Gel (Life Technologies). SDS-PAGE was performed using NuPAGE MOPS SDS Running Buffer (Life Technologies) and transferred onto PVDF membranes in NuPAGE Transfer Buffer (Novex).

The membranes were first probed for either p-Smad or p-p38 and were subsequently stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) for 5 minutes at RT and re-probed for β-Actin. Blocking steps were performed in TBST containing 5% BSA (prior to stripping) or 5% milk (following stripping) for 1 hour at room temperature (RT). Next the membranes were probed with one of the following primary antibodies: p-p38 MAPK (T180/Y182) Rabbit antibody (Cell Signaling) 1:1000 dilution , p-Smad1/5(S463/465)/Smad8(S426/428) Rabbit antibody (Cell Signaling) 1:500 dilution overnight at 4°C or Ms Anti-β-Actin 1:10,000 dilution for 1 hour at RT in TBST containing 0.1% BSA or 1% milk. This was followed by 1 hour secondary antibody incubation at RT with Goat Anti-Rabbit-HRP Conjugate (Bio-Rad), 1:4000 dilution or Goat Anti-Mouse-HRP Conjugate (Bio-Rad), 1:5000 dilution. Membranes were exposed to SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) for 5 minutes to detect the chemiluminescent signal, which was imaged using a Kodak In-vivo Imaging System.

Quantitative RT-PCR. C2C12 cells were seeded on tissue culture plastic in serum-containing media (10% FBS) for 6 hours to allow cells to adhere. The cell media was then removed and replaced with either BMP-2 or BMP-2/PA mixtures (BMP-2: 10 nM, PA: 0.001%(w/v)) in serum-containing media (2.5% FBS) for three days. Their gene expression was determined by real-time PCR using an iQ5 Real-Time PCR Detection System (Bio-Rad). RNA isolation was performed using TRIzol (Invitrogen) followed by a reverse-transcription step using iScript Reverse Transcription Supermix (Bio-Rad), and finally PCR amplification was achieved using iQ SYBR Green Supermix (Bio-Rad). Primers for each target gene are as follows: Osteocalcin (OCN), forward primer: 5'-CAA GTC CCA CAC AGC AGC TT-3', reverse primer: 5'-AAA GCC GAG CTG CCA GAG TT-3' and alkaline phosphatase (ALP), forward primer: 5'-GTT GCC AAG CTG GGA AGA ACA C-3', reverse primer: 5'-CCC ACC CCG CTAT TCC AAA C-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward primer: 5'-TGA AGG TCG GTG TGA ACG GAT TGG C-3', reverse primer: 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3' (IDT).[46, 47] PCR conditions were as follows: cDNA denaturation at 94 °C for 5 min, followed by 40 repeated cycles at 94 °C for 45 s, annealing at 55 °C for 1 min, and extension at 68 °C for 1min. To confirm the specificity of the amplified products, melting curves

were performed by cooling samples at 55 °C for 30 s and then increasing the temperature to 94 °C at 0.5 °C/sec with continuous fluorescence measurement. Data were normalized to endogenous GAPDH levels using the ΔΔCt method. Three separate experiments with 4 replicates each were averaged for statistical analysis.

For ST2 cells, RNA was isolated using RNAqueous®-4PCR Kit (Ambion) following the manufacturer's instructions. Synthesis of cDNA was performed using RETROscript® Kit (Ambion). For cDNA template preparation, 1 μg of total RNA was used in a 20 μL reaction. Quantitative PCR was performed according to the manufacturer's protocol. Briefly, a 25 μL reaction was prepared for each sample. Included in this reaction volume was 1 μL of the resulting cDNA, iQ SYBR green supermix (Bio-Rad) containing dNTP and iTaq DNA polymerase, and the appropriate primers. The resulting threshold cycle (C_T) value from each primer pair was normalized with the C_T value for *18S RNA*, which serves as an internal control. After amplification, melting curve analysis was performed as described in the manufacturer's protocol, and samples with aberrant melting curves were excluded. The corresponding primer sequences are 18S RNA (forward, 5′-CGATGCTCTTAGCTGAGTGT-3′; reverse, 5′-GGTCCAAGAATTTCACCTCT-3′), Runx2 (forward 5'-CCGTGGCCTTCAAGGTTGT-3', reverse 5'-TTCATAACAGCGGAGGCATTT-3'), osterix (forward 5'-CCCTTCTCAAGCACCAATGG-3', reverse 5'-AAGGGTGGGTAGTCATTTGCATA-3'), Dlx5 (forward 5'-GTCCCAAGCATCCGATCCG-3', reverse 5'-GCGATTCCTGAGACGGGTG-3'), collagen I (forward 5'-GCTCCTCTTAGGGGCCACT-3', reverse 5'-CCACGTCTCACCATTGGGG-3'), C/EBPα (forward, 5′-TGAACAAGAACAGCAACGAG-3′; reverse, 5′-TCACTGGTCACCTCCAGCAC-3′), PPARγ (forward, 5′-GGAAAGACAACGGACAAATCAC-3′; reverse, 5′-TACGGATCGAAACTGGCAC-3′), Wnt10b (forward 5'-TTCTCTCGGGATTTCTTGGATTC-3', reverse 5'-TGCACTTCCGCTTCAGGTTTTC-3').

Fluorescence Recovery after Photobleaching. FRAP experiments were performed using an ANDOR spinning disk confocal microscope with a 100X objective. Cells were cultured on glass bottom petri dishes (Mattek) overnight with serum-containing medium. For FRAP experiments, cells were stained with recombinant cholera toxin subunit B (CTxB)-Alexa Fluor® 488 (10 μg/mL, Life Technologies) in PBS for 5 minutes at room temperature. The cells were washed three times with PBS, and maintained in serum-containing medium supplemented with 25 mM HEPES for live-cell imaging. The fluorescently labeled cells were transferred to Tokai-HIT stage-top CO_2 incubator to maintain humidified culture conditions at 37°C and 5% CO_2. After equilibration, circular bleach regions 2.9 μm in diameter were selected along the leading edge of the cell to achieve reproducible recovery of CTxB. In the same culture dish, the culture media was removed and media containing serum, HEPES, and either Strong PA or Weak PA (0.001%) was added. Following a 5-10 minute equilibration period, bleach regions were selected along the leading edge of each cell.

FRAP timelapse images were acquired using an iXon3 140bit EM CCD camera. Three pre-bleach images were taken followed by 100 frames taken every 2 seconds to monitor fluorescence recovery. Photobleaching was achieved using a 488 nm laser at full power for one pulse of 40 μs.

FRAP data analysis was performed using an equation that considers the half time of recovery and bleach diameter to extract the diffusion coefficients.

(1) $$D = \frac{r_n^2 + r_e^2}{8\tau_{1/2}}$$

D is the diffusion coefficient, r_n is the nominal radius of the circular bleach region, r_e is the experimental bleach radius of the circular bleach region, and $\tau_{1/2}$ is the time for the fluorescent profile to recover half of the way.

To calculate the diffusion coefficients, line profiles were drawn across the bleached region of interest in pre- and post-bleach images. The normalized intensity was fit to a Gaussian using the Levenberg-Marquardt algorithm (or damped least-squares fit) with Mathematica. An experimental or effective radius (r_e) was calculated as half of the width of the Gaussian at 86% of the total well depth. The fluorescence was plotted as a function of time and corrected for fading of the fluorescent signal in the same set of timelapse images as follows:

(2) $$F_{Corrected}(t) = \frac{F_{Raw}(t) - F_{Background}}{F_{Fading}(t) - F_{Background}}$$

(3) $$F(t) = \frac{F_{Corrected}(t)}{F_{Corrected}^{prebleach}}$$

The time at half fluorescence was linearly extrapolated and used in equation (1).

Statistical analysis. All data are represented as the mean ± standard deviation. Statistical analysis was performed using Prism software.