Guanine nucleotide exchange factor Epac2–dependent activation of the GTP-binding protein Rap2A mediates cAMP-dependent growth arrest in neuroendocrine cells

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First messenger-dependent activation of MAP kinases in neuronal and endocrine cells is critical for cell differentiation and function and requires guanine nucleotide exchange factor (GEF)-mediated activation of downstream Ras family small GTPases, which ultimately lead to ERK, JNK, and p38 phosphorylation. Because there are numerous GEFs and also a host of Ras family small GTPases, it is important to know which specific GEF–small GTPase dyad functions in a given cellular process. Here we investigated the upstream activators and downstream effectors of signaling via the GEF Epac2 in the neuroendocrine NS-1 cell line. Three cAMP sensors, Epac2, PKA, and neuritogenic cAMP sensor–Rapgef2, mediate distinct cellular outputs: p38-dependent growth arrest, cAMP response element–binding protein–dependent cell survival, and ERK-dependent neuritogenesis, respectively, in these cells. Previously, we found that cAMP-induced growth arrest of PC12 and NS-1 cells requires Epac2-dependent activation of p38 MAP kinase, which posed the important question of how Epac2 engages p38 without simultaneously activating other MAP kinases in neuronal and endocrine cells. We now show that the small GTP-binding protein Rap2A is the obligate effector for, and GEF substrate of, Epac2 in mediating growth arrest through p38 activation in NS-1 cells. This new pathway is distinctly parcellated from the G protein—coupled receptor → G_s → adenylate cyclase → cAMP → PKA → cAMP response element–binding protein pathway mediating cell survival and the G protein—coupled receptor → G_s → adenylate cyclase → cAMP → neuritogenic cAMP sensor–Rapgef2 → B-Raf → MEK → ERK pathway mediating neuritogenesis in NS-1 cells.

First messenger-dependent activation of MAP[2] kinases in neuronal and endocrine cells requires guanine nucleotide exchange factors (GEFs) to activate downstream Ras family small GTPases, which ultimately lead to ERK, JNK, and p38 phosphorylation (1). There are numerous GEFs as well as a host of Ras family small GTPases. Therefore, it is of critical interest to know which specific GEF–small GTPase dyad functions in a given specific cellular process.

GEFs activated by neurotrophins and G protein-coupled receptors (GPCRs) include Sos, RasGRF1, RasGRF2, RasGRP1, RasGRP2, RasGRP3, RasGRP4, C3G, Epac1, Epac2, NCS-Rapgef2 (alternatively referred to as PDZGEF1, CNrasGEF, or nRap GEP) and RapGEF6 (PDZGEF2). Ras and Rap comprise the major subfamilies of small GTPases considered to be involved in MAP kinase activation (2, 3). Despite the fact that the members of the Ras and Rap subfamilies are highly homologous proteins, it is now generally accepted that there is a high degree of specificity between GEF activation of Ras *versus* Rap proteins, albeit with some exceptions (4). For example, the Ras GEFs Sos, RasGRF1, RasGRF2, RasGRP1, and RasGRP4 have all been shown to be specific activators of Ras compared with Rap (5–9). However, the Ras GEFs RasGRP2 and RasGRP3 are considered to activate both Ras and Rap1 (10, 11). Likewise, C3G (also called RapGEF1 and characterized as a GEF for Rap) has been shown to activate R-Ras (9).

The degree of substrate specificity for Rap GEFs for the Rap isoforms Rap1A, 1B, 2A, 2B, and 2C is still unclear. Epacs 1 and 2 (also known as RapGEF3 and RapGEF4) were initially characterized as GEFs for Rap1 but have been shown to catalyze GDP release from Rap2 *in vitro* and in cell-based systems using transient transfection (12). However, we are not aware of evidence for Epac-mediated regulation of natively expressed Rap2 in intact cells. Upon its initial identification, RapGEF2 (PDZGEF1) was considered to be the first dually specific GEF for Rap1 and Rap2, based predominantly on evidence from experiments using cell-free assay systems (13). Subsequent studies of the related GEF RapGEF6 (PDZGEF2) have shown

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[2] The abbreviations used are: MAP, mitogen-activated protein; GPCR, G protein–coupled receptor; NS-1, Neuroscreen-1; PACAP, pituitary adenylate cyclase–activating polypeptide; FTS-A, farnesylthiosalicylic acid amide; GEF, guanine nucleotide exchange factor; ANOVA, analysis of variance; 8-CPT-cAMP, 8-(4-chlorophenylthio)-methyladenosine-3′,5′-cyclic monophosphate; 8-CPT-2′-*O*-Me-cAMP, 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate; Epac, exchange protein activated by cAMP; ESI-09, α-[(2-(3-chlorophenyl) hydrazinylidene]-5-(1,1-dimethylethyl)-β-oxo-3-isoxazolepropanenitrile; GGTI-298, *N*-[4-[2(*R*)-amino-3-mercaptopropyl]amino-2-(1-naphthalenyl)benzoyl]-l-leucine methyl ester; NCS, neuritogenic cyclic AMP sensor; SB239063, *trans*-4-[4-(4-fluorophenyl)-5-(2-methoxy-4-pyrimidinyl)-1*H*-imidazol-1-yl]cyclohexanol.

that it is, in fact, the most likely candidate dual specificity GEF for Rap1 and Rap2 (14), although evidence for activation of native Rap2 by RapGEF6 in intact cells is lacking.

In summary, it appears that the substrate specificity of Ras GEFs and Rap GEFs can be underestimated when assessed using overexpression of dominant negative or constitutively active congeners of signaling molecules (15, 16). In addition, Ras and Rap family members are recruited to unique cellular locations because of differential lipid modification in intact cells. We have therefore recently developed a battery of neuroendocrine and non-neuroendocrine cell lines designed to allow detection of GEF/small GTPase interactions at physiologically relevant signaling molecule stoichiometries and with physiologically appropriate posttranslational modifications.

Here we report the use of the neuroendocrine Neuroscreen-1 (NS-1) cell line to "prenylation-profile" various Ras/Rap GEFs, *i.e.* show their dependence on either farnesyl or geranylgeranyl lipid modification, to discriminate the various cAMP → GEF → small GTPase → MAP kinase pathways controlling distinct cellular outputs, including changes in cell morphology, proliferation, and gene expression. Based on its prenylation profiling pattern, we demonstrate that signaling for growth arrest by G_s-coupled GPCR-initiated cAMP elevation in NS-1 cells is mediated via Epac2-dependent activation of Rap2 and is independent of Rap1. The prenylation profile for cAMP *versus* neurotrophin signaling to p38, and the underlying growth arrest by both GPCR and neurotrophin receptor ligands, also reveals that both pathways converge on p38 activation through Rap2 and Ras, respectively.

Results

Differential farnesylation requirement for PACAP-initiated signaling to ERK and p38 MAP kinase

We have shown previously that cyclic AMP elevation causes growth arrest in neuroendocrine cells through activation of Epac-dependent signaling to p38 MAP kinase. Rap1, the classical substrate for Epac, is not involved in this signaling pathway (17). We therefore wished to determine the downstream effector molecule that mediates cAMP-dependent p38 phosphorylation. The MAP kinase ERK, a parallel signaling molecule to p38, is regulated by cAMP in a Rap-dependent manner, and its activation results in neurite elongation in neuroendocrine cells. As seen in Fig. 1*A*, p38 phosphorylation caused by either the G_s–GPCR ligand, 100 nM pituitary adenylate cyclase–activating polypeptide (PACAP-38), or a neurotrophin, 100 ng/ml NGF, were differentially inhibited by addition of 10 μM farnesylation inhibitor, farnesylthiosalicylic acid amide (FTS-A). This suggests that neurotrophin-dependent but not cAMP-dependent p38 phosphorylation requires farnesylation. By contrast, FTS-A blocked the effects of both PACAP and NGF on ERK phosphorylation (Fig. 1*B*), indicating that both pathways are farnesylation-dependent.

We next assessed the phosphorylation status of p38 following 30-min treatments with the Epac-selective agonist 8-CPT-2′-*O*-Me-cAMP (100 μM) in the absence or presence of either FTS-A (10 μM) or the Epac inhibitor ESI-09 (10 μM). As seen in Fig. 2, selective activation of Epac with 8-CPT-2′-*O*-Me-cAMP

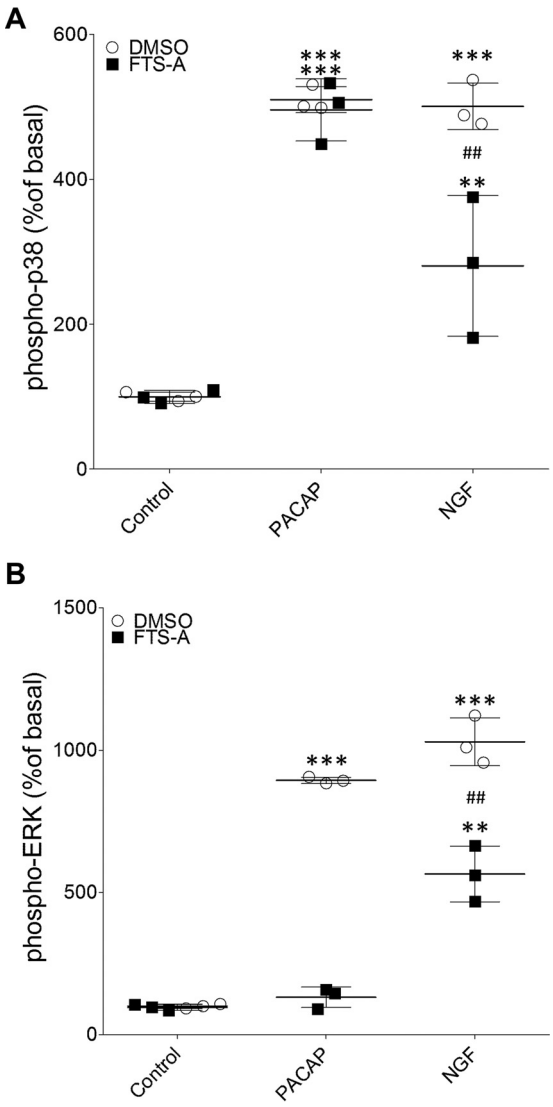


Figure 1. Differential farnesylation requirement for PACAP-initiated signaling to ERK and p38 MAP kinases. *A*, measurements of p38 phosphorylation by a phospho-specific antibody cell-based ELISA in NS-1 cells pretreated with 0.02% DMSO or 10 μM FTS-A, followed by treatment with either 100 nM PACAP-38 or 100 ng/ml NGF. *B*, measurements of phosphorylated ERK in NS-1 cells following identical treatments to those shown in *A*. *Bars* represent means from three experiments, and *error bars* correspond to standard deviations. Data were analyzed by two-way ANOVA and Bonferroni-corrected post hoc tests: ***, $p < 0.001$; **, $p < 0.01$; comparing the effects of each condition with its respective untreated control. Statistical significance of inhibitory effects of FTS-A on either PACAP or NGF are denoted by ##, $p < 0.01$.

caused a robust increase in p38 phosphorylation that was blocked by ESI-09 but was insensitive to FTS-A, suggesting that farnesylation is not a requirement for this signaling to occur.

A small GTPase, Rit1, that undergoes neither farnesylation nor any other lipid modification has been shown to act as an indirect substrate of Epac (18). Thus, Rit1 is a potential candidate as an Epac-dependent GEF, functioning in the Epac → p38 → growth arrest pathway. We created an NS-1 cell line in which the expression of Rit1 was stably reduced via RIT1 shRNA expression (Fig. 3*A*) and compared the effects of 100 μM 8-CPT-2′-*O*-Me-cAMP on growth arrest in shRIT1- *versus* scrambled shRNA– expressing NS-1 cells (Fig. 3, *B* and *C*). As seen in Fig. 3*C*, Rit1 did not appear to play a role in either

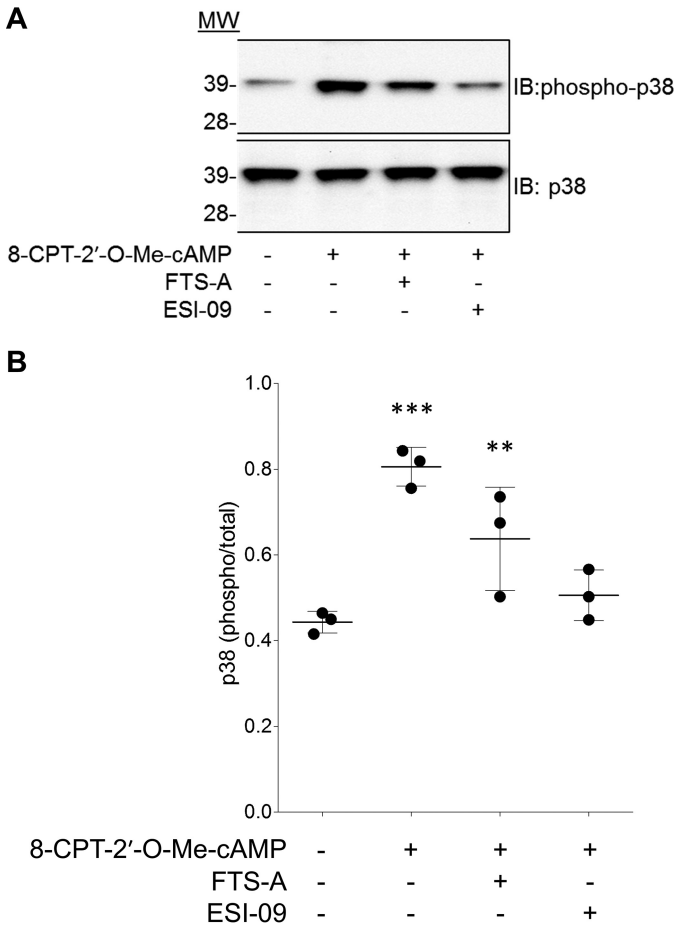


Figure 2. Epac-dependent p38 phosphorylation does not require farnesylation. *A*, representative Western blots of phosphorylated and total p38 in NS-1 cells pretreated for 30 min with vehicle (0.02% DMSO), FTS-A (10 μM), or ESI-09 (10 μM), followed by addition of 8-CPT-2′-*O*-Me-cAMP (100 μM) for 30 min. *MW*, molecular weight; *IB*, immunoblot. *B*, quantifications of three independent experiments. *Bars* represent means, and *error bars* correspond to the standard deviations. Data were analyzed by ANOVA and Bonferroni-corrected *t* tests. ***, $p < 0.001$; **, $p < 0.01$; comparing the effect of 8-CPT-2′-*O*-Me-cAMP with vehicle-treated controls.

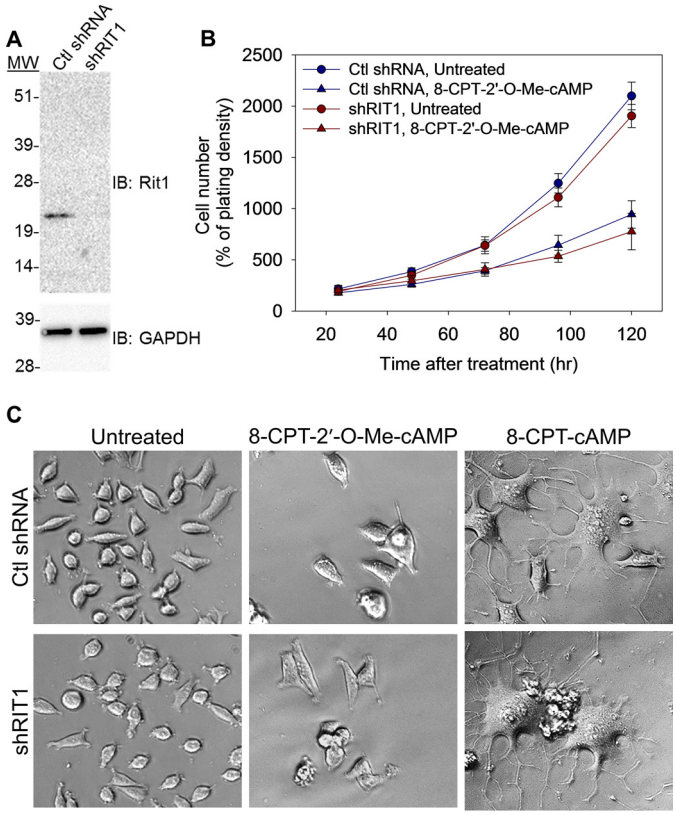


Figure 3. Rit1 is not involved in NS-1 cell growth arrest. *A*, NS-1 cells were transduced with a lentiviral shRNA targeting RIT1 mRNA (*shRIT1*) or a control construct expressing scrambled control shRNA (*Ctl shRNA*). After stable cell lines were established, Rit1 protein was compared in lysates from cells either expressing scrambled shRNA (*lane 1*) or shRNA targeting RIT1 mRNA (*lane 2*). *Bottom panel*, the membrane was stripped and reprobed for GAPDH to confirm equivalent protein loading. *MW*, molecular weight; *IB*, immunoblot. *B*, growth curves from NS-1 sublines expressing scrambled shRNA or RIT1 shRNA grown in 96-well plates and treated with 100 μM 8-CPT-2′-*O*-Me-cAMP for 5 days. *C*, representative images of NS-1 cells expressing scrambled shRNA or shRNA targeting RIT1 obtained after treatment for 5 days with 100 μM 8-CPT-2′-*O*-Me-cAMP or 100 μM 8-CPT-cAMP.

growth arrest or neuritogenesis in NS-1 cells treated with 8-CPT-cAMP or to affect exclusively Epac activation of growth arrest by treatment with 8-CPT-2′-*O*-methyl-cAMP.

Cyclic AMP- and Epac-mediated p38 activation is dependent on geranylgeranylation

To determine whether a prenylation pathway other than farnesylation mediates cAMP- and Epac-dependent signaling to p38, NS-1 cells were pretreated for 30 min with varying concentrations of the geranylgeranyltransferase I inhibitor GGTI-298, with FTS-A treatment as a control, followed by addition of either 3 μM forskolin (an adenylate cyclase activator) (Fig. 4*A*) or 100 μM 8-CPT-2′-*O*-Me-cAMP (an Epac agonist) (Fig. 4*B*). GGTI-298 significantly inhibited p38 phosphorylation caused by either forskolin (Fig. 4*A*) or 8-CPT-2′-*O*-Me-cAMP (Fig. 4*B*), suggesting that geranylgeranylation is necessary for at least one component in the signaling pathway for Epac-dependent p38 activation. As expected, FTS-A did not significantly inhibit the effect of either forskolin (Fig. 4*A*) or 8-CPT-2′-*O*-Me-cAMP (Fig. 4*B*) on p38 activation.

Activation of Rap2A requires geranylgeranylation

Three isoforms of Rap2 have been identified: Rap2A, Rap2B, and Rap2C. Rap2 was one of the first Ras superfamily proteins identified as geranylgeranylated (19). Microarray analysis of the relative abundancies of mRNAs encoding Rap proteins in NS-1 cells reveals that Rap2A mRNA expression is relatively high, and transcripts corresponding to both Raps 2B and 2C are low to nonexistent in these cells.[3] Indeed, we probed NS-1 cell lysates with an antibody that recognizes the electrophoretically distinguishable forms of Rap2 and identified a single band at the estimated molecular weight for Rap2A (Fig. 5*A*). We next assessed whether, in NS-1 cells, Rap2A is activated by the Epac agonist 8-CPT-2′-*O*-Me-cAMP, and if so, whether its activation is sensitive to FTS-A. NS-1 cells were treated for 30 min with FTS-A (10 μM), followed by 10-min treatment with 8-CPT-2′-*O*-Me-cAMP (100 μM). Levels of activated Rap2 (Fig. 5*B*), specifically Rap2A (Fig. 5*C*), were increased following treatment with 8-CPT-2′-*O*-Me-cAMP. Furthermore, the effect of 8-CPT-2′-*O*-Me-cAMP on Rap2–GTP/Rap2A–GTP was not blocked by FTS-A (Fig. 5, *B* and *C*).

[3] A. Emery, A. Elkahloun, and L. Eiden, unpublished observations.

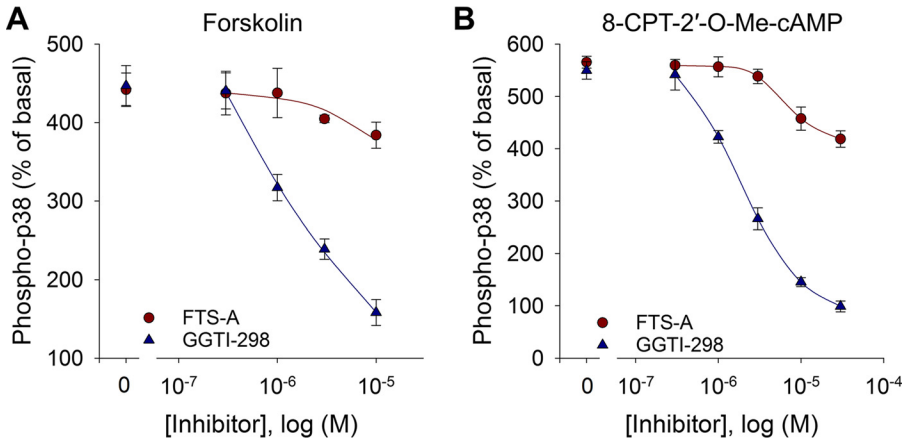


Figure 4. Cyclic AMP/Epac-dependent p38 activation requires geranylgeranylation. *A* and *B*, NS-1 cells were pretreated with the farnesylation inhibitor FTS-A or the geranylgeranyltransferase I inhibitor GGTI-298, followed by stimulation with either 10 μM forskolin (*A*) or 100 μM 8-CPT-2′-*O*-Me-cAMP (*B*). p38 phosphorylation was determined by phospho-specific antibody cell-based ELISA. Data points are means from six determinations, with *error bars* corresponding to the standard errors of the mean.

To establish whether Epac-dependent Rap2A activation is geranylgeranylation-dependent, NS-1 cells were pretreated with 10 μM GGTI-298, or with 10 μM FTS-A as a control, followed by treatment with 100 μM 8-CPT-2′-*O*-Me-cAMP. As seen in Fig. 5, *D* and *E*, inhibition of geranylgeranyltransferase I with GGTI-298 significantly blocked Epac-induced Rap2A activation, whereas inhibition of farnesylation was without effect. As a further control for potential off-target effects of GGTI-298, the same samples were run on separate gels and probed using antibodies for Rap1 (Fig. 5*F*). As seen in Fig. 5*G*, GGTI-298 did not significantly obtund the effect of 8-CPT-2′-*O*-Me-cAMP on Rap1-GTP, whereas FTS-A blocked it, indicating the prenylation specificity of this pair of pharmacological reagents.

Blockade of PACAP-dependent growth arrest in NS-1 cells by inhibition of Epac or p38

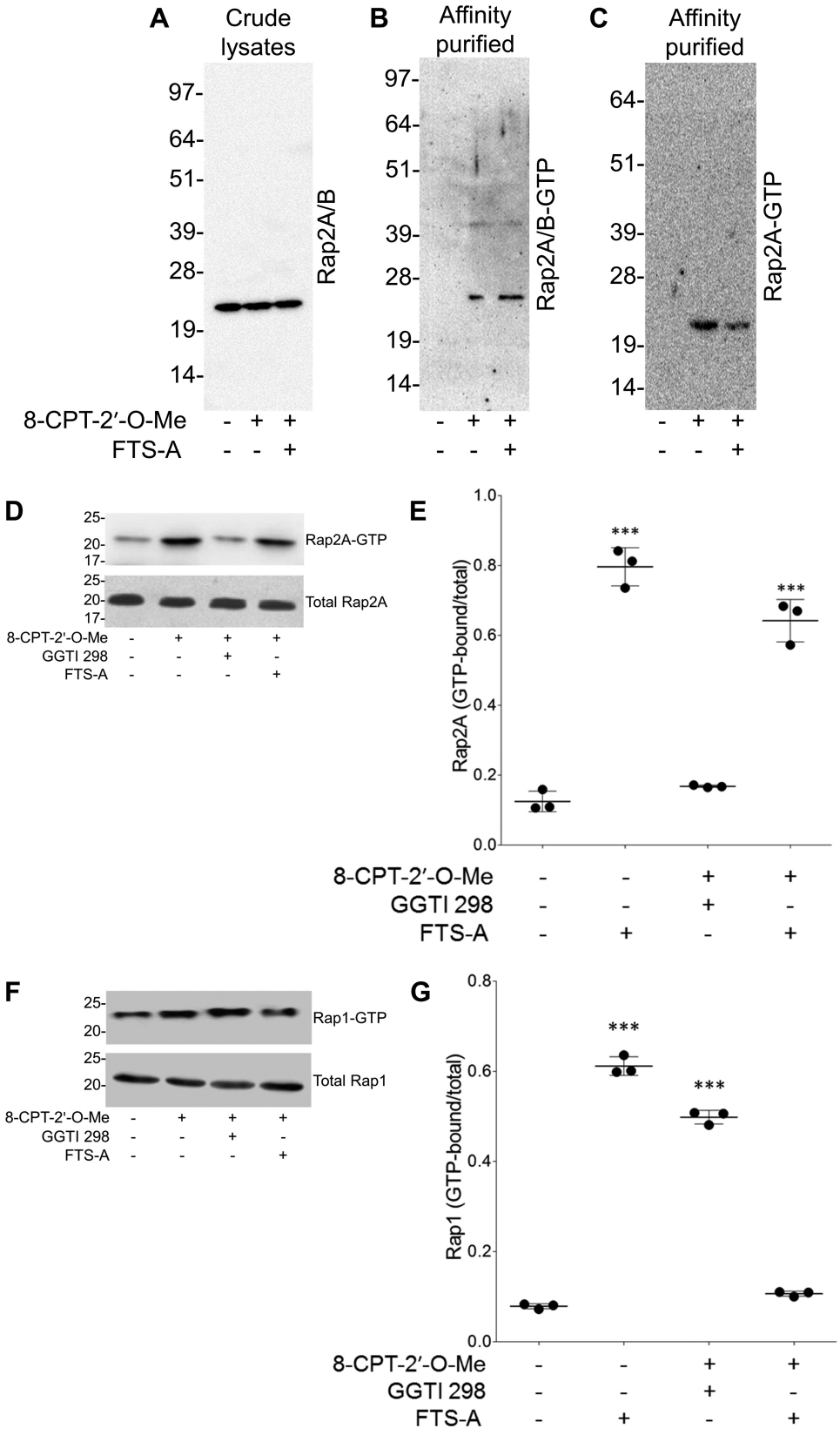
Growth arrest is a p38-dependent phenotypic outcome in NS-1 and PC12 cells that is dissociable from the ERK-dependent effects on neuroendocrine cell differentiation, such as neuritogenesis (17). We employed automated microscopy and high-content analysis to monitor cell growth rates over 5 days following treatment with 100 nM PACAP-38 in the absence or presence of the Epac inhibitor ESI-09 (10 μM) or the p38 inhibitor SB239063 (10 μM). As seen in Fig. 6, PACAP-38 caused a reduction in proliferation (growth arrest) relative to untreated controls. The effect of PACAP was reversed more than 50% by inhibiting either Epac or p38. Likewise, as seen in Fig. 7, the pan-specific 8-CPT-cAMP, applied at 100 μM, causes a reduction in cell proliferation relative to untreated controls. Growth arrest caused by 8-CPT-cAMP was also reversed by ESI-09 or SB239063. We interpret less than complete reversal of growth arrest caused by PACAP-38 (Fig. 6*B*) or 8-CPT-cAMP (Fig. 7*B*) by ESI-09 or SB239063, at doses that do completely inhibit Epac and p38 respectively, as a result of lack of reversal of neurite extension, which may itself impede cell growth relative to untreated cells without neuritic processes.

Direct Epac activation promotes growth arrest in NS-1 cells

To establish that Epac is the sole cAMP sensor mediating growth arrest, we compared the effects of the specific Epac agonist 8-CPT-2′-*O*-Me-cAMP (100 μM) with 8-CPT-cAMP (100 μM), which is a pan-specific activator of Epac, NCS-Rapgef2, and PKA, in our quantitative growth arrest assay. As seen in Fig. 8, both 8-CPT-cAMP and 8-CPT-2′-*O*-Me-cAMP caused growth arrest to an equivalent extent. Furthermore, although both agents caused growth arrest, cells treated with 8-CPT-cAMP responded with a full neuritogenic response, whereas no detectable neurite elongation was observed in cells treated with 8-CPT-2′-*O*-Me-cAMP (Fig. 8*B*).

Rap2A is necessary for Epac2-dependent p38 activation

To evaluate whether Rap2A is required for Epac2 signaling to p38, we generated stable NS-1 cell lines expressing lentiviral vector-expressing shRNAs spanning the 491-nt mRNA sequence of rat RAP2A as well as a scrambled control shRNA. Crude lysates were harvested for detection of Rap2A in cells stably expressing scrambled shRNA (Fig. 9*A*, *lane 1*) or RAP2A-targeting shRNAs 1, 2, and 3 (Fig. 9*A*, *lanes 2–4*). Densitometric analysis indicated that Rap2A protein levels were reduced >95% in cells expressing the RAP2A-targeting shRNAs. As a control for equal protein loading, blots were reprobed with antibodies raised against Rap1 (Fig. 9*C*), the abundance of which did not vary among the four cell lines. To ascertain whether Rap2A expression is necessary for Epac-dependent p38 activation, NS-1 cells expressing either scrambled or Rap2A-targeting shRNAs were treated for 30 min with varying concentrations of 8-CPT-2′-*O*-Me-cAMP (Fig. 9*D*). None of the cell lines deficient in Rap2A activated p38 in an Epac2-dependent manner. In contrast to cAMP-dependent p38 activation, NGF-dependent p38 activation seems to require farnesylation (Fig. 1), and a separate, cAMP-independent NGF–p38 pathway also causes growth arrest (17). We therefore compared the effects of NGF-induced p38 activation in NS-1 cells expressing either scrambled or Rap2A-expressing shRNAs. As seen in Fig. 9*D*, NGF promoted robust p38 activation that was statistically indistinguishable across all cell


A
Crude lysates
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64-
51-
39-
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19-
14-
Rap2A/B
8-CPT-2′-O-Me - + +
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B
Affinity purified
97-
64-
51-
39-
28-
19-
14-
Rap2A/B-GTP
- + +
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C
Affinity purified
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8-CPT-2'-O-Me - + + +
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8-CPT-2′-O-Me - - + +
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F
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20-
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Total Rap1
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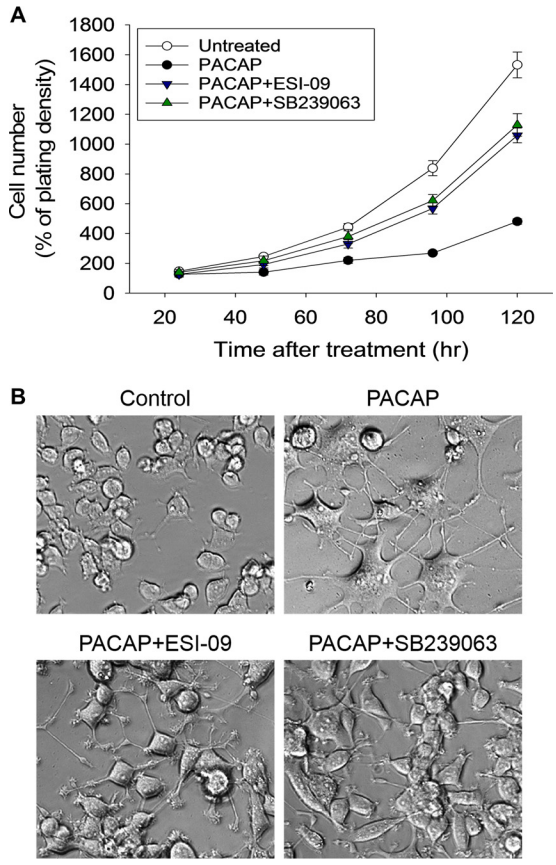

Figure 6. Blockade of PACAP-dependent growth arrest in NS-1 cells by inhibition of Epac or p38. *A*, growth curves of NS-1 cells grown for 5 days in 96-well plates in the absence or presence of 100 nM PACAP-38 with or without the Epac inhibitor ESI-09 (10 μM) or the p38 inhibitor SB239063 (10 μM). Five images per well were acquired every 24 h by automated microscopy. *Data points* represent means, and *error bars* correspond to the standard error of the mean from quadruplicate determinations. *B*, representative images of phase-contrast photomicrographs (×20 objective) obtained after 5 days of treatment.

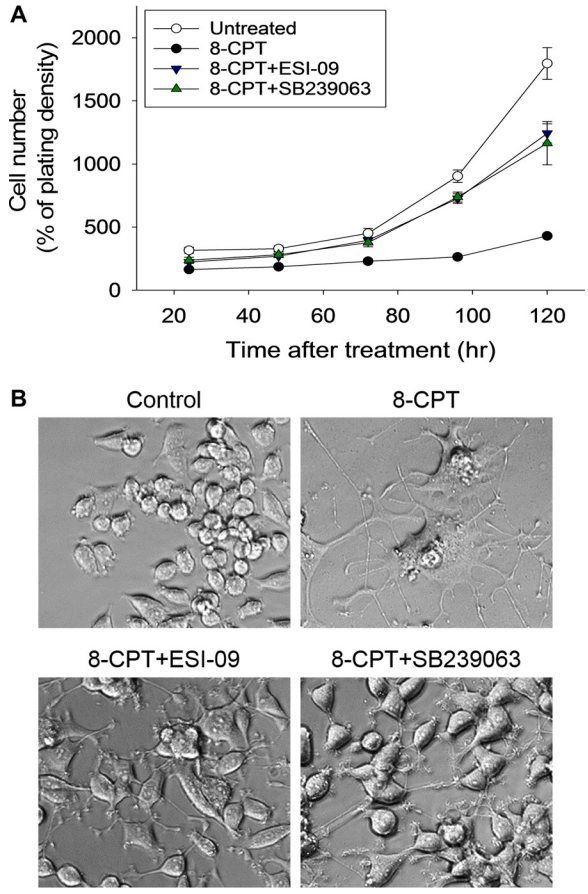

Figure 7. Blockade of cyclic AMP–dependent growth arrest in NS-1 cells by inhibition of Epac or p38. *A*, growth curves of NS-1 cells grown for 5 days in 96-well plates in the absence or presence of 100 μM 8-CPT-cAMP (*8-CPT*) in the absence or presence of either the Epac inhibitor ESI-09 (10 μM) or the p38 inhibitor SB239063 (10 μM). Five images per well were acquired every 24 h by automated microscopy. *Data points* represent means, and *error bars* correspond to the standard error of the mean from eight independent determinations. *B*, representative phase-contrast photomicrographs (×20 objective) obtained after 5 days of treatment.

lines, demonstrating that Rap2A is not involved in NGF signaling to p38.

Rap2A mediates Epac2-dependent growth arrest

Finally, to evaluate whether Rap2A is necessary for Epac2-dependent growth arrest, NS-1 cells stably expressing scrambled shRNA were treated with either 100 μM 8-CPT-2′-*O*-Me-cAMP or 100 ng/ml NGF (Fig. 10*A*). Both agents caused growth arrest to a similar extent in these cells. By contrast (Fig. 10*B*), NS-1 cells stably expressing RAP2A-targeting shRNA and devoid of Rap2A protein had no growth arrest response to treatment with 8-CPT-2′-*O*-Me-cAMP. NGF effects on growth arrest were unaffected in these cells compared with wild-type (normally Rap2A-expressing) NS-1 cells.

Our results, summarized in Fig. 11, highlight the functional insulation of the cyclic AMP pathways for neuroendocrine cell differentiation (cAMP → NCS-Rapgef2 → Rap1 → MEK/ERK → neuritogenesis and cAMP → Epac2 → Rap2A → p38 → growth arrest) as well as the identification of points of convergence for GPCR and neurotrophin receptor signaling for differentiation (ERK for neuritogenesis and p38 for growth arrest). Thus, although both cAMP and NGF activate ERK and p38, we now show that cAMP signaling engages the signaling cassettes for these two MAP kinases through activation of separate small GTPase effector proteins, Rap1 (for ERK) and Rap2A (for p38). NGF, on the other hand, appears to access the two MAP kinases, ERK and p38, through a common small GTP-binding effector protein, Ras.

Figure 5. Activation of Rap2A requires geranylgeranylation. *A*, profiling of Rap2 isoform expression levels in three cultures of NS-1 cells using a Rap2A/B antibody. *B*, measurements of active Rap2 in NS-1 cells pretreated with 10 μM FTS-A for 30 min, followed by treatment with 100 μM 8-CPT-2′-*O*-Me-cAMP (*8-CPT-2′-O-Me*) for 10 min. Active Rap GTPases were purified from lysates with a recombinant fusion protein comprising the Ras binding domain of the Ras/Ral GEF RalGDS. *C*, detection of active Rap2A using the treatments and method described for *B*. *D*, representative Western blot depicting GTP-bound and total Rap2A following pretreatment for 30 min with 10 μM GGTI-298 or 10 μM FTS-A, followed by treatment with 8-CPT-2′-*O*-Me-cAMP (100 μM) for 10 min. Total levels of Rap2 protein prior to affinity purification of samples in *B* and *C* are shown in *A*. *E*, quantification of three independent experiments. Levels of active Rap are expressed as the ratio of total Rap, and data were analyzed by ANOVA and Bonferroni-corrected *t* tests. ***, $p < 0.001$ compared with controls. *F*, measurements of active and total Rap1 following the same treatments as used in *D* and *E*. *G*, quantification of three independent experiments.

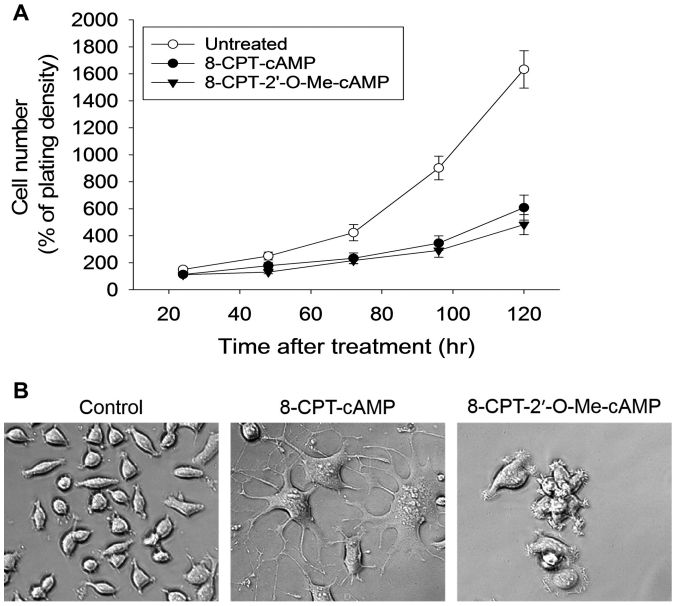

Figure 8. Epac activation promotes growth arrest in NS-1 cells. *A*, NS-1 cells growing in 96-well plates were treated with either 100 μM 8-CPT-cAMP (pan-specific) or 100 μM 8-CPT-2′-*O*-Me-cAMP (Epac-specific). Cells were imaged every 24 h, and cell number was determined for each set of images. *Data points* represent means from quadruplicate determinations, and *error bars* show standard errors of the mean. *B*, representative phase-contrast ×20 photomicrographs acquired after treatment for 5 days.

Discussion

Delineating the myriad physiological functions of MAP kinases in neuronal and endocrine cells, particularly in response to GPCR- and neurotrophin/Trk–initiated signaling, has been a focus of many laboratories over the past several decades (20–26). Activation of MAP kinase signaling by GPCRs and neurotrophin receptors is critical across the lifespan for the processes of neuronal plasticity, proliferation, differentiation, extension of dendrites and axons, long-term potentiation and depression (27–30), and secretion from endocrine tissues (31–33). The signal transduction required for a number of these physiological processes is either wholly dependent on MAP kinase signaling or occurs through coordinated signaling between MAP kinases and molecules such as PI3K, PKA, and calmodulin (34–37).

GPCR signaling activates MAP kinases through the second messengers cyclic AMP, calcium, and inositol trisphosphate, which, in turn, activate an array of small molecule–sensitive GEFs. In contrast, neurotrophin receptor-induced MAP kinase activation occurs through the physical coordination of Trk receptors with adaptor proteins and GEFs for small GTPases. Signaling from both GPCRs and neurotrophin receptors leads to the activation of small GTPases such as those in the Ras and Rap families. These small GTPases, in turn, regulate the activation of MAP kinase cascades for ERK1/2, p38$\alpha/\beta/\gamma/\delta$, JNK1–3, and others (38–40).

It has been known for some time that cAMP elevation causes activation of the MAP kinases p38 and ERK in neurons and endocrine cells (27, 41). However, progress in understanding the molecular links between G_s-coupled GPCRs and MAP kinases has been modest (42). In particular, identification of the cAMP sensors mediating Ras/Rap activation and identification of the specific Ras/Rap isoforms activated for specific cellular MAP kinase signaling events has not yet been accomplished in most cases (21). It was hypothesized previously that Rap activation by PKA plays a critical role in cAMP-dependent MAP kinase signaling (43, 44). However, no direct mechanism for PKA activation of MAP kinases has been demonstrated in neuroendocrine cells (45). Likewise, Epac, although activating Rap in a cAMP-dependent manner, does not appear to engage downstream MAP kinases in this cell type (45). In fact, only mutation of Epac1 with a prenylation-promoting *CAAX* motif fused to its C terminus allows this GEF to couple cAMP signaling to ERK activation in PC12 cells (46).

These observations have focused the attention of our laboratory on GEF-mediated Rap activation, specifically on the cAMP sensor NCS-Rapgef2, a plasma membrane–localized Rap GEF that mediates downstream ERK activation in a cAMP-dependent manner (47). NCS-Rapgef2 and Epac2 both play important roles in neuroendocrine cell differentiation: ERK-dependent neuritogenesis and p38-dependent growth arrest, respectively (17). To investigate the mechanism by which these two cAMP-responsive GEFs activate different MAP kinases, we examined their potential substrates (Rap1 and Rap2). This presented the possibility of the physiological importance of specific prenylation pathways in cAMP signaling to MAP kinases via differential engagement of cAMP-responsive GEFs, potentially acting upon specific Rap isoforms to activate specific MAP kinases. Alternative prenylation enzyme pathways have been shown previously to differentiate most Ras isoforms (both geranylgeranylated and farnesylated), Rap1 (farnesylated) (48), and Rap2 (geranylgeranylated) (19).

Through the use of well-characterized pharmacological reagents (FTS-A and GGTI-298) and the appropriate controls, we initially characterized differential prenylation profiles for the signaling pathways through which cAMP activates p38 and ERK. We next found that Epac mediates the activation of Rap2A in NS-1 cells. Moreover, Epac-dependent Rap2A activation had the identical prenylation profile (*i.e.* sensitive to GGTI-298 although insensitive to FTS-A) as what we observed for cAMP- and Epac-dependent p38 phosphorylation. We then determined that Rap2A expression is necessary for Epac-dependent p38 activation in NS-1 cells. We have thus now identified cAMP → Epac2 → Rap2A → p38 as well as cAMP → NCS-Rapgef2 → Rap1 → ERK as two cyclic AMP-dependent, cAMP sensor-specific, and Rap isoform-specific pathways that distinctly activate two separate MAP kinases dedicated to parallel and distinct physiological aspects of neuronal differentiation: growth arrest and neuritogenesis.

Investigating the roles of the recently identified Epac2–Rap2A–p38 signaling pathway

This signaling cassette is most likely to have a prominent physiological function in neurons and endocrine cells because the expression of Epac2 is highly enriched in these tissue types (49, 50). In fact, PACAP-induced Epac and p38 activation have been implicated in cerebellar and hippocampal long-term depression (27, 41).

In contrast to the dual modes of MAP kinase regulation seen in cAMP signaling, our results suggest that the neurotrophin

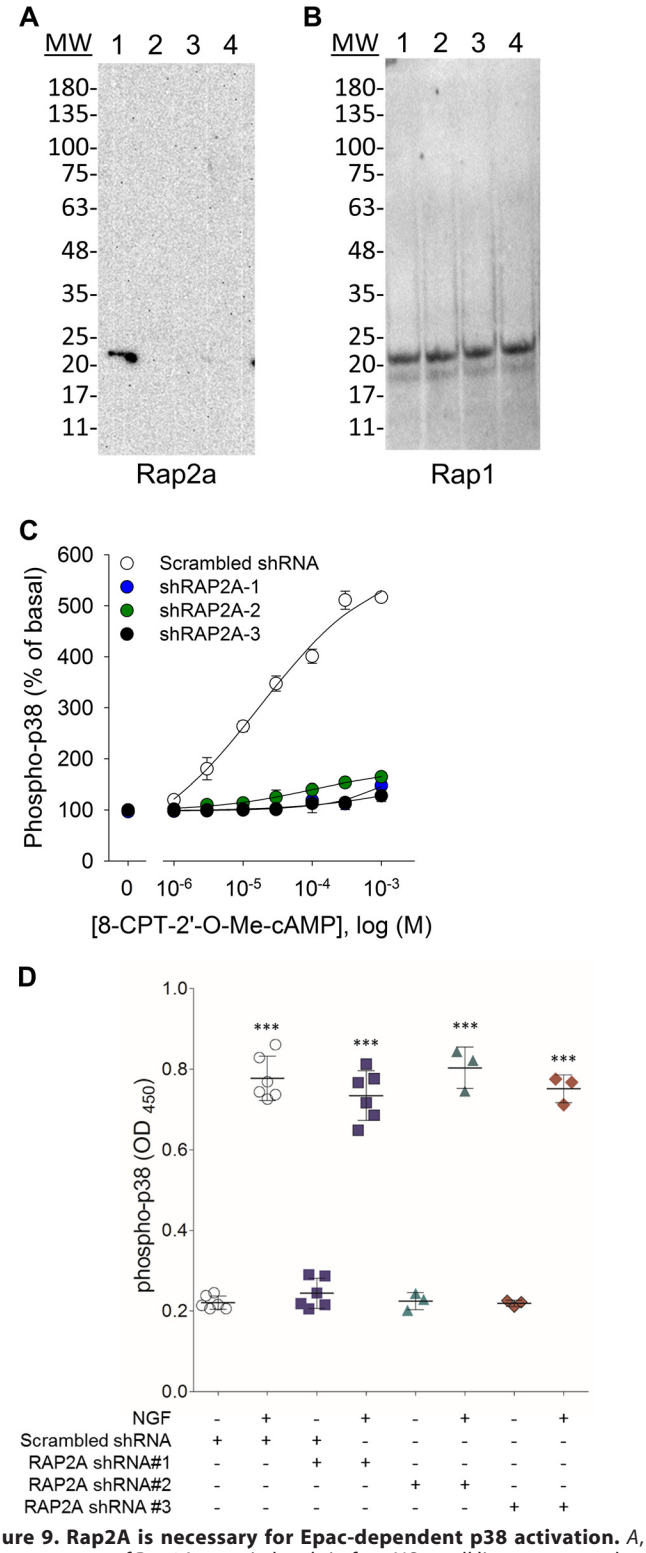

Figure 9. Rap2A is necessary for Epac-dependent p38 activation. *A*, measurements of Rap2A protein levels in four NS-1 cell lines generated to stably express either scrambled shRNA or shRNAs targeting various regions of RAP2A-encoding mRNA. *Lane 1*, NS-1 cells expressing RAP2A-targeting shRNA. *Lane 2*, NS-1 cells expressing shRAP2A-1. *Lane 3*, NS-1 cells expressing shRAP2A-2. *Lane 4*, NS-1 cells stably expressing shRAP2A-3. *MW*, molecular weight. *B*, levels of Rap1 protein in samples from *A*. *C*, measurements of p38 phosphorylation in NS-1 cells stably expressing scrambled shRNA or shRNA targeting RAP2A mRNA. Cells were treated with varying concentrations of 8-CPT-2′-*O*-Me-cAMP for 30 min. *Data points* are means from three experiments performed in duplicate, with *error bars* representing standard error. *D*, p38 phosphorylation in NS-1 cells expressing either scrambled shRNA or shRNA targeting RAP2A following treatment with NGF (100 ng/ml) for 30 min. Data were analyzed by ANOVA and Bonferroni-corrected *t* tests. ***, $p < 0.001$ relative to comparable untreated control cells.

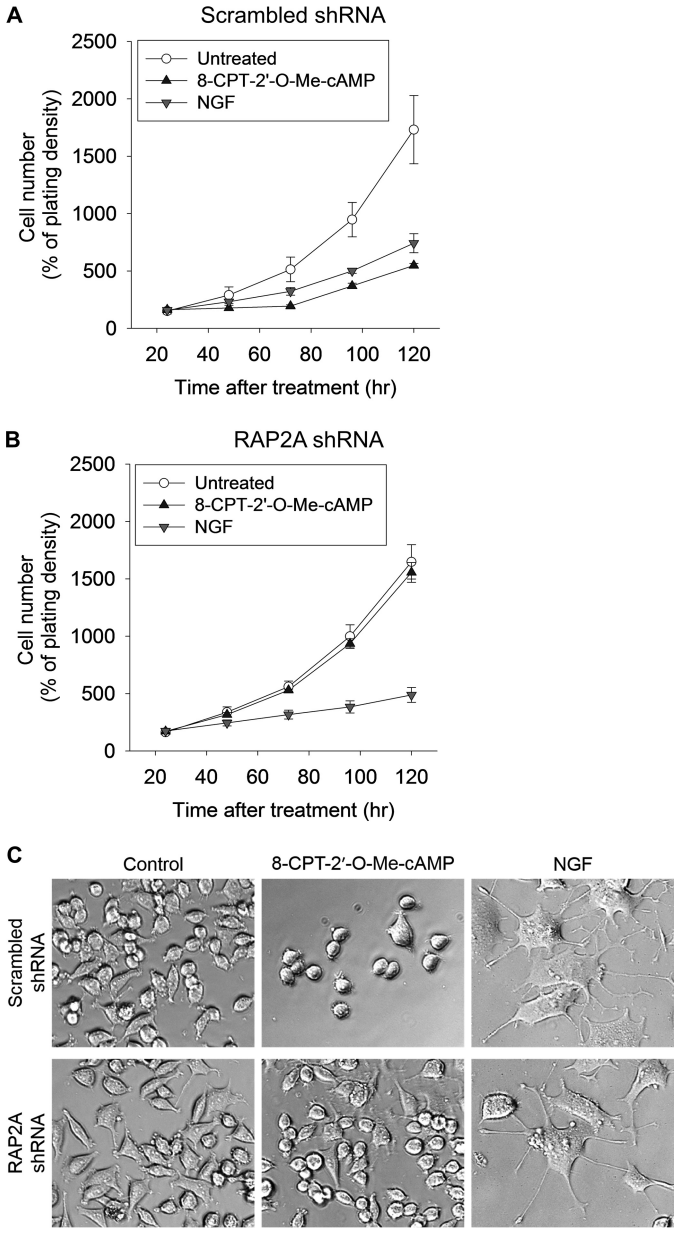

Figure 10. Rap2A mediates Epac-dependent growth arrest. *A* and *B*, growth properties of NS-1 cells stably expressing either (*A*) scrambled shRNA or (*B*) shRNA targeting RAP2A mRNA grown in 96-well plates and treated with 8-CPT-2′-*O*-Me-cAMP (100 μM) or NGF (100 ng/ml) for 5 days. *C*, representative images obtained on the final measurement shown in *A* and *B*.

NGF promotes signaling through only one small GTPase to activate both p38 and ERK. These observations are consistent with others that have implicated Ras as the necessary and sufficient component connecting signaling from activated neurotrophin receptors to MAP kinases (51, 52). In fact, an important implication of this report regarding MAP kinase signaling in neuroendocrine cells is the utilization of a single small GTPase for activation of both ERK and p38 pathways, leading to neuritogenesis and growth arrest, by the neurotrophin NGF.

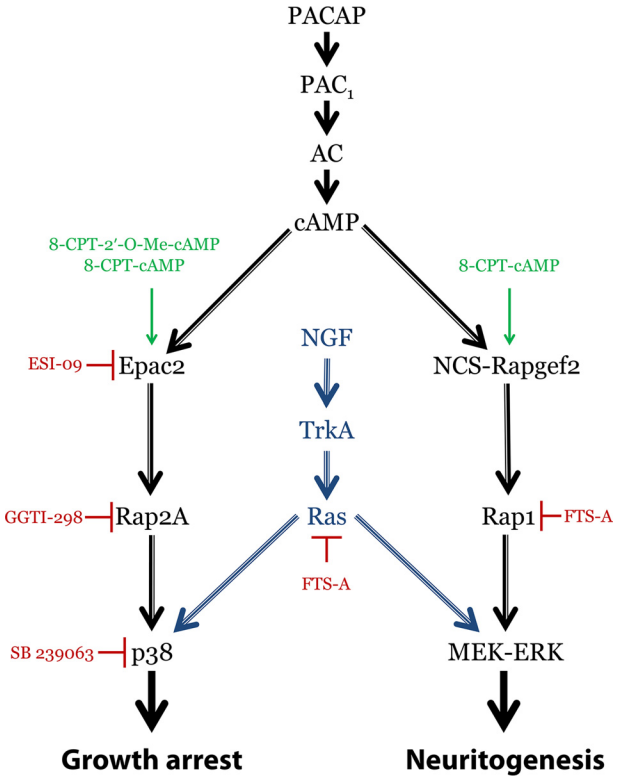

Figure 11. Parcellation of neuropeptide- and neurotrophin-initiated signaling to growth arrest and neuritogenesis in NS-1 cells. *Rap1* denotes Rap1A or B (not yet determined); *MEK-ERK* denotes MEK1/2 and ERK1/2 or both (not yet determined); *p38* is most likely p38α, although minor contributions of p38β or others cannot be ruled out.

Differentiation by cAMP, on the other hand, relies on two distinguishable small GTPases: Rap1 and Rap2A. These have been identified as substrates for Epac2 to cause growth arrest and NCS-Rapgef2 to cause neuritogenesis, respectively. Characterizing distinct Epac2 → Rap2A → p38 and NCS-Rapgef2 → Rap1 → ERK signaling pathways for cellular function in primary neuronal and endocrine cells, analogous to growth arrest and neuritogenesis in NS-1 cells (see above) will be a further fruitful avenue for investigation of neuroendocrine MAP kinase signaling.

Experimental procedures

Drugs and reagents

8-CPT-cAMP and 8-CPT-2′-*O*-Me-cAMP were synthesized by the Biolog Life Sciences Institute and purchased through Axxora. The Epac inhibitor ESI-09, the p38 inhibitor SB239063, and the geranylgeranyltransferase I inhibitor GGTI-298 were purchased from Tocris Bioscience. FTS-A was from the Cayman Chemical Co. NGF was from BD Biosciences, and PACAP-38 was purchased from Anaspec. Inhibitors were prepared as fresh stocks in DMSO. Cyclic AMP analogs, PACAP-38, and NGF were dissolved in culture medium.

Cell culture

NS-1 cells are a subclone of PC12 cells purchased from Cellomics. All solutions used for cell culture were purchased from Invitrogen unless otherwise noted. NS-1 cells were cultured in RPMI 1640 medium supplemented with 10% horse serum (HyClone), 5% heat-inactivated fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in flasks (Techno Plastic Products) coated with collagen type I from rat tail at 37 °C in a humidified incubator containing 5% CO_2. Cultures routinely tested negative for mycoplasma and cells were used between passages 4 and 13 for the experiments reported here.

High-content analysis

Growth arrest and neuritogenesis were measured in NS-1 cells stably expressing enhanced GFP using automated microscopy and automated image analysis as described previously (53). Cells were seeded in collagen type I– coated 96-well plates in growth medium at a density of 1×10^3 per well. Two hours later, cells were imaged using a ×20 objective using an inverted fluorescence microscope outfitted with motorized objectives, shutters, filters, stage, automated focusing, and focus correction (TiE Eclipse, Nikon). Images were captured with a Hamamatsu C11440 digital camera. Five pairs of phase-contrast and fluorescent images were captured per well during each acquisition. Following each acquisition, plates were returned to an incubator. Eighteen hours after plating, cells were treated as indicated. Images were then acquired every 24 h for 5 days. Cells were counted using Nikon NIS-Elements High Content software. To account for possible unevenness of plating, cell growth data were normalized to the values obtained from each field at initial acquisition.

Western blotting

NS-1 cells were seeded and grown overnight. When indicated, inhibitors were added for 30 min, followed by agonist treatment for 30 min. Media were removed, and cells were collected in ice-cold lysis buffer (150 mM NaCl, 25 mM Tris-HCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with Halt protease and phosphatase inhibitors (Thermo, 78446) and snap-frozen. Protein concentrations were determined using Micro BCA protein assays (Thermo, 23235) following the protocol of the manufacturer. Samples were diluted in water, lithium dodecyl sulfate sample buffer (Invitrogen), and NuPAGE reducing reagent (Invitrogen) to a final protein concentration of 1 μg/μl. Proteins (25 μg/lane) were then separated by SDS-PAGE using precast 5–15% polyacrylamide gels (Nacalai USA). Gels were blotted onto nitrocellulose membranes (Invitrogen) using a semidry transfer apparatus (Invitrogen) at 30 V for 2 h at room temperature. Membranes were then blocked with 5% skim milk dissolved in Tris-buffered saline with 1% Tween 20 (TBST) for 1 h. Membranes were incubated overnight at 4 °C with primary antibodies to label phospho-p38 (which recognizes dual-phosphorylated p38α, β, δ, and γ; Cell Signaling Technology, 4511). Membranes were then washed five times in TBST and incubated with an anti-rabbit HRP-coupled secondary antibody (Cell Signaling Technology) in blocking buffer for 1 h. Membranes were washed five times in TBST and exposed to a chemiluminescent HRP substrate (Super Signal West Pico, Thermo, 34078). Membranes were imaged using a cooled charge-coupled device camera (Protein Simple). Bound antibodies were stripped from membranes by a 30-min incubation in Restore Plus Western blot

stripping buffer (Thermo, 46430), followed by five washes in TBST. Membranes were incubated for 1 h in blocking buffer and reprobed with primary antibodies raised against total p38 (Cell Signaling Technology, 8690).

Cell-based ELISA

Phosphorylated p38 and ERK were measured using a cell-based ELISA according to a protocol described previously (54) with minor modifications. NS-1 cells were seeded at density of 3×10^5 per well in 96-well plates. The following day, cells were pretreated for 30 min with inhibitors or vehicle followed by stimulation with agonists for 30 min. After treatment, media were aspirated, and cells were fixed in 4% formaldehyde in PBS for 20 min at room temperature. Following fixation, cells were permeabilized by three washes in PBST, and endogenous peroxidase activity was quenched by a 20-min incubation in PBS containing 0.6% H_2O_2. Plates were washed three times in PBST and blocked using 10% FBS in PBST for 1 h. Primary antibodies against phospho-p38 (Cell Signaling Technology, 4155) or phospho-ERK (Cell Signaling Technology, 9101), each diluted 1:500, were incubated overnight at 4 °C with gentle agitation. The next day, unbound primary antisera were removed by three washes three times in PBST and two washes in PBS. Bound antibodies were labeled with an HRP-coupled anti-rabbit secondary antibody (Cell Signaling Technology, 7074), diluted 1:500 in PBST containing 5% BSA, for 1 h at room temperature. After five washes in PBST, samples were exposed to the colorimetric substrate 1-Step Ultra TMB-ELISA (Thermo, 34029). After development in the dark for 10 min, the reaction was stopped by adding 4 M sulfuric acid, and absorbance was read at 450 nm.

Silencing of signaling proteins

shRNAs were expressed in psi-Lv-HIVH1 lentiviral vectors (Genecopoeia). The expression of rat Rit1 (NM_001109185) was silenced by targeting gcagcactctcaagggaat. Expression of rat RAP2A-encoding mRNA (NM_053741.1) was silenced using three shRNAs expressed in separate cell lines: shRAP2–1 targeted nucleotides 59–78, shRAP2–2 targeted nucleotides 80–99, and shRAP2A-3 targeted nucleotides 305–224. For generation of stable shRNA-bearing NS-1 cell lines, lentiviral particles were generated by co-transfection of plasmids encoding shRNA expression vectors (10 μg), gag-pol-rev (6.5 μg), and vesicular stomatitis virus glycoprotein (3.5 μg) in HEK293T cells using the ProFection calcium phosphate system (Promega) according to the instructions of the manufacturer. 48 h after transfection, supernatants were harvested, filtered through 0.45-micron filters, and used to transduce 60–70% confluent cultures of NS-1 cells. Transductions were carried out for 12 h, and then cells were split and grown in medium containing puromycin (1 μg/ml). Following two passages in selection medium, >98% of transduced NS-1 expressed visible GFP upon epifluorescence illumination. Protein knockdown was confirmed by Western blotting as described above using antibodies for Rit1 (Atlas Antibodies, catalog no. HPA053249) and Rap2A (Thermo, catalog no. PA5-28905). Where appropriate, protein content was normalized by stripping membranes and reprobing with an anti-rabbit GAPDH antibody (Cell Signaling Technology, catalog no. 174) at a dilution of 1:1000. Between four and eight shRNAs constructs targeting different regions of the transcript of interest were evaluated for reduction of the protein product of the mRNA of interest. Cell lines were generated that express scrambled shRNA (target sequence, gcttcgcgccgtagtctta) from the same vector to be used as an experimental control.

Measurements of Rap activation

Rap-GTP was measured using the Active Rap1 Pull-Down and Detection Kit (Thermo, Catalog no. 16120) according to the instructions of the manufacturer. NS-1 cells were grown to near confluency in 60-mm dishes. Cells were treated for 10 min as indicated and then collected in ice-cold lysis/binding/wash buffer provided in the kit. Protein concentrations in lysates were determined by Micro BCA assays (Thermo, catalog no. 23235) following the protocol provided by the manufacturer. 500 μg of protein from each dish was gently mixed in a glutathione resin slurry containing the recombinant fusion protein GST-RalGDS-RBD (20 μg) for 1 h at 4 °C. Resins were then centrifuged through spin cups, washed three times with lysis/binding/wash buffer, followed by the addition of reducing buffer provided by the manufacturer. Samples were then vortexed and centrifuged through spin cups for separation from resins. Eluted samples were heated to 95 °C for 5 min, followed by Western blotting, as described above. Membranes were probed with antibodies raised against Rap1A/B diluted 1:1000 (Pierce, catalog no. 89872D), Rap2 diluted 1:1000 (BD Biosciences, catalog no. 610216), or Rap2A diluted 1:1000 (Thermo, catalog no. PA5-28905). To account for possible differences in Rap content between samples, unpurified protein samples (25 μg) corresponding to affinity-purified samples were analyzed on separate gels using the same antibodies.

Calculations and statistics

All statistical analyses were performed using Sigma Plot (Systat). Curves were fit to dose-response data by four-parameter logistic regressions. Statistical comparisons were done by one- or two-way ANOVA, followed by Bonferroni-corrected *t* tests comparing each condition to its appropriate control.

Author contributions—A. C. E. and L. E. E. designed the experiments. A. C. E. and W. X. conducted the experiments. M. V. E. and W. X. contributed new cell and molecular biological reagents. A. C. E. and L. E. E. wrote the manuscript. L. E. E. oversaw the project.

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Note added in proof—In the version of this article that was published as a Paper in Press on May 25, 2017, Fig. 5*G* was incorrect. It was a duplicate of Fig. 5*E*. The correct version of Fig. 5*G* has been restored. The conclusions of the report are now consistent with the actual data, as presented in the current and final manuscript. The authors regret the previous incorrect Fig. 5*G*.

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