Chemistry & Biology
Article

# Identification of Biologically Active PDE11-Selective Inhibitors Using a Yeast-Based High-Throughput Screen

Ozge Ceyhan,[1] Kivanc Birsoy,[2] and Charles S. Hoffman[1,*]
[1]Biology Department, Boston College, 140 Commonwealth Avenue, Higgins Hall Room 401B, Chestnut Hill, MA 02467, USA
[2]Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, USA
*Correspondence: hoffmacs@bc.edu
DOI 10.1016/j.chembiol.2011.12.010

## SUMMARY

**The biological roles of cyclic nucleotide phosphodiesterase 11 (PDE11) enzymes are poorly understood, in part due to the lack of selective inhibitors. To address the need for such compounds, we completed an ~200,000 compound high-throughput screen (HTS) for PDE11 inhibitors using a yeast-based growth assay, and identified 4 potent and selective PDE11 inhibitors. One compound, along with two structural analogs, elevates cAMP and cortisol levels in human adrenocortical cells, consistent with gene association studies that link PDE11 activity to adrenal function. As such, these compounds can immediately serve as chemical tools to study PDE11 function in cell culture, and as leads to develop therapeutics for the treatment of adrenal insufficiencies. Our results further validate this yeast-based HTS platform for the discovery of potent, selective, and biologically active PDE inhibitors.**

## INTRODUCTION

The second messengers cyclic AMP (cAMP) and cyclic GMP (cGMP) regulate a myriad of processes such as cell proliferation, differentiation, apoptosis, inflammation, hormone secretion, muscle contraction, and cognitive functions (Bender and Beavo, 2006; Conti and Beavo, 2007). Intracellular cAMP and cGMP levels are determined by the balance between their synthesis by adenylate or guanylate cyclases and their degradation by phosphodiesterases (PDEs). In mammals, 21 genes encode ~100 PDE isoforms that are grouped into 11 families based on their substrate specificity, overall sequence conservation, and regulatory properties. The unique tissue expression and subcellular localization patterns of PDE enzymes, together with their diversity and differences in enzymatic properties, allow individual isoforms to control specific physiological functions and link them to different pathological conditions. Therefore, selective PDE inhibitors have the potential to provide therapeutic benefit to a wide range of diseases (Bender and Beavo, 2006; Conti and Beavo, 2007).

PDE11 is the most recently discovered PDE family (Fawcett et al., 2000). In humans the *PDE11A* gene encodes four isoforms that are dual-specificity enzymes, hydrolyzing both cAMP and cGMP (Fawcett et al., 2000; Hetman et al., 2000; Yuasa et al., 2000). PDE11A is expressed in skeletal muscle, prostate, testis, brain, kidney, liver, pancreas, lymphoid cells, and pituitary and adrenal glands (D'Andrea et al., 2005; Dong et al., 2010; Lakics et al., 2010; Loughney et al., 2005; Seftel, 2005), but the biological roles of PDE11 in these tissues are poorly understood due to the lack of selective inhibitors. PDE11A knockout mice display subtle alterations in sperm function (Wayman et al., 2005) and psychiatric disease-related phenotypes (Kelly et al., 2010). The absence of more dramatic phenotypes may be due to compensation by other PDEs during development that masks normal roles for PDE11.

Genetic defects of *PDE11A* have been linked to major depression, bipolar disorder, asthma, adrenal, testicular, and prostatic cancers in genome-wide association studies (DeWan et al., 2010; Fatemi et al., 2010; Faucz et al., 2011; Horvath et al., 2009; Libé et al., 2011; Wong et al., 2006). In addition, inactivating mutations of *PDE11A* are found in patients with several forms of adrenal hyperplasia and Cushing syndrome, which results from excess cortisol release from adrenocortical tumors (Boikos et al., 2008; Carney et al., 2010; Horvath et al., 2006a, 2006b; Libé et al., 2011). Adrenal tumor homogenates from these patients have elevated cyclic nucleotide levels and increased CREB phosphorylation, suggesting that PDE11 plays a major role in controlling cAMP and cGMP levels in these tissues (Horvath et al., 2006a). In addition the PDE5 inhibitor tadalafil, currently used to treat erectile dysfunction, has significant activity against PDE11 (Weeks et al., 2005). Thus, PDE11-specific inhibitors would be useful for studying the biological roles of PDE11 and in clarifying any PDE11-related side effects of this commonly prescribed drug.

We previously reported the development of a fission yeast cell-based screening platform to identify small molecule inhibitors of mammalian PDEs (Alaamery et al., 2010; Demirbas et al., 2011a, 2011b; Ivey et al., 2008). The screen employs genetically engineered yeast strains whose growth behavior reflects the activity of heterologously expressed PDEs. The cells express a *ura4* reporter that is regulated by PDE activity and is counterselectable for growth in medium containing 5-fluoroorotic acid (5FOA). Cells lacking both adenylate cyclase and PDE activity respond to low levels of exogenous cAMP or cGMP to activate PKA and, thus, repress *ura4* expression, conferring 5FOA resistance ($5FOA^R$). Cells that express PDEs that hydrolyze the exogenously added cGMP (or cAMP) remain 5FOA

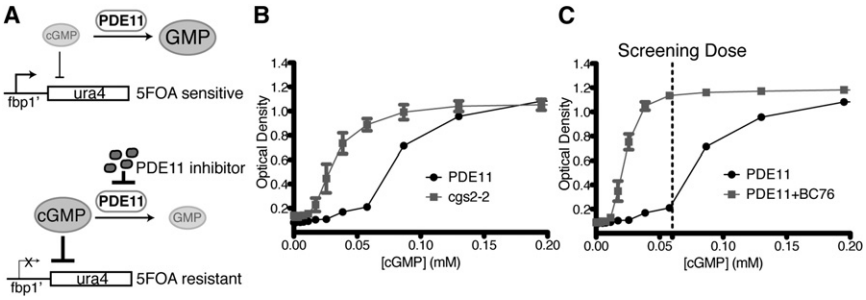



**Figure 1. Optimization of Growth Assay to Identify PDE11 Inhibitors**

(A) Screening strains possess the *fbp1-ura4* reporter, whose expression can be repressed by PKA, which is activated by the addition of cGMP to the growth medium. PDE11 hydrolysis of cGMP allows *ura4* expression, producing a $5FOA^S$ phenotype (top panel). PDE11 inhibition elevates intracellular cGMP levels to confer $5FOA^R$ growth (bottom panel).

(B) 5FOA growth assays with strains lacking PDE activity (*cgs2-2* is a frameshift allele of the only *S. pombe* PDE gene; Wang et al., 2005b) or expressing human PDE11A4 were performed in 0–2 mM cGMP.

(C) 5FOA growth assays with a strain expressing human PDE11A4 in medium containing either 0.2% DMSO or 25 μM BC76, with varying concentrations of cGMP (0–0.2 mM). The vertical line indicates the cGMP concentration that produces the maximum difference in average OD between the DMSO-treated and BC76-treated cultures. Values are the mean of three experiments (with three wells per condition in each experiment) ± SEM.

See also Figure S1.

sensitive ($5FOA^S$; Figure 1A, top panel), whereas the addition of a PDE inhibitor confers $5FOA^R$ growth (Figure 1A, bottom panel). Using strains that express 10 of the 11 PDE families, this yeast-based assay allows us to identify and profile PDE inhibitors. We have used this platform to identify PDE4 and PDE7 inhibitors that are biologically active in mammalian cells (Alaamery et al., 2010).

In this study we present the use of this screening platform to develop and perform a high-throughput screen (HTS) for PDE11 inhibitors. We identified four highly selective and potent compounds, as judged by in vitro enzyme assays and yeast-based growth assays. To our knowledge, these are the first PDE11-selective inhibitors to be reported. We further use these compounds to demonstrate a biological role of PDE11 in cortisol production in H295R adrenocortical carcinoma cells. One of these compounds, BC11-38, increases cAMP levels, PKA-mediated ATF-1 phosphorylation, and cortisol production in these cells. By comparing the effects of BC11-38 and structural analogs on H295R cells versus HeLa cells, which have little or no PDE11A expression, we demonstrate that the biological effects of these compounds are due to PDE11 inhibition.

## RESULTS

### Optimization and Performance of a Yeast-Based HTS for PDE11 Inhibitors

We used a fission yeast-based assay to develop and conduct a HTS for PDE11 inhibitors. The screening strain was generated by replacing the open reading frame of the only *S. pombe* PDE gene, *cgs2*$^+$, with a human *PDE11A4* cDNA, via homologous recombination (Demirbas et al., 2011b). Compared with cells that lack PDE activity, cells expressing PDE11A4 require more cyclic nucleotide in the growth medium to achieve $5FOA^R$ (Figure 1B; Figure S1 available online), indicating that the expressed enzyme is functionally active in yeast. Because expression of PDE11A4 creates a greater increase in the amount of exogenous cGMP (Figure 1B) than cAMP (Figure S1) required to promote growth, HTSs for PDE11A4 inhibitors were carried out in the presence of cGMP.

We next optimized the growth and assay conditions for the detection of PDE11 inhibitors, using a previously identified nonselective PDE inhibitor, BC76 (Demirbas et al., 2011b), as a positive control. The optimal preassay growth conditions, initial cell density, and cGMP concentration in the screening medium that confer saturated growth only in the presence of BC76 were determined. A total of 60 μM cGMP allows cells to grow to saturation (optical density [OD] ~1.1) after 48 hr at 30°C in the presence of 25 μM BC76 but fails to promote growth of PDE11-expressing cells in the absence of BC76 (OD ~0.2; Figure 1C).

In the HTS, PDE11-expressing cells were grown in 5FOA medium for 48 hr in the presence of ~20 μM test compounds, and growth in each well was assessed by measuring OD. Robustness of the screening conditions was determined by a Z′ factor analysis. The optimized screening conditions produced Z′ factors of 0.7–0.9, indicative of a robust screen. A total of 198,382 compounds were screened in duplicate in the primary screen. The Z scores of the duplicate wells are shown in Figure 2A. Candidate "hit" compounds were defined based on their composite Z scores, which reflect both the level of growth stimulation and the reproducibility of the replicate assays (Figure 2B). The composite Z scores for BC76 positive control wells ranged from 26 to 148. Test compounds that promoted significant $5FOA^R$ growth were grouped by their composite Z scores as strong (>35), moderate (26–35), or weak (20–26) hits (Figure S2).

### Selection of PDE11-Specific Inhibitors

The process by which we analyzed 198,382 compounds to identify 4 PDE11-specific inhibitors is presented in Figure 3. Data from our previous screens for inhibitors of PDE8 (D. Demirbas and C.S.H., unpublished data), PDE4, and PDE7 (Alaamery et al., 2010) were used to identify nonselective PDE inhibitors and compounds that stimulate cell growth via PDE-independent mechanisms from among the 1,143 hit compounds in this screen (Figure 3). For confirmation of hits and to further eliminate nonspecific inhibitors, the top 595 hits based on composite Z scores (0.3% of the total number of compounds screened) were subjected to a confirmatory screen using the PDE11-expressing strain, and 2 counterscreens using PDE5- and PDE10-expressing strains (PDE5 and PDE10 are the 2 PDEs most structurally similar to PDE11). Although data from previous screens allowed us to exclude many nonselective and off-target hits, a small group of these primary hit compounds was found to

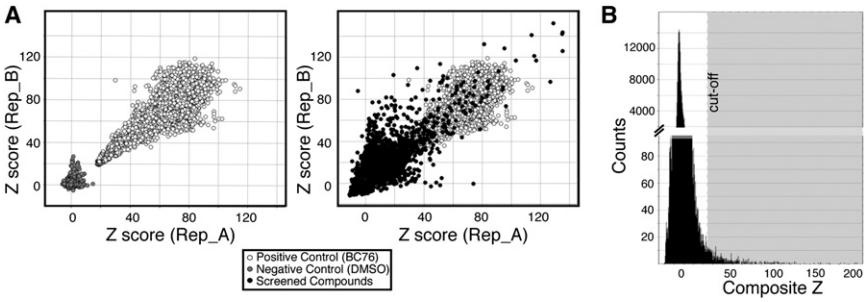



**Figure 2. HTS Data Summary**
(A) Scatterplot represents Z scores for duplicate wells pinned with 0.2% DMSO (negative controls; gray circles), 25 μM BC76 (positive controls; white circles), or screening compounds (black circles).
(B) Composite Z score distribution of the screened compounds. The height of each bar represents the number of screened compounds displaying the corresponding composite Z score in the x axis. The x axis is binned into 537 bins with intervals of 0.4. Compounds with composite Z scores >20 are defined as hits.
See also Figure S2.

inhibit PDE5 and PDE10 in the counterscreens (Figure S3). From the 99 compounds that promoted growth of the PDE11-expressing strain but not the PDE5- or PDE10-expressing strains, 39 lead candidates were selected for secondary assays, based on their potency and selectivity. In addition only the most effective one or two members of a set of compounds that share a large substructure were studied.

The 39 lead compounds were characterized by 5FOA growth assays using yeast strains that express PDEs representing 10 of the 11 mammalian PDE families (with the exception of PDE6) and in a PDE11 in vitro enzyme assay. Compounds that promoted growth of only the PDE11-expressing strain in the yeast growth assays and had $IC_{50}$ values <2 μM against PDE11 were further tested using in vitro enzyme assays against PDEs representing the other 10 PDE families (PDE1–PDE10). In this manner we identified four highly selective and potent PDE11 inhibitors. In 5FOA growth assays these compounds stimulate growth of the PDE11-expressing strain at <10 μM, whereas having little to no effect on any of the strains expressing other PDEs at ≤100 μM (Figure 4A; data from exposure to >30 μM compound are not shown to avoid compression of data at low compound concentrations). They display $IC_{50}$ values ≤330 nM for PDE11 and >100-fold selectivity for PDE11 relative to other PDEs as judged by in vitro enzyme assays, with the exception of BC11-19 that is only 30-fold selective for PDE11 relative to PDE1 (Figure 4B). To our knowledge, these compounds are the first identified PDE11-selective inhibitors.

## PDE11-Selective Inhibitors Elevate cAMP Levels and Cortisol Production in Adrenocortical Cells

PDE11 is expressed in adrenal glands, and PDE11-inactivating mutations, as well as elevated cAMP levels, have been identified in patients with adrenocortical tumors and Cushing syndrome, a condition resulting from excess cortisol release from adrenal tumors (Boikos et al., 2008; Carney et al., 2010; Horvath et al., 2006a, 2006b). To test whether phenotypes associated with PDE11 inactivation in Cushing syndrome (i.e., elevated cAMP and cortisol levels) could be mimicked by treatment of adrenocortical cells with our PDE11-specific inhibitors, we examined the effect of these compounds on H295R human adenocarcinoma cells (Rainey et al., 1994). We found that BC11-38 significantly increased cAMP levels and cortisol production in H295R cells, both in the absence and presence of the adenylate cyclase activator forskolin (Figure 5). As a control, the nonselective PDE inhibitor IBMX (500 μM) was used to eliminate all PDE activity, with the exception of PDE8 and PDE9, in order to assess the relative importance of PDE11 on cAMP hydrolysis in these cells. These data suggest that PDE11 represents a significant proportion of the cAMP PDE activity and has a major role in regulating cortisol production in H295R cells. Because the $IC_{50}$ values for IBMX against various PDEs range from 2 to 50 μM (Bender and Beavo, 2006; Conti and Beavo, 2007), we used 500 μM IBMX to measure the effect of near-complete inhibition of all PDEs with the exception of PDE8 and PDE9. However, at this concentration, off-target effects of IBMX treatment may have prevented

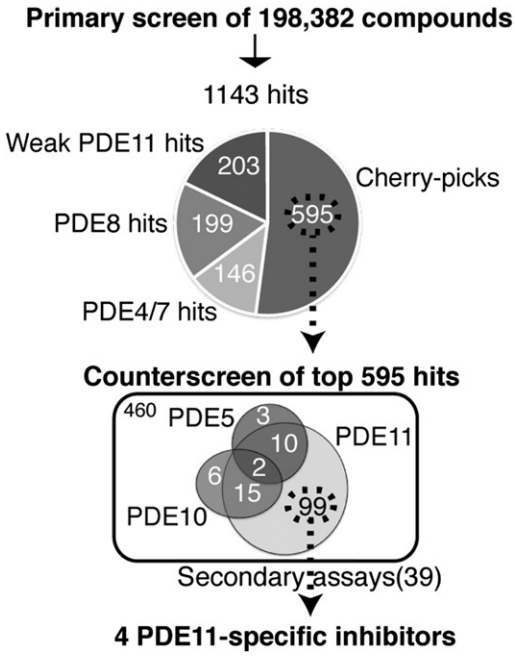
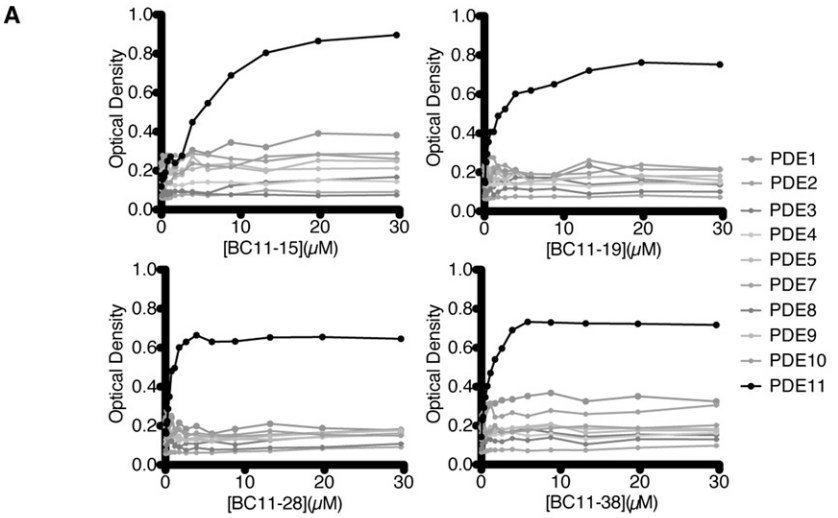



**Figure 3. Overview of the Processing of Initial Hit Compounds**
Of 198,382 compounds screened, 1,143 hits were considered for further characterization. Compounds that were also detected as hits in our previous PDE8, PDE4, and PDE7 screens were excluded, as indicated in the pie chart. Of the remaining 798 compounds, the most effective 595 candidates (cherry-picks; as determined by composite Z score) were rescreened using the PDE11-expressing strain and counterscreened using PDE5- and PDE10-expressing strains. A total of 99 compounds conferred $5FOA^R$ growth in the rescreen using the PDE11-expressing strain, but not in the counterscreens. The most selective and potent 39 compounds were acquired and tested in secondary assays (a full panel of in vitro enzyme assays and 5FOA growth assays), leading to the identification of 4 PDE11-specific inhibitors. See also Figure S3.




B

| | BC11-15 | BC11-19 | BC11-28 | BC11-38 |
|---|---|---|---|---|
| MW | 404.5 | 295.3 | 394.4 | 304.4 |
| $IC_{50}$ (μM) PDE11 | 0.18 | 0.33 | 0.11 | 0.28 |
| PDE1 | >100 | 10 | >100 | >100 |
| PDE2 | >100 | >100 | >100 | >100 |
| PDE3 | >100 | 51 | >100 | >100 |
| PDE4 | 70 | >100 | >100 | >100 |
| PDE5 | 43 | >100 | >100 | >100 |
| PDE6 | 25 | >100 | >100 | >100 |
| PDE7 | 43 | >100 | >100 | >100 |
| PDE8 | >100 | >100 | >100 | >100 |
| PDE9 | >100 | >100 | >100 | >100 |
| PDE10 | 35 | >100 | >100 | >100 |

**Figure 4. Four Potent and Selective PDE11 Inhibitors Identified by the HTS**

(A) Profiling of PDE11-specific inhibitors in yeast growth assays. Four PDE11-selective inhibitors were profiled using strains expressing 10 of the 11 PDE families. Dose-response growth curves in the presence of ≤30 μM compound from one representative (with duplicate wells for each data point) of triplicate experiments are presented.

(B) Structures and $IC_{50}$ values of PDE11-specific inhibitors in in vitro enzyme assays. Because the assays were carried out at substrate concentrations of one-tenth the $K_M$ of the enzymes, the $IC_{50}$ values approximate the $K_i$ values for these compounds. MW, molecular weight.

cells from increasing cortisol production. PDE11 inhibitors were used at 20 μM, the maximal effective concentration in these assays. The failure to elevate cAMP and cortisol levels by BC11-15, BC11-19, and BC11-28 suggests that these compounds either fail to enter the cells due to poor solubility in tissue culture media or have a detrimental effect on cells. Consistent with this idea, compounds BC11-15 and BC11-28 have high LogP values (>4.7), suggesting that they may be too lipophilic for these assays. Alternatively, the effect of BC11-38 may be due to an off-target activity.

### Biological Effects of BC11-38 and Related Compounds Are Due to PDE11 Inhibition

To address the possibility that BC11-38 elevates cAMP levels and cortisol release by H295R cells in a PDE11-independent manner, we analyzed four structural derivatives of BC11-38 that differ by a single methyl or methoxy group. The potency of these compounds against PDE11 (Figure 6A) is consonant with their ability (at 20 μM) to elevate cAMP levels and cortisol production (Figure 6C) in H295R cells. Transcription of genes encoding several key enzymes involved in cortisol production by adrenocortical cells is activated by the cAMP-response element (CRE)-binding proteins (Almeida and Stratakis, 2011; Stratakis, 2009). Because ATF-1 and CREM are the major proteins that bind the CRE in H295R cells, which lack CREB, and are phosphorylated by PKA in response to elevated cAMP levels to activate transcription, we analyzed the phosphorylation state of ATF-1 in H295R cells (Groussin et al., 2000; Rosenberg et al., 2002; Wang et al., 2000). BC11-38 and its derivatives elevate ATF-1 phosphorylation in a manner that corresponds with their potency against PDE11 (Figure 6B).

To further test whether the biological activity of BC11-38 and related compounds is due to PDE11 inhibition, we examined *PDE11A* transcription in a group of cell lines, finding very low levels of *PDE11A* mRNA in several lines including HeLa and MDA-MB-231 cells (Figure 6D). Consistent with PDE11 inhibition as the primary effect of these compounds, neither BC11-38 nor its derivatives affect cAMP levels or CREB phosphorylation in HeLa cells (Figure 6E). Similarly, BC11-38 fails to elevate cAMP levels and CREB phosphorylation in MDA-MB-231 cells (Figure S4). These results suggest that the biological effects of BC11-38 and related compounds in adrenocortical cells are due to PDE11 inhibition.

## DISCUSSION

Increasingly, yeast-based HTSs are being used to discover compounds for the study and treatment of human disorders (Couplan et al., 2011; Marjanovic et al., 2010). Here, we demonstrate the use of a fission yeast-based screen to identify potent and selective human PDE11 inhibitors. One inhibitor discovered in our screens, BC11-38, along with two derivatives, elevates cAMP levels and cortisol production in adrenocortical cells in a PDE11-specific manner, mimicking the phenotypes observed in Cushing syndrome. Our results demonstrate that these

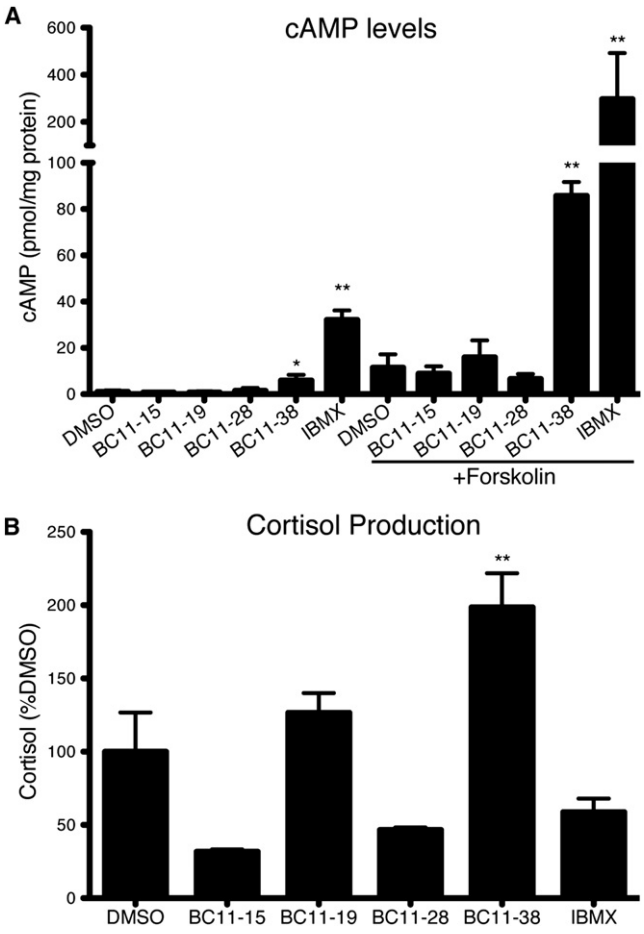



**Figure 5. PDE11-Selective Inhibitors Elevate cAMP Levels and Cortisol Production in H295R Adrenocortical Cells**

(A) cAMP levels of H295R cells following a 2 hr treatment with PDE11-specific inhibitors (20 μM) in the absence or presence of 10 μM forskolin. The nonselective PDE inhibitor IBMX was used at 500 μM as a positive control.

(B) Cortisol release by H295R cells following a 24 hr treatment with PDE11-specific inhibitors (20 μM) or IBMX (500 μM) in the presence of 10 μM forskolin. Data are presented as percentage (%) of forskolin plus DMSO-treated cells. Values represent the averages of at least two independent experiments for each assay performed in duplicate ± SEM (*$p < 0.05$, **$p < 0.01$; as determine by one-way ANOVA).

compounds could serve immediately as useful research tools to study the biological roles of PDE11 in mammalian cells.

This screening platform has several features that aid in the identification of biologically active PDE inhibitors. First, the target PDEs are full-length proteins expressed in eukaryotic cells, requiring inhibition to occur in the protein-dense yeast cytosol, which resembles the human cytoplasmic environment. Second, compounds are detected by their ability to stimulate cell growth, so that the identified compounds must be cell permeable, chemically stable during the 48 hr growth period, and nontoxic to *S. pombe*. This last feature is a proxy for high specificity because compounds that promiscuously bind proteins would likely retard or inhibit growth. Other favorable features of this screen include the ability to detect both active site and allosteric inhibitors and the use of a simple readout to rapidly screen large compound libraries. This 384-well format assay allowed us to screen ~200,000 compounds, which is larger than any previously published yeast-based HTS, to our knowledge. Our ability to identify potent PDE inhibitors that are biologically active in mammalian cells in this and other HTSs confirms that *S. pombe* is well suited for chemical screening. Using strains that express 10 of the 11 PDE families, we were able to profile inhibitor specificity and eliminate the majority of nonselective compounds prior to performing in vitro enzyme assays. Similarly, future PDE inhibitor screens will benefit from the database developed from this and previous screens to filter out nonselective PDE inhibitors and compounds that act in a PDE-independent manner.

Although the overall frequency of strong and moderate hits in this screen was 0.36%, this frequency varied significantly among the compound libraries (Figure S2A). Of the large libraries screened, the highest frequencies of hits were observed in the Actimol TimTec 1 (0.8%; 68 hits from 8,518 compounds), Chembridge 3 (0.7%; 74 hits from 10,560 compounds), and ChemDiv 6 (0.64%; 283 from 44,000 compounds) libraries. In contrast the lowest frequency of hits was observed in the ChemDiv 1 library (0.04%; 6 hits from 16,544 compounds), which may reflect the fact that this was one of the oldest libraries screened, and repeated freezing and thawing during previous screens may have adversely affected these compounds. Interestingly, the four compounds presented in Figure 4 came from four different libraries produced by four different companies (BC11-15, Enamine T0515-1965; BC11-19, Maybridge BTB 12009; BC11-28, ChemDiv K405-0344; BC11-38, Life Chemicals F0579-0060).

One unexpected result was that many of the compounds identified in the initial screen were not validated as PDE11 hits in cherry-picking experiments. This is likely due to the different delivery method because the cherry-pick screens use pocket tips rather than the steel pin arrays used in the primary screens. It appears that the pocket tip method of introducing compounds to microtiter dishes fails to allow some compounds to dissolve in the growth medium. Thus, selection of only validated hits for secondary assays may have excluded compounds with poor solubility in growth medium.

PDE11A-inactivating mutations were suggested as predisposing factors in the development of adrenocortical hyperplasia (Horvath et al., 2006a). In these tumors, elevated cAMP levels and increased CREB phosphorylation result in excess cortisol release, leading to Cushing syndrome. Here, we confirm the role of PDE11 in cortisol production by adrenocortical cells, using selective inhibitors. We observe the disease phenotypes linked to inactivating mutations in the *PDE11A* gene using pharmacological inhibitors of the enzyme in adrenocortical cells, thus phenocopying a genetic disorder using small molecules. This directly demonstrates a link between the loss of PDE11 function with phenotypes related to adrenal hyperplasia and Cushing syndrome.

Among the four initial PDE11-specific inhibitors, one compound, BC11-38, along with two derivatives, significantly elevates cAMP and cortisol levels in H295R cells. The lack of this effect by the other compounds does not argue against the PDE11-specific effect of BC11-38 because it is most likely related to problems with entry of the compounds into mammalian cells, either due to poor solubility or being bound up by

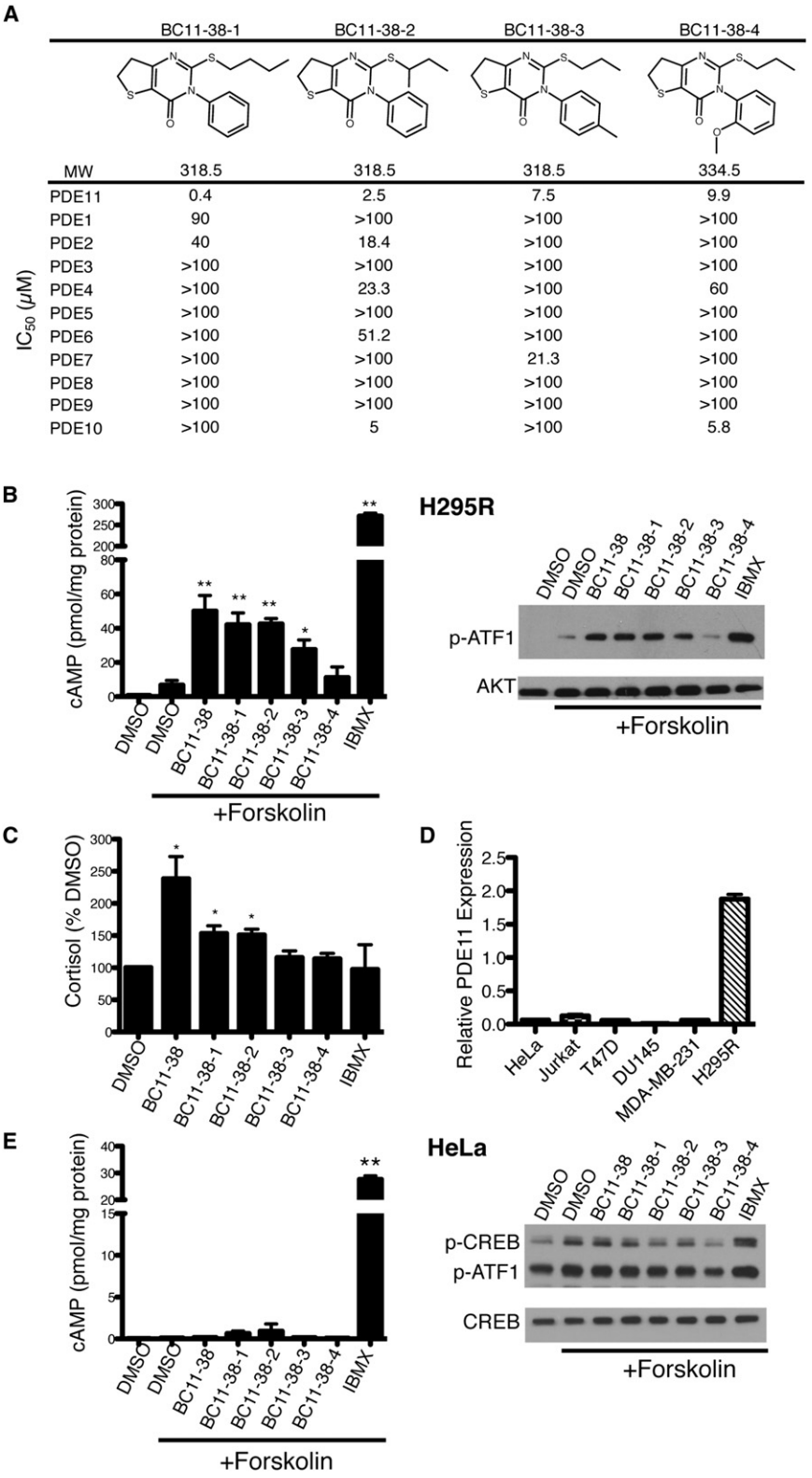



**Figure 6. Biological Activities of BC11-38 and Derivatives Correspond with Their Potency for PDE11 Inhibition**

(A) Structures and $IC_{50}$ values of BC11-38 derivatives in in vitro enzyme assays. MW, molecular weight.

(B) cAMP levels (left) and ATF-1 phosphorylation (right) in H295R adrenocortical cells following a 2 hr treatment with BC11-38 and derivatives (20 μM) in the presence of 10 μM forskolin. Cells were lysed, and proteins were subjected to immunoblotting with a p-CREB/ATF-1 antibody.

(C) Cortisol release by H295R cells following a 24 hr treatment with BC11-38 and derivatives (20 μM) or IBMX (500 μM) in the presence of 10 μM forskolin. Data are presented as percentage (%) of forskolin plus DMSO-treated cells.

(D) Semiquantitative RT-PCR for *PDE11A* mRNA in various cell lines. *PDE11A* expression was normalized to the expression level of the *RPLP0* reference gene.

(E) BC11-38 and related compounds do not increase cAMP levels or CREB phosphorylation in HeLa cells. Compound treatment, cAMP assays, and immunoblots were performed as described for H295R cells. Values represent the averages of three separate experiments for each assay performed in duplicate ± SEM (*$p < 0.05$, **$p < 0.01$; determined by one-way ANOVA).

See also Figure S4.

components of the growth media. The 5FOA growth media used in the yeast-based assays are more acidic (pH ~5.0) than tissue culture media (pH ~7.0), which might contribute to the differences in the solubility of the compounds in yeast-based versus mammalian assays. Indeed, compound crystals were observed in the cell culture media in wells containing the three ineffective compounds, indicating solubility problems. Due to their biological activity as well as their biochemical potency and specificity, BC11-38 and BC11-38-1 represent a logical starting point for medicinal chemistry approaches to enhance potency, specificity, and pharmacokinetic properties, to develop compounds suitable for whole-animal studies of PDE11 function. Such compounds could produce an acute loss of PDE11 activity as a way of identifying biological roles for PDE11, which might be overlooked in knockout mouse studies due to compensation of activity by other PDEs or developmental alterations caused by an early loss of PDE11 activity. These compounds could also serve as therapeutic candidates to treat adrenal insufficiencies that lead to cortisol deficiency, such as Addison's disease. This study serves as a "proof of principle" that the yeast-based platform described here can be used in chemical screens to identify potent and selective PDE inhibitors that may be effective in cell culture studies even prior to medicinal chemistry efforts. Such specific compounds

will be powerful tools to enhance our understanding of PDEs and to develop therapeutics for several diseases related to cyclic nucleotide signaling defects.

## SIGNIFICANCE

**The functional importance of PDE11 has been suggested via gene association studies that link it to a variety of cancers, psychiatric diseases, asthma, and Cushing syndrome. However, studies to examine its biological role in different tissues and these disease states have been hampered by the lack of selective pharmacological reagents. To our knowledge, we report the identification of the first selective PDE11 inhibitors through the use a yeast cell-based HTS of ~200,000 compounds. Compounds BC11-38, BC11-38-1, and BC11-38-2 are commercially available reagents that we have shown to be suitable for mammalian cell culture studies to evaluate PDE11 function. These compounds increase cAMP levels and cortisol production in human adrenocortical cells, mimicking the phenotypes associated with Cushing syndrome. Our results demonstrate a direct role of PDE11 in regulating cortisol production by adrenal cells, consistent with the presence of missense mutations in the *PDE11A* alleles of patients suffering from Cushing syndrome. These compounds can serve as research tools even prior to further medicinal chemistry approaches and as leads for the development of therapeutics for the treatment of adrenal insufficiencies. This study also highlights the use of the fission yeast *Schizosaccharomyces pombe* as a host for the screening of large small molecule libraries to discover potent, selective, and biologically active PDE inhibitors. Our ability to use a positive growth selection in a 384 well format suggests that other HTSs based on the repression of gene expression could be carried out in *S. pombe* using a similar approach.**

## EXPERIMENTAL PROCEDURES

### Yeast Strains, Media, and Growth Conditions

Constructions of *S. pombe* strains that express mammalian PDEs were previously described (Demirbas et al., 2011b). Yeast cells were grown and maintained using YEA-rich and EMM-defined media as described by Demirbas et al. (2011b).

### 5FOA Growth Assays, HTS, and Statistical Analysis

5FOA growth assays were performed using strains that express human PDE1B1, PDE3A1, PDE4A1, PDE7A1, PDE9A5, PDE10A1, and PDE11A4, murine PDE2A2 and PDE8A1, and bovine PDE5A1, as described (Demirbas et al., 2011b).

HTS was performed at the ICCB-Longwood Screening Facility of Harvard Medical School. Yeast cells that express human PDE11A4 were grown in EMM medium with 0.25 mM cAMP for 24 hr to ~$10^7$ cells/ml. The screening 5FOA medium is SC based and contains 0.4 g/l 5FOA. A total of 25 μl 5FOA medium was transferred into duplicate 384-well flat, clear-bottom microtiter dishes, and 100 nl of compounds (from stock solutions of generally ~10–15 mM) was pinned into the wells. Cells were collected by centrifugation, resuspended in 5FOA medium with 120 μM cGMP, and 25 μl was transferred into each well at an initial cell density of 0.75 × $10^5$ cells/ml. Control plates consisted of positive control cultures containing 25 and 40 μM BC76 and negative control cultures containing 0.2% DMSO. Each screening plate included internal positive and negative control wells. Plates were incubated at 30°C for 48 hr in a closed container with moist paper towels to prevent evaporation. ODs of the cultures were measured at 600 nm. In cherry-picking experiments, 100 nl compounds were added using pocket tips instead of steel pin arrays.

Z′ factors of assays were determined as described (Ivey et al., 2008). An assay with a Z′ factor >0.5 is considered sufficiently robust for HTS. Within a screen, individual wells are assigned a Z score, representing the number of SDs above or below the mean of the negative control wells. Duplicate Z scores for each compound are plotted onto a grid and projected perpendicularly to the diagonal. A composite Z score is the distance from this point on the diagonal to the origin.

### In Vitro Enzyme Assays

In vitro enzyme assays were conducted via the $Ba(OH)_2$ precipitation method of Wang et al. (2005a) using recombinant human PDE1C, PDE3B, PDE5A1, PDE6C, PDE8A, PDE9A2, PDE10A1, PDE11A4, rat PDE2A (BPS Bioscience), human PDE7A (BIOMOL International), and human PDE4A10 enzymes (gift from Dr. Hengming Ke). Substrate concentrations used were 100 nM cGMP (PDE1C), 1 μM cGMP (PDE2A), 30 nM cGMP (PDE3B), 625 nM cAMP (PDE4A), 500 nM cGMP (PDE5A), 1.7 μM cGMP (PDE6C), 15 nM cAMP (PDE7A), 10 nM cAMP (PDE8A), 70nM cGMP (PDE9A), 30 nM cAMP (PDE10A), and 100 nM cGMP (PDE11A). Inhibitor concentrations that reduce enzyme activity by 50% ($IC_{50}$) are presented. The values are means of at least three independent experiments. Substrate concentrations were ~0.1 × $K_M$ for each enzyme; thus, $IC_{50}$ values approximate the $K_i$ values.

### Mammalian Cell Culture, cAMP Assays, and Immunoblot Analysis

Human NCI-H295R cells were maintained as described (Rainey et al., 1993). HeLa cells were maintained in DMEM with 10% FBS. Approximately 90% confluent cells in 12-well dishes were incubated in serum-starved media for 1 hr, then in 0.5 ml serum-starved media with 20 μM compounds or 0.2% DMSO for 2 hr in the absence or presence of 10 μM forskolin (Sigma-Aldrich). Media were collected, and cAMP levels were measured as described (Rainey et al., 1993), using a cAMP ELISA kit (Enzo). cAMP levels were normalized to protein content measured using a BCA Protein Assay Kit (Pierce). Immunoblot analysis of protein lysates was performed as described (Sarbassov et al., 2006), using phospho-CREB(9198), Akt(4685), or CREB(9197) primary antibodies (Cell Signaling Technology) and goat anti-rabbit IgG-HRP secondary antibody (sc-2030; Santa Cruz Biotechnology).

### Cortisol Assays

H295R cells were treated with compounds as described in the cAMP assays, for 24 hr. Media were collected, and cortisol content was quantified using a Cortisol EIA Kit (Oxford Biomedical Research). Cortisol levels were normalized to protein concentrations in cell extracts, as described above.

### Semiquantitative RT-PCR

Total cellular RNA was isolated using RNeasy Mini Kits (QIAGEN). cDNA was synthesized from 2 μg of total RNA using SuperScript II reverse transcriptase with random hexamers. *PDE11A* expression was determined by a SYBR Green Real-Time PCR assay (ABI) using *PDE11A*-specific primers (5′-TGGAGTG GATTGATAGCATCTG-3′ and 5′-TTTGGTGTAGCTCTTCCCAC-3′). Expression levels were normalized to *RPLP0* expression (IDT).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.chembiol.2011.12.010.

## ACKNOWLEDGMENTS

We thank Caroline Shamu, David Wrobel, Jen Nale, Katrina Rudnicki, and Doug Flood at ICCB-Longwood for their assistance in screening, Hengming Ke for the generous gift of recombinant PDE4A enzyme, Didem Demirbas and Arlene Wyman for helpful discussions, and Anthony West and Paul Epstein for critical reading of the manuscript. We also thank David Sabatini for cell lines used in this study. This work was supported by a grant from Boston College and by the Peter Rieser Lectureship Fund.

Received: September 23, 2011
Revised: December 13, 2011
Accepted: December 15, 2011
Published: January 26, 2012

## REFERENCES

Alaamery, M.A., Wyman, A.R., Ivey, F.D., Allain, C., Demirbas, D., Wang, L., Ceyhan, O., and Hoffman, C.S. (2010). New classes of PDE7 inhibitors identified by a fission yeast-based HTS. J. Biomol. Screen. *15*, 359–367.

Almeida, M.Q., and Stratakis, C.A. (2011). How does cAMP/protein kinase A signaling lead to tumors in the adrenal cortex and other tissues? Mol. Cell. Endocrinol. *336*, 162–168.

Bender, A.T., and Beavo, J.A. (2006). Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol. Rev. *58*, 488–520.

Boikos, S.A., Horvath, A., Heyerdahl, S., Stein, E., Robinson-White, A., Bossis, I., Bertherat, J., Carney, J.A., and Stratakis, C.A. (2008). Phosphodiesterase 11A expression in the adrenal cortex, primary pigmented nodular adrenocortical disease, and other corticotropin-independent lesions. Horm. Metab. Res. *40*, 347–353.

Carney, J.A., Gaillard, R.C., Bertherat, J., and Stratakis, C.A. (2010). Familial micronodular adrenocortical disease, Cushing syndrome, and mutations of the gene encoding phosphodiesterase 11A4 (PDE11A). Am. J. Surg. Pathol. *34*, 547–555.

Conti, M., and Beavo, J. (2007). Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu. Rev. Biochem. *76*, 481–511.

Couplan, E., Aiyar, R.S., Kucharczyk, R., Kabala, A., Ezkurdia, N., Gagneur, J., St Onge, R.P., Salin, B., Soubigou, F., Le Cann, M., et al. (2011). A yeast-based assay identifies drugs active against human mitochondrial disorders. Proc. Natl. Acad. Sci. USA *108*, 11989–11994.

D'Andrea, M.R., Qiu, Y., Haynes-Johnson, D., Bhattacharjee, S., Kraft, P., and Lundeen, S. (2005). Expression of PDE11A in normal and malignant human tissues. J. Histochem. Cytochem. *53*, 895–903.

Demirbas, D., Ceyhan, O., Wyman, A.R., and Hoffman, C.S. (2011a). A fission yeast-based platform for phosphodiesterase inhibitor HTSs and analyses of phosphodiesterase activity. Handb. Exp. Pharmacol. *204*, 135–149.

Demirbas, D., Ceyhan, O., Wyman, A.R., Ivey, F.D., Allain, C., Wang, L., Sharuk, M.N., Francis, S.H., and Hoffman, C.S. (2011b). Use of a *Schizosaccharomyces pombe* PKA-repressible reporter to study cGMP metabolising phosphodiesterases. Cell. Signal. *23*, 594–601.

DeWan, A.T., Triche, E.W., Xu, X., Hsu, L.I., Zhao, C., Belanger, K., Hellenbrand, K., Willis-Owen, S.A., Moffatt, M., Cookson, W.O., et al. (2010). PDE11A associations with asthma: results of a genome-wide association scan. J. Allergy Clin. Immunol. *126*, 871–873.

Dong, H., Zitt, C., Auriga, C., Hatzelmann, A., and Epstein, P.M. (2010). Inhibition of PDE3, PDE4 and PDE7 potentiates glucocorticoid-induced apoptosis and overcomes glucocorticoid resistance in CEM T leukemic cells. Biochem. Pharmacol. *79*, 321–329.

Fatemi, S.H., Folsom, T.D., Reutiman, T.J., and Vazquez, G. (2010). Phosphodiesterase signaling system is disrupted in the cerebella of subjects with schizophrenia, bipolar disorder, and major depression. Schizophr. Res. *119*, 266–267.

Faucz, F.R., Horvath, A., Rothenbuhler, A., Almeida, M.Q., Libé, R., Raffin-Sanson, M.L., Bertherat, J., Carraro, D.M., Soares, F.A., Molina, Gde.C., et al. (2011). Phosphodiesterase 11A (PDE11A) genetic variants may increase susceptibility to prostatic cancer. J. Clin. Endocrinol. Metab. *96*, E135–E140.

Fawcett, L., Baxendale, R., Stacey, P., McGrouther, C., Harrow, I., Soderling, S., Hetman, J., Beavo, J.A., and Phillips, S.C. (2000). Molecular cloning and characterization of a distinct human phosphodiesterase gene family: PDE11A. Proc. Natl. Acad. Sci. USA *97*, 3702–3707.

Groussin, L., Massias, J.F., Bertagna, X., and Bertherat, J. (2000). Loss of expression of the ubiquitous transcription factor cAMP response element-binding protein (CREB) and compensatory overexpression of the activator CREMtau in the human adrenocortical cancer cell line H295R. J. Clin. Endocrinol. Metab. *85*, 345–354.

Hetman, J.M., Robas, N., Baxendale, R., Fidock, M., Phillips, S.C., Soderling, S.H., and Beavo, J.A. (2000). Cloning and characterization of two splice variants of human phosphodiesterase 11A. Proc. Natl. Acad. Sci. USA *97*, 12891–12895.

Horvath, A., Boikos, S., Giatzakis, C., Robinson-White, A., Groussin, L., Griffin, K.J., Stein, E., Levine, E., Delimpasi, G., Hsiao, H.P., et al. (2006a). A genome-wide scan identifies mutations in the gene encoding phosphodiesterase 11A4 (PDE11A) in individuals with adrenocortical hyperplasia. Nat. Genet. *38*, 794–800.

Horvath, A., Giatzakis, C., Robinson-White, A., Boikos, S., Levine, E., Griffin, K., Stein, E., Kamvissi, V., Soni, P., Bossis, I., et al. (2006b). Adrenal hyperplasia and adenomas are associated with inhibition of phosphodiesterase 11A in carriers of PDE11A sequence variants that are frequent in the population. Cancer Res. *66*, 11571–11575.

Horvath, A., Korde, L., Greene, M.H., Libe, R., Osorio, P., Faucz, F.R., Raffin-Sanson, M.L., Tsang, K.M., Drori-Herishanu, L., Patronas, Y., et al. (2009). Functional phosphodiesterase 11A mutations may modify the risk of familial and bilateral testicular germ cell tumors. Cancer Res. *69*, 5301–5306.

Ivey, F.D., Wang, L., Demirbas, D., Allain, C., and Hoffman, C.S. (2008). Development of a fission yeast-based high-throughput screen to identify chemical regulators of cAMP phosphodiesterases. J. Biomol. Screen. *13*, 62–71.

Kelly, M.P., Logue, S.F., Brennan, J., Day, J.P., Lakkaraju, S., Jiang, L., Zhong, X., Tam, M., Sukoff Rizzo, S.J., Platt, B.J., et al. (2010). Phosphodiesterase 11A in brain is enriched in ventral hippocampus and deletion causes psychiatric disease-related phenotypes. Proc. Natl. Acad. Sci. USA *107*, 8457–8462.

Lakics, V., Karran, E.H., and Boess, F.G. (2010). Quantitative comparison of phosphodiesterase mRNA distribution in human brain and peripheral tissues. Neuropharmacology *59*, 367–374.

Libé, R., Horvath, A., Vezzosi, D., Fratticci, A., Coste, J., Perlemoine, K., Ragazzon, B., Guillaud-Bataille, M., Groussin, L., Clauser, E., et al. (2011). Frequent phosphodiesterase 11A gene (PDE11A) defects in patients with Carney complex (CNC) caused by PRKAR1A mutations: PDE11A may contribute to adrenal and testicular tumors in CNC as a modifier of the phenotype. J. Clin. Endocrinol. Metab. *96*, E208–E214.

Loughney, K., Taylor, J., and Florio, V.A. (2005). 3′,5′-cyclic nucleotide phosphodiesterase 11A: localization in human tissues. Int. J. Impot. Res. *17*, 320–325.

Marjanovic, J., Chalupska, D., Patenode, C., Coster, A., Arnold, E., Ye, A., Anesi, G., Lu, Y., Okun, I., Tkachenko, S., et al. (2010). Recombinant yeast screen for new inhibitors of human acetyl-CoA carboxylase 2 identifies potential drugs to treat obesity. Proc. Natl. Acad. Sci. USA *107*, 9093–9098.

Rainey, W.E., Bird, I.M., Sawetawan, C., Hanley, N.A., McCarthy, J.L., McGee, E.A., Wester, R., and Mason, J.I. (1993). Regulation of human adrenal carcinoma cell (NCI-H295) production of C19 steroids. J. Clin. Endocrinol. Metab. *77*, 731–737.

Rainey, W.E., Bird, I.M., and Mason, J.I. (1994). The NCI-H295 cell line: a pluripotent model for human adrenocortical studies. Mol. Cell. Endocrinol. *100*, 45–50.

Rosenberg, D., Groussin, L., Jullian, E., Perlemoine, K., Bertagna, X., and Bertherat, J. (2002). Role of the PKA-regulated transcription factor CREB in development and tumorigenesis of endocrine tissues. Ann. N Y Acad. Sci. *968*, 65–74.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., and Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol. Cell *22*, 159–168.

Seftel, A.D. (2005). Phosphodiesterase 11 (PDE11) regulation of spermatozoa physiology. J. Urol. *174*, 1043–1044.

Stratakis, C.A. (2009). New genes and/or molecular pathways associated with adrenal hyperplasias and related adrenocortical tumors. Mol. Cell. Endocrinol. *300*, 152–157.

Wang, H., Liu, Y., Chen, Y., Robinson, H., and Ke, H. (2005a). Multiple elements jointly determine inhibitor selectivity of cyclic nucleotide phosphodiesterases 4 and 7. J. Biol. Chem. *280*, 30949–30955.

Wang, L., Griffiths, K., Jr., Zhang, Y.H., Ivey, F.D., and Hoffman, C.S. (2005b). *Schizosaccharomyces pombe* adenylate cyclase suppressor mutations suggest a role for cAMP phosphodiesterase regulation in feedback control of glucose/cAMP signaling. Genetics *171*, 1523–1533.

Wang, X.L., Bassett, M., Zhang, Y., Yin, S., Clyne, C., White, P.C., and Rainey, W.E. (2000). Transcriptional regulation of human 11beta-hydroxylase (hCYP11B1). Endocrinology *141*, 3587–3594.

Wayman, C., Phillips, S., Lunny, C., Webb, T., Fawcett, L., Baxendale, R., and Burgess, G. (2005). Phosphodiesterase 11 (PDE11) regulation of spermatozoa physiology. Int. J. Impot. Res. *17*, 216–223.

Weeks, J.L., Zoraghi, R., Beasley, A., Sekhar, K.R., Francis, S.H., and Corbin, J.D. (2005). High biochemical selectivity of tadalafil, sildenafil and vardenafil for human phosphodiesterase 5A1 (PDE5) over PDE11A4 suggests the absence of PDE11A4 cross-reaction in patients. Int. J. Impot. Res. *17*, 5–9.

Wong, M.L., Whelan, F., Deloukas, P., Whittaker, P., Delgado, M., Cantor, R.M., McCann, S.M., and Licinio, J. (2006). Phosphodiesterase genes are associated with susceptibility to major depression and antidepressant treatment response. Proc. Natl. Acad. Sci. USA *103*, 15124–15129.

Yuasa, K., Kotera, J., Fujishige, K., Michibata, H., Sasaki, T., and Omori, K. (2000). Isolation and characterization of two novel phosphodiesterase PDE11A variants showing unique structure and tissue-specific expression. J. Biol. Chem. *275*, 31469–31479.