Ishikawa et al., http://www.jcb.org/cgi/content/full/jcb.201101050/DC1

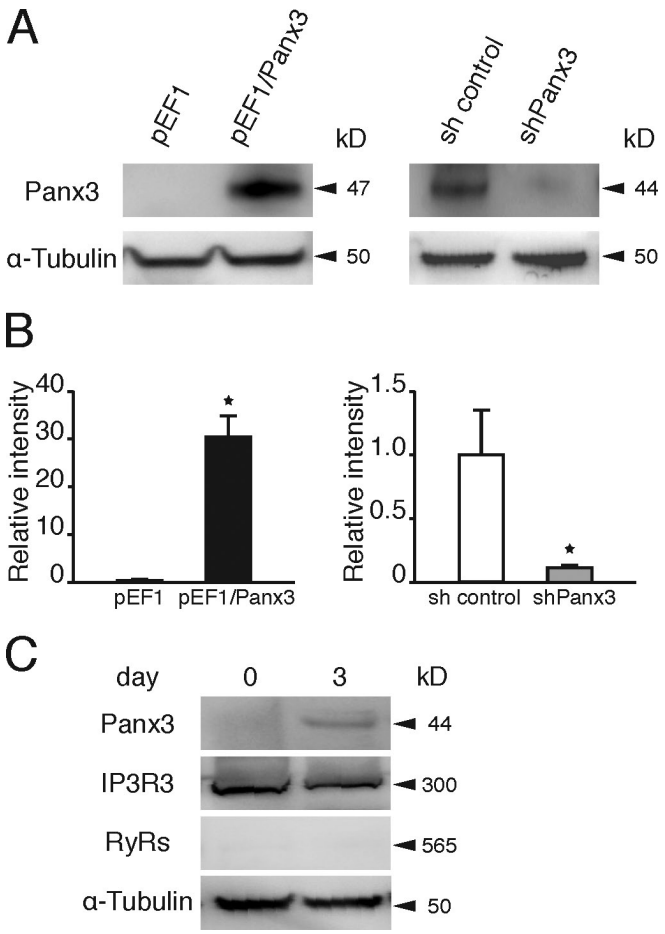


Figure S1. **Panx3 protein expression in pEF1/Panx3- or shPanx3 RNA–transfected C2C12 cells, and expression of IP3R and RyRs during differentiation of C2C12 cells into osteoblasts.** C2C12 cells were stably transfected with the control empty vector (pEF1) or the Panx3 expression vector (pEF1/Panx3). Pooled C2C12 cells, stably transfected with either the sh control vector (sh control) or the Panx3 shRNA vector, were cultured with BMP2 for 3 d. (A) Expression of Panx3 protein. Pooled transfectants were analyzed by Western blotting using anti-Panx3. Panx3 expression in pEF1/Panx3 cells was much higher than in pEF1 cells (A and B, left). Panx3 expression was reduced in Panx3 shRNA–transfected C2C12 cells (A and B, right). (B) Quantification of the protein bands. Image J 1.40g was used to quantify the bands. *, $P < 0.05$. Error bars represent the mean ± SD; $n = 3$. (C) C2C12 cells were seeded at ~90% confluence. After 1 d in culture (day 0), the cells were induced to differentiate by BMP2 for 2 d. Pooled cells were analyzed by Western blotting using anti-IP3R3 or anti-RyRs. No significant change was detected in the expression levels of the IP3R3 and RyRs during osteoblast differentiation of C2C12 cells.

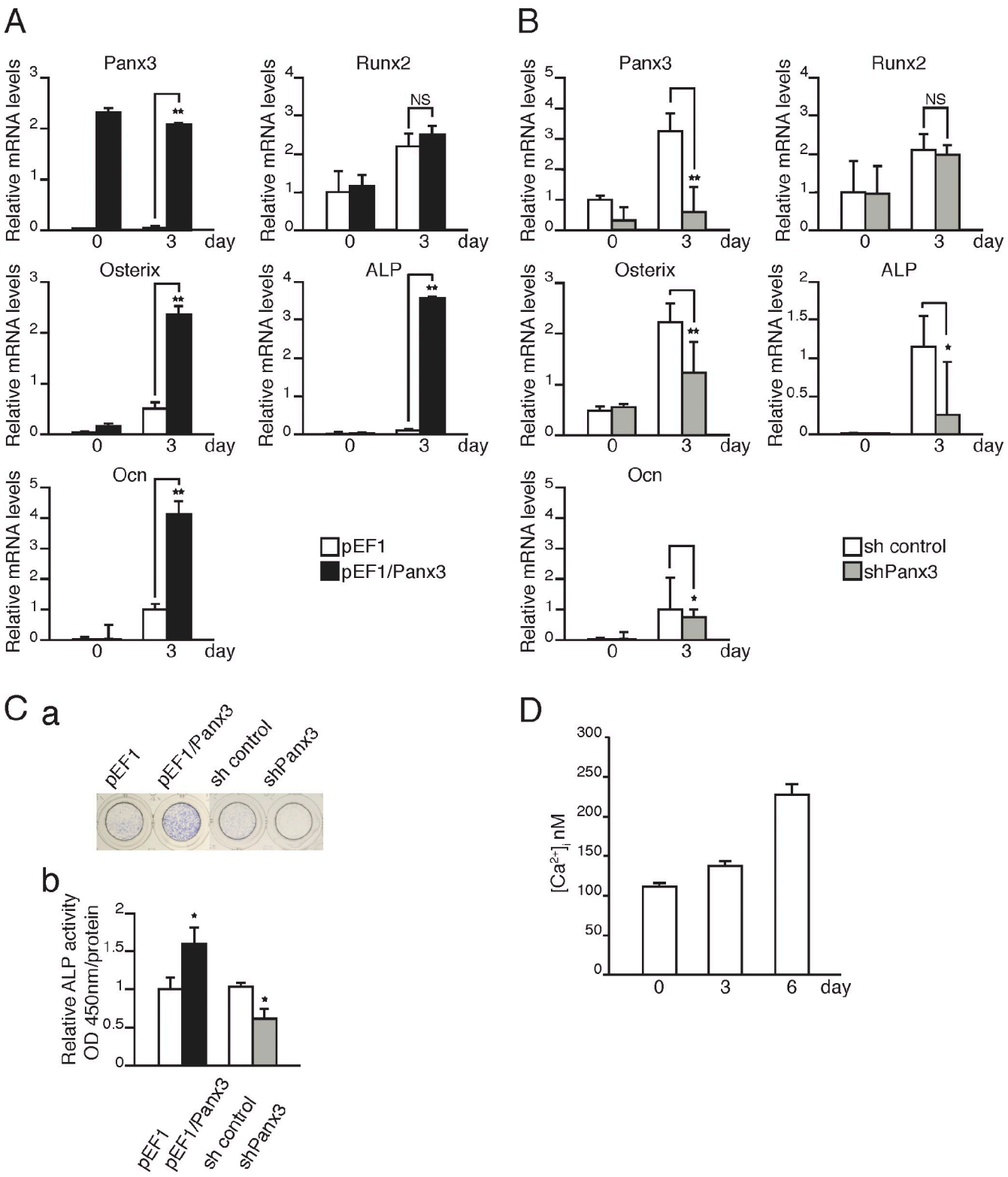


Figure S2. **Panx3 promotes osteoblastic differentiation of primary calvarial cells and increases $[Ca^{2+}]_i$ levels.** Primary calvarial cells were transiently transfected with a control pEF1 vector, pEF1/Panx3, a control sh vector, or a Panx3 shRNA vector, and these cells were cultured with osteoinduction media that included 50 µg/ml ascorbic acid and 5 mM β-glycerophosphate for 3 d. Total RNA was extracted at day 0 and day 3, and mRNA levels were analyzed by quantitative RT-PCR. (A) Panx3 overexpression promoted the expression of osteoblast marker genes for osterix, ALP, and Ocn, except that the expression of Runx2 remained the same. (B) shPanx3 suppressed the induction of these genes, except for Runx2. (C) Panx3 overexpression promoted ALP activity whereas shPanx3 inhibited it. (bottom) Quantitative data of ALP activity. pEF1/Panx3- and shPanx3-transfected primary calvarial cells were cultured with osteoinduction media for 3 d. (D) $[Ca^{2+}]_i$ levels during differentiation of primary calvarial cells into osteoblasts. Cells were cultured with osteoinduction media at the indicated days. $[Ca^{2+}]_i$ levels were increased during osteoblast differentiation. *, $P < 0.05$; **, $P < 0.01$. Error bars represent the mean ± SD; $n = 3$.

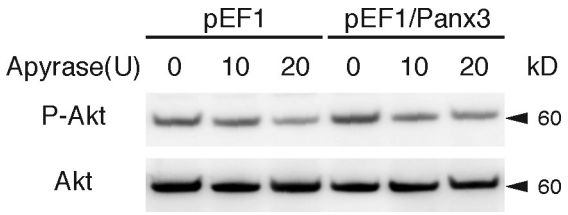
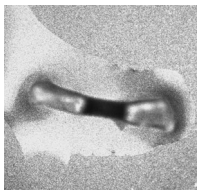
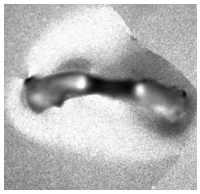


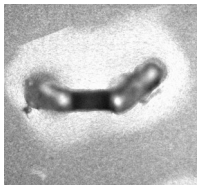
Figure S3. **Apyrase, an ATP antagonist concentration for a Ca^{2+} wave assay.** C2C12 cells stably transfected with pEF1 or with pEF1/Panx3 were incubated for 1 h with BMP2 in the presence of apyrase, and levels of phosphorylation of Akt and Akt protein were analyzed by Western blotting. Apyrase inhibited P2R/Akt signaling in pEF1/Panx3-transfected C2C12 cells.



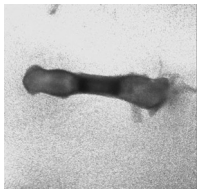
Video 1. **Ex vivo metatarsal growth infected with control adenovirus.** Metatarsal cultures were infected with control adenovirus. Live images were captured with a camera (Infinity2; Lumenera) attached to a microscope (Axiovert 25; Carl Zeiss). Frames were taken every 20 min for 3 d. The playback rate is 10 frames per second.



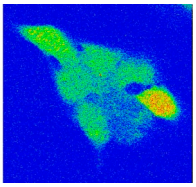
Video 2. **Ex vivo metatarsal growth infected with control Panx3 adenovirus.** Metatarsal cultures were infected with Panx3 adenovirus. Live images were captured with a camera (Infinity2; Lumenera) attached to a microscope (Axiovert 25; Carl Zeiss). Frames were taken every 20 min for 3 d. The playback rate is 10 frames per second.



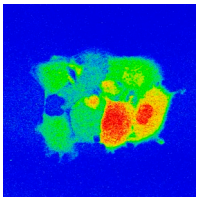
Video 3. **Ex vivo metatarsal growth incubated with a scramble peptide.** Metatarsal cultures were incubated with a scramble peptide. Live images were captured with a camera (Infinity2; Lumenera) attached to a microscope (Axiovert 25; Carl Zeiss). Frames were taken every 20 min for 3 d. The playback rate is 10 frames per second.



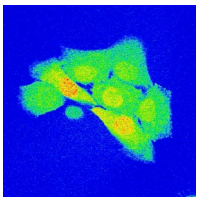
Video 4. **Ex vivo metatarsal growth incubated with the Panx3 peptide.** Metatarsal cultures were incubated with the Panx3 peptide. Live images were captured with a camera (Infinity2; Lumenera) attached to a microscope (Axiovert 25; Carl Zeiss). Frames were taken every 20 min for 3 d. The playback rate is 10 frames per second.



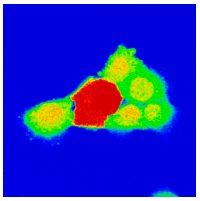
Video 5. **Ca^{2+} wave propagation in C2C12 cells transfected with control pEF1 vector.** Time-lapse confocal imaging of Ca^{2+} wave propagation. The Ca^{2+} wave was measured in C2C12 cells stably transfected with the control pEF1 vector, then loaded with Fluo-4 and NP-EGTA (caged Ca^{2+}) by initiating photolysis of NP-EGTA in a single cell using a flash of UV light. The playback rate is 8 frames per second.



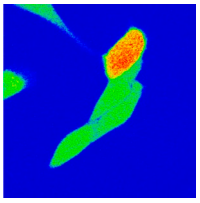
Video 6. **Ca^{2+} wave propagation in C2C12 cells transfected with pEF1/Panx3 vector.** Time-lapse confocal imaging of Ca^{2+} wave propagation. The Ca^{2+} wave was measured in C2C12 cells stably transfected with pEF1/Panx3 vector, then loaded with Fluo-4 and NP-EGTA (caged Ca^{2+}) by initiating photolysis of NP-EGTA in a single cell using a flash of UV light. The playback rate is 8 frames per second.



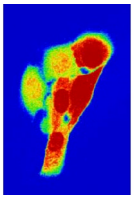
Video 7. **Ca^{2+} wave propagation in C2C12 cells transfected with control pEF1 vector in the presence of CBX.** Time-lapse confocal imaging of Ca^{2+} wave propagation in C2C12 cells stably transfected with control pEF1 vector, loaded with Fluo-4 and NP-EGTA (caged Ca^{2+}), and incubated with CBX (gap junction inhibitor) in Ca^{2+}-depleted media. The playback rate is 8 frames per second.



Video 8. **Ca^{2+} wave propagation in C2C12 cells transfected with pEF1/Panx3 vector in the presence of CBX.** Time-lapse confocal imaging of Ca^{2+} wave propagation in C2C12 cells stably transfected with pEF1/Panx3 vector, loaded with Fluo-4 and NP-EGTA (caged Ca^{2+}), and incubated with CBX (gap junction inhibitor) in Ca^{2+}-depleted media. The playback rate is 8 frames per second.



Video 9. **Ca^{2+} wave propagation in C2C12 cells transfected with control pEF1 vector in the presence of apyrase.** Time-lapse confocal imaging of Ca^{2+} wave propagation in C2C12 cells stably transfected with control pEF1 vector, loaded with Fluo-4 and NP-EGTA (caged Ca^{2+}), and incubated with apyrase (ATP receptor inhibitor) in Ca^{2+}-depleted media. The playback rate is 8 frames per second.



Video 10. **Ca^{2+} wave propagation in C2C12 cells transfected with pEF1/Panx3 vector in the presence of apyrase.** Time-lapse confocal imaging of Ca^{2+} wave propagation in C2C12 cells stably transfected with pEF1/Panx3 vector, loaded with Fluo-4 and NP-EGTA (caged Ca^{2+}), and incubated with apyrase (ATP receptor inhibitor) in Ca^{2+}-depleted media. The playback rate is 8 frames per second.

Table S1. **Primer sequences for semiquantitative and quantitative RT-PCR**

Gene name	Sequence
Panx3 (forward)[a]	5′-GCCCCTGGATAAGATGGTCAAG-3′
Panx3 (reverse)[a]	5′-GCGGATGGAACGGTTGTAAGAC-3′
Panx3 (forward)[b]	5′-ACTGCCCCTGGATAAGATGGTC-3′
Panx3 (reverse)[b]	5′-AGCCTGCCTGACACTGAAGTTG-3′
Runx2 (forward)[a]	5′-AACCGAGTCATTTAAGGCTGC-3′
Runx2 (reverse)[a]	5′-GGCTCACGTCGCTCATCTTGC-3′
Runx2 (forward)[b]	5′-GATGACACTGCCACCTCTGACTTC-3′
Runx2 (reverse)[b]	5′-AACTGCCTGGGGTCTGAAAAAG-3′
Osterix (forward)[a]	5′-CTGGGGAAAGGAGGCACAAAGAAG-3′
Osterix (reverse)[a]	5′-GGGTTAAGGGGAGCAAAGTCAGAT-3′
Osterix (forward)[b]	5′-ATACTCTGGGGGCTCTCTCTGTTC-3′
Osterix (reverse)[b]	5′-AAGAAAAAGTTGAGGAGGTCGGAG-3′
ALP (forward)[ab]	5′-AACAACCTGACTGACCCTTCGC-3′
ALP (reverse)[ab]	5′-CATTTTCCCGTTCACCGTCC-3′
Ocn (forward)[ab]	5′-CAGGAGGGCAATAAGGTAGTGAAC-3′
Ocn (reverse)[ab]	5′-CAGAGTTTGGCTTTAGGGCAGC-3′
Nat1 (forward)[ab]	5′-ATTCTTCGTTGTCAAGCCGCCAAAGTGGAG-3′
Nat1 (reverse)[ab]	5′-AGTTGTTTGCTGCGGAGTTGTCATCTCGTC-3′
Hprt (forward)[ab]	5′-GTTAAGCAGTACAGCCCCAAA-3′
Hprt (reverse)[ab]	5′-AGGGCATATCCAACAACAAACTT-3′

[a]Semiquantitative RT-PCR

[b]Quantitative RT-PCR