

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

Andrew C. Emery¹, Wenqin Xu¹, Maribeth V. Eiden², and Lee E. Eiden¹

From ¹Section on Molecular Neuroscience and ²Office of the Scientific Director, National Institute of Mental Health Intramural Research Program, Bethesda, MD 20892

Running Title: Epac2 signaling to p38 through Rap2A

To whom correspondence should be addressed: Lee E. Eiden, Section on Molecular Neuroscience, National Institute of Mental Health Intramural Research Program, 9000 Rockville Pike, Building 49, Room 5A38, Bethesda, MD, USA, 20892, Tel.: (301) 496-4110, E-mail, eidenl@mail.nih.gov

Keywords: cyclic AMP, Epac2, ERK, GEF, small GTPase, NCS-Rapgef2, p38 MAPK, Rap2

ABSTRACT

First messenger-dependent activation of MAPKs 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 NCS-Rapgef2 mediate distinct cellular outputs: p38-dependent growth arrest, CREB-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 MAPK, which posed the important question of how Epac2 engages p38 without simultaneously activating other MAPKs 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 GPCR

→ Gs → AC → cAMP → PKA → CREB pathway mediating cell survival, and the GPCR → Gs → AC → cAMP → NCS-Rapgef2 → B-Raf → MEK → ERK pathway mediating neuritogenesis in NS-1 cells.

INTRODUCTION

First messenger-dependent activation of MAP 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 to 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).

However, the degree of substrate specificity for Rap GEFs for Rap isoforms Rap1A, 1B, 2A, 2B, and 2C is 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 upon evidence from experiments using cell-free assay systems (13). Subsequent studies on the related GEF RapGEF6 (PDZGEF2) have shown 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, due to 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 post-translational 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 Gs-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 underlying growth arrest by both GPCR and neurotrophic 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 previously shown 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 both cAMP in a Rap-dependent manner and its activation results in neurite elongation in neuroendocrine cells. As seen in Figure 1A, p38 phosphorylation caused by either the Gs-GPCR ligand, 100 nM pituitary adenylate cyclase-activating polypeptide (PACAP-38), or a neurotrophin, 100 ng/ml nerve growth factor (NGF), were differentially inhibited by the addition of 10 μM of the 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 (Figure 1B), 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 Figure 2, selective activation of Epac with 8-CPT-2'-O-Me-cAMP 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 \rightarrow p38 \rightarrow growth arrest pathway. We created an NS-1 cell line in which the expression of Rit1 was stably reduced via RIT1 shRNA expression (Figure 3A), and compared the effects 100 μ M 8-CPT-2'-O-Me-cAMP on growth arrest in shRIT1- versus scrambled shRNA-expressing NS-1 cells (Figures 3B and 3C). As seen in Figure 3C, Rit1 did not appear to play a role in either growth arrest or neuritogenesis in NS-1 cells treated with 8-CPT-cAMP, nor to affect exclusively Epac activation of growth arrest by treatment with 8-CPT-2'-O-methyl-cAMP.

Cyclic AMP and Epac-mediated p38 activation are dependent on geranylgeranylation – In order 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 the addition of either 3 μ M of the adenylate cyclase activator forskolin (Figure 4A) or 100 μ M of the Epac agonist 8-CPT-2'-O-Me-cAMP (Figure 4B). GGTI-298 significantly inhibited p38 phosphorylation caused by either forskolin (Figure 4A) or 8-CPT-2'-O-Me-cAMP (Figure 4B), suggesting 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 (Figure 4A) or 8-CPT-2'-O-Me-cAMP (Figure 4B) on p38 activation.

Activation of Rap2A requires geranylgeranylation – Three isoforms of Rap2 have been identified: Rap2A, Rap2B, and Rap2C. Rap2 was among the first of the Ras superfamily protein identified as geranylgeranylated (19). Microarray analysis of 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 (A. Emery, A. Elkahouloun and L. Eiden, unpublished observations). 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 (Figure 5A). 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 μ M) followed by 10 min treatment with 8-CPT-2'-O-Me-cAMP (100 μ M). Levels of activated Rap2 (Figure 5B), specifically Rap2A (Figure 5C), 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 (Figures 5B, 5C).

To establish whether Epac-dependent Rap2A activation is geranylgeranylation-dependent, NS-1 cells were pretreated with 10 μ M GGTI-298, or with 10 μ M FTS-A as a control, followed by treatment with 100 μ M 8-CPT-2'-O-Me-cAMP. As seen in Figures 5D and 5E, inhibition of geranylgeranyltransferase I with GGTI-298 significantly blocked Epac-induced Rap2A activation, while 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 (Figure 5F). As seen in Figure 5G, GGTI-298 did not significantly obtund the effect of 8-CPT-2'-O-Me-cAMP on Rap1-GTP, while 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 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 five days following treatment with 100 nM PACAP-38 in the absence or presence of the Epac inhibitor ESI-09 (10 μ M) or the p38 inhibitor SB239063 (10 μ M). As seen in Figure 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 Figure 7, the pan-specific 8-CPT-cAMP, applied at 100 μ M, 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 (Figure 6B) or 8-CPT-cAMP (Figure 7B) by ESI-09 or SB239063, at doses that do completely inhibit Epac and p38 respectively, as due to 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 μ M) with 8-CPT-cAMP (100 μ M), which is a pan-specific activator of Epac, NCS-Rapgef2, and PKA, in our quantitative growth arrest assay. As seen in Figure 8, both 8-CPT-cAMP and 8-CPT-2'-O-Me-cAMP caused growth arrest to an equivalent extent. Furthermore, while both agents caused growth arrest, cells treated with 8-CPT-cAMP responded with a full neurotogenic response, while no detectable neurite elongation was observed in cells treated with 8-CPT-2'-O-Me-cAMP (Figure 8B).

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 shRNAs spanning the 491 nucleotide 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 (Figure 9A, lane 1) or RAP2A-targeting shRNAs 1, 2, and 3 (Figure 9A, lanes 2-4). Densitometric analysis indicated that Rap2A protein levels were reduced > 95% in cells expressing either of the RAP2A-targeting shRNAs. As a control for equal protein loading, blots were re-probed with antibodies raised against Rap1 (Figure 9C), the abundance of which did not vary between 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 (Figure 9D). 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 (Figure 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 Figure 9D, NGF promoted robust p38 activation that was statistically indistinguishable across all cell 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 μ M 8-CPT-2'-O-Me-cAMP or 100 ng/ml NGF (Figure 10A). Both agents caused growth arrest to a similar extent in these cells. By contrast (Figure 10B), 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 to wild-type (normally Rap2A-expressing) NS-1 cells.

Our results, summarized in Figure 11, highlight the functional insulation of the cyclic AMP pathways for neuroendocrine cell differentiation (cAMP \rightarrow NCS-Rapgef2 \rightarrow Rap1 \rightarrow MEK/ERK \rightarrow neuritogenesis and cAMP \rightarrow Epac2 \rightarrow Rap2A \rightarrow p38 \rightarrow growth arrest), as well as the identification of points of convergence for GPCR and neurotrophin signaling for differentiation (ERK for neuritogenesis, and p38 for growth arrest). Thus, while 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.

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 neurotrophins is critical across the lifespan for the processes of neuronal plasticity, proliferation, differentiation, extension of dendrites and axons, long-term potentiation (LTP) and depression (LTD), (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-induced MAP kinase activation occurs through the physical coordination of Trk receptors with adaptor proteins and GEFs for small GTPases. Signaling from by both GPCRs and neurotrophins leads to the activation of small GTPases such as those in the Ras and Rap families. These small GTPases in turn regulate 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 Gs-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 isoform activated for specific cellular MAP kinase signaling events has not yet been accomplished in most cases (21). It was previously hypothesized 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 confers cAMP-dependent ERK activation properties to this GEF, 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 previously shown 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 activation of Rap2A in NS-1 cells. Moreover, Epac-dependent Rap2A activation had the identical 'prenylation profile', (i.e. sensitive to GGTI-298 while insensitive to FTS-A) as we observed for cAMP- and Epac-dependent p38 phosphorylation. We then determined that Rap2A expression is necessary Epac-dependent p38 activation in NS-1 cells. We have thus now identified cAMP \rightarrow Epac2 \rightarrow Rap2A \rightarrow p38, as well as cAMP \rightarrow NCS-Rapgef2 \rightarrow Rap1 \rightarrow 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 since the expression levels of Epac2 are highly enriched in these tissue types (49,50). In fact, PACAP-induced Epac and p38 activation have been implicated in cerebellar and hippocampal LTD (27,41).

In contrast to the dual modes of MAP kinase regulation seen in cAMP signaling, our results suggest that the neurotrophin 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 contrast observed between 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. 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 \rightarrow Rap2A \rightarrow p38, and NCS-Rapgef2 \rightarrow Rap1 \rightarrow ERK signaling pathways for cellular function in primary neuronal and endocrine cells, analogous to growth arrest and neuritogenesis in NS-1 cells, (vide supra) will be a further fruitful avenue for investigation of neuroendocrine MAP kinase signaling.

EXPERIMENTAL PROCEDURES

Drugs and Reagents – 8-(4-Chlorophenylthio)-methyladenosine-3',5'-cyclic monophosphate (8-CPT-cAMP) and 8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8-CPT-2'-O-Me-cAMP) were synthesized by the Biolog Life Sciences Institute and purchased through Axxora. The Epac inhibitor α -[(2-(3-Chlorophenyl)hydrazinylidene]-5-(1,1-dimethylethyl)- β -oxo-3-isoxazolepropanenitrile (ESI-09), p38 inhibitor *trans*-4-[4-(4-Fluorophenyl)-5-(2-methoxy-4-pyrimidinyl)-1H-imidazol-1-yl]cyclohexanol (SB 239063), and geranylgeranyltransferase I inhibitor *N*-[4-[2(*R*)-Amino-3-mercaptopropyl]amino-2-(1-naphthalenyl)benzoyl]-L-leucine methyl

ester (GGTI 298) were purchased from Tocris Bioscience. Farnesylthiosalicylic acid amide (FTS-A) was from the Cayman Chemical Company. Nerve growth factor (NGF) was from BD Biosciences and pituitary adenylate cyclase-activating polypeptide-38 (PACAP-38) was purchased from Anaspec. Inhibitors were prepared as fresh stocks in dimethyl sulfoxide (DMSO). Cyclic AMP analogs, PACAP-38, and NGF were dissolved in culture media.

Cell culture – Neuroscreen-1 (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 Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% horse serum (HyClone), 5% heat-inactivated fetal bovine serum (Atlanta Biologicals), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/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% CO₂. Cultures routinely tested negative for mycoplasma and cells were used between passages four and 13 for the experiments reported here.

High-content analysis (HCA) – Growth arrest and neuritogenesis were measured in NS-1 cells stably expressing eGFP 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 x20 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 hours for five 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 ice-cold lysis buffer (150 mM NaCl, 25 mM Tris-HCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Halt protease and phosphatase inhibitors (Thermo, #78446) and snap frozen. Protein concentrations determined using Micro BCA Protein Assays (Thermo, #23235) following the protocol of the manufacturer. Samples were diluted in water, LDS 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 semi-dry 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 (recognizes dual-phosphorylated p38alpha, beta, delta and gamma; 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 hour in blocking buffer and re-probed with primary antibodies raised against total p38 (Cell Signaling, #8690).

Cell-based ELISA – Phosphorylated p38 and ERK were measured using 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 for 30 min with inhibitors or vehicle followed by 30 min with agonists. 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 0.1% Triton X-100 in PBS (PBST), and endogenous peroxidase activity was quenched by a 20 min incubation in PBS containing 0.6% H₂O₂. 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 one hour 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 minutes, the reaction was stopped by adding 4 M sulfuric acid, and absorbance was read at 450 nm.

Silencing of signaling proteins – Short hairpin RNAs (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 VSV-G (3.5 µg) in HEK293T cells using the ProFection calcium phosphate system (Promega) according to the manufacturer's instructions. 48 hours 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 hours, after which time cells were split and grown in media containing puromycin (1 µg/ml). Following 2 passages in selection media, >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 # HPA053249) and Rap2A (Thermo, Catalog # PA5-28905). Where appropriate, protein content was normalized by stripping membranes and re-probing with an anti-rabbit GAPDH antibody (Cell Signaling Technology, Catalog #5174) 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 # 16120) according to the manufacturer's instructions. NS-1 cells were grown to near confluency in 60 mm dishes. Cells were treated for 10 min as indicated and were 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 # 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 hour 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 #89872D), Rap2, diluted 1:1000 (BD Bioscience, Catalog # 610216), or Rap2A, diluted 1:1000 (Thermo, Catalog # 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.

Acknowledgments: The authors thank David Huddleston and Chang-Mei Hsu for their technical assistance and Dr. Jhihong Sunny Zhang for her insightful comments about this manuscript.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this report.

Author contributions: A.C.E. and L.E.E. designed experiments, A.C.E. and W.X. conducted 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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FOOTNOTES

This work was supported by the National Institute of Mental Health Intramural Research Program, Projects ZIAMH002386 to L.E.E. and ZIAMH002592 to M.V.E., and by the Brain and Behavior Research Foundation by NARSAD Young Investigator Grant # 21356 to A.C.E.

The abbreviations used are: 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; cAMP, 3'-5'-cyclic adenosine monophosphate; Epac, exchange protein activated by cAMP; ERK, extracellular signal-regulated kinase; ESI-09, α -[(2-(3-Chlorophenyl)hydrazinylidene)-5-(1,1-dimethylethyl)- β -oxo-3-isoxazolepropanenitrile]; FTS-A, farnesylthiosalicylic acid amide; GEF, guanine nucleotide exchange factor; GGTI-298, *N*-[4-[2(R)-Amino-3-mercaptopropyl]amino-2-(1-naphthalenyl)benzoyl]-L-leucine methyl ester; GPCR, G protein-coupled receptor; JNK, c-Jun N-terminal kinases; MAP, mitogen-activated protein; NCS, neuritogenic cyclic AMP sensor; NGF, nerve growth factor; NS-1, Neuroscreen-1; p38, P38 mitogen-activated protein kinase; PACAP, pituitary adenylate cyclase-activating polypeptide; PKA, protein kinase A; SB 239063, *trans*-4-[4-(4-Fluorophenyl)-5-(2-methoxy-4-pyrimidinyl)-1*H*-imidazol-1-yl]cyclohexanol; SEM, standard error of the mean; shRNA, short hairpin RNA; Trk, tyrosine receptor kinase

FIGURE LEGENDS

FIGURE 1. Differential farnesylation requirement for PACAP-initiated signaling to ERK and p38 MAP kinase. *A*, Measurements of p38 phosphorylation by phospho-specific antibody cell-based ELISA in NS-1 cells pretreated with 0.02% DMSO or 10 μ M farnesylthiosalicylic acid amide (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 2-way ANOVA and Bonferroni-corrected post hoc tests: *** $P < 0.001$, ** $P < 0.01$ comparing the effects of each condition to its respective untreated control. Statistical significance of inhibitory effects of FTS-A on either PACAP or NGF are denoted by ## $P < 0.01$.

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 minutes with vehicle (0.02% DMSO), FTS-A (10 μ M), or ESI-09 (10 μ M) followed by addition of 8-CPT-2'-O-Me-cAMP (100 μ M) for 30 minutes. *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 to 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 (Ctl shRNA; Lane 1) or shRNA targeting RIT1 mRNA (shRIT1; Lane 2). Below, membrane was stripped and re-probed for GAPDH to confirm equivalent protein loading. *B*, Growth curves from NS-1 sublines expressing scrambled shRNA or RIT1 shRNA grown in 96-well plates and treated with 100 μ M 8-CPT-2'-O-Me-cAMP for five days. *C*, Representative images of NS-1 cells expressing scrambled shRNA (Ctl shRNA) or shRNA targeting RIT1 (shRIT1) obtained after treatment for five days with 100 μ M 8-CPT-2'-O-Me-cAMP or 100 μ M 8-CPT-cAMP.

FIGURE 4. Cyclic AMP/Epac-dependent p38 activation requires geranylgeranylation. NS-1 cells were pretreated with farnesylation inhibitor FTS-A or the geranylgeranyltransferase I inhibitor GGTI-298, followed by stimulation with either (10 μ M) forskolin (*A*) or 100 μ M 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.

FIGURE 5. Activation of Rap2A requires geranylgeranylation. *A*, Profiling of expression 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 μ M FTS-A for 30 minutes followed by treatment with 100 μ M 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 (RBD) 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 μ M GGTI-298 or 10 μ M FTS-A followed by treatment with 8-CPT-2'-O-Me-cAMP (100 μ M) 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 ratio of total Rap and data were analyzed by ANOVA and Bonferroni-corrected t-tests, *** $P < 0.001$ compared to controls. *F*, Measurements of active and total Rap1 following the same treatments used in *D* and *E*. *G*, Quantification of 3 independent experiments.

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 five days in 96-well plates in the absence or presence of 100 nM PACAP-38 with or without the Epac inhibitor ESI-09 (10 μ M) or the p38 inhibitor SB239063 (10 μ M). Five images per well were acquired every 24 hours 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 (20x objective) obtained after five 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 five days in 96-well plates in the absence or presence of 100 μ M 8-CPT-cAMP (8-CPT) in the absence or presence of either the Epac inhibitor ESI-09 (10 μ M) or the p38 inhibitor SB239063 (10 μ M). Five images per well were acquired every 24 hours 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 (20x objective) obtained after five days of treatment.

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 μ M 8-CPT-cAMP (pan-specific) or 100 μ M 8-CPT-2'-O-Me-cAMP (Epac-specific). Cells were imaged every 24 hours and cell number was determined for each set of images. Data points represent means from quadruplicate determinations and error bars are standard errors of the mean. *B*, Representative phase-contrast 20x photomicrographs acquired after treatment for five days.

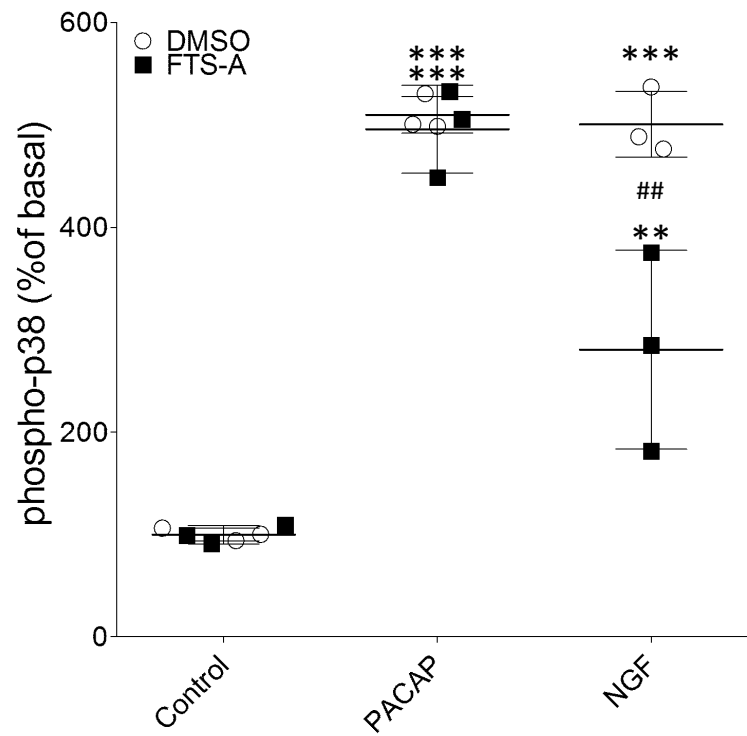
FIGURE 9. Rap2A is necessary for Epac-dependent p38 activation. *A*, Measurements of Rap2A protein levels in four NS-1 cell lines generated to stably express either scrambled shRNA or shRNAs targeting various regions of RAP2A-encoding mRNA. Lane 1, NS-1 cells expressing RAP2A-targeting shRNA; Lane 2, NS-1 cells expressing shRAP2A-1; Lane 3, NS-1 cells expressing shRAP2A-2; Lane 4, NS-1 cells stably expressing shRAP2A-3. *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 minutes. Data points are means from three experiments performed in duplicate with error bars representing the S.E.M. *D*, P38 phosphorylation in NS-1 cells expressing either scrambled shRNA or shRNA targeting RAP2A following treatment with NGF (100 ng/ml) for 30 minutes. 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. 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 μ M) or NGF (100 ng/ml) for five days. *C*, Representative images obtained on the final measurement shown in *A* and *B*.

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 p38 α , although minor contributions of p38 β or others cannot be ruled out.

Figure 1

A



B

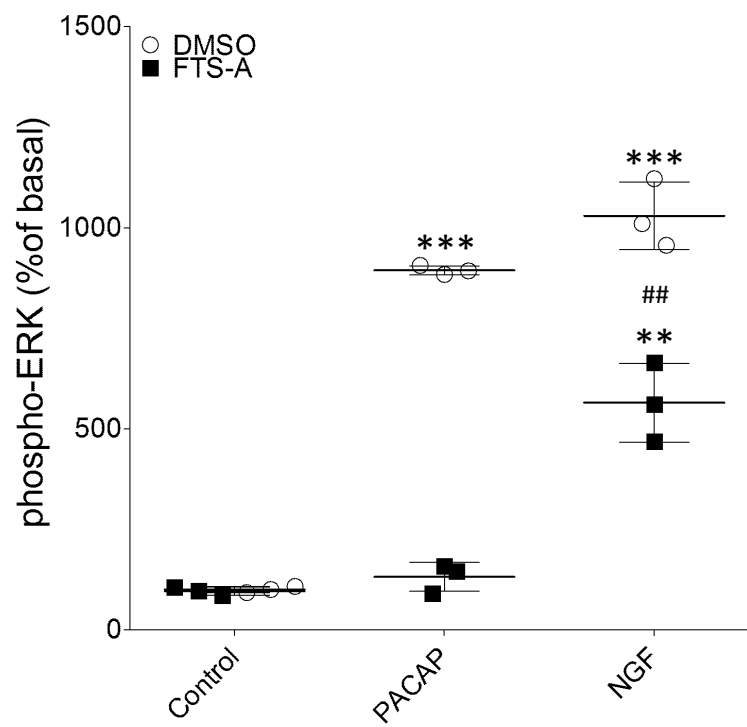


Figure 2

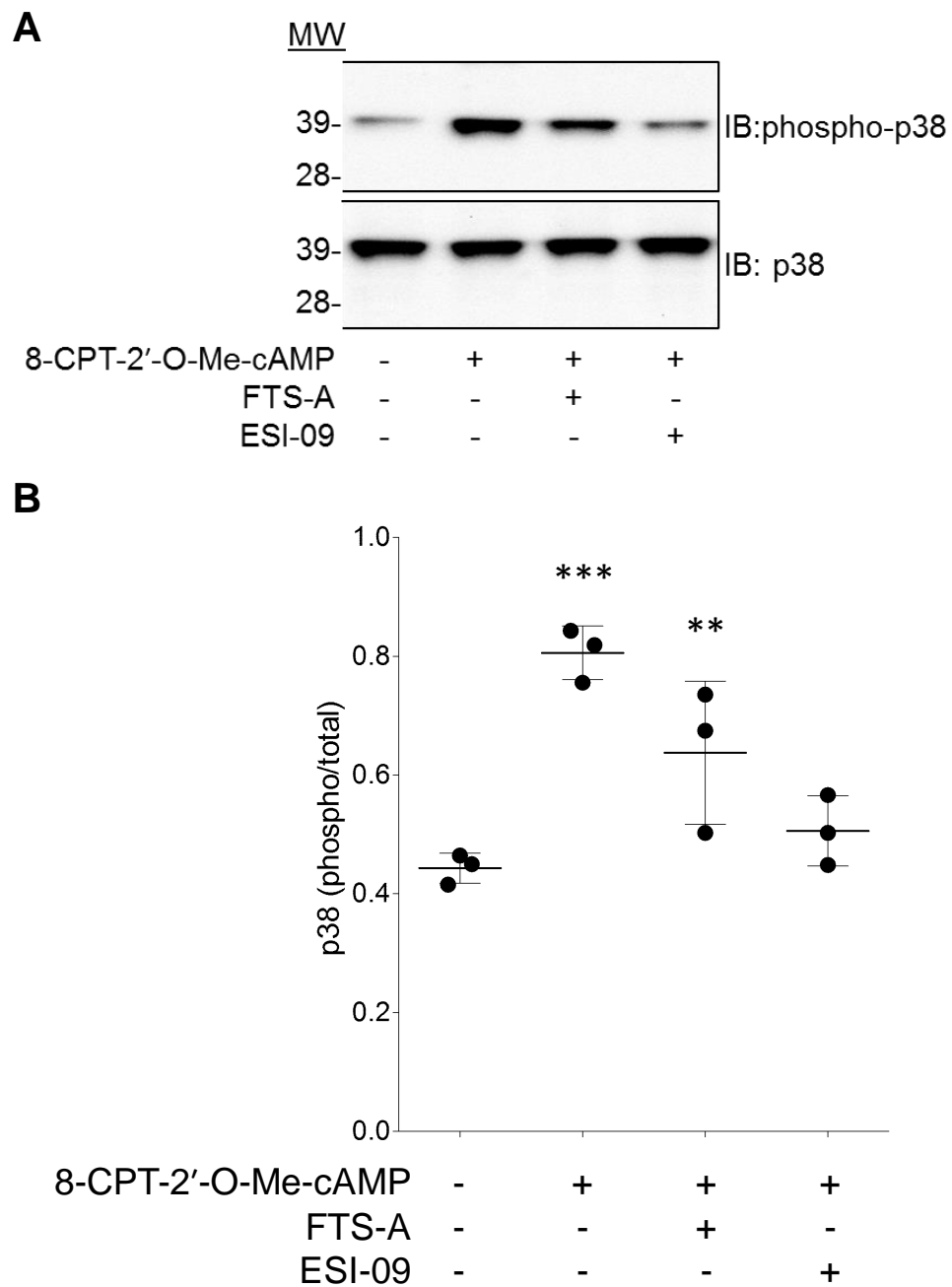


Figure 3

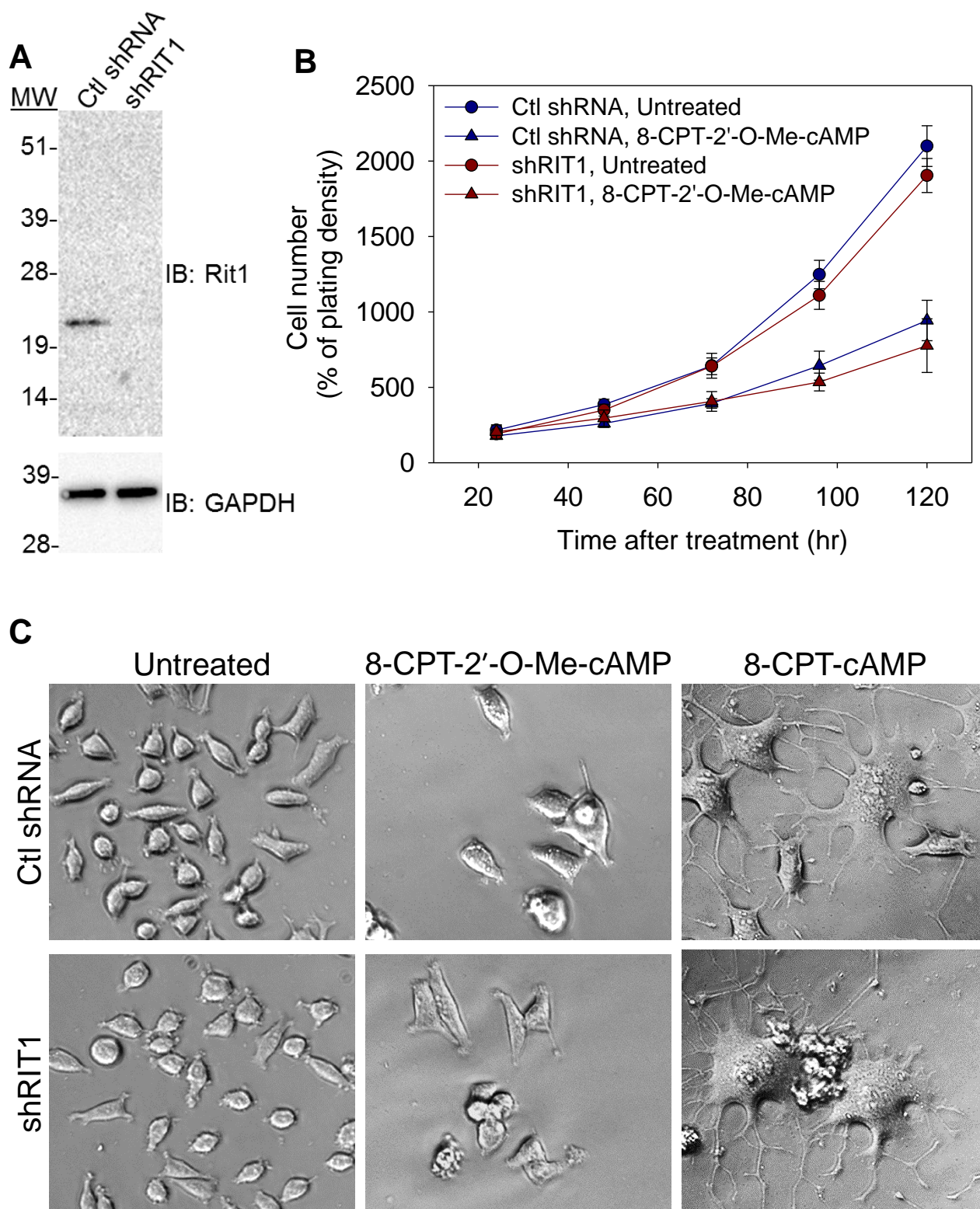


Figure 4

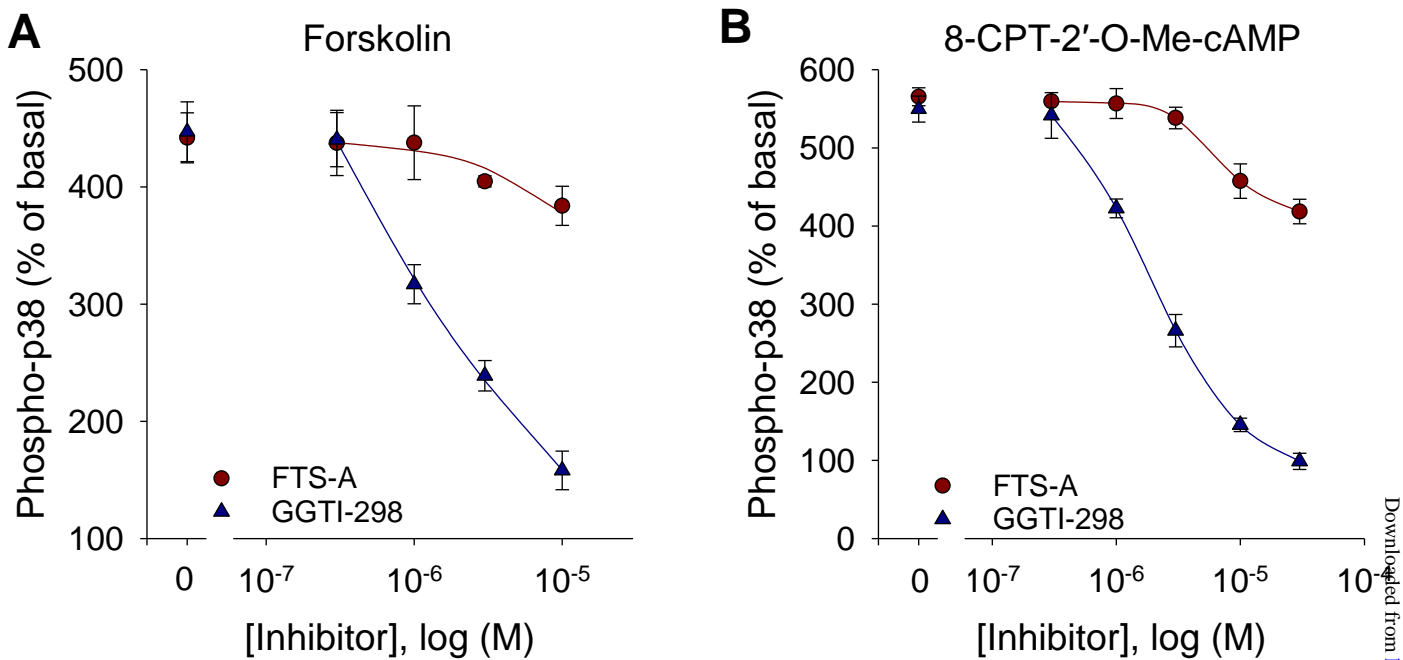


Figure 5

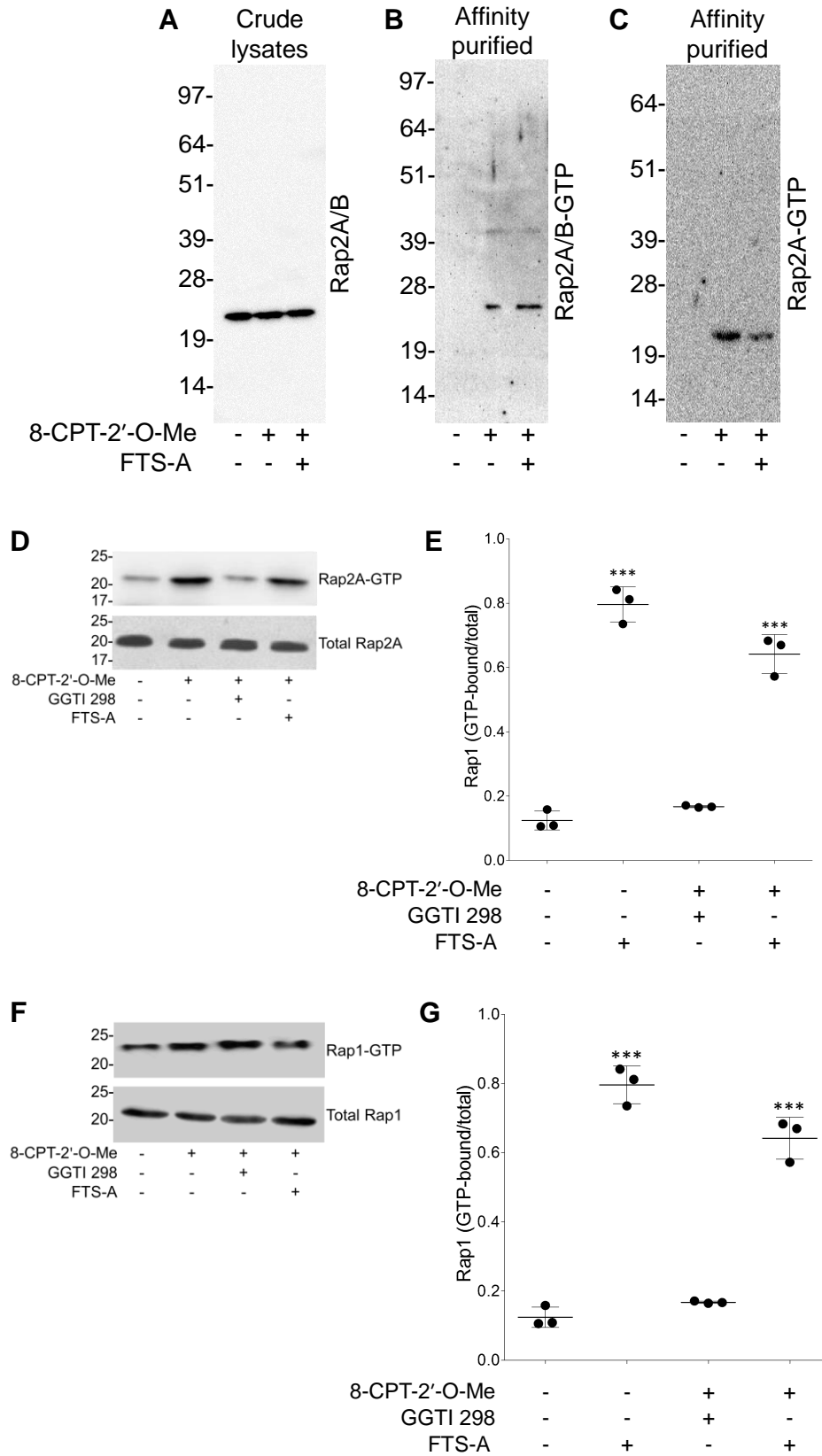


Figure 6

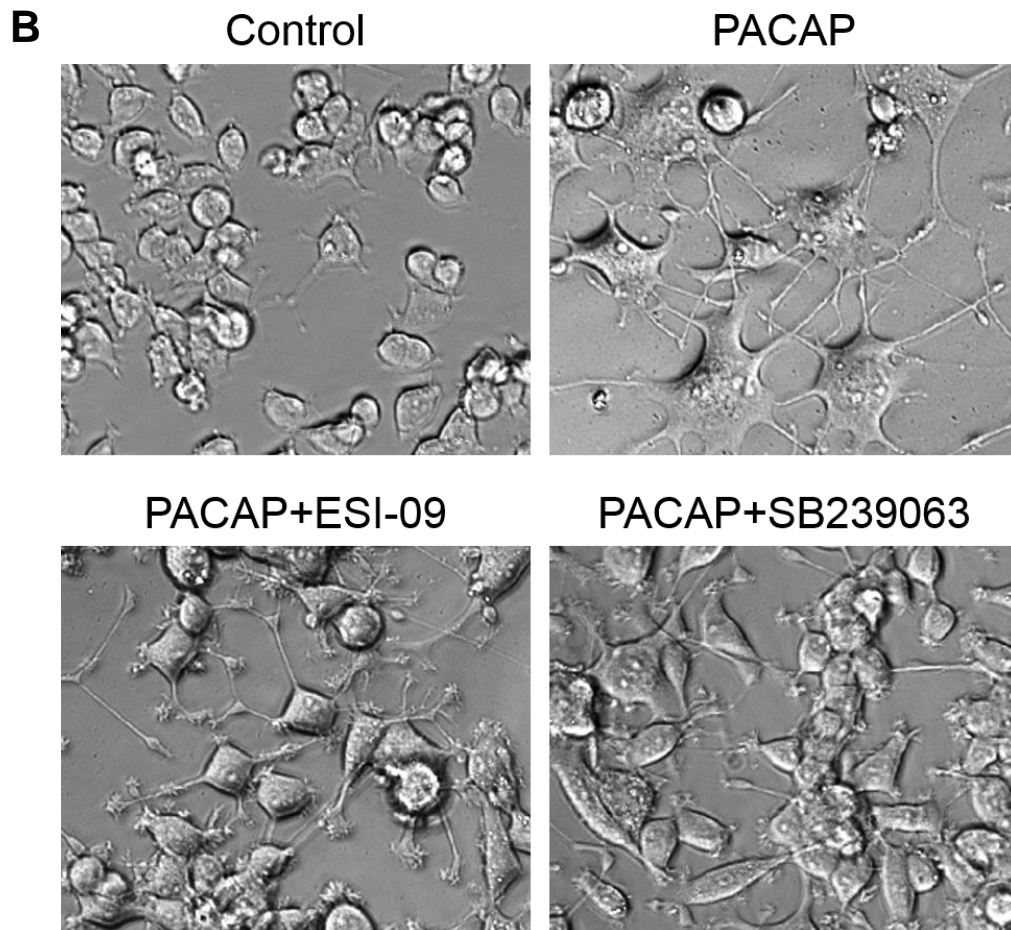
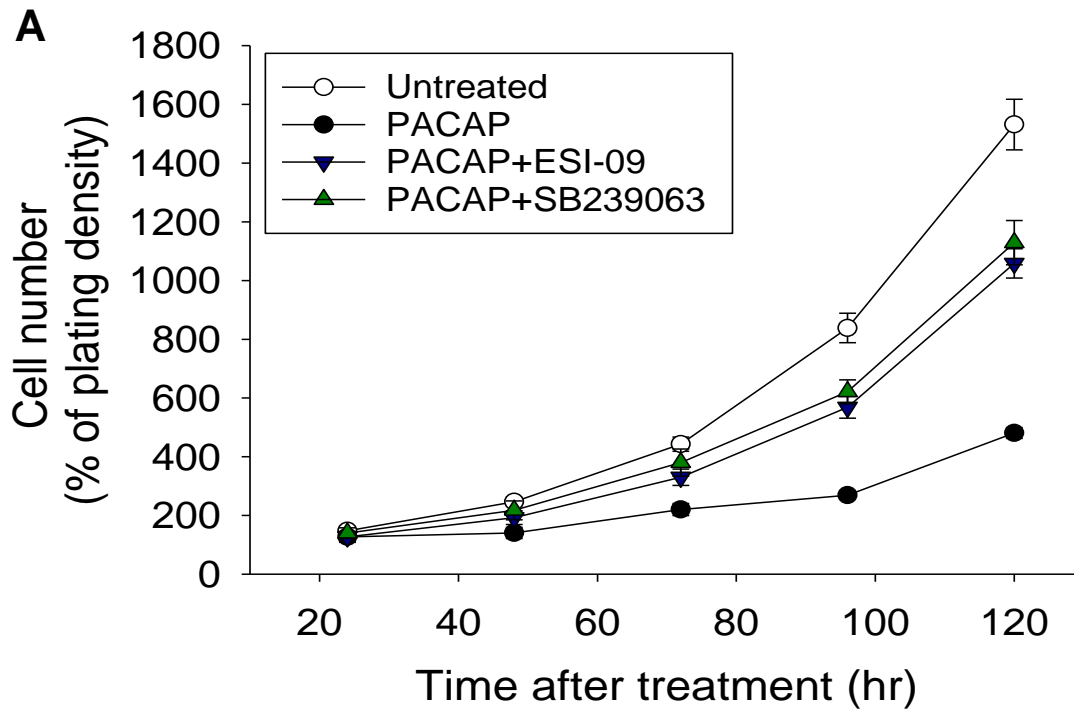


Figure 7

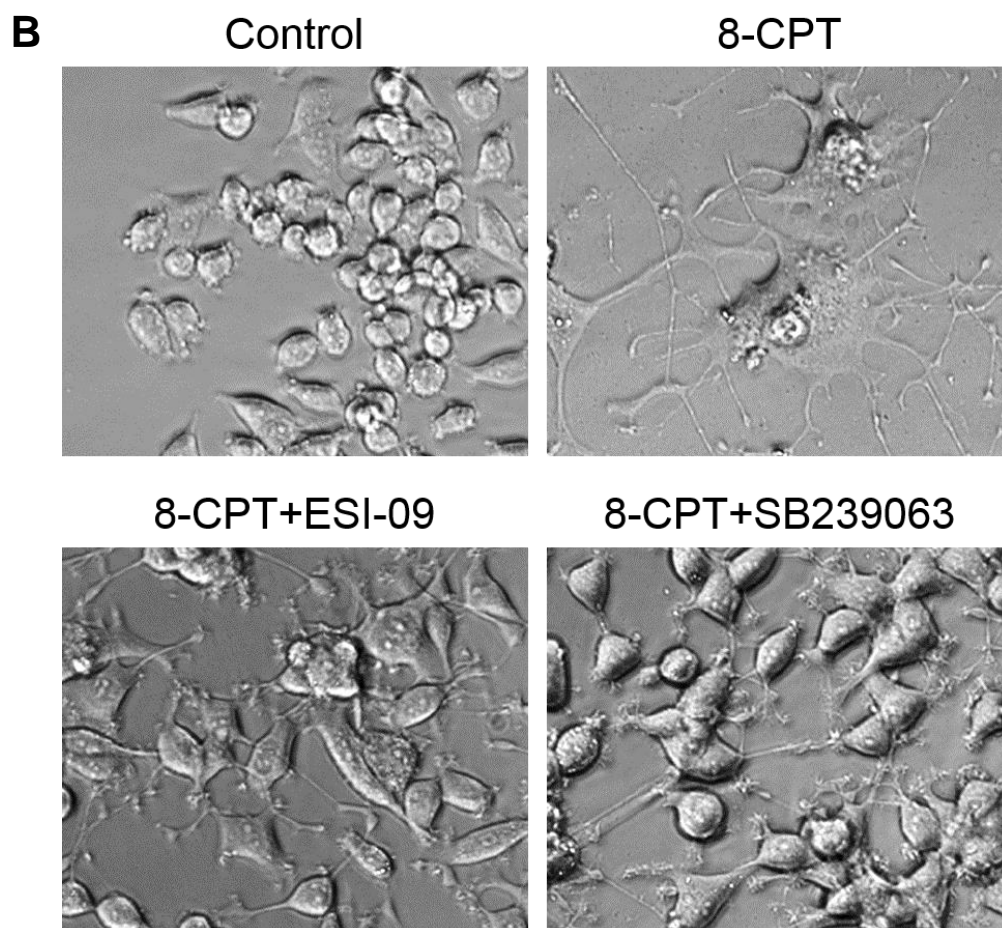
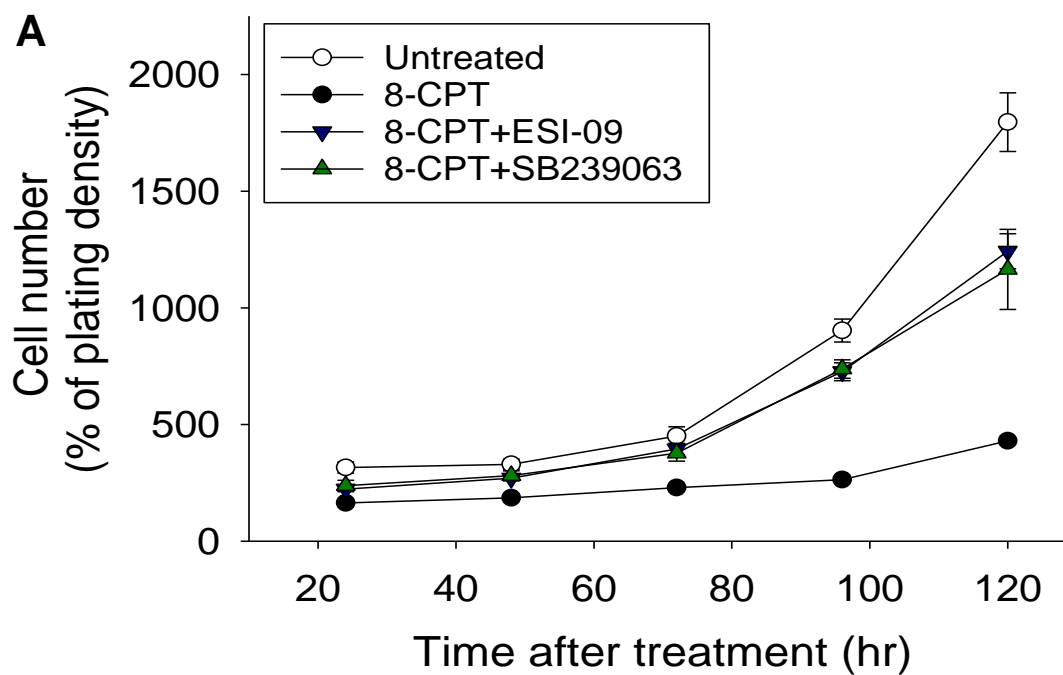


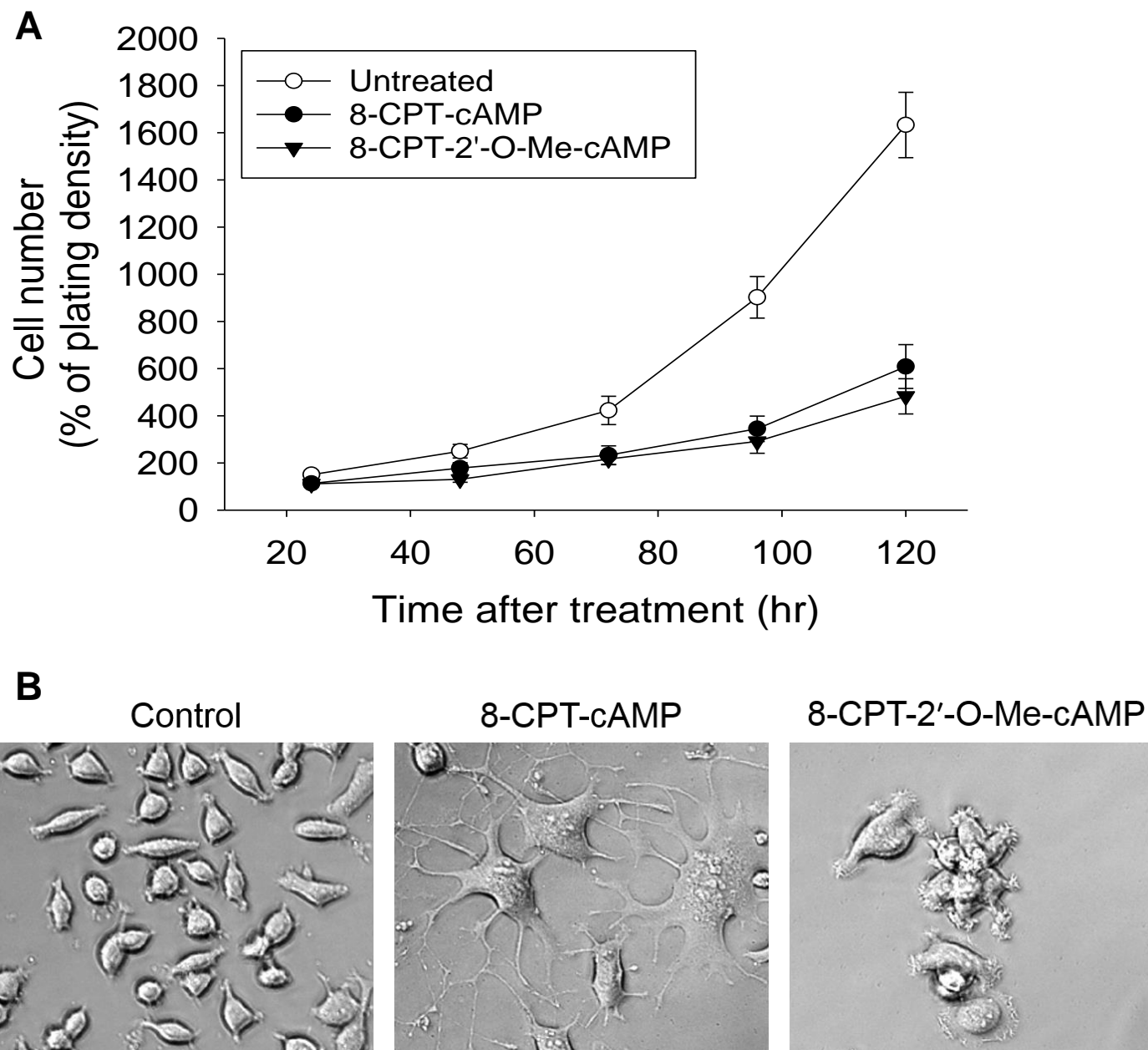
Figure 8

Figure 9

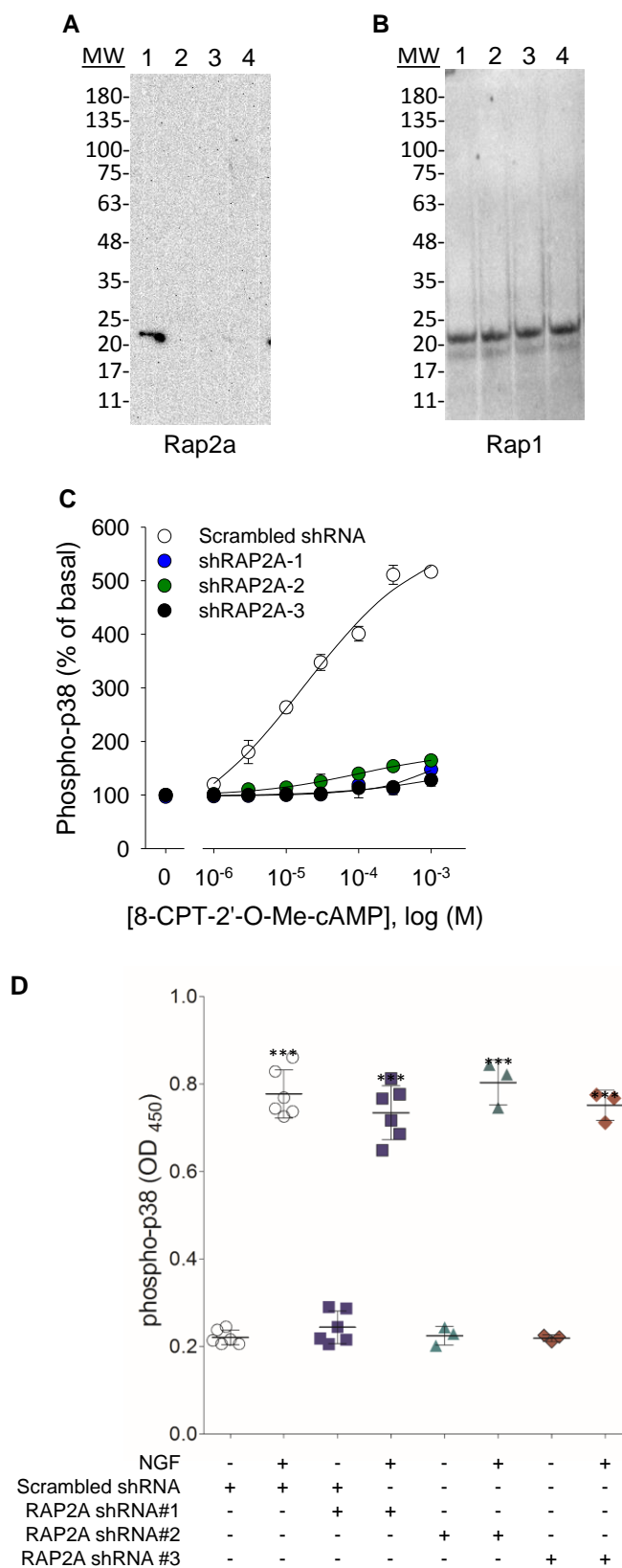


Figure 10

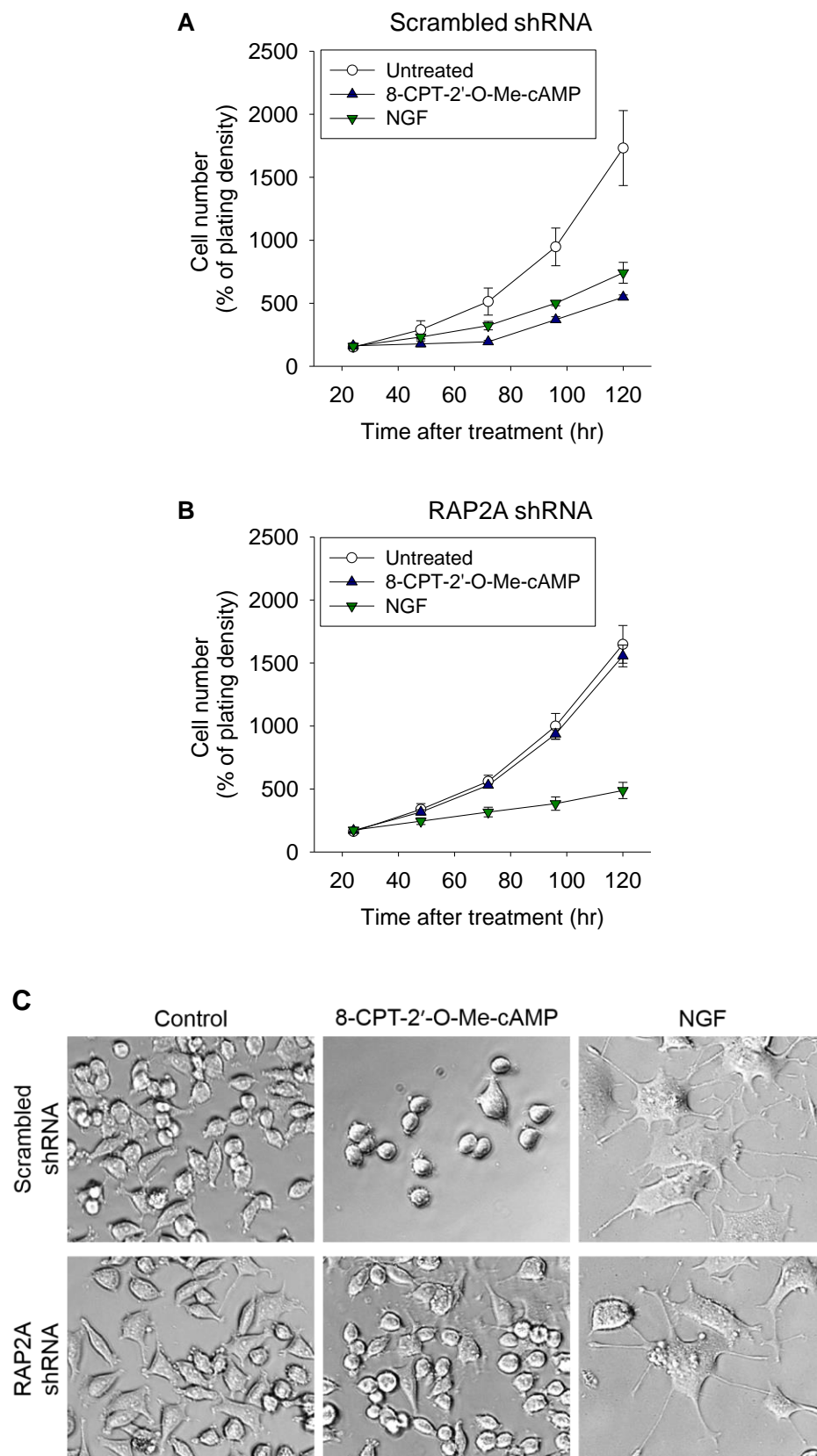
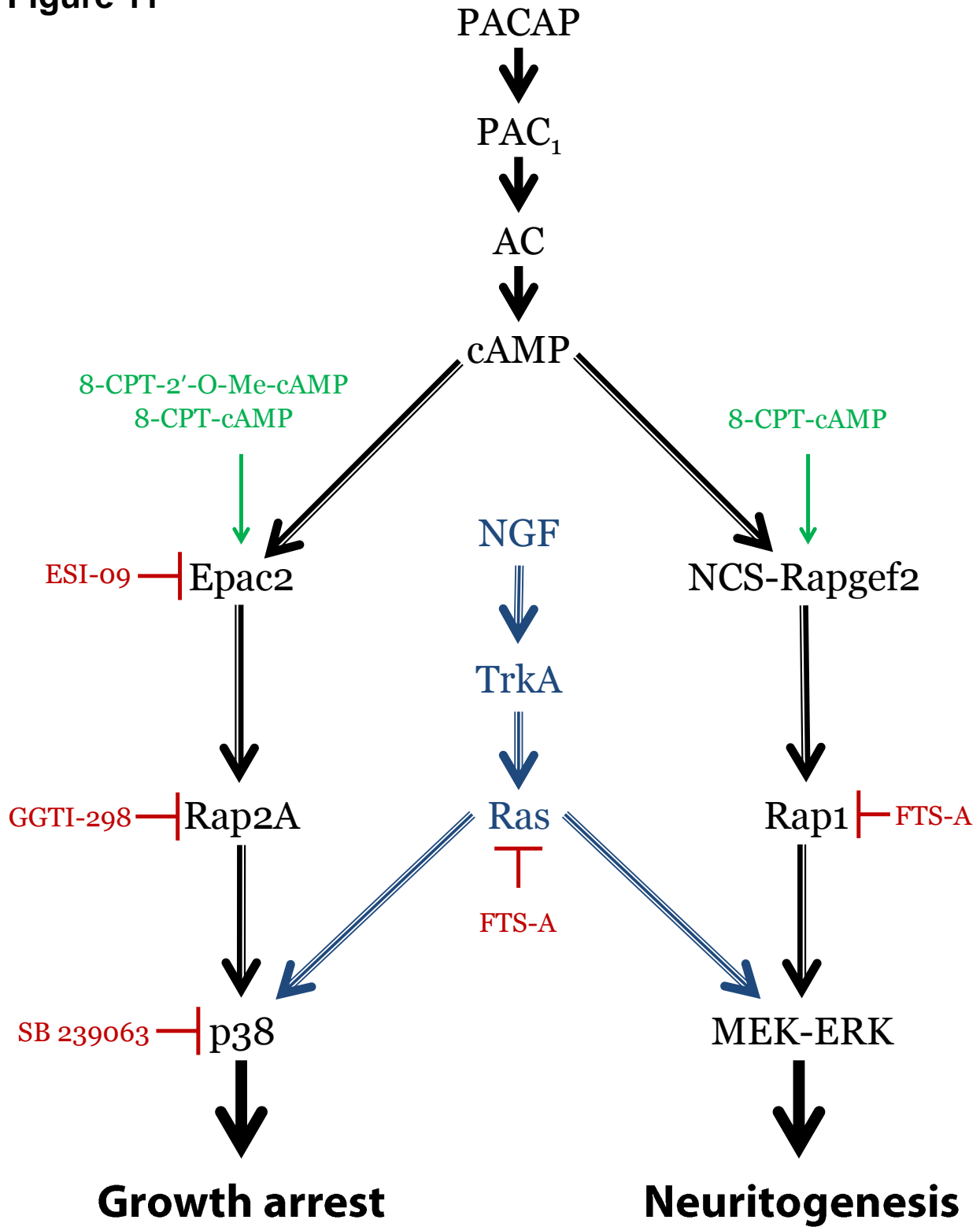


Figure 11



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

Andrew C Emery, Wenqin Xu, Maribeth V Eiden and Lee E Eiden

J. Biol. Chem. published online May 25, 2017

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