ligament (PDL) cells during experimental tooth movement. *J Dent Res.* 76:1357-1366.

Unger, V.M., N.M. Kumar, N.B. Gilula, and M. Yeager. 1999. Three-dimensional structure of a recombinant gap junction membrane channel. *Science.* 283:1176-1180.

Vanden Abeele, F., G. Bidaux, D. Gordienko, B. Beck, Y.V. Panchin, A.V. Baranova, D.V. Ivanov, R. Skryma, and N. Prevarskaya. 2006. Functional implications of calcium permeability of the channel formed by pannexin 1. *J Cell Biol.* 174:535-546.

Vinken, M., T. Vanhaecke, P. Papeleu, S. Snykers, T. Henkens, and V. Rogiers. 2006. Connexins and their channels in cell growth and cell death. *Cell Signal.* 18:592-600.

Wang, X., H.Y. Kua, Y. Hu, K. Guo, Q. Zeng, Q. Wu, H.H. Ng, G. Karsenty, B. de Crombrugghe, J. Yeh, and B. Li. 2006. p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. *J Cell Biol.* 172:115-125.

Wang, X.H., M. Streeter, Y.P. Liu, and H.B. Zhao. 2009. Identification and characterization of pannexin expression in the mammalian cochlea. *J Comp Neurol.* 512:336-346.

Wicks, S.J., S. Lui, N. Abdel-Wahab, R.M. Mason, and A. Chantry. 2000. Inactivation of smad-transforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II. *Mol Cell Biol.* 20:8103-8111.

Zayzafoon, M. 2006. Calcium/calmodulin signaling controls osteoblast growth and differentiation. *J Cell Biochem.* 97:56-70.

Zayzafoon, M., K. Fulzele, and J.M. McDonald. 2005. Calmodulin and calmodulin-dependent kinase IIalpha regulate osteoblast differentiation by controlling c-fos expression. *J Biol Chem.* 280:7049-7059.

Ziganshin, A.U., C.H. Hoyle, G. Lambrecht, E. Mutschler, H.G. Bumert, and G. Burnstock. 1994. Selective antagonism by PPADS at P2X-purinoceptors in rabbit isolated blood vessels. *Br J Pharmacol.* 111:923-929.

Figure Legends

Figure 1. Expression of Panx3 in growth plates, C2C12 cells, and primary calvarial cells. (A) Immunostaining of newborn mouse growth plates. Image under light microscopy (a), Panx3 (b), osteocalcin (Ocn) (c), Hoechst nuclear staining (blue), and merged image (d). Arrowheads, perichondrium/periosteum. (B) Semiquantitative RT-PCR. C2C12 cells (a) and primary calvarial cells (b) were cultured with BMP2 and ascorbate, respectively, at day 0. Panx3 was induced during osteoblast differentiation in both cell types. Runx2, osterix, ALP (alkaline phosphatase) and Ocn are osteoblast differentiation marker genes. Nat1 was used as a control. (C) (a) Cellular localization of endogenous Panx3 in undifferentiated (a,b,c) and differentiated C2C12 cells (d,e,f) after 4-day culture with BMP2. Panx3 (red) was localized in the plasma membrane, cell-cell junctions, and ER membranes in differentiated cells. Calnexin was used as an ER marker. (b) Measurements show a percentage of colocalization between Panx3 with calnexin (top panel), and calnexin with Panx3 (bottom panel). Asterisk: $P < 0.05$. Error bars represent the mean ± s.d., $n = 12$.

Figure 2. Panx3 promotes osteoblast differentiation. C2C12 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 BMP2 for various durations, as indicated. Total RNA was extracted each day for 4 days 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, while shPanx3 inhibited it. Representative ALP staining (upper panel) and its quantitative data (bottom panel). C2C12 cells, pEF1/Panx3- and shPanx3-transfected C2C12 cells were cultured with BMP2 for 3 days. (D) Representative Alizarin Red S staining (upper panel) and its quantitative data (bottom panel) of C2C12, pEF1/panx3- and shPanx3-transfected C2C12 cells cultured with BMP2 for 15 days. Two asterisks: $P < 0.01$. Error bars represent the mean ± s.d., $n = 3$.

Figure 3. Panx3 promotes the growth of metatarsus ex vivo. (A) Live images of ex vivo metatarsal growth (top panels) and histology (bottom panels). (a) Newborn mouse metatarsal bones were cultured and infected with Panx3 adenovirus (AdPanx3) or control adenovirus. (b) Metatarsus cultures were incubated with the Panx3 peptide for 3days. Metatarsal bone growth was measured by real time imaging. AdPanx3 promoted metatarsal bone growth (a), whereas the Panx3 peptide inhibited it (b). (B) Relative change in metatarsal length (Ba) or width (Bb) after 3days culture compared with day 0. The bone length was measured from edge to edge (Aa, red dashed line). The width was measured from side to side (Aa, red line). AdPanx3 promoted the growth of both length and width, while Panx3 peptide inhibited both. (C) Quantitative RT-PCR. Metatarsus cultures were incubated for 3 days with AdPanx3 or the Panx3 peptide. AdPanx3 promoted the expression of osteoblast marker genes, osterix, ALP and Ocn. The Panx3 peptide inhibited this marker gene expression. Asterisk: $P < 0.05$, two asterisks: $P < 0.01$. Error bars represent the mean ± s.d., $n = 3$.

Figure 4. Panx3 functions as an ER Ca^{2+} channel, and activates the CaM and Akt pathways. (Aa and b) Panx3 ER Ca^{2+} channel. C2C12 cells stably transfected with pEF1 (black), or pEF1/Panx3 (red) expression vectors, were analyzed for ATP-stimulated $[Ca^{2+}]_i$ in a time course (Aa). Primary calvarial cells transiently transfected with pEF1 (black), or pEF1/Panx3 (red) expression vectors (Ab). (Ac) $[Ca^{2+}]_i$ levels during differentiation od C2C12 cells. Untransfected and stably transfected cells with pEF1/Panx3 or shPanx3 vectors were cultured with BMP2 as indicated days. $[Ca^{2+}]_i$ levels in pEF1/Panx3 transfected cells were much higher than in C2C12 cells, while those in shPanx3 cells were lower. Asterisk: $P < 0.05$. Two asterisks: $P < 0.01$. Error bars represent the mean ± s.d., $n = 3$. (B and C) Panx3 activates the CaM/NFATc1 signaling pathways. C2C12 cells or primary calvarial cells were transfected with pEF1 and pEF1/Panx3 vectors, incubated for 1h with BMP2, and the levels of signal molecules were analyzed by Western blotting. For shPanx3 inhibition experiments, stably transfected C2C12 cells or transiently transfected primary calvarial cells with sh control and shPanx3 RNA were cultured for 1 day in the presence of BMP2.

Figure 5. Panx3 ER Ca^{2+} channel activation and its downstream signaling. C2C12 cells stably transfected with pEF1, or pEF1/Panx3 expression vectors, were analyzed for ATP-stimulated $[Ca^{2+}]_i$. Inhibitors were added to the cell culture for 30 min prior to ATP stimulation. In inhibition of endogenous IP3R3 expression, $[Ca^{2+}]_i$ was analyzed after 3days of transfection of C2C12 cells with siRNA for IP3R3. (A) Panx3 ER Ca^{2+} channel independent of the IP3R ER Ca^{2+} channel. 2-APB (IP3R inhibitor)(a) or U-73122 (IP3

synthesis inhibitor)(b) completely inhibited Ca^{2+} release from the IP3R ER Ca^{2+} channel. The Panx3 ER Ca^{2+} channel was inhibited by 2-APB (a), while it was partially inhibited by U-73122 (b). siRNA for IP3R3 inhibited the IP3R3 ER Ca^{2+} channel, but not the Panx3 ER Ca^{2+} channel (c). Thapsigargin (SERCA ER Ca^{2+} pump inhibitor) completely inhibited Ca^{2+} release from the ER in pEF1/Panx3-transfected cells, while it partially inhibited it in pEF1-transfected cells (d). (B) PPADS inhibited the Panx3 ER Ca^{2+} channel but not the IP3R ER Ca^{2+} channel (a). Suramin completely inhibited the IP3R ER Ca^{2+} channel but partially inhibited the Panx3 ER Ca^{2+} channel (b). A combination of PPADS and suramin blocked both ER Ca^{2+} channels (c). Arrows indicate the time of ATP addition. (C) PPADS inhibition of CaM-downstream signaling. Stably transfected C2C12 cells with pEF1 and pEF1/Panx3 vectors were incubated for 1h with BMP2, with or without PPADS, and levels of phosphorylation of CaMKII (a) and Smad1/5 (b) phosphorylation were analyzed by Western blotting. The left panel in (b) indicates Smad1/5 phosphorylation levels in cells without BMP2 and PPADS. In the middle and right panels of (b), cells were induced by BMP2.

Figure 6. Panx3 activates the Akt pathway. (Aa and b) Panx3 activates Akt signaling. Stably transfected C2C12 cells or transiently transfected primary calvarial cells with pEF1 and pEF1/Panx3 vectors were incubated for 1h with BMP2 and levels of signal molecules were analyzed by Western blotting. Panx3 expression increased phosphorylation of Akt and MDM2 and promoted p53 degradation. (B) Akt inhibition reduced Panx3-promoted expression of osterix (a) and ALP expression (b). The transfected cells were cultured with BMP2 for 3 days, and the expression of osterix and

ALP was analyzed by real-time PCR. The Akt inhibitor and Akt-DN inhibited the expression of Panx3-mediated induction of these genes, while Akt-CA increased the expression levels in control and Panx3 overexpressing cells.

Figure 7. Activation of Panx3 ER Ca^{2+} channel by PI3K/Akt signaling. (A) pEF1 or pEF1/Panx3 transfected C2C12 cells were incubated with the Akt inhibitor (a), or transfected with the dominant negative Akt (Akt DN) (b) or the activated Akt (Akt CA) vector (c), or LY294002 (PI3K inhibitor)(d). $[Ca^{2+}]_i$ was measured by the fluorescence intensity ratio of Fura-2 (F_{340nm}/F_{380nm}) in each condition. The Akt inhibitor blocked the Panx3 ER Ca^{2+} channel (a). Akt DN inhibited the Panx3 ER Ca^{2+} channel, not IP3R ER channel (b). Akt CA promoted the Panx3 ER Ca^{2+} channel, not the IP3R ER channel (c). LY294002 inhibited Ca^{2+} release from both Panx3 and IP3R ER Ca^{2+} channels (d). Arrows indicate the time of ATP addition. (B) The $[Ca^{2+}]_i$ level was increased by Akt activation. The basal levels of $[Ca^{2+}]_i$ in Panx3 overexpressing C2C12 (pEF1/Panx3) cells are higher than in control vector transfected (pEF1) cells. Akt CA increased $[Ca^{2+}]_i$ in pEF1/Panx3 cells, while Akt DN reduced $[Ca^{2+}]_i$ to similar levels in control cells. (C) The transfected cells were induced by BMP2 for 1 hour and phosphorylation of CaMKII and NFATc1 was analyzed by Western blotting. Akt increased CaMKII and NFATc1 phosphorylation levels in control cells and Panx3 overexpression further enhanced its activation. Akt-DN inhibited these activations to the levels in control cells.

Figure 8. Panx3 hemichannel releases intracellular ATP and promotes differentiation. (A) Imaging of intracellular ATP levels in pEF1- or pEF1/Panx3-transfected C2C12 cells. Cells were incubated with the caged luciferin, and then exposed to a flash of UV light for photolysis to convert active luciferin. Fluorescence excitation images (red) caused by luciferin-ATP interactions at 5s and 15s after a UV flash were shown. Higher red fluorescence images were observed in control pEF1-transfected cells compared with Panx3-overexpressing cells, indicating that Panx3 overexpression reduced intracellular ATP levels. (Ab and c) Measurement of ATP release. The transfected C2C12 cells or primary calvarial cells were treated with or without KGlu for 2 min, and then ATP released into the media was measured. (B) Inhibition of ATP release. The transfected cells were incubated with Panx3 antibody or Panx3 peptide for 30min, and ATP release was measured. The Panx3 antibody (1.5μg/ml) inhibited ATP release in pEF1/Panx3 transfected cells (a). This inhibition was blocked by various concentrations of the Panx3 peptide. The Panx3 peptide also inhibited the ATP release (a). Control sh- or shPanx3-transfected cells were cultured with BMP2 for 2 days and ATP release was measured. ATP release was reduced in shPanx3-transfected cells compared to control sh-transfected cells (b). (C) Inhibition of osterix (a) and ALP (b) expression by the anti-Panx3 antibody.

Figure 9. Panx3 functions as a gap junction. (A) Real time imaging of Ca^{2+} wave propagation. The Ca^{2+} wave was measured in cells loaded with Fluo-4 and NP-EGTA (caged Ca^{2+}) by starting photolysis of NP-EGTA in a single cell using a flash of UV light. (a) pEF1/Panx3-transfected cells, but not pEF1-transfected cells, propagated Ca^{2+}

waves to neighboring cells. (b) Inhibition of the Ca^{2+} wave propagation by carbenoxolone (CBX, gap junction inhibitor). (c) Apyrase, ATP receptor antagonist, did not inhibit Ca^{2+} propagation in pEF1/Panx3-transfected cells. (B) CBX inhibited C2C12 cell differentiation. (a) ALP staining. (b) Quantitative data from the ALP staining. CBX inhibited osteoblast differentiation in pEF1/Panx3 transfected cells (a and b). asterisk: $P < 0.05$, two asterisks: $P < 0.01$. Error bars represent the mean ± s.d., $n = 3$.

Figure 10. Panx3 pathways in osteoblast differentiation. The Panx3 hemichannel releases intracellular ATP. The released ATP binds to purinoreceptors (P2Rs) in its own cell and/or neighboring cells, and activates the PI3K-Akt pathway. Akt then activates the Panx3 ER Ca^{2+} channel to increase $[Ca^{2+}]_i$ levels, which leads to activation of the calmodulin (CaM)/calmodulin kinase II (CaMKII) pathway. The ATP also activates the PLC/PIP2/IP3R ER Ca^{2+} channel pathway, which is distinct from that of the Panx3 ER Ca^{2+} channel. The Akt activation also phosphorylates MDM2, which induces degradation of p53, an inhibitor for osteogenic differentiation, and promotes differentiation. CaM also activates calcineurin (CN), which dephosphorylates inactive phosphorylated NFAT in cytosol. Dephosphorylated NFATc1 enters the nucleus and binds to the promoter regions of differentiation genes such as osterix and alkaline phosphatase (ALP). Activated CaMKII also increases c-fos and NFAT expression, and activation of Ap-1 and Smad1/5. Panx3 gap junction activity promotes Ca^{2+} wave propagation between adjacent cells for differentiation.

Supplemental Figures.

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 days. (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 panel). Panx3 expression was reduced in Panx3 shRNA-transfected C2C12 cells (A and B right panel). (B) Quantification of the protein bands. Image J 1.40g was used to quantify the bands. Asterisk: $P < 0.05$. Error bars represent the mean ± s.d., $n = 3$. (C) C2C12 cells were seeded at ~90% confluence. After 1 day culture (day 0), the cells were induced to differentiate by BMP2 for 2 days. Pooled cells were analyzed by Western blotting using anti-IP3R3 or anti-Ryanodine receptors (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 included 50 μg/mL ascorbic acid and 5mM β-glycerophosphate for 3days. Total RNA was extracted in day0 and day3 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 while shPanx3 inhibited it. Quantitative data of ALP activity (bottom panel). pEF1/Panx3- and shPanx3-transfected primary calvarial cells were cultured with osteoinduction media for 3 days. (D) $[Ca^{2+}]_i$ levels during differentiation of primary calvarial cells into osteoblast. Cells were cultured with osteoinduction media as the indicated days. $[Ca^{2+}]_i$ levels were increased during osteoblast differentiation. Asterisk: $P < 0.05$, two asterisks: $P < 0.01$. Error bars represent the mean ± s.d., $n = 3$.

Figure S3. Apyrase, an ATP antagonist concentration for Ca2+ wave assay. C2C12 cells stably transfected with pEF1 or with pEF1/Panx3 were incubated for 1h 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.

Figure S4. Videos 1-4. Imaging of ex vivo metatarsal growth. (Videos 1-2) Metatarsal bones from newborn mice were cultured and infected with control adenovirus (Video1) or with Panx3 adenovirus (AdPanx3) (Video2). Videos 2-3, Metatarsal cultures were incubated with the Panx3 peptide (Video 4) or with a scramble peptide (Video 3). Live

images were captured with a Lumenera Infinity2 camera attached to a Zeiss Axiovert 25 microscope. Frames were taken every 20 minutes for 3 days.

Figure S5. Videos 5-8. Imaging of Ca^{2+} wave propagation. (Videos 5-6) C2C12 cells stably transfected with pEF1 (Video 5) or with pEF1/Panx3 (Video 6) were loaded with Fluo-4 and NP-EGTA (caged Ca^{2+}) in Ca^{2+}-depleted media. Videos 7, C2C12 cells transfected with pEF1 were incubated with carbenoxolone (CBX, gap junction inhibitor) alone (Video 7). Videos 8, C2C12 cells transfected with pEF1/Panx3 were incubated with CBX (Video 8). Images were captured by a time-lapse confocal microscopy (LSM 510; Carl Zeiss, Inc.). Frames were taken every second for 1 min.

Table S1. Primer sequences for semiquantitative (1) and quantitative (2) RT-PCR.

Gene name	Sequence
Panx3 (1) (forward)	5'GCCCCTGGATAAGATGGTCAAG3'
Panx3 (1) (reverse)	5'GCGGATGGAACGGTTGTAAGAC3'
Panx3 (2) (forward)	5'ACTGCCCCTGGATAAGATGGTC3'
Panx3 (2) (reverse)	5'AGCCTGCCTGACACTGAAGTTG3'
Runx2 (1) (forward)	5'AACCGAGTCATTTAAGGCTGC3'
Runx2 (1) (reverse)	5'GGCTCACGTCGCTCATCTTGC3'
Runx2 (2) (forward)	5'GATGACACTGCCACCTCTGACTTC3'
Runx2 (2) (reverse)	5'AACTGCCTGGGGTCTGAAAAAG3'
Osterix (1) (forward)	5'CTGGGGAAAGGAGGCACAAAGAAG3'
Osterix (1) (reverse)	5'GGGTTAAGGGGAGCAAAGTCAGAT3'
Osterix (2) (forward)	5'ATACTCTGGGGGCTCTCTCTGTTC3'
Osterix (2) (reverse)	5'AAGAAAAAGTTGAGGAGGTCGGAG3'
ALP (1)(2) (forward)	5'AACAACCTGACTGACCCTTCGC3'
ALP (1)(2) (reverse)	5'CATTTTCCCGTTCACCGTCC3'
Osteocalcin (1)(2) (forward)	5'CAGGAGGGCAATAAGGTAGTGAAC3'
Osteocalcin (1)(2) (reverse)	5'CAGAGTTTGGCTTTAGGGCAGC3'
Nat1 (1) (2)(forward)	5'ATTCTTCGTTGTCAAGCCGCCAAAGTGGAG3'
Nat1 (1)(2) (reverse)	5'AGTTGTTTGCTGCGGAGTTGTCATCTCGTC3'
Hprt (1)(2) (forward)	5'GTTAAGCAGTACAGCCCCAAA3'
Hprt (1)(2)(reverse)	5'AGGGCATATCCAACAACAAACTT3'

Fig. 1

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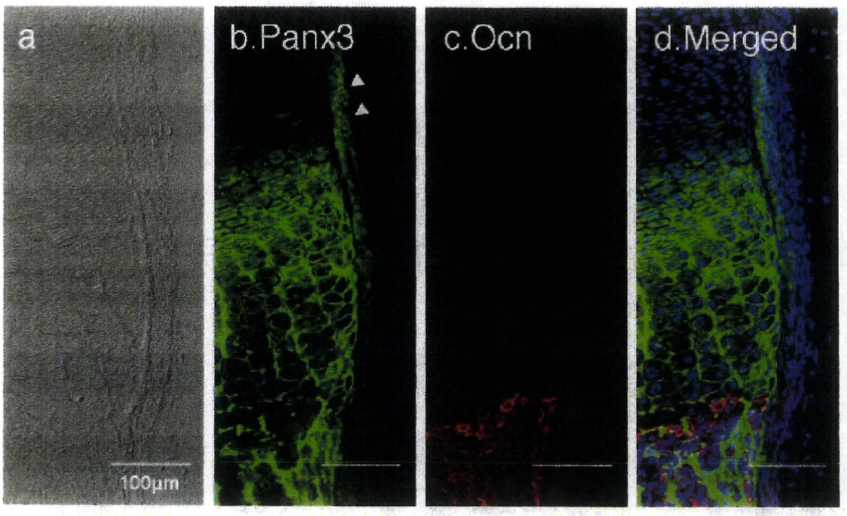
A



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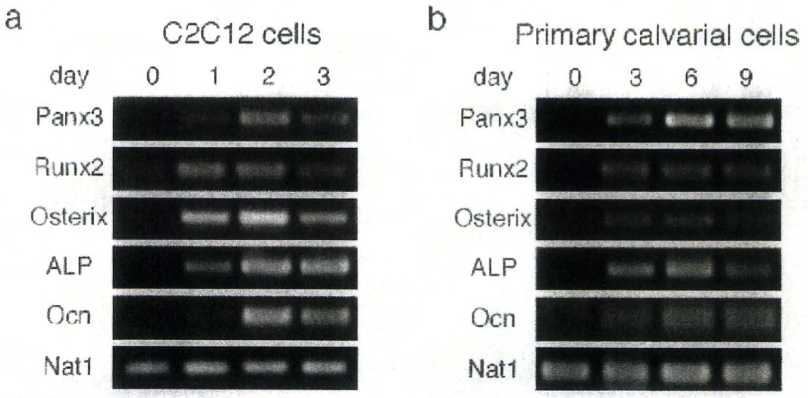

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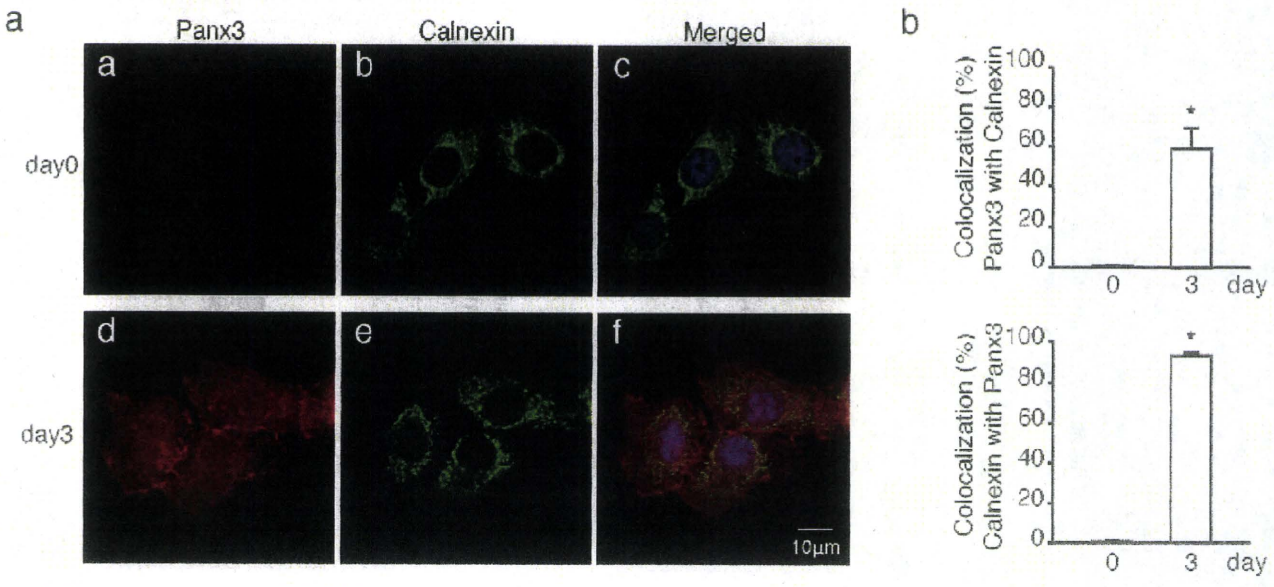

Fig. 2

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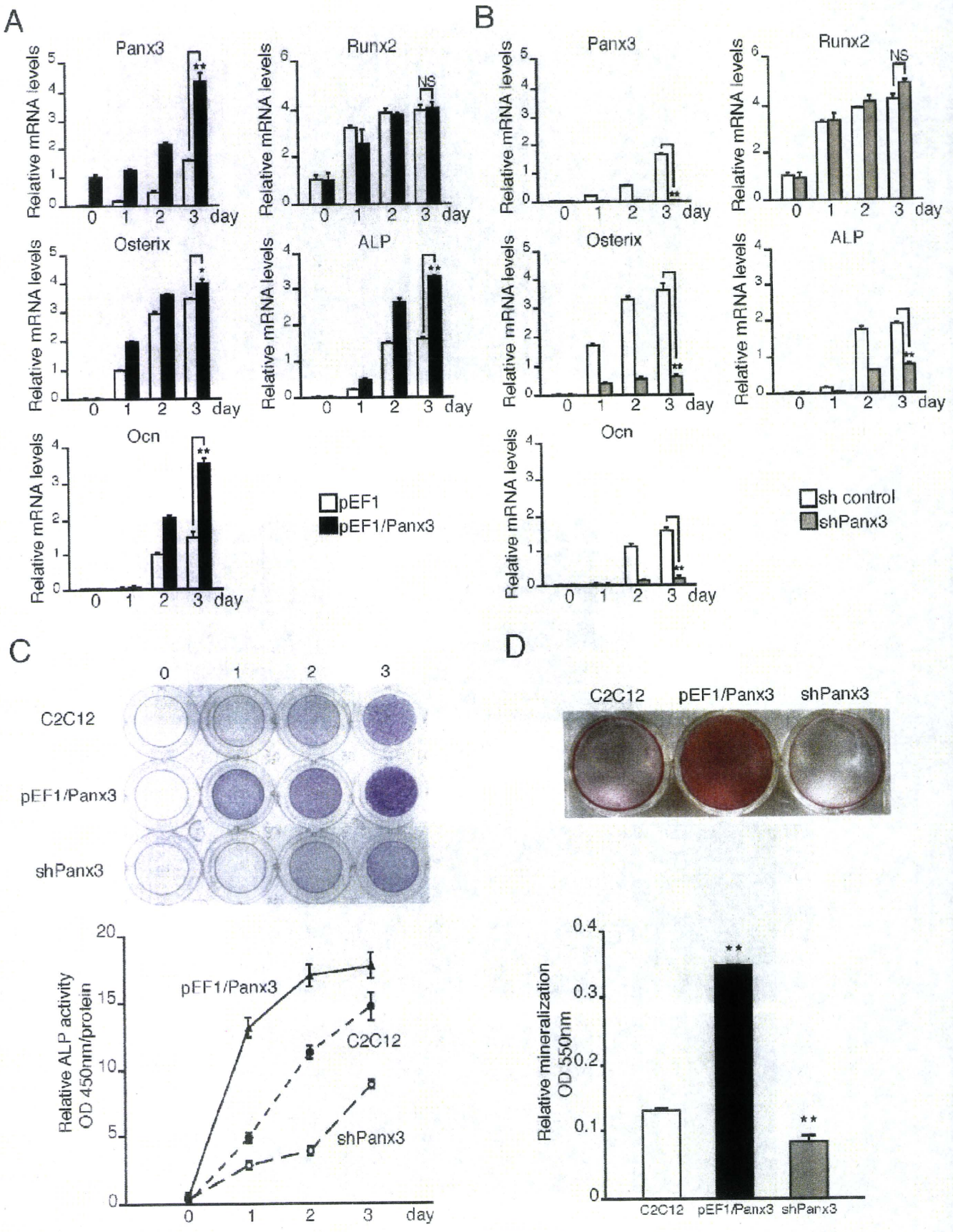

Fig. 3

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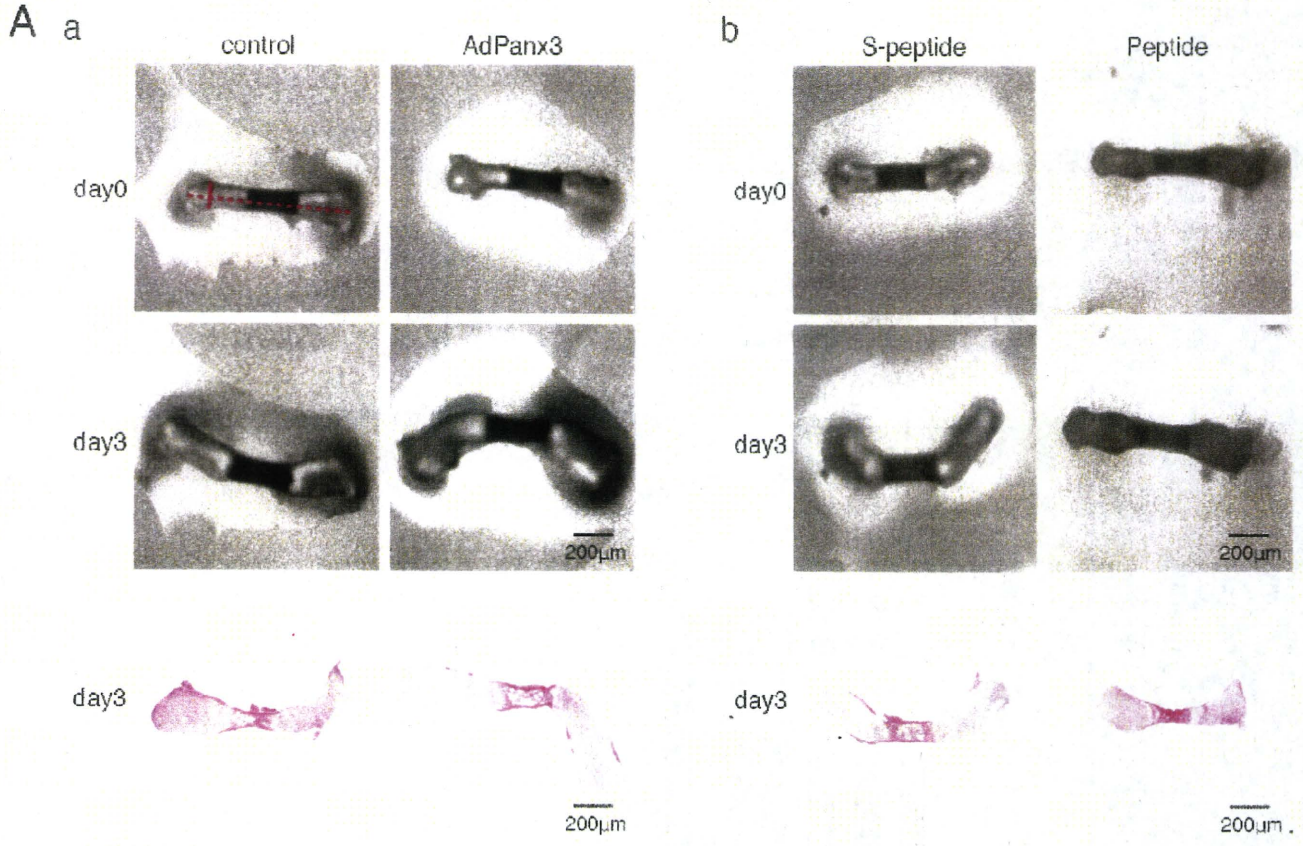

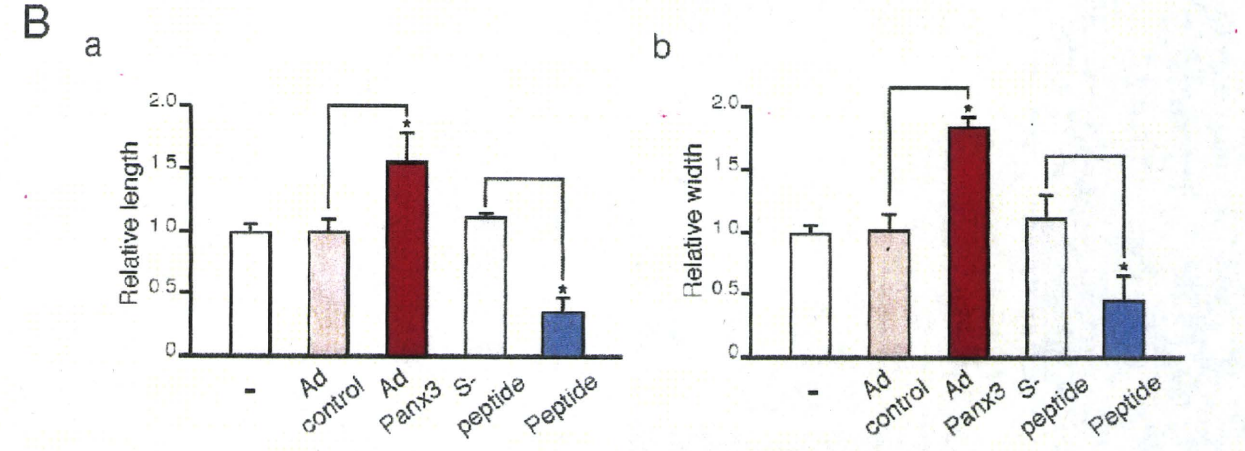

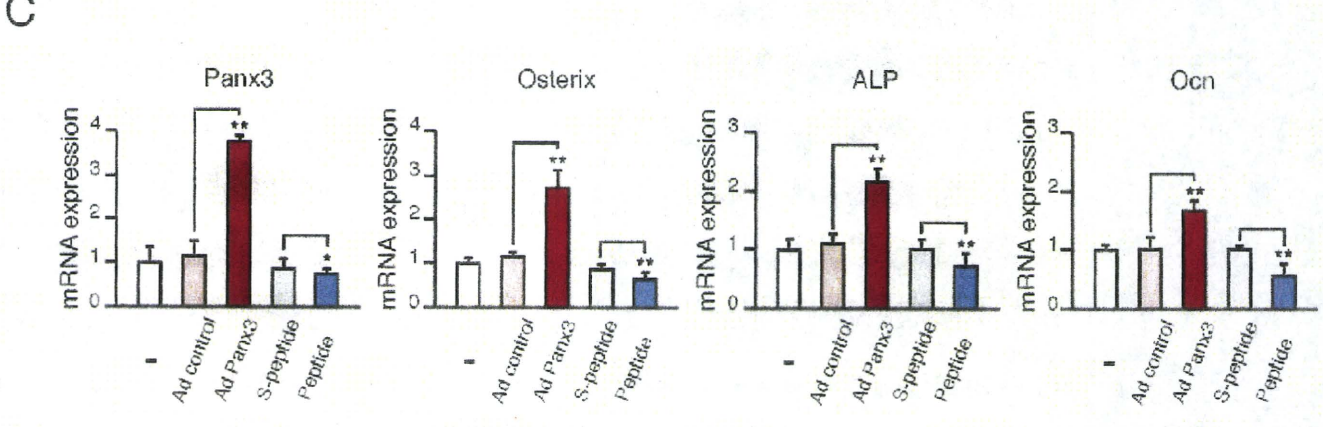

Fig. 4

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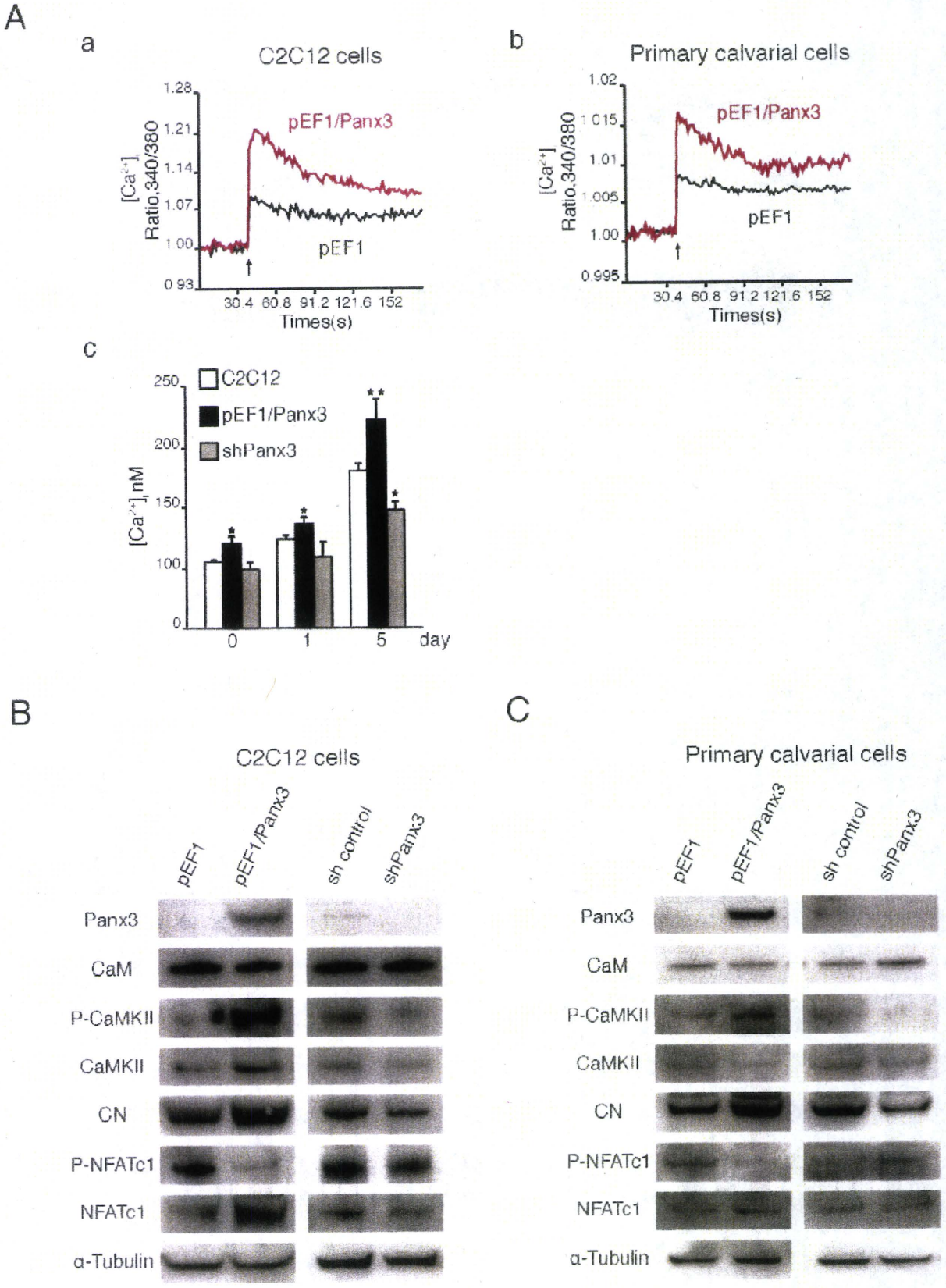

Fig. 5

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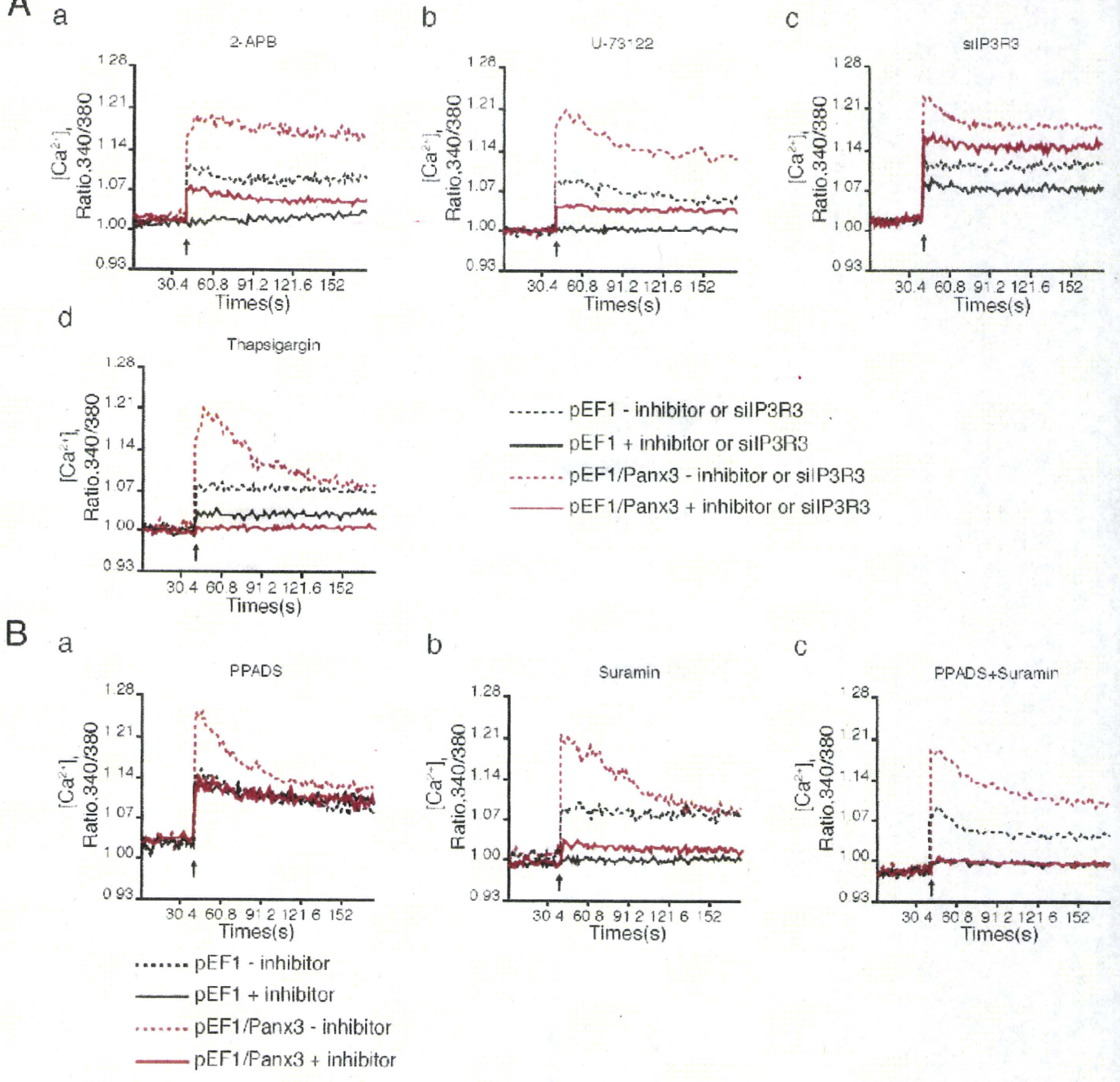

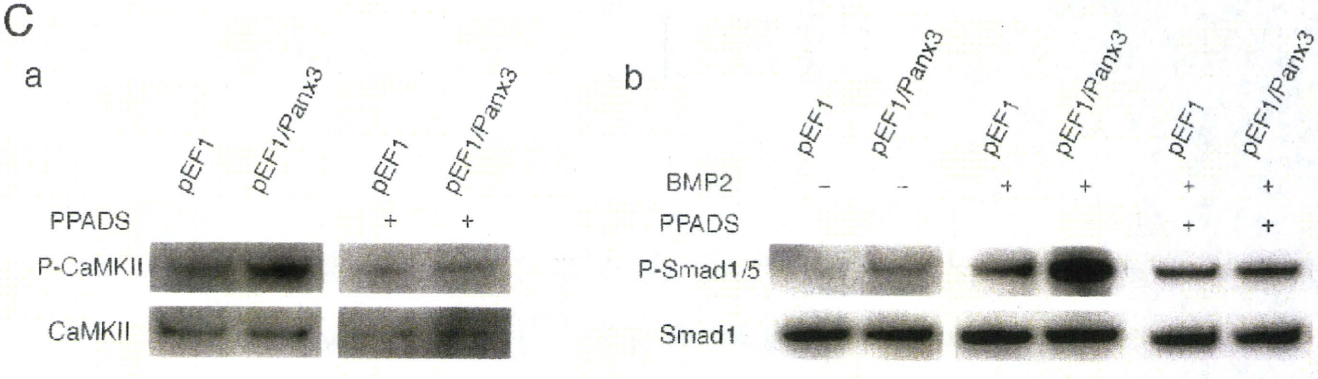

Fig. 6

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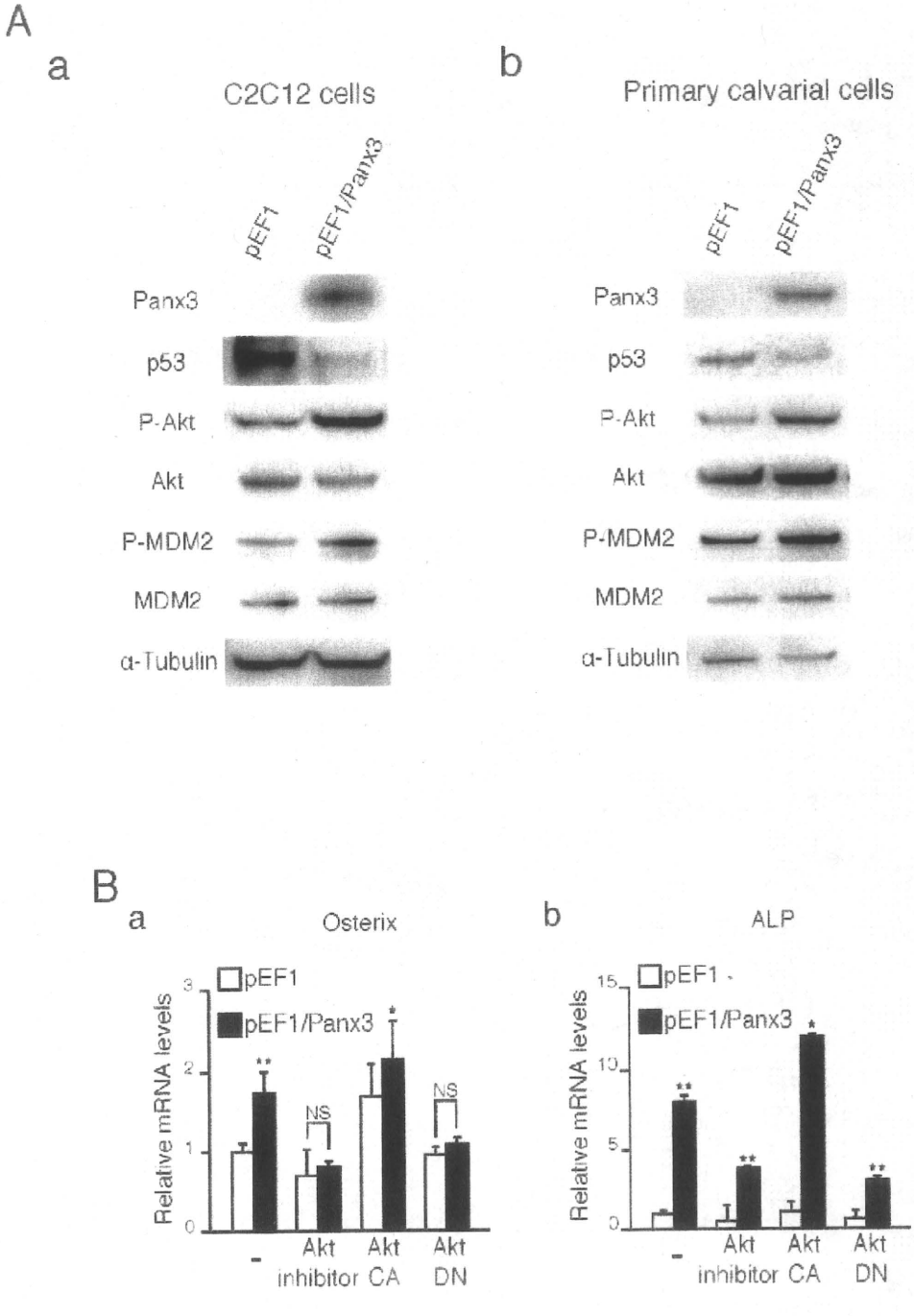

Fig. 7

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