Supplemental Material: Goto *et al.*

MATERIALS AND METHODS

FRET biosensors

The pEpacSensor-2052x was derived from pcDNA3-CFP-Epac1(δDEP)-YFP (Ponsioen et al., 2004) and encoded an Epac biosensor, which uses monomeric teal fluorescent protein (Ai et al., 2006) in place of CFP (unpublished data).

Plasmid

pIMR21-CdGAP and pIRM21-FLAG-N-WASP-CRIB have been described previously (Aoki *et al*. 2004).

Reagents and Antibodies

LY294002 was purchased from Sigma-Aldrich. PACAP was obtained from Calbiochem. Anti-PKA subunit Cα (C-20), anti-Tiam1 (C-16), and anti-Epac2 (M-18) polyclonal antibodies were purchased from Santa Cruz Biotechnology. The anti-STEF polyclonal antibodies were described previously (Matsuo *et al.*, 2002). The anti-α tubulin monoclonal antibody was obtained from Roche Diagnostics. The anti-c-Raf (53) monoclonal antibody was purchased from BD Biosciences (Franklin Lakes, NJ).

Quantitative Real-Time QRT-PCR Assays

Real-time RT-PCR was carried out as described previously (Guo *et al.*, 2007) using SYBR GreenER qPCR SuperMix (Invitrogen). Total RNA was isolated using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). The cDNA was generated using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA). The cDNA was PCR-amplified using primers specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), PKA subunit Cβ (PRKACB), and Epac1. PCR amplification was carried out using a PRISM 7000 (Applied Biosystems). The threshold cycle (Ct) value of PRKACB and Epac1 amplification were normalized to that of the GAPDH control. The primers for QRT-PCR were as follows: GAPDH forward (F), 5′-GAGTCTACTGGCGTCTTCAC-3′; GAPDH reverse (R), 5′-GTTCACACCCATCACAAACA-3′; PRKACB (F), 5′-TGGTTTGCTACTACCGACTGG-3′; PRKACB (R), 5′-AGTTGCTGGTATCGCCAGAG-3′; Epac1 (F), 5′-GGTCAATTCTGCCGGTGAT-3′; and Epac1 (R), 5′-TTGAGCCCCAGGGATGTG-3′.

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Guo, W. J., Datta, S., Band, V., and Dimri, G. P. (2007). Mel-18, a polycomb group protein, regulates cell proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc oncoproteins. Mol. Biol. Cell *18*, 536-546.

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Ponsioen, B., Zhao, J., Riedl, J., Zwartkruis, F., van der Krogt, G., Zaccolo, M., Moolenaar, W. H., Bos, J. L., Jalink, K. (2004). Detecting cAMP-induced Epac activation by fluorescence resonance energy transfer: Epac as a novel cAMP indicator. EMBO Rep. *5*, 1176-1180.

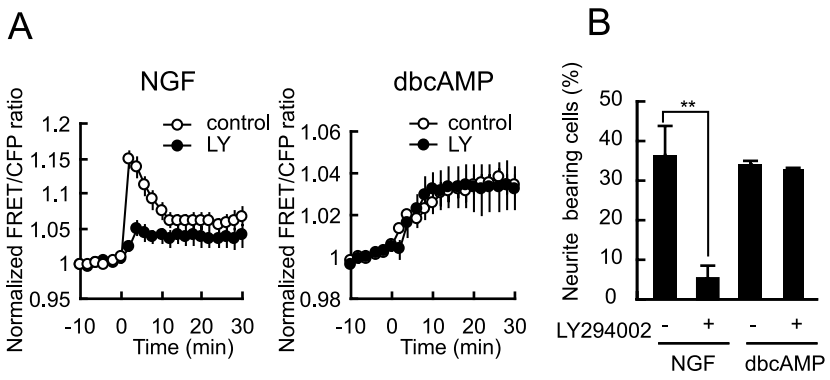
VIDEO LEGENDS

Video 1. Spatiotemporal changes in Rac1 activity for 5 h after dbcAMP addition. PC12D cells expressing Raichu-Rac1 were starved for 2 h and then treated with 1 mM dbcAMP. Images were obtained every 5 min for 300 min after dbcAMP addition. The numbers indicate the elapsed time (min). Bars, 10 μm.

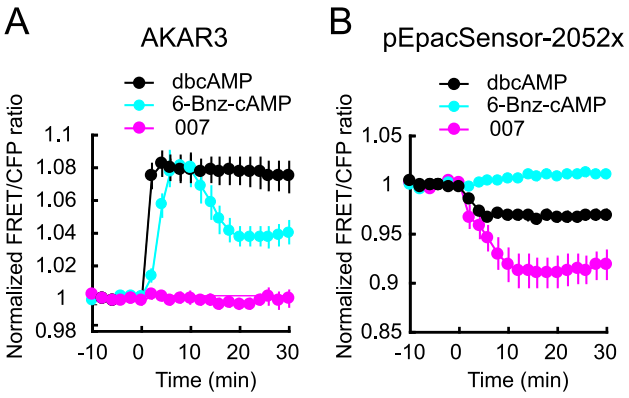
Video 2. Spatiotemporal changes in Cdc42 activity for 5 h after dbcAMP addition. PC12D cells expressing Raichu-Cdc42 were starved for 2 h and then treated with 1 mM dbcAMP. Images were obtained every 5 min for 300 min after dbcAMP addition. The numbers indicate the elapsed time (min). Bars, 10 μm.

Video 3. Morphological changes in PC12D cells stimulated with NGF. PC12D cells were starved for 2 h and then treated with 50 ng/ml NGF. Images were obtained every 5 min for 300 min after NGF stimulation. The numbers indicate the elapsed time (min). Bars, 10 μm.

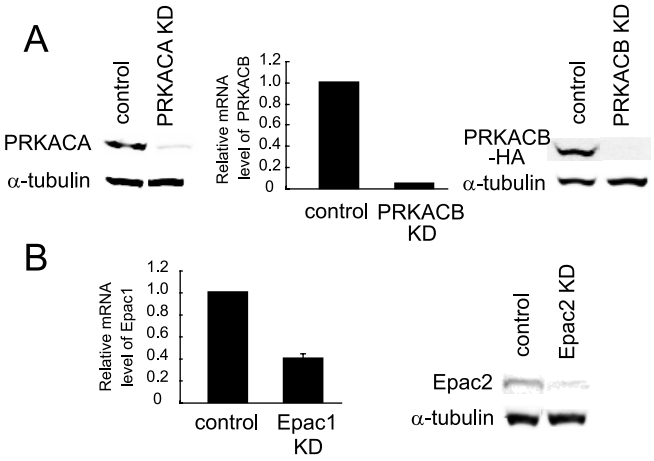
Video 4. Morphological change in PC12D cells treated with dbcAMP. PC12D cells were starved for 2 h and then treated with 1 mM dbcAMP. Images were obtained every 5 min for 300 min after dbcAMP addition. The numbers indicate the elapsed time (min). Bars, 10 μm.



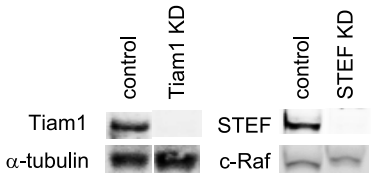
Supplemental Figure 1. The effect of LY294002 on Rac1 activation and neurite outgrowth in response to NGF or dbcAMP. (A) PC12D cells expressing Raichu-Rac1 were starved for 2 h and then treated with 50 ng/ml NGF (left) or 1 mM dbcAMP (right) in the presence or absence of LY294002. Images were obtained every 2 min for 30 min after the addition of NGF or dbcAMP. The mean FRET/CFP ratios averaged over the whole cell are expressed. The number of experiments was as follows: NGF without (n = 7) or with LY294002 (n = 8), dbcAMP without (n = 20) or with LY294002 (n = 8). Error bars show the SE. (B) PC12D cells were incubated with NGF or dbcAMP in the presence or absence of LY294002 for 2 d, and then fixed for microscopy. Cells with neurites whose lengths were at least 2-fold longer than their cell body lengths were scored as neurite-bearing cells. At least 50 cells were assessed in each experiment, and the experiments were repeated three times. The results are expressed as the mean percentage of neurite-bearing cells, with SE. The symbol indicates the result of a t-test; $^{**}p < 0.01$ compared with control.



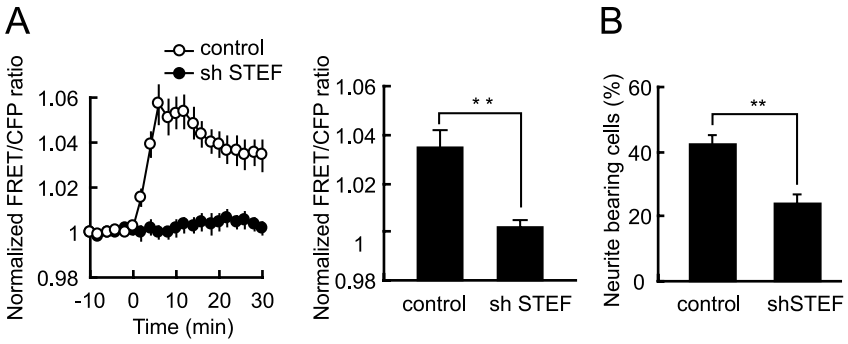
Supplemental Figure 2. The effect of dbcAMP, 6-Bnz-cAMP, or 007 on the activation of PKA or Epac. (A) PC12D cells expressing pmAKAR3 were starved for 2 h and then treated with 1 mM dbcAMP, 100 μM 6-Bnz-cAMP, or 100 μM 007. Images were obtained every 2 min for 30 min after drug treatment. The number of experiments was as follows: dbcAMP (n = 10), 6-Bnz-cAMP (n = 17), 007 (n = 13). The mean FRET/CFP ratios averaged over the whole cell are expressed. Error bars show the SE. (B) PC12D cells expressing pEpacSensor-2052x were starved for 2 h and then treated with 1 mM dbcAMP, 100 μM 6-Bnz-cAMP, or 100 μM 007. Images were obtained every 2 min for 30 min after drug treatment. The number of experiments was as follows: dbcAMP (n = 13), 6-Bnz-cAMP (n = 20), 007 (n = 14). The mean FRET/CFP ratios averaged over the whole cell are expressed. Error bars show the SE.



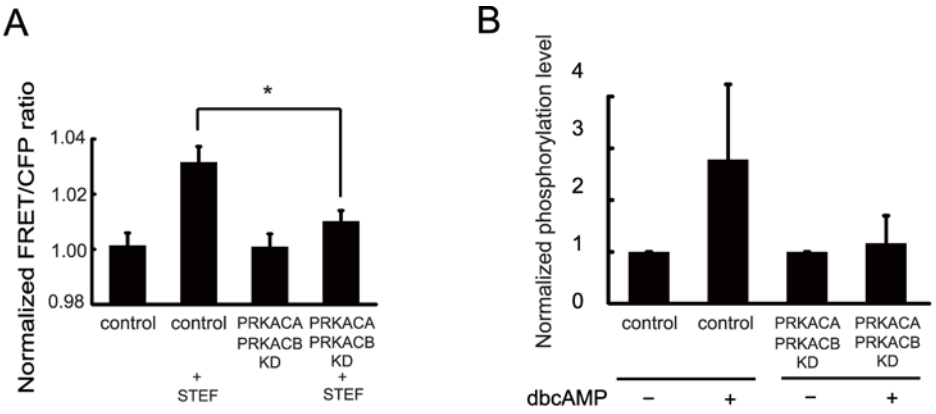
Supplemental Figure 3. The efficiency of depletion of PKA and Epac. (A) PKA subunit Cα (PRKACA) and PKA subunit Cβ (PRKACB) expression in control and knockdown cells was analyzed by immunoblotting (for PRKACA, left) or quantitative real-time RT-PCR (for PRKACB, middle). The efficiency of depletion of exogenous PRKACB protein by shRNA was confirmed by immunoblotting (right). (B) Epac1 and Epac2 expression in control and knockdown cells was analyzed by quantitative real-time RT-PCR (for Epac1, left) or immunoblotting (for Epac2, right).



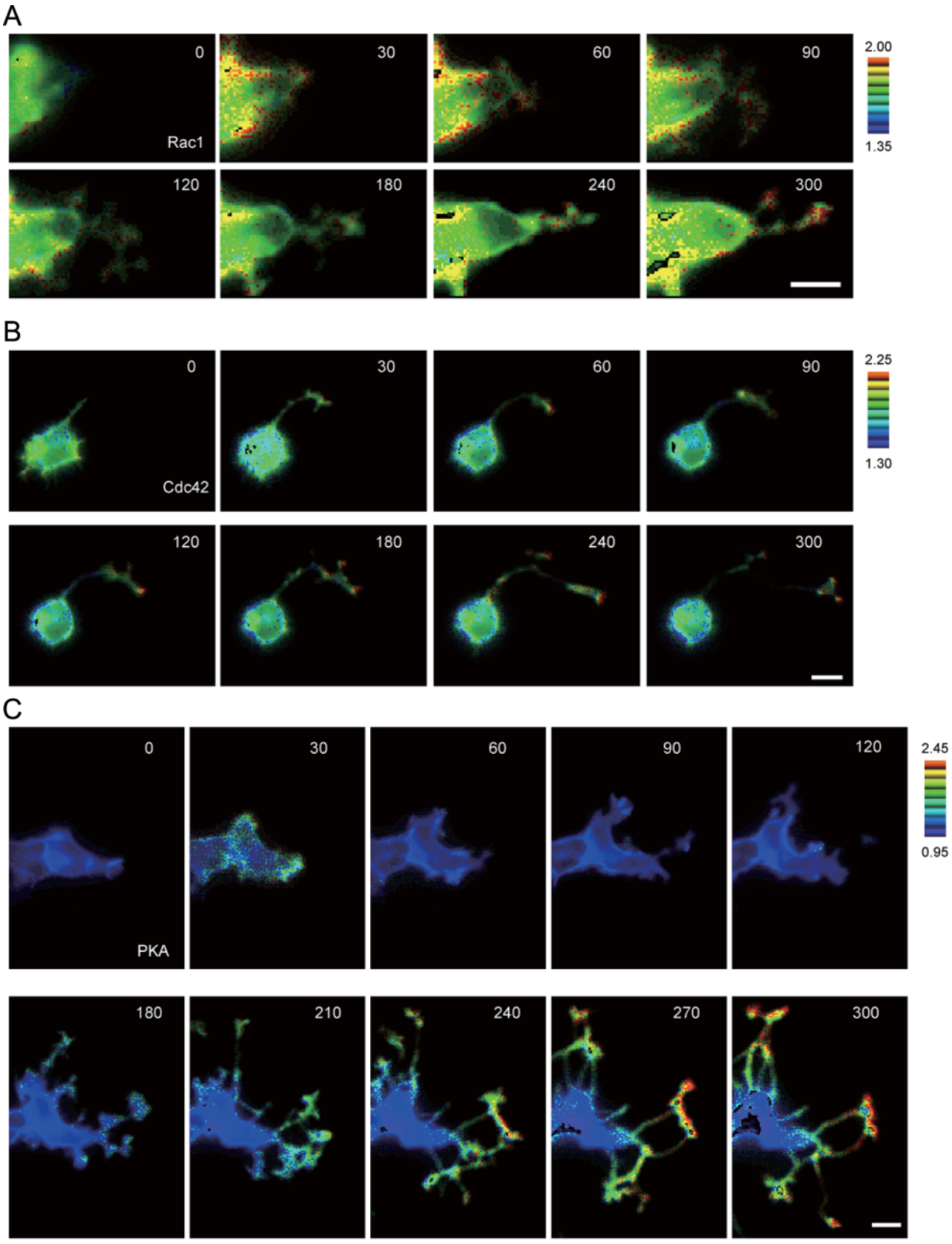
Supplemental Figure 4. The efficiency of depletion of Tiam1 and STEF. Tiam1 and STEF expression in control and knockdown cells was analyzed by immunoblotting.



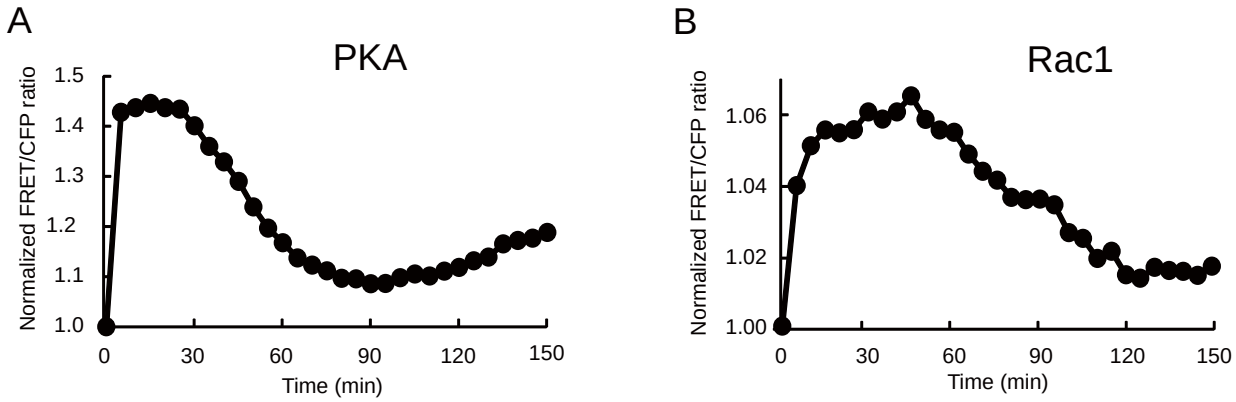
Supplemental Figure 5. The Effect of depletion of STEF on PACAP-induced Rac1 activation and neurite outgrowth. (A) PC12D cells were transfected with an empty pSUPER vector or pSUPER-STEF. After selection with puromycin, the cells were further transfected with pRaichu-Rac1. After starvation for 2 h, images were obtained every 2 min for 30 min after the addition of 100 nM PACAP. The number of experiments was as follows: control (n = 8), STEF KD (n = 12). Left: the mean FRET/CFP ratios averaged over the whole cell. Error bars show the SE. Right: the bar graph represents the average of the highest values of Rac1 activation during the 30 min in the indicated samples, with SE. The symbol indicates the result of a t-test (**$p < 0.01$). (B) PC12D cells were transfected with an empty pSUPER vector or pSUPER-STEF. After recovery, the cells were incubated with puromycin for 2 d. Then, the selected cells were cultured with 100 nM PACAP for 2 d and fixed for microscopy. At least 50 cells were assessed in each experiment, and the experiments were repeated three times. Cells with neurites whose lengths were at least 2-fold longer than their cell body lengths were scored as neurite-bearing cells. The results are expressed as the mean percentage of neurite-bearing cells with the SE. The symbol indicates the result of a t-test; **$p < 0.01$ compared with control.



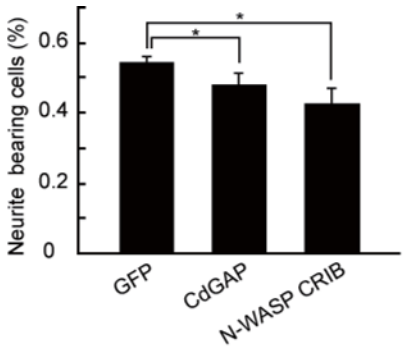
Supplemental Figure 6. The effect of PKA depletion on STEF-mediated Rac1 activation and STEF phosphorylation. (A) Control or PKA-depleted COS-7 cells expressing Raichu-Rac1 in the absence or presence of exogeneous STEF protein were starved 2 hr and then treated with 1 mM dbcAMP. Images were obtained every 2 min for 30 min. The bar graph represents the average of the highest values of the normalized FRET/CFP ratios during the 30 min in the indicated samples, with SE. The number of experiments was as follows: control ($n = 21$), control+STEF ($n = 15$), PKA KD ($n = 20$), PKA KD+STEF ($n = 18$). The symbols indicate the results of a t-test ($*p < 0.05$). (B) Control or PKA-depleted COS-7 cells expressing STEF-HA protein were mock-treated or treated with 1 mM dbcAMP for 30 min and lysed for immunoprecipitation. The bar graph represents the averages of the relative levels of STEF phosphorylation with SE ($n = 4$). The level of STEF phosphorylation in untreated cells was set to 1.



Supplemental Figure 7. The long-term response of Rac1, Cdc42, and PKA activity following dbcAMP treatment was examined as described for Figure 1A. Bars, 10 μm.



Supplemental Figure 8. The long-term response of PKA and Rac1 activity following dbcAMP treatment was examined as described for Figure 1A. The FRET/CFP ratios averaged over the whole cell are expressed.



Supplemental Figure 9. The effect of the expression of CdGAP and the CRIB domain of N-WASP on dbcAMP-induced neurite outgrowth in PC12D cells. PC12D cells were transfected with an empty pCAGGS vector, pIMR21-CdGAP, or pIRM21-FLAG-N-WASP-CRIB. After recovery, the cells were cultured with 1 mM dbcAMP for 2 d and fixed for microscopy. At least 50 cells were assessed in each experiment, and the experiments were repeated three times. Cells with neurites whose lengths were at least 2-fold longer than their cell body lengths were scored as neurite-bearing cells. The results are expressed as the mean percentage of neurite-bearing cells with SE. The symbol indicates the result of a t-test; *$p < 0.05$ compared with the control.