Analyses of PDE-regulated phosphoproteomes reveal unique and specific cAMP-signaling modules in T cells

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Contributed by Joseph A. Beavo, May 28, 2017 (sent for review March 10, 2017; reviewed by Paul M. Epstein, Donald H. Maurice, and Kjetil Tasken)

Specific functions for different cyclic nucleotide phosphodiesterases (PDEs) have not yet been identified in most cell types. Conventional approaches to study PDE function typically rely on measurements of global cAMP, general increases in cAMP-dependent protein kinase (PKA), or the activity of exchange protein activated by cAMP (EPAC). Although newer approaches using subcellularly targeted FRET reporter sensors have helped define more compartmentalized regulation of cAMP, PKA, and EPAC, they have limited ability to link this regulation to downstream effector molecules and biological functions. To address this problem, we have begun to use an unbiased mass spectrometry-based approach coupled with treatment using PDE isozyme-selective inhibitors to characterize the phosphoproteomes of the functional pools of cAMP/PKA/EPAC that are regulated by specific cAMP-PDEs (the PDE-regulated phosphoproteomes). In Jurkat cells we find multiple, distinct PDE-regulated phosphoproteomes that can be defined by their responses to different PDE inhibitors. We also find that little phosphorylation occurs unless at least two different PDEs are concurrently inhibited in these cells. Moreover, bioinformatics analyses of these phosphoproteomes provide insight into the unique functional roles, mechanisms of action, and synergistic relationships among the different PDEs that coordinate cAMP-signaling cascades in these cells. The data strongly suggest that the phosphorylation of many different substrates contributes to cAMP-dependent regulation of these cells. The findings further suggest that the approach of using selective, inhibitor-dependent phosphoproteome analysis can provide a generalized methodology for understanding the roles of different PDEs in the regulation of cyclic nucleotide signaling.

Significance

We have coupled mass spectrometry-based phosphoproteomic analyses with treatment using various selective PDE inhibitors to characterize the PDE-regulated phosphoproteome of CD3/CD28-stimulated Jurkat cells. Predictive algorithms were used to identify likely upstream regulatory kinases, metabolic pathways, and biological processes that can be regulated by different PDEs. Here we compare the phosphoproteomes of different functional compartments subserved by combinations of individual PDE isozymes in a T-cell model. We observed unique phosphoproteomes associated with specific combinations of PDEs. These data allow one to prioritize future experiments to understand further how these pathways are regulated by specific PDEs. The results also have substantial implications for the design and use of selective PDE inhibitors in clinical practice.


Reviewers: P.M.E., University of Connecticut Health Center; D.H.M., Queen’s University; and K.T., University of Oslo.

The authors declare no conflict of interest.

Data deposition: Phosphoproteomic raw data have been deposited with the Mass Spectrometry Interactive Virtual Environment (MassIVE) maintained by the Center for Computational Mass Spectrometry (CCMS) in the Computer Science and Engineering Department of the University of California, San Diego (accession no. MSV000081115).

See Commentary on page 7741.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1703939114/-/DCSupplemental.
identification of 13,589 sites, of which 618 were sensitive to PDE inhibition. In doing so, we identified two distinct functional compartments regulated by two different combinations of PDE inhibitors.

Results

Global cAMP Levels Are Elevated Only by Specific Combinations of PDE Inhibitors. To determine which sets of PDE inhibitors to use in phosphoproteomic studies, we first treated Jurkat cultures with individual isozyme-selective PDE inhibitors or various combinations of these inhibitors and measured the resulting changes in cAMP levels. Previous studies identified PDEs 1B, 1C, 3B, 4A, 7A, and 8A as the predominant cAMP-hydrolyzing PDE mRNA transcripts expressed in Jurkat cells (25–27). In theory, any of these PDEs or any combination of PDEs could regulate different functional cAMP compartments in the cell. We also used 50 μM and 200 μM isobutylmethylxanthine (IBMX), a potent, general, nonselective PDE inhibitor, in combination with 200 nM PF-04957325 (a PDE3-selective inhibitor) to inhibit the majority of cAMP-hydrolyzing PDEs. For these initial studies, we then chose the inhibitor combinations that seemed most likely to influence the greatest number of PDE-regulated functional compartments for follow-up by phosphoproteomic analysis (Fig. 1).

Somewhat unexpectedly, treatment with an individual PDE inhibitor alone did not cause a significant increase in total cAMP in either the absence or the presence of low PGE2 (1 nM) (SI Appendix, Fig. S1). However, when combined with the low concentration of PGE2 (1 nM), combined treatment with cilostamide (a PDE3 inhibitor) and rolipram (a PDE4 inhibitor) greatly increased cAMP, from 9.7 to 144 pmol/mL (Fig. 1). Interestingly, the combination of PGE2 plus 50 μM IBMX and 200 nM PF-04957325 increased cAMP to a lesser degree (from 9.7 to ∼50 pmol/mL) than the combination of PGE2 plus cilostamide and rolipram, perhaps because of the known antagonistic effect of IBMX on adenosine-stimulated cAMP. However, a higher concentration of IBMX (200 μM) and 200 nM PF-04957325 plus PGE2 (1 nM) increased cAMP ∼35-fold (from 6.6 to 250 pmol/mL) (Fig. 1). In these conditions, the highest level of cAMP was seen in the presence of a saturating concentration of 200 μM PGE2 (Fig. 1). Overall, the results suggested that most putative cAMP compartments would be saturated only, if at all, in the high PGE2 condition, thus implying that it should be feasible to use lower combinations of PDE inhibitors to begin to resolve the phosphorylations that occur in the PDE-specific functional compartments of the cell.

Phosphoproteomic Interrogation of PDE Functional Compartments (the PDE-Regulated Phosphoproteomes). To identify the constituents of putative PDE-regulated compartments, we designed a phosphoproteomic approach using the combination of PDE inhibitors that caused the greatest increases in global cAMP based on our cAMP assay results. This approach is illustrated in SI Appendix, Fig. S2A. Preliminary analysis of the number of phosphopeptides identified in the basal condition (SI Appendix, Fig. S2B) suggested that nearly maximal identification could be achieved with nine LC-MS/MS runs for each treatment condition (with three or more biological replicates and three analytical replicates each). A single LC-MS/MS run yielded an average 5,146 unique quantified phosphopeptides, and increasing the number of LC-MS/MS runs to nine approached a plateau number of ~10,000 phosphopeptides per biological condition (SI Appendix, Fig. S2B). The Pearson correlation coefficients between biological replicates and analytical replicates were between 0.5–0.9 and ≥0.9, respectively (SI Appendix, Fig. S2C). In total, we identified 13,589 phosphopeptides and 3,241 proteins. For further downstream functional analyses, we included only phosphopeptides that were observed in at least 60% of the LC-MS/MS runs in the respective treatment groups. Of the 13,589 phosphopeptides identified, we found 618 phosphosites distributed among 461 unique proteins that were significantly regulated by the PDE inhibitor treatments [false discovery rate (FDR) ≤0.05, two-tailed t test, permutation-based FDR]. (Please refer to Dataset S1 for the complete list).

Different PDEs Regulate Distinct Functional Compartments. Consistent with the cAMP assays, no phosphosites were significantly altered by individual PDE inhibitor treatments alone under the basal condition (no PGE2) (Dataset S1) or in the 1-nM PGE2-stimulated state (Fig. 2A). However, we observed a synergistic increase in phosphopeptides identified when two or more PDEs were inhibited (Fig. 2C). To corroborate the proteomics data, we used commercially available (Abcam and Cell Signaling) antibodies to probe for changes in phosphorylation in phosphosites identified in our proteomics study, Stat3/TN (STMN1) S63 and Rho/Rac guanine nucleotide exchange factor 2 (ARHGEF2) S858. In agreement with the proteomics study, we found that neither PDE3 or PDE4 inhibitors alone, nor the combination of PDE1, PDE7, and PDE8 inhibitors, caused a change in STMN1 S63 phosphorylation, but inhibition of both PDE3 and PDE4 increased STMN1 S63 phosphorylation (Fig. 2F, see also SI Appendix, Fig. S7). In this case also, treatment with 200 μM IBMX plus the PDE8 inhibitor caused the greatest increase in phosphorylation. The individual PDE1, PDE7, and PDE8 inhibitors alone did not increase ARHGEF2 S858 phosphorylation, but the combination of all three inhibitors did (Fig. 2C).

In an attempt to define the functional compartments also regulated by PDEs other than PDE3 and PDE4, we performed the same analysis with 50 μM IBMX plus 200 nM PF-04957325 and 200 μM IBMX plus 200 nM PF-04957325. The expectation was that the 200 μM IBMX plus PF-04957325 condition would likely define the total PDE-regulated functional compartments. Each of these conditions was assessed in the absence or presence of a low concentration (1 nM) of PGE2. Indeed, many more regulated phosphosites were modulated when all PDEs were inhibited (Fig. 3). We
Membranes were probed with anti-STMN1 antibody (S) (1:2,000) (Abcam) and anti-GAPDH antibody (G) (1:4,000) (Cell Signaling). Error bars in Fig. 2, B and C, indicate SD. Statistical analysis was performed using a Student t test; *P = 0.02, **P = 0.006. (C) Immunoblot analysis of changes in STMN1 phosphorylation at S63. Cells (1 × 10^6) were treated as previously described. Cells were harvested and boiled in 200 μL Laemmli buffer and transferred to nitrocellulose. Membranes were probed with anti-STMN1 antibody (S) (1:2,000) (Abcam) and anti–β-actin (B) (1:200,000) (GeneTex). Membranes were quantified on the Odyssey Scanner Clx (LI-COR). The blot is shown on the left, and quantification is shown on the right. Error bars show SD. Statistical analysis was performed using a Student’s t test; *P = 0.02, **P = 0.006. (C) Immunoblot analysis of changes in Rho/RAC guanine nucleotide exchange factor 2 phosphorylation at S858. Cells (1 × 10^6) were treated as previously described, harvested, boiled in 200 μL Laemmli buffer, and transferred to nitrocellulose. Membranes were probed with anti-ARHGEF2 antibody (A) (1:2,000) (Cell Signaling) or anti-GAPDH antibody (G) (1:4,000) (Cell Signaling). Error bars indicate SD. Statistical analysis was performed using a Student’s t test; *P = 0.04; ns, not significant.

It was surmised that the remaining PDEs (i.e., not PDE3 and PDE4), which included PDEs 1, 7, and 8 (or some combination thereof), might also subserve functional compartments different from those regulated by PDE3 and/or PDE4. Therefore, we also treated Jurkat cells with a combination of 200 nM ITI-078 (a PDE1 inhibitor), 30 μM BRL50481 (a PDE7 inhibitor), and 200 nM PF-04957325 (a PDE8 inhibitor), also in the absence or presence of a low (1 nM) level of PGE2.

The subset of cilostamide- and rolipram-regulated phosphorysites made up a portion of the total IBMX- and PGE2-stimulated phosphorysites (Fig. 3, Left). Forty phosphorysites were selectively regulated by inhibiting PDE3 and PDE4 and not by inhibiting the combination of PDEs 1, 7, and 8 (Fig. 3, Right). Forty-three phosphorysites were regulated by the PDE3 and PDE4 treatment, and 123 phosphorysites were regulated by the PDE1/7/8 treatment (Fig. 3, Right). However, even with increased peptide identification, only one common phosphosite, S811 on Slingshot protein phosphatase 2 (SSH2), was statistically altered by both PDE inhibitor treatment groups. This general absence of overlap strongly suggested that the pool(s) of cAMP regulated by the combination of PDE1/7/8 inhibitors are functionally different from the pool(s) regulated by the combination of PDE3 and PDE4 inhibitors and that perhaps SSH2 is regulated by cAMP in more than one functional compartment (Fig. 3, Right and Table 1).

Identifying Kinases That Modulate PDE-Regulated Phosphoproteomes. The distinct functional pools of PDE-regulated phosphorysites were further characterized by analyzing which regulatory kinases would be most likely to phosphorylate the PDE inhibitor-dependent sites. Analysis of the sequences of the phosphorysites with the program NetPhorest (28), a web-based tool for kinase prediction (https://omictools.com), suggested that the majority of phosphorysites regulated by combined inhibition of PDEs 1, 7, and 8 are primarily phosphorylated by casein kinase II or by a kinase with a similar substrate recognition motif in both the basal and PGE2-stimulated conditions (Fig. 4, Left). In contrast, the same algorithm suggested that most of the phosphorysites increased by inhibiting both PDE3 and PDE4 are primarily phosphorylated by PKA (Fig. 4, Right). We observed a similar trend in PGE2-stimulated cells. The majority of sites predicted to be phosphorylated by PKA contain a modified S/T residue contained in the classic PKA consensus motif R/K, R/K, X, S/T (red bars in Fig. S3 B and D). However, 132 of 151 phosphorysites in the cells treated with combined PDE1/7/8 inhibitors did not contain this predicted PKA consensus site (Fig. S3 A and C).
and mutagenesis approaches to corroborate possible new regulatory sites are prime candidates for further follow-up with genetic treatment (Table 3) and 30 potential regulatory sites in the group of PDE1/7/8 inhibitors are shown in Table 4. We found 50 potential regulatory sites in the group of PDE1/7/8 inhibitors are shown in Table 3, and examples of treatment with 200 μM IBMX plus the PDE8 inhibitor (SI Appendix, Fig. S4). Under default network visualization settings, with the inhibition of PDE1, 7, and 8 there are clusters of interacting proteins around RANBP2 (Fig. 5). The combined inhibition of PDE3 and PDE4 shows a cluster of interacting proteins around RANBP2 (Fig. 5). Last, when all PDEs were inhibited by IBMX plus the PDE8 inhibitor, additional clusters of proteins interacting with HDAC1, SIN3A, MAPRE1, ARHGEF7, ABL1, LCK, CHEK1, and PRKAB1 are visible (SI Appendix, Fig. S4). Each of these clusters is indicative of proteins associated with particular pathways or functions. The observation that CAMP modulates several members of a cluster most likely suggests that cAMP regulates multiple points of the pathway/process.

Identification of Biological Processes Regulated by Specific Combinations of PDE Inhibitors. Gene Ontology (GO) analysis of the list of phosphoproteins regulated by PDE1/7/8 inhibitor treatment grouped 90 of 133 genes into 17 functional clusters (SI Appendix, Fig. S5 and Table S1). Fig. 64 shows an example of six identified functional clusters and the genes associated with their respective processes. GO analysis of the combination of PDE3 and PDE4 inhibitor treatment sites resulted in 20 of 74 genes grouped into six functional clusters (Fig. 6b and SI Appendix, Table S2), GO analysis of the PDE inhibitor treatment group and to prioritize further which phosphosites warranted further investigation. STRING identifies proteins that have been empirically shown to interact (experiment option) or that are known components of an annotated common pathway (database option). This analysis showed a greater number of interacting proteins in the group treated with PDE1/7/8 inhibitors than in the group treated with PDE3 and PDE4 inhibitors (Fig. 5). As expected, the greatest numbers of interactions were observed in the group treated with 200 μM IBMX plus the PDE8 inhibitor (SI Appendix, Fig. S4). Under default network visualization settings, with the inhibition of PDE1, 7, and 8 there are clusters of interacting proteins around RANBP2 (Fig. 5). The combined inhibition of PDE3 and PDE4 shows a cluster of interacting proteins around RANBP2 (Fig. 5). Last, when all PDEs were inhibited by IBMX plus the PDE8 inhibitor, additional clusters of proteins interacting with HDAC1, SIN3A, MAPRE1, ARHGEF7, ABL1, LCK, CHEK1, and PRKAB1 are visible (SI Appendix, Fig. S4). Each of these clusters is indicative of proteins associated with particular pathways or functions. The observation that CAMP modulates several members of a cluster most likely suggests that cAMP regulates multiple points of the pathway/process.

**Table 1.** Top phosphorylated proteins in response to PDE3 plus PDE4 inhibition in the absence (−) and presence (+) of 1 nM PGE2 compared with changes seen in response to the other PDE inhibitors under the same conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Position no.</th>
<th>Sequence</th>
<th>Fold change over control</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANBP2</td>
<td>E3 SUMO-protein ligase RanBP2</td>
<td>1,509</td>
<td>PRKQSLPAT</td>
<td>2.20 4.51 5.01 5.21</td>
</tr>
<tr>
<td>HIST1H1C</td>
<td>Histone H1.2</td>
<td>36</td>
<td>PRKASGPPV</td>
<td>1.94 3.25 3.58 5.42</td>
</tr>
<tr>
<td>SEC22B</td>
<td>Vesicle-trafficking protein SEC22b</td>
<td>137</td>
<td>RNLGSINTE</td>
<td>1.21 2.16 2.68 2.89</td>
</tr>
<tr>
<td>GAS2L1</td>
<td>GAS2-like protein 1</td>
<td>316</td>
<td>ERRGRSPEM</td>
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<td><strong>Response to PDE3 and PDE4 inhibitor (+) PGE2</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RANBP2</td>
<td>E3 SUMO-protein ligase RanBP2</td>
<td>1,509</td>
<td>PRKQSLPAT</td>
<td>2.20 4.51 5.01 5.21</td>
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<tr>
<td>HIST1H1C</td>
<td>Histone H1.2</td>
<td>36</td>
<td>PRKASGPPV</td>
<td>1.94 3.25 3.58 5.42</td>
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<td>ARRASRGPI</td>
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<td>Vesicle-trafficking protein SEC22b</td>
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<td>ARNNSISGP</td>
<td>1.27 3.29 4.42 3.20</td>
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<tr>
<td>STMN1</td>
<td>Stathmin</td>
<td>63</td>
<td>ERKKSHelda</td>
<td>0.42 3.25 1.57 1.59</td>
</tr>
</tbody>
</table>
| **Interaction Networks Defined by STRING Analysis.** We used STRING analysis (31) as another method to suggest which biological processes or pathways might be regulated in each PDE inhibitor treatment group and to prioritize further which phosphosites warranted further investigation. STRING identifies proteins that have been empirically shown to interact (experiment option) or that are known components of an annotated common pathway (database option). This analysis showed a greater number of interacting proteins in the group treated with PDE1/7/8 inhibitors than in the group treated with PDE3 and PDE4 inhibitors (Fig. 5). As expected, the greatest numbers of interactions were observed in the group treated with 200 μM IBMX plus the PDE8 inhibitor (SI Appendix, Fig. S4). Under default network visualization settings, with the inhibition of PDE1, 7, and 8 there are clusters of interacting proteins around RANBP2 (Fig. 5). The combined inhibition of PDE3 and PDE4 shows a cluster of interacting proteins around RANBP2 (Fig. 5). Last, when all PDEs were inhibited by IBMX plus the PDE8 inhibitor, additional clusters of proteins interacting with HDAC1, SIN3A, MAPRE1, ARHGEF7, ABL1, LCK, CHEK1, and PRKAB1 are visible (SI Appendix, Fig. S4). Each of these clusters is indicative of proteins associated with particular pathways or functions. The observation that CAMP modulates several members of a cluster most likely suggests that cAMP regulates multiple points of the pathway/process.
200-μM IBMX treatment data resulted in 317 of 368 genes grouped into 34 clusters. Therefore it is quite likely that these different combinations of PDE inhibitors subserve different functional pools of CAMP and that the different functional pools in turn regulate the different functions identified by the GO analysis. It should be noted that, particularly in the presence of PGE2, several common gene products are identified in both the PDE3 plus PDE4 inhibitor condition and the PDE1/7/8 inhibitor condition. In general, however, different sites are phosphorylated by different combinations of PDE inhibitors. Several possibilities for this observation are discussed in the next section.

Discussion

In this study, we have used a nonbiased MS phosphoproteomics approach coupled with isozyme-selective PDE inhibitors to identify some of the PDE-regulated phosphoproteomes modulated by several different CAMP PDEs in a model T-cell line. According to Ponomarenko (32), there are more than 20,000 different proteins in the human proteome, not including splice variants and post-translationally modified proteins. No single cell is expected to translate all possible proteins at all times. In our datasets, we identified 3,241 phosphorylated proteins containing 13,589 phosphorylation sites identified with the combined use of multiple cAMP PDEs in a model T-cell line. According to Ponomarenko (32), there are more than 20,000 different proteins in the human proteome, not including splice variants and post-translationally modified proteins. No single cell is expected to translate all possible proteins at all times. In our datasets, we identified 3,241 phosphorylated proteins containing 13,589 phosphorylation sites.

Sensitivity and Phosphopeptide Coverage. No one yet knows how many phosphosites are regulated by PDEs in T cells. Using multiple (nine or more) LC/MS runs deepened our proteomic coverage in this context. However, because it has been previously reported that little or no PDE1 activity or protein is present in Jurkat cells, it is quite likely that the majority of effects observed in the PDE1/7/8-inhibited condition are in fact caused by PDE7 and/or PDE8 regulation (26). For a full understanding of all of the potential PDE synergies present in T cells and to understand fully the different PDE phosphoproteomes, phosphorylation profiles from each PDE inhibitor and PDE inhibitor combination will need to be determined in future studies. For example, the effects of PDE3 and PDE4 inhibitors will need to be tested in all combinations with PDE1/7/8 inhibitors. It is known, for example, that a combination of PDE4 and PDE8 inhibitors is particularly effective in MA-10 cells (35, 36), so it seems quite likely that additional PDE-regulated functional compartments will be identified as different combinations of PDE inhibitors are investigated.
effect also suggests that other functional compartments regulated by different combinations of PDEs remain to be identified and annotated. Both the phosphoproteomic results and the changes in cAMP seen in response to the PDE inhibitor treatments also suggested that major functional compartments in these cells are most likely not regulated by single PDEs. In addition, it is, of course, possible that some functional cAMP compartments may be subserved by three or more PDEs. This possibility was not tested. Dong et al. (26) reported that inhibiting PDE3, PDE4, and PDE7 together maximally potentiated glucocorticoid-mediated apoptosis. Unfortunately, time and resources did not allow phosphoproteomic studies under all the possible combinations of PDEs expressed in the Jurkat cell line. Eventually these studies will need to be done to understand the full PDE-regulated phosphoproteomes.

It is perhaps worth repeating that the largest changes both in the phosphoproteome and cAMP data were seen in the PGE2-stimulated conditions rather than in the basal condition. Thus it appears that these functional compartments are dynamic and can be influenced by the source of cAMP. Bloom et al. (37) reported that pools of cAMP in distinct subcellular locales could be detected in T cells stimulated with different agonists. Therefore, it is plausible, for example, that the functional compartment(s) defined by the inhibition of PDEs 1, 7, and 8 or by the inhibition of PDE3 plus PDE4 could be different in adenosine-stimulated cells versus PGE2-stimulated cells. Additionally, the time-course phosphoproteomic studies by Giansanti et al. (17) and Golkowski et al. (35) suggest that these functional compartments change in time as well, indicating an additional parameter that will need to be explored to understand fully the scope of the functional PDE-regulated phosphoproteomes. Clearly a more comprehensive study of cAMP/PDE functional compartments is needed to investigate all the possible combinations of PDE inhibitors under multiple agonist stimulation paradigms.

**Direct Phosphorylation by PKA.** Of the 618 significantly regulated phosphosites, 55 are annotated in PhosphositePlus, indicating that, when phosphorylated, these sites have been empirically determined to regulate protein function directly or indirectly. Of these 55 phosphosites 13 were at canonical PKA consensus sequences and therefore are likely to be directly phosphorylated by PKA. These include ARFF1 S132 (38, 39), ARHGEF2 S886 (40), BAD S152 (41, 42), BRAF S446 (43), CAD S1406 (44), CAMKK1 S458 (45), KIF1A S63 (46, 47), LASP1 S146 (48–50), PTPN7 S44 (51, 52), RCC1 S113 (53–55), RPS6 S235 (56), STMN1 S63 (57–59), and TAL1 172 (60, 61). In fact, eight of these sites—in ARHGEF2, BAD, CAD, CAMKK1, LASP1, PTPN7, STMN1, and TAL1—have been reported to be substrates of PKA in other cell types. These results strongly corroborate the combined inhibitor-phosphoproteomic approach outlined in this article. Moreover, these proteins are associated with a wide array of biological functions and thus suggest that cAMP coordinates not only a few rate-limiting steps but rather a large number of sites that in turn regulate multiple processes in the T cells. In fact, this observation may call into question the generally taught concept that only a single rate-limiting step is likely to be the major regulatory site for cAMP/PKA in many pathways.

As indicated above, these regulatory sites should not be interpreted as exclusively PKA substrates. For example, STMN1 S63, RCC1 S11, and PTPN7 S44 also can be phosphorylated by CaMKIV (58), CDK1 (55), and PKCζ (51), respectively. Therefore it is plausible, and perhaps even likely, that the sequences of these sites have evolved so that different kinases can regulate the same site under specific but differing cellular contexts. Nonetheless, the identification of these phosphosites also tends to validate the approach of using shotgun MS to identify biologically relevant phosphosites in response to treatment with combinations of PDE inhibitors. Therefore, we were encouraged to expand our analysis beyond sites with canonical PKA consensus sequences to identify other PDE-dependent phosphosites with probable regulatory actions, because they could lead to the identification of novel

**Table 2. Sites in our dataset modulated by any PDE inhibitor condition that are identified as regulatory in the PFP database (28)**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
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<tr>
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<td>CAMKK1</td>
<td>C+-calmodulin-dependent protein kinase kinase 1</td>
<td>S</td>
<td>485</td>
<td>- - - - - +</td>
</tr>
<tr>
<td>USP20</td>
<td>Ubiquitin carboxyl-terminal hydrolase 20</td>
<td>S</td>
<td>333</td>
<td>- - - - - +</td>
</tr>
</tbody>
</table>

A truncated peptide sequence of four amino acid residues flanking the regulated phosphosite was used to screen the PFP proteomic database for predicted functional phosphosites. Predictive models used by PFP are Bayes (B), logistic (L), multilayer (M), and random (R). Empirically determined regulatory sites (RP) as derived from the PhosphositePlus database (29) are reported in the last column.
molecular mechanisms by which cAMP and PDEs coordinate cellular responses.

**PDE Synergies: Inhibition of Multiple PDEs Is Needed to Regulate Individual Sites and Processes.** The majority of phosphosites were not regulated until more than one PDE was inhibited. This finding was corroborated by Western blots of STMN1 S63 and ARHGEF2 S858 (Fig. 2 B and C). Of note, probing by Western blot revealed increased phosphorylation of ARHGEF2 S858 by combined treatment with PDE3 and PDE4 inhibitors which was not noted in the initial analysis of the phosphoproteomics data because a single statistical outlier confounded the analysis (SI Appendix, Fig. S6). Giebbycz et al. (62) have reported the pro-

The many examples of PDE synergy in the current datasets at both the cAMP and PDE-regulated phosphoproteome level also likely have substantial implications for drug design. Data from a clinical study by Franciosi et al. (63) showed that RPL554, a dual PDE3 and PDE4 inhibitor, was an effective bronchodilator and reduced inflammation in patients with chronic obstructive pulmonary disease. Although perhaps not yet fully embraced by the pharmaceutical industry, an increasing number of such functional examples are being elucidated in which multiple PDEs need to be inhibited to elicit a pharmacological response. To our knowledge very few such screening studies have been carried out previously. The current data demonstrate this same principle of PDE synergy at a molecular level and suggest that a phosphoproteomic approach could be used as a part of an initial preclinical screen to determine which PDEs need to be inhibited to maximize a therapeutic effect or to minimize an unwanted side effect.

**PDE1/7/8-Regulated Functional Compartment(s).** Interestingly, the characteristics of the functional compartment(s) regulated by combined inhibition of PDEs 1, 7, and 8 differ from the characteristics of the compartment(s) regulated by PDE 3 and PDE4. The stark differences between these two functional compartments were not fully expected. We found, for example, that the majority of sites regulated by PDE3 and PDE4 have a PKA consensus sequence, and NetPhorest analysis predicted that the majority of these sites would be phosphorylated by PKA. This

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Amino acid</th>
<th>Position no.</th>
<th>Predicted kinase</th>
<th>Predictive models</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS1</td>
<td>Protein C-ets-1</td>
<td>S</td>
<td>285</td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td>HDAC4</td>
<td>Histone deacetylase 4</td>
<td>S</td>
<td>453</td>
<td>CK2</td>
<td>—</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma-associated protein</td>
<td>S</td>
<td>624</td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td>ANAPC2</td>
<td>Anaphase-promoting complex subunit 2</td>
<td>S</td>
<td>314</td>
<td>MOK</td>
<td>+</td>
</tr>
<tr>
<td>HIST1H1B</td>
<td>Histone H1.5</td>
<td>S</td>
<td>18</td>
<td>N/A</td>
<td>+</td>
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<tr>
<td>NBEAL2</td>
<td>Neurobeachin-like protein 2</td>
<td>T</td>
<td>1,683</td>
<td>N/A</td>
<td>—</td>
</tr>
<tr>
<td>TPS3B1</td>
<td>Tumor suppressor p53-binding protein 1</td>
<td>S</td>
<td>552</td>
<td>MAPK1</td>
<td>+</td>
</tr>
<tr>
<td>BCL11B</td>
<td>B-cell lymphoma leukemia 11B</td>
<td>T</td>
<td>131</td>
<td>CDK2</td>
<td>—</td>
</tr>
</tbody>
</table>

A truncated peptide sequence of four amino acid residues flanking the regulated phosphosite was used to screen the PFP proteomic database for predicted functional phosphosites (28). Sites were considered positive if at least one of four prediction models suggested function. Predictive models used by PFP are Bayes (B), logistic (L), multilayer (M), and random (R). The same sequence was used in NetPhorest (27) to predict the regulatory kinase. The threshold was set at 0.21. N/A, not applicable.

Table 3. Examples of sites of unknown function in the dataset modulated by PDE1/7/8 inhibitors that are predicted to be regulatory in the PFP database (28)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Amino acid</th>
<th>Position no.</th>
<th>Predicted kinase</th>
<th>Predictive models</th>
</tr>
</thead>
<tbody>
<tr>
<td>NUMA1</td>
<td>Nuclear mitotic apparatus protein 1</td>
<td>S</td>
<td>1,955</td>
<td>N/A</td>
<td>+</td>
</tr>
<tr>
<td>MKI67</td>
<td>Antigen Ki-67</td>
<td>S</td>
<td>2,033</td>
<td>N/A</td>
<td>—</td>
</tr>
<tr>
<td>FLNA</td>
<td>Filamin-A</td>
<td>S</td>
<td>2,309</td>
<td>ATM</td>
<td>—</td>
</tr>
<tr>
<td>AGT16L1</td>
<td>Autophagy-related protein 16-1</td>
<td>T</td>
<td>2,659</td>
<td>PKA</td>
<td>—</td>
</tr>
<tr>
<td>PLEXF2</td>
<td>Pleckstrin homology domain-containing F member 2</td>
<td>S</td>
<td>16</td>
<td>PKA</td>
<td>—</td>
</tr>
<tr>
<td>TEX2</td>
<td>Testis-expressed sequence 2 protein</td>
<td>S</td>
<td>295</td>
<td>PKA</td>
<td>—</td>
</tr>
<tr>
<td>ABL1</td>
<td>Tyrosine-protein kinase ABL1</td>
<td>S</td>
<td>16</td>
<td>PKA</td>
<td>—</td>
</tr>
<tr>
<td>RIF1</td>
<td>Telomere-associated protein RIF1</td>
<td>S</td>
<td>2,205</td>
<td>PKA</td>
<td>+</td>
</tr>
<tr>
<td>SNX1</td>
<td>Sorting nexin-1</td>
<td>S</td>
<td>188</td>
<td>PKA</td>
<td>+</td>
</tr>
<tr>
<td>CUL4A</td>
<td>Cullin-4</td>
<td>S</td>
<td>10</td>
<td>PKA</td>
<td>+</td>
</tr>
<tr>
<td>PRKDC</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
<td>S</td>
<td>893</td>
<td>PKA</td>
<td>+</td>
</tr>
<tr>
<td>CAMKK2</td>
<td>Ca2+/calmodulin-dependent protein kinase 2</td>
<td>S</td>
<td>468</td>
<td>N/A</td>
<td>—</td>
</tr>
<tr>
<td>CDC2A</td>
<td>Cell division cycle-associated protein 2</td>
<td>S</td>
<td>962</td>
<td>PKA</td>
<td>+</td>
</tr>
<tr>
<td>MACF1</td>
<td>Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5</td>
<td>S</td>
<td>7,068</td>
<td>N/A</td>
<td>—</td>
</tr>
</tbody>
</table>

A truncated peptide sequence of four amino acid residues flanking the regulated phosphosite was used to screen the PFP proteomic database for predicted functional phosphosites (28). Sites were considered positive if at least one of four prediction models suggested function. Predictive models used by PFP are Bayes (B), logistic (L), multilayer (M), and random (R). The same sequence was used in NetPhorest (27) to predict the regulatory kinase. The threshold was set at 0.21. N/A, not applicable.

Table 4. Examples of sites of unknown function in the dataset modulated by PDE3 and PDE4 inhibitors that are predicted to be regulatory in the PFP database (28)
finding strongly suggested that they are direct substrates of PKA and/or perhaps also other AGC-type kinases. In contrast, a much greater diversity of kinases was predicted to regulate the phosphatases in the PDE1/7/8 compartment(s), and the majority of sites regulated in these compartments do not have a PKA consensus site. In both the basal and PGE2-stimulated state, CK2 is the predominantly predicted kinase for this functional compartment. One possible mechanism that might be operative here is that cAMP might activate PKA or EPAC upstream of CK2 so that the effects of cAMP, although real, are indirect. If so, this activity represents an unexplored mechanism of cAMP action. PDE1/7/8 inhibitors also may have effects on phosphatase activity.

**Biological Processes Regulated by Different PDE-Regulated Phosphoproteomes.** GO analysis was performed to provide insight into which biological processes might be regulated by each series of PDE inhibitor combinations. We observed that the biological processes regulated by inhibition of PDEs 1, 7, and 8 were largely distinct from those regulated by inhibition of PDE 3 and PDE4, as might be expected because different proteins are phosphorylated. It is worth noting that, although functional clusters such as spindle organization, regulation of cytoskeleton, and repair of double-stranded breaks are common to both treatment groups, the genes present in each node are different among treatment groups, suggesting that inhibiting different combinations of PDEs can affect the same biological process but likely does so via different mechanisms. A number of the biological processes identified by GO analysis have been previously reported to be regulated by cAMP. These include mRNA splicing (64–66), spindle organization (67, 68), fibroblast migration (69, 70), lamellipodium assembly (71, 72), ATM signaling (77, 78), gene silencing via microRNA (79–82), T-cell selection (83), and cytoskeletal reorganization (84).

This study suggests which molecular substrates might participate in the regulation of these processes. To date the roles of cAMP/PKA signaling are even less well defined for most of the other biological processes implicated, such as chromatin remodeling (85–87) and chromosomal segregation. Again, the phosphoproteids that map to these processes should be a good place to start mechanistic studies of cAMP/PDE effects on these processes.

The combined approach of using selective inhibitors with phosphoproteomic analysis builds on previous classical and phosphoproteomic studies (17) in several key aspects. First, the majority of previous phosphoproteomic studies used high concentrations of agents such as cAMP analogs or receptor agonists to increase cAMP globally. In other studies, relatively nonselective phosphatase inhibitors were used. In general, the investigators in these studies were most interested in defining a maximal cAMP-regulated or phosphatase-regulated phosphoproteome. Our data suggest that a much more nuanced understanding, particularly regarding the physiological roles of different subsets of PDEs, can be achieved by using selective PDE inhibitors at their selective concentrations to interrogate the phosphoproteome of a cell.

Second, using this combination approach, we have identified a number of proteins known to be key regulators of important pathways/processes that have been largely understudied in the context of cAMP regulation. For example, as also seen in our recent description of a PDE-regulated phosphoproteome of MA-10 cells (35), a number of small G protein-regulated pathways were identified. As in the MA-10 system, the regulation of these
pathways is likely to be at the level of the guanine nucleotide exchange factors and GTPase-activating proteins that modulate the small Rho-type GTPases rather than a direct phosphorylation of the GTPase itself. In both studies, inhibition of a combination of PDEs increased ARHGEF2 phosphorylation on S1509. This site has been previously reported to regulate ARHGEF2 activity (40). Moreover, ARHGEF2-dependent RhoA activity also has been shown to regulate the uropod of migrating T cells (71), in accordance with the report by Van et al. (88) that PDE8 inhibition caused a decrease in T-cell motility. In the current study, the combination of PDEs 1, 7, and 8 showed the largest increase in ARHGEF2 phosphorylation. In the context of T-cell biology, cytoskeletal reorganization has been intimately linked with T-cell recepting (8). An increased phosphorylation of ARHGEF1, ARHGEF7, ARHGAP4, BRAF, CFL1, MAPK3, PIK3C2A, SLC9A1, and SSH2 (all proteins associated with cytoskeletal reorganization), suggested that PDE8, possibly in combination with another PDE (likely PDE7), may regulate T-cell migration at multiple points (see SI Appendix, Supplemental Methods and Materials for further discussion of other PDE-modulated functional compartments). However, further studies will be necessary to prove this hypothesis.

Finally, we have identified a number of PDE-modulated phosphosites on proteins not previously known to be regulated by cAMP/PDEs. For example, RANBP2 has been identified as an EPAC1 interactor. Gloorich et al. (89) showed that EPAC1 bound to RAN-GTP, which in turn bound to the cluster of zinc finger domains of RANBP2. They also showed that phosphatase inhibitor treatment increased the phosphorylation of RANBP2 zinc finger domains and prevented EPAC binding. However, the exact site/zinc finger domain was not identified. Inhibition of PDE3 and PDE4 caused a significant increase in RANBP2-S1509 phosphorylation (Tables 2 and 4) under both basal and PGE2 conditions. S1509 is immediately C terminal of the third zinc finger domain and is within a canonical PKA consensus sequence. It is therefore likely that PKA phosphorylates RANBP2 at S1509 and disrupts RAN-GTP/EPAC binding to the third zinc finger domain. Again, further studies will be necessary to prove this notion.

In summary, we have used MS phosphoproteomic analysis on Jurkat cultures treated with various selective concentrations of inhibitors of PDEs 1, 3, 4, 7, and 8 to begin to characterize the phosphoproteome of the functional pools of cAMP subserved by these PDEs. Predictive algorithms were used to identify upstream regulatory kinases and to prioritize potential regulatory phosphosites for future investigation. The big advantage of this approach lies in the unbiased identification of regulated phosphosites and the sheer number of sites identified. This study compares the phosphoproteomes of two functional compartments subserved by any individual PDE isozyme or by any combinations of PDEs in a T-cell model, not only in the basal state but in an adenylyl cyclase-stimulated state as well. We observed at least two functional pools of cAMP in Jurkat cells that are distinct from one another. The data underscore the need to understand the exact pharmacological response to individual and combinations of PDE inhibitors. Currently we do not know how many functional PDE-regulated cAMP compartments exist in Jurkat cells, nor do we understand if similar functional PDE-regulated cGMP pools exist in these cells. We also need to know if these cyclic nucleotide pools regulate one another and which biological processes each of these functional pools controls. The answers to these questions will undoubtedly be complex and assuredly will be cell-context and time dependent. However, addressing these questions using the combined selective PDE inhibitor approach coupled with phosphoproteomic analyses is well within our current abilities. The volume of data to be gleaned is staggering, but with enough experimental iterations it should be possible, using phosphosites as molecular signatures, to construct a functional atlas of PDE-regulated cyclic nucleotide signaling not only in Jurkat cells but also in any other model cell culture system. Using other agonists and antagonists, the same approach should be possible for the cyclic nucleotide synthetic pathways.

Materials and Methods
The methods and sources of materials can be found in SI Appendix, Supplemental Methods and Materials.
Supplemental Methods and Materials

Cell culture

Jurkat cell stocks (ATCC) \((5\times10^6)\) were thawed from cells stored in liquid nitrogen (LN2). Cells were cultured at 37°C, 5% CO\(_2\) for one week in RPMI media, containing 10% FBS, 2mM Glutamine, 100 U/ml Penicillin, and 100 µg/ml Streptomycin. Twenty-four hours prior to an experiment cells were washed and serum starved for 16 hours by culturing in RPMI in bacterial plates to prevent adhesion. After 16 hours, cells were synchronized by culturing in 50% FBS for two hours, washed twice with RPMI, followed by 6 hours of culture in serum free RPMI. To start the experiment, cells were concurrently treated with the following reagents as described in the figures: 0.5 µg/ml CD3, 0.5 µg/ml CD28 (Miltenyi #s 130-093-387, 130-093-375), and 1 nM prostaglandin E\(_2\) (Cayman Chemical #14010). One or more of the following PDE inhibitors were also added at this time; 5 µM cilostamide (PDE3i) (Tocris #0915), 10 µM rolipram (PDE4i) (Tocris #1349), 30µM BRL50481 (PDE7i) (Tocris #2237), 200 nM PF-04957325 (PDE8i) (Pfizer), 50 µM IBMX (non-selective PDE inhibitor for all cAMP-PDEs except PDE8) (Sigma), or 200 µM IBMX. Some cells were also treated with 200 nM ITI-078 (PDE1i) (Intracellular Therapeutics)[1]. The structure of this compound is shown as compound # 38 in this reference[1]. Treated cultures were incubated in 2 ml of media, in 2 ml microfuge tubes, at 37°C, 5% CO\(_2\) for 20 minutes.

cAMP measurements

Jurkat cells \((1\times10^7)\) were treated as previously stated. Cells were briefly centrifuged and the supernate discarded. Cell pellets were snap frozen in LN2, and stored at -80°C until analysis. Cell pellets were lysed by adding 1ml of 1:99 mixture of 11.65 M HCL: 95% ethanol. Pellets were dispersed using a P1000 Eppendorf pipet tip, and vortexed. Lysate was incubated at room temperature for 30 min. Following incubation, the extraction volume was transferred to a fresh microfuge tube, and dried in a speed vacuum. The cAMP was re-suspended in 150 µl of 0.1 M HCl, acetylated, and assayed using a cAMP ELISA kit according to manufacturer’s recommendations (Cayman Biochemical).

Immunoblot analysis

Jurkat cells \((1\times10^7)\) were treated with PDE inhibitors as previously stated. Cells were briefly centrifuged and the supernate discarded. Cell pellets were snap frozen in LN2, and stored at -80°C until analysis. Cells were lysed with 200 µl of Laemmli 2% SDS buffer containing 1 mM dithiothreitol (DTT), and 3% glycerol, vortexed briefly, and immediately placed in a boiling water bath. Samples were incubated for 7 minutes, vortexed, and centrifuged briefly. Twenty µl was loaded onto 6 or 10% polyacrylamide Tris Glycine SDS (1.5 mm thickness) gels. Electrophoresis was run at 150 V for 1 hour. Proteins were transferred to nitrocellulose at 34V for 16 hours. Immunoreactive bands were visualized and quantified using an Odyssey CLx imaging system using the 600 nm and 800 nm laser intensities set at 4 and 6 respectively. Membranes were blocked with PBS based Odyssey blocking buffer and then probed with primary antibodies in ½ diluted Odyssey blocking buffer containing 0.1% Tween20. Secondary antibodies (Licor) 1:25,000 in ½ diluted Odyssey blocking buffer, containing 0.1% Tween20, and 0.05 % SDS. All incubations were 1 hour. Membranes were washed with PBS, 0.1% Tween20, for 5 minutes each wash, for a total of 3 washes. A final wash before visualization was in PBS. Image Studio Light v5.2 was used to quantify scans, and Graphpad Prism was used to graph results.

Mass Spectrometry sample preparation
A label-free mass spectrometry approach was used for these studies[2]. Each biological condition was repeated a minimum of 3 times and at least a total of 3 analytical replicates each (minimum of 9 LC-MS/MS runs per condition) (Refer to Supplemental Figure 2 for experimental summary, and supplemental materials for details). After a 20-minute incubation with the indicated PDE inhibitors, adenylyl cyclase agonist, and CD3/CD28 antibodies, cells were centrifuged for 30 seconds at 20K x g. The supernate was aspirated and 250 µl of boiling lysis buffer was added: 6M guanidinium hydrochloride, 100 µl Tris-HCl pH 8.0, 5 mM Tris (2-carboxyethyl) phosphine (TCEP), and 10 mM chloroactamide (CAM). Tubes were vortexed briefly, and placed in a boiling water bath for 10 minutes. Tubes were again vortexed briefly, and then centrifuged at 20K x g for 5 minutes. Supernates were applied to Waters Oasis 10mg extraction cartridges that were activated with IMAC resin with 150 µl of boiling lysis buffer (Sigma). Peptides were incubated at 25°C for 1 hour with 1500 rpm shaking. Post incubation, tubes were centrifuged briefly, and the IMAC resin was washed with 500 µl of 80% ACN, 0.1% TFA. Washes were repeated for a total of two washes. IMAC resin was applied to C18 StageTips[3] (two punches of C18 membrane, Empore C18 #2315) and the volume was passed through with a syringe. The C18 was washed twice with 150 µl of 1% formic acid. Phosphopeptides were eluted twice from the IMAC resin with 150 µl of 500 mM K2PO4. C18 resin was washed twice with 150 µl of 1% formic acid. StageTips were stored at 4°C.

**LC-MS/MS and data analysis**

Peptides were eluted from StageTips with 50 µl of 80% ACN, 0.1% TFA, into a 96 well, conical bottom, polypropylene plate. Phosphopeptides were dried in a speed vacuum and resuspended in 12 µl of 5% ACN, 0.1% TFA. Three µl of phosphopeptides were applied to a self-pulled, 360 µm OD x 100 µm ID 20 cm column (with a 7 µm tip) packed with 3 µm diameter Reprosil C18 resin (Dr. Maisch GmbH, Germany). Peptides were analyzed in a 120 min, 5% to 30% acetonitrile gradient in 0.1% acetic acid at 300 nL/min on a nanoLC-MS (Thermo Dionex RSLCnano) and injected into an Orbitrap Elite. Orbitrap FTMS spectra (R = 30 000 at 400 m/z; m/z 350–1600; 3e6 target; max 500 ms ion injection time) and Top15 data dependent CID MS/MS spectra (1e4 target; max
100 ms injection time) were collected with dynamic exclusion for 30 s and an exclusion list size of 50. The normalized collision energy applied for CID was 35% for 10 ms. Mass spectra were searched against the Uniprot human reference proteome downloaded on July 29, 2015 and quantified using MaxQuant v1.5.2.8[4]. Under group-specific parameters, all default parameters were retained. Under global parameters, match between runs were selected: match time window and alignment time window were set to 0.7 and 20 respectively. Data analysis was performed using the Perseus[5] software suite.

**Kinase Prediction**

The web based NetPhorest program identifies probable kinases likely to phosphorylate queried sequences, based on the substrate consensus sequence motifs of 179 kinases. A truncated peptide sequence of 4 amino acid residues flanking both sides of the regulated phosphosite was used as an input sequence in NetPhorest[6]. The predictive threshold was set at 0.21.

**Functional Prediction**

The Predict Functional Phosphosites (pfp) database evaluates whether a phosphorylation at a specified amino acid is likely to alter protein function by evaluating the evolutionary conservation, degree of disorder, presence of structural features, and kinase association of the amino acid sequence containing the modification site. The same truncated peptide sequence of 4 amino acid residues flanking the regulated phosphosite was used as an input sequence to query the database:pfp_database_release_1_2_update_1__20160126. Downloaded from http://pfp.biosino.org/pfp/[7]

**STRING (Search Tool for the Retrieval of INteracting Genes) analysis**

For each series of PDE inhibitor treatments, all statistically significant regulated phosphosites were compiled and lists of unique proteins were generated. Each protein list was used to query the STRING interaction database[8] (http://string-db.org/). “Experiments” and “Databases” source options were selected, and the minimum interaction score was set to 0.700. For visual clarity, disconnected nodes were omitted from the interaction map.

**Gene ontology analysis**

Gene ontology (GO) analysis was performed using the ClueGo Cytoscape plug in[9]. Lists of unique proteins, for each series of PDE inhibitor treatments, were generated from the statistically significant regulated phosphosites. Each list was used to query Kegg, Gene Ontology—biological function database, and Wiki pathways. ClueGo parameters were set as indicated: Go Term Fusion selected; only display pathways with p values ≤ 0.05; GO tree interval, all levels; GO term minimum # genes, 3; threshold of 4% of genes per pathway; and a kappa score of 0.42. Gene ontology terms are presented as nodes and clustered together based on the similarity of genes present in each term or pathway. Node size is proportional to the P value for GO term enrichment, i.e. a larger node is generated from a smaller P value. Proteins are presented as smaller circles. Multicolored circles indicate proteins associated with more than one process.
Supplemental Figure 1

Effects of individual PDE inhibitors on cAMP in the presence of 1 nM PGE₂.

Cyclic AMP Measurements. 1X10⁷ Jurkat cells were treated as indicated. Cells were briefly centrifuged and the supernate discarded. Cell pellets were lysed by adding 1 ml of 1:99 mixture of 11.65 M HCL, and 95% EtOH. Pellets were dispersed using a P1000 pipet tip, and vortexed. Lysate was incubated at room temperature for 30 min. Following incubation, the extraction volume was transferred to a fresh microfuge tube, and dried in a speed vacuum. The cAMP was re-suspended in 150 μl of 0.1 M HCl, acetylated, and assayed using a cAMP ELISA kit according to the manufacturer’s recommendations (Cayman Biochemical). A two-tailed, t-test was performed to assess for statistically significant changes. No statistically significant changes in cAMP were observed. ITI-078 was used at 200 nM; cilostamide was used at 5 μM; rolipram was used at 10 μM, BRL50481 was used at 30 μM; and PF-04957325 was used at 200 nM.
**Experimental Design** (A) Jurkat (5x10^6) cells were cultured at 37°C, 5% CO₂ for one week in RPMI, 10% FBS, 2mM Glutamine, 100 U/ml Penicillin, and 100 µg/ml Streptomycin. Twenty-four hours prior to experiment cells were washed and serum starved for 16 hours. After 16 hours, cells were synchronized by culturing in 50% FBS for 2 hours, followed by 1 hour of culture in serum free RPMI. Cells then were concurrently treated as indicated: 0.5 µg/ml CD3, 0.5 µg/ml CD28, 1 nM PGE₂, 200 nM ITI-078, 5 µM cilostamide, 10 µM rolipram, 30 µM BRL50481, 200 nM PF-04957325, 50 µM IBMX, or 200 µM IBMX. Treated cultures were incubated at 37°C, 5% CO₂ for 20 minutes. (B) The total number of phosphopeptides identified in DMSO and PGE₂ conditions, with reported intensities, were counted and plotted as a function of the cumulative number of LC-MS/MS runs ● DMSO, ■ PGE₂ (C). Scatter plots assessing the reproducibility of LC-MS/MS runs. The Pearson’s correlation between phosphopeptide intensities between the PGE₂ IBMX/PF-04957325 LC-MS/MS runs are listed in blue. Red boxes highlight biological replicates, and analytical LC-MS/MS runs.
Frequency of canonical PKA sequences that occur at sites predicted to be phosphorylated by PKA (or other kinases). A truncated peptide sequence of 4 amino acid residues flanking the regulated phosphosite was used as an input sequence in NetPhorest to predict kinases responsible for phosphorylating identified sites. Threshold score was set at 0.21. ATM = ataxia telangiectasia mutated kinase; CDK1 = Cyclin dependent kinase 1; CLK1 = CDC-like kinase; DMPK2 = Myotonic Dystrophy protein kinase-like protein (CDC42 binding protein kinase gamma); ICK = intestinal cell kinase; MAPK3 = mitogen activated protein kinase 3; MST4 = mammalian STE20-like protein kinase; PKA = cAMP-dependent protein kinase; PKB = protein kinase B (AKT), PKC = protein kinase C; TGFbR2 = transforming growth factor beta receptor 2; N/A = Not identified.
Supplemental Figure 4

STRING analysis interaction network of proteins with phosphosites regulated by combination of 200 µM IBMX and 200 nM PDE8 inhibitor.

STRING analysis to identify interacting proteins. A gene list was generated from statistically significantly regulated phosphosites modulated by 200 µM IBMX, and PDE8 inhibition, both in the basal and PGE₂ stimulated condition. The list was submitted to the STRING analysis web portal to query the “Experiments” and “Databases” source options, with a minimum interaction score of 0.700. For visual clarity, disconnected nodes were omitted from the interaction map.
Supplemental Figure 5

GO analysis showing networks of pathways and biological processes whose constituent proteins have phosphosites that are regulated by the combination PDE 1, 7, and 8 inhibition.

Biological processes likely to be regulated by the combination PDE1, 7, and 8 inhibitors. Gene ontology (GO) analysis was performed using the ClueGo Cytoscape plug in. A list of unique proteins, regulated by PDE 1, 7, and 8 inhibition were generated from the statistically significant regulated phosphosites. Each list was used to query the KEGG, Gene Ontology—biological function database, and Wikipathways. ClueGo parameters were set as indicated: Go Term Fusion selected; only display pathways with p values ≤ 0.05; GO tree interval, all levels; GO term minimum # genes, 3; threshold of 4% of genes per pathway; and kappa score of 0.42. Gene ontology terms are presented as nodes and clustered together based on the similarity of genes present in each term or pathway.
**Supplemental Figure 6**

Fold increase in phosphorylation of ARHGEF2 S858 measured by mass spectrometry.

Analysis of ARHGEF2 mass spectrometry data. Values are normalized to the DMSO/CD3/CD28 control value (set to 1). Significant differences are seen only in the presence of prostaglandin plus the PDE8 inhibitor, the combination of PDE3+4 inhibitors, or the combination of IBMX and PDE8 inhibitor. No effect of the PDE1 inhibitor was noted either alone or in combination. Intensities from all LC/MS and all experimental conditions are plotted. Statistical outliers were identified in PRISM using the ROUT function, with a Q value of 1%. Outliers are highlighted in red.
Supplemental Figure 7

Comparison of changes in STMN1 S63 phosphorylation in response to two different combinations of PDE inhibitor treatment.

Immunoblot analysis of changes in Stathmin1 phosphorylation at S63. Jurkat (1×10^7) cells were treated as previously described. Cells were harvested and boiled in 200 µl Laemmeli buffer and transferred to nitrocellulose. Membranes were probed with anti-STMN1 antibody “S” 1:2000 (Abcam), and anti-Beta actin “B” 1:200,000 (Genetex). Membranes were quantified on the Odyssey Scanner Clx (Licor). Blot shown left, and quantified on the right. Statistical analysis was performed using a Student’s t test. * P≤0.02,
Supplemental Table 1

List of genes comprising GO terms in functional cluster regulated by PDE1, 7, and 8 inhibition

<table>
<thead>
<tr>
<th>Cluster</th>
<th>GO Term</th>
<th>Term P Value</th>
<th>Group P Value</th>
<th>Associated Genes Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>alternative mRNA splicing, via spliceosome</td>
<td>21.0E-3</td>
<td>3.0E-3</td>
<td>[CDK13, DDX17, SFPQ, TRA2B]</td>
<td></td>
</tr>
<tr>
<td>nucleotide-excision repair, DNA incision, 3’-to lesion</td>
<td>28.0E-3</td>
<td>3.5E-3</td>
<td>[BIVM-ERCC5, DDB2, ERCC5]</td>
<td></td>
</tr>
<tr>
<td>Fas Ligand (FasL) pathway and Stress induction of Heat Shock Proteins (HSP) regulation</td>
<td>15.0E-3</td>
<td>2.1E-3</td>
<td>[ARHGDF1, ARHGDF7, BRAF, CFL1, MAPK3, PIK3C2A, SLC9A1, SSH2]</td>
<td></td>
</tr>
<tr>
<td>Regulation of Actin Cytoskeleton</td>
<td>1.1E-3</td>
<td>200.0E-6</td>
<td>[ARHGAP4, ARHGDF7, BRAF]</td>
<td></td>
</tr>
<tr>
<td>spindle organization</td>
<td>8.9E-3</td>
<td>1.4E-3</td>
<td>[ASUN, ATRX, CHD3, CHMP2B, NCO1, SEPT1, TPR]</td>
<td></td>
</tr>
<tr>
<td>regulation of fibroblast migration</td>
<td>49.0E-3</td>
<td>1.8E-3</td>
<td>[ARHGAP4, ARHGDF7, BRAF]</td>
<td></td>
</tr>
<tr>
<td>lamellipodium assembly</td>
<td>39.0E-3</td>
<td>3.4E-3</td>
<td>[ASUN, ATRX, CHD3, CHMP2B, NCO1, SEPT1, TPR]</td>
<td></td>
</tr>
<tr>
<td>DNA geometric change</td>
<td>3.9E-3</td>
<td>620.0E-6</td>
<td>[ARHGAP4, ARHGDF7, BRAF]</td>
<td></td>
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<tr>
<td>positive regulation of viral process</td>
<td>46.0E-3</td>
<td>3.3E-3</td>
<td>[ARHGAP4, ARHGDF7, BRAF]</td>
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<tr>
<td>ATM Signaling Network in Development and Disease</td>
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<td>3.7E-3</td>
<td>[ARHGAP4, ARHGDF7, BRAF]</td>
<td></td>
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<tr>
<td>double-strand break repair via nonhomologous end joining</td>
<td>47.0E-3</td>
<td>3.7E-3</td>
<td>[ARHGAP4, ARHGDF7, BRAF]</td>
<td></td>
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<tr>
<td>T cell selection</td>
<td>16.0E-3</td>
<td>1.4E-3</td>
<td>[BCL11B, BRAF, FASN, ITPKB]</td>
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<tr>
<td>alpha-beta T cell differentiation</td>
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<td>1.4E-3</td>
<td>[BCL11B, BRAF, ITPKB, SASH3, SATB1]</td>
<td></td>
</tr>
<tr>
<td>gene silencing by RNA</td>
<td>5.1E-3</td>
<td>580.0E-6</td>
<td>[E4F4G1, HNRNPA2B1, NCO1, NUP50, POLR2A, SNIP1, TPR]</td>
<td></td>
</tr>
<tr>
<td>negative regulation of translation</td>
<td>8.9E-3</td>
<td>580.0E-6</td>
<td>[E4F4G1, E4F2AK2, E4F4G1, HNRNPA2B1, NCO1, SNIP1, TPR]</td>
<td></td>
</tr>
<tr>
<td>gene silencing by miRNA</td>
<td>36.0E-3</td>
<td>580.0E-6</td>
<td>[E4F4G1, HNRNPA2B1, NCO1, SNIP1]</td>
<td></td>
</tr>
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</table>

Supplemental Table 1. List of genes comprising GO terms in functional clusters regulated by combined PDE1, 7, and 8 inhibitors. Gene ontology (GO) analysis was performed using the ClueGo Cytoscape plug in. Lists of unique proteins for PDEs 1, 7, and 8 inhibitor treatment were generated from the statistically significant regulated phosphosites. Each list was used to query KEGG, Gene Ontology—biological function database, and WikiPathways. ClueGo parameters were set as indicated: Go Term Fusion selected; only display pathways with p values ≤ 0.05; Go tree interval, all levels; GO term minimum # genes, 3; threshold of 4% of genes per pathway; and kappa score of 0.42. Bolded genes have regulated phosphosites predicted to be functional. In addition, Red bolded genes are sites with a PKA consensus site.
**Supplemental Table 1 (continued)**

List of genes comprising GO terms in functional cluster regulated by PDE1, 7, and 8 inhibition

<table>
<thead>
<tr>
<th>Cluster</th>
<th>GO Term</th>
<th>Term P Value</th>
<th>Group P Value</th>
<th>Associated Genes Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroid cancer</td>
<td>regulation of chromosome segregation</td>
<td>41.0E-3</td>
<td>62.0E-6</td>
<td>[BRAF, MAPK3, TPR]</td>
</tr>
<tr>
<td></td>
<td>regulation of chromatin organization</td>
<td>3.2E-3</td>
<td>62.0E-6</td>
<td>[ATRX, DYNC1LI1, MKI67, RB1, SFPQ, TPR]</td>
</tr>
<tr>
<td></td>
<td>regulation of sister chromatid cohesion</td>
<td>14.0E-3</td>
<td>62.0E-6</td>
<td>[ATRX, MAPK3, MKI67, RIF1, RTF1, TP53BP1, TPR]</td>
</tr>
<tr>
<td></td>
<td>positive regulation of chromatin organization</td>
<td>21.0E-3</td>
<td>62.0E-6</td>
<td>[ATRX, RB1, SFPQ]</td>
</tr>
<tr>
<td></td>
<td>positive regulation of chromosome organization</td>
<td>31.0E-3</td>
<td>62.0E-6</td>
<td>[MAPK3, RIF1, RTF1, TP53BP1, TPR]</td>
</tr>
<tr>
<td>mRNA Processing</td>
<td>mRNA processing</td>
<td>3.8E-3</td>
<td>11.0E-9</td>
<td>[CLASRP, HNRNPA2B1, POLR2A, SFPQ, SRRM1, SUGP2, TRA2B]</td>
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<tr>
<td>RNA splicing</td>
<td>regulation of RNA splicing</td>
<td>6.7E-9</td>
<td>11.0E-9</td>
<td>[BUD13, CDK13, CLASRP, DDX17, FIP1L1, HNRNPA2B1, LIN9, PNN, POLR2A, SAFB, SAFB2, SFPQ, SPEN, SRRM1, SRRM2, SUGP2, TFIP11, TRA2A, TRA2B, WDR33, YTHDC1]</td>
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<tr>
<td></td>
<td>regulation of mRNA processing</td>
<td>34.0E-9</td>
<td>11.0E-9</td>
<td>[DDX17, HNRNPA2B1, POLR2A, TRA2B, YTHDC1]</td>
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<td>mRNA splicing, via spliceosome</td>
<td>45.0E-3</td>
<td>11.0E-9</td>
<td>[DDX17, HNRNPA2B1, SAFB, SAFB2, TRA2B, YTHDC1]</td>
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<tr>
<td></td>
<td>mRNA splicing, via spliceosome</td>
<td>7.3E-3</td>
<td>11.0E-9</td>
<td>[DDX17, HNRNPA2B1, SAFB, SAFB2, TRA2B, YTHDC1]</td>
</tr>
</tbody>
</table>

**Supplemental Table 1 (continued).** List of genes comprising GO terms in functional cluster regulated by combined PDE1, 7, and 8 inhibitors. Gene ontology (GO) analysis was performed using the ClueGo Cytoscape plug in. Lists of unique proteins for PDEs 1, 7, and 8 inhibitor treatment were generated from the statistically significant regulated phosphosites. Each list was used to query KEGG, Gene Ontology—biological function database, and Wikipathways. ClueGo parameters were set as indicated: Go Term Fusion selected; only display pathways with p values ≤0.05; GO tree interval, all levels; GO term minimum # genes, 3; threshold of 4% of genes per pathway; and kappa score of 0.42. **Bolded genes** have regulated phosphosites predicted to be functional. In addition, **Red bolded genes** are sites with a PKA consensus site.
## Supplemental Table 1 (continued)

List of genes comprising GO terms in functional cluster regulated by PDE1, 7, and 8 inhibition

<table>
<thead>
<tr>
<th>Cluster</th>
<th>GO Term</th>
<th>Term P Value</th>
<th>Group P Value</th>
<th>Associated Genes Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA transport</td>
<td>GO Term</td>
<td>14.0E-3</td>
<td>2.4E-6</td>
<td>[EIF2S2, EIF4G1, <strong>NUP50</strong>, PNN, SRRM1, TPR, XPO1]</td>
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<tr>
<td>RNA transport</td>
<td>ribonucleoprotein complex localization</td>
<td>46.0E-6</td>
<td>2.4E-6</td>
<td>[BUD13, FIP1L1, HNRNPA2B1, LIN9, <strong>NUP50</strong>, SRRM1, TPR, <strong>WDR33</strong>, XPO1]</td>
</tr>
<tr>
<td>RNA transport</td>
<td>intracellular transport of virus</td>
<td>25.0E-6</td>
<td>2.4E-6</td>
<td>[<strong>NUP50</strong>, TPR, XPO1]</td>
</tr>
<tr>
<td>mRNA transport</td>
<td>protein export from nucleus</td>
<td>690.0E-6</td>
<td>2.4E-6</td>
<td>[BUD13, FIP1L1, HNRNPA2B1, <strong>NUP50</strong>, SRRM1, TPR, <strong>WDR33</strong>, XPO1]</td>
</tr>
<tr>
<td>protein export from nucleus</td>
<td>regulation of chromosome segregation</td>
<td>3.2E-3</td>
<td>120.0E-9</td>
<td>[ATRX, DYN1L1, MKI67, <strong>RB1, SFPQ</strong>, TPR]</td>
</tr>
<tr>
<td>regulation of chromosome segregation</td>
<td>sister chromatid segregation</td>
<td>640.0E-6</td>
<td>120.0E-9</td>
<td>[ATRX, CHAMP1, <strong>CHMP2B</strong>, DYNC1L1, KIF22, <strong>RB1, SFPQ</strong>, STAG2, TPR, XPO1]</td>
</tr>
<tr>
<td>metaphase plate congresion</td>
<td>sister chromatid cohesion</td>
<td>27.0E-3</td>
<td>120.0E-9</td>
<td>[CHAMP1, <strong>CHMP2B</strong>, KIF22, SEPT1]</td>
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<tr>
<td>metaphase plate congresion</td>
<td>mitotic sister chromatid segregation</td>
<td>19.0E-3</td>
<td>120.0E-9</td>
<td>[ATRX, KIF22, <strong>RB1, SFPQ</strong>, STAG2, XPO1]</td>
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<tr>
<td>metaphase plate congresion</td>
<td>regulation of mitotic nuclear division</td>
<td>6.0E-3</td>
<td>120.0E-9</td>
<td>[ATRX, CHAMP1, <strong>CHMP2B</strong>, DYN1L1, KIF22, <strong>RB1</strong>, TPR]</td>
</tr>
<tr>
<td>metaphase plate congresion</td>
<td>regulation of sister chromatid cohesion</td>
<td>9.5E-3</td>
<td>120.0E-9</td>
<td>[ATRX, CDK13, <strong>CHMP2B</strong>, DYN1L1, MKI67, <strong>RB1</strong>, TPR]</td>
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<tr>
<td>metaphase plate congresion</td>
<td>positive regulation of chromosome organization</td>
<td>21.0E-3</td>
<td>120.0E-9</td>
<td>[ATRX, HNRRNPA2B1, <strong>MAPK3</strong>, <strong>RB1</strong>, RIF1, <strong>RTF1, SFPQ</strong>, <strong>TP53BP1</strong>, TPR]</td>
</tr>
</tbody>
</table>

**Supplemental Table 1 (continued).** List of genes comprising GO terms in functional cluster regulated by combined PDE1, 7, and 8 inhibitors. Gene ontology (GO) analysis was performed using the ClueGo Cytoscape plug-in. Lists of unique proteins for PDEs 1, 7, and 8 inhibitor treatment were generated from the statistically significant regulated phosphosites. Each list was used to query KEGG, Gene Ontology—biological function database, and Wikipathways. ClueGo parameters were set as indicated: Go Term Fusion selected; only display pathways with p values ≤0.05; GO tree interval, all levels; GO term minimum # genes, 3; threshold of 4% of genes per pathway; and kappa score of 0.42. **Bolded genes** have regulated phosphosites predicted to be functional. In addition, **Red bolded genes** are sites with a PKA consensus site.
Supplemental Table 1 (continued)
List of genes comprising GO terms in functional cluster regulated by PDE1, 7, and 8 inhibition

<table>
<thead>
<tr>
<th>Cluster</th>
<th>GO Term</th>
<th>Term P Value</th>
<th>Group P Value</th>
<th>Associated Genes Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorso-ventral axis formation</td>
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<td>39.0E-3</td>
<td>860.0E-12</td>
<td>[BRAF, ETS1, MAPK3]</td>
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<tr>
<td>B cell receptor signaling pathway</td>
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<td>16.0E-3</td>
<td>860.0E-12</td>
<td>[MAPK3, NFATC3, PRKCB]</td>
</tr>
<tr>
<td>Fc gamma R-mediated phagocytosis</td>
<td></td>
<td>26.0E-3</td>
<td>860.0E-12</td>
<td>[CFL1, CRKL, MAPK3, MARCKSL1, PRKCB]</td>
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<tr>
<td>Long-term potentiation</td>
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<td>40.0E-3</td>
<td>860.0E-12</td>
<td>[BRAF, MAPK3, PRKCB]</td>
</tr>
<tr>
<td>Thyroid hormone signaling pathway</td>
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<td>12.0E-3</td>
<td>860.0E-12</td>
<td>[MAPK3, NCOR1, PRKCB, SLC9A1, STAT1, TBC1D4]</td>
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<tr>
<td></td>
<td>Hepatitis B</td>
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<td>860.0E-12</td>
<td>[DDB2, DDX3X, MAPK3, NFATC3, PRKCB, RB1, STAT1]</td>
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<tr>
<td></td>
<td>Renal cell carcinoma</td>
<td>46.0E-3</td>
<td>860.0E-12</td>
<td>[BRAF, CRKL, ETS1, MAPK3]</td>
</tr>
<tr>
<td></td>
<td>Pancreatic cancer</td>
<td>45.0E-3</td>
<td>860.0E-12</td>
<td>[BRAF, MAPK3, RB1, STAT1]</td>
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<tr>
<td></td>
<td>Glioma</td>
<td>45.0E-3</td>
<td>860.0E-12</td>
<td>[BRAF, MAPK3, PRKCB, RB1]</td>
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<tr>
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<td>Thyroid cancer</td>
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<td>[BRAF, MAPK3, TPR]</td>
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<tr>
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<td>Melanoma</td>
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<tr>
<td></td>
<td>Chronic myeloid leukemia</td>
<td>11.0E-3</td>
<td>860.0E-12</td>
<td>[BRAF, CRKL, MAPK3, RB1, RUNX1]</td>
</tr>
<tr>
<td></td>
<td>Non-small cell lung cancer</td>
<td>3.7E-3</td>
<td>860.0E-12</td>
<td>[BRAF, MAPK3, PRKCB, RB1, STK4]</td>
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<tr>
<td></td>
<td>Human Thyroid Stimulating Hormone (TSH signaling pathway)</td>
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<td>[BRAF, MAPK3, RAP1GAP, RB1, STAT1]</td>
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<td>Signaling Pathways in Glioblastoma</td>
<td>17.0E-3</td>
<td>860.0E-12</td>
<td>[BRAF, MAPK3, PIK3C2A, PRKCB, RB1]</td>
</tr>
<tr>
<td></td>
<td>B Cell Receptor Signaling Pathway</td>
<td>30.0E-3</td>
<td>860.0E-12</td>
<td>[BRAF, CRKL, ETS1, NFATC3, PRKCB]</td>
</tr>
<tr>
<td></td>
<td>Kit receptor signaling pathway</td>
<td>36.0E-3</td>
<td>860.0E-12</td>
<td>[CRKL, MAPK3, PRKCB, STAT1]</td>
</tr>
<tr>
<td></td>
<td>EGF/EGFR Signaling Pathway</td>
<td>11.0E-3</td>
<td>860.0E-12</td>
<td>[ARHGEF1, ATXN2, BRAF, CFL1, CRKL, PRKCB, STAT1]</td>
</tr>
<tr>
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<td>epithelial cell apoptotic process</td>
<td>31.0E-3</td>
<td>860.0E-12</td>
<td>[BRAF, FASN, HIPK1, RB1, STK4]</td>
</tr>
<tr>
<td></td>
<td>positive regulation of chromatin organization</td>
<td>31.0E-3</td>
<td>860.0E-12</td>
<td>[MAPK3, RIF1, RTF1, TP53BP1, TPR]</td>
</tr>
<tr>
<td></td>
<td>hepatocyte apoptotic process</td>
<td>11.0E-3</td>
<td>860.0E-12</td>
<td>[FASN, RB1, STK4]</td>
</tr>
</tbody>
</table>

Supplemental Table 1 (continued). List of genes comprising GO terms in functional cluster regulated by PDE1, 7, and 8 inhibition. Gene ontology (GO) analysis was performed using the ClueGo Cytoscape plug in. Lists of unique proteins for PDEs 1, 7, and 8 inhibitor treatment were generated from the statistically significant regulated phosphosites. Each list was used to query KEGG, Gene Ontology—biological function database, and Wikipathways. ClueGo parameters were set as indicated: Go Term Fusion selected; only display pathways with p values ≤0.05; GO tree interval, all levels; GO term minimum # genes, 3; threshold of 4% of genes per pathway; and kappa score of 0.42. Bolded genes have regulated phosphosites predicted to be functional. In addition, Red bolded genes are sites with a PKA consensus site.
# Supplemental Table 2

## List of genes comprising GO terms in functional cluster regulated by PDE3, and 4 inhibition

<table>
<thead>
<tr>
<th>Cluster</th>
<th>GO Term</th>
<th>Term P Value</th>
<th>Group P Value</th>
<th>Associated Genes Found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>double-strand break repair via nonhomologous end joining</td>
<td>5.3E-3</td>
<td>2.6E-3</td>
<td>[MDC1, RIF1, SMC5]</td>
</tr>
<tr>
<td>Pathways Affected in Adenoid Cystic Carcinoma</td>
<td>7.3E-3</td>
<td>4.9E-3</td>
<td>[ARID1A, HIST1H1E, MAP2K2]</td>
<td></td>
</tr>
<tr>
<td>establishment of spindle orientation</td>
<td>1.1E-3</td>
<td>490.0E-6</td>
<td>[ARHGEF2, MAP4, NUMA1]</td>
<td></td>
</tr>
<tr>
<td>Chronic myeloid leukemia</td>
<td>3.2E-3</td>
<td>2.8E-3</td>
<td>[ABL1, BAD, MAP2K2]</td>
<td></td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis (ALS)</td>
<td>2.9E-3</td>
<td>2.8E-3</td>
<td>[ALS2, BAD, MAP2K2]</td>
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<tr>
<td>histone H3-K27 trimethylation</td>
<td>98.0E-6</td>
<td>53.0E-6</td>
<td>[HIST1H1C, HIST1H1D, HIST1H1E]</td>
<td></td>
</tr>
<tr>
<td>peptidyl-lysine trimethylation</td>
<td>200.0E-6</td>
<td>53.0E-6</td>
<td>[HIST1H1C, HIST1H1D, HIST1H1E, PWP1]</td>
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</tr>
<tr>
<td>histone lysine methylation</td>
<td>100.0E-6</td>
<td>53.0E-6</td>
<td>[HIST1H1C, HIST1H1D, HIST1H1E, PWP1, RIF1, SNW1]</td>
<td></td>
</tr>
<tr>
<td>histone H3-K4 methylation</td>
<td>730.0E-6</td>
<td>53.0E-6</td>
<td>[HIST1H1C, HIST1H1D, HIST1H1E, SNW1]</td>
<td></td>
</tr>
<tr>
<td>Regulation of Microtubule Cytoskeleton</td>
<td>4.1E-3</td>
<td>270.0E-6</td>
<td>[ABL1, CLIP1, STMN1]</td>
<td></td>
</tr>
<tr>
<td>regulation of microtubule cytoskeleton organization</td>
<td>200.0E-6</td>
<td>270.0E-6</td>
<td>[ABL1, ARHGEF2, CLIP1, GAS2L1, RANBP2, STMN1]</td>
<td></td>
</tr>
<tr>
<td>negative regulation of microtubule polymerization or depolymerization</td>
<td>1.6E-3</td>
<td>270.0E-6</td>
<td>[ARHGEF2, GAS2L1, STMN1]</td>
<td></td>
</tr>
<tr>
<td>regulation of microtubule polymerization</td>
<td>1.7E-3</td>
<td>270.0E-6</td>
<td>[ABL1, CLIP1, STMN1]</td>
<td></td>
</tr>
<tr>
<td>protein depolymerization</td>
<td>3.5E-3</td>
<td>270.0E-6</td>
<td>[ARHGEF2, GAS2L1, MICAL1, STMN1]</td>
<td></td>
</tr>
</tbody>
</table>

**Legend -- List of genes comprising GO terms in functional cluster regulated by PDE3, and 4 inhibition.**

Gene ontology (GO) analysis was performed using the ClueGo Cytoscape plug in. Lists of unique proteins for PDEs 3 and 4inhibitor treatment were generated from the statistically significant regulated phosphosites. Each list was used to query KEGG, Gene Ontology—biological function database, and Wikipathways. ClueGo parameters were set as indicated: Go Term Fusion selected; only display pathways with p values ≤ 0.05; GO tree interval, all levels; GO term minimum # genes, 3; threshold of 4% of genes per pathway; and kappa score of 0.42. **Bolded genes** have regulated phosphosites predicted to be functional. In addition, Red **bolded genes** are sites with a PKA consensus site.
Expanded discussion of PDE-modulated functional compartments.

STRING analysis of the PDE 1,7, and 8 inhibitor phosphosites, suggests a cAMP mediated regulatory action on the phosphatase, PTPN7, a known regulator of MAPK3 and other kinases in this cascade. There are 15 phosphosites modulated under the PDE 1, 7, and 8 inhibitor condition that are predicted to be phosphorylated by MAPK3. Likely examples of indirect cAMP signaling via phosphatase regulation include: BAP18 S76, CHAMP1 S286, CHD3 S1660, CHD3 S1660, ITPKB S43, LIN9 S380, LMNB1 S391, NCOR1 S2048, PIK3C2A 1553, RB1 T778, RING1 S254, SMEK1 S728, SNIP1 S54, SNTB2 S393, AND STAT1 S727.

Interestingly, Conche et al.[10] previously implicated PTPN7, ERK, and a cAMP mediated signal as an early enhancer of the T cell receptor response promoted by cellular adhesion. We found that phosphosites on ARHGEF2, BRAF, LASP1 and PTPN7 were regulated by inhibiting PDEs 1, 7, and 8; whereas sites on CAD, BAD, CAMKK1, PTPN7, and STMN1 were regulated by inhibiting PDEs 3 and 4. ARHGEF2[11], BRAF[12], and LASP1[13] have been associated with regulation of cytoskeletal organization and cell adhesion/migration. It is plausible that inhibiting a combination of PDEs 1, 7, and 8 could therefore regulate T cell migration and activation. Since PDE1 protein and activity is reported to be low in several T cell models, this perhaps suggests that the most important combination will be PDE7 and PDE8 inhibitors.

PDE 3 and 4 inhibition also significantly increased the phosphorylation of CAD (Table 2), the rate limiting protein in the pyrimidine biosynthetic pathway. Increased glutamine metabolism in lymphocytes is thought to be required to support proliferation, and provide metabolites for biosynthetic pathways[14-16]. CAD is a multi-domain protein that catalyzes the first reactions of de novo pyrimidine biosynthesis, converting glutamine to carbamoyl phosphate. UTP binding to the BH3 regulatory domain of CAD, causes feedback inhibition. Phosphorylation of S1406 prevents UTP binding and increases activity of the enzyme[17]. Paradoxically, it seems that phosphorylation of S1406 on CAD would increase glutamine metabolism and promote T cell activation. However, the combination treatment of PDEs 3 and 4 inhibitor has been shown to inhibit T cell function. One possible explanation is that an overactive pyrimidine biosynthetic pathway reduces the available glutamine that can be converted to glutamate and shuttled into the TCA cycle or converted to glutathione. This should reduce the cells ability to buffer against reactive oxygen species generated by rapid proliferation, and could be a novel mechanism of cAMP-dependent inhibition of T cell function.

Similarly, S163 of DGKZ is one of the most consistently phosphorylated sites in the PDE3 plus PDE4 inhibitor condition. A number of T cell functions are known to be controlled by DGKZ[18]. For example, it is well established that redistribution of diacylglycerol kinase to the immunological synapse can regulate several aspects of T cell activation[19] and function including T cell adhesion and migration[20]. Activation of DGKZ would be expected to lower diacylglycerol and increase phosphatidic acid, both of which are likely to decrease T cell activation. Much less is known about a possible role for cAMP-dependent phosphorylation of S163 as a regulator of T cells though one would predict that activation of the enzyme would be consistent with an inhibitory effect of PDE 3 plus 4 inhibition on T cell activation. Further studies will be needed to test how much of the inhibitory effect of cAMP on T cell function might be localized to this phosphosite and regulated by a combination of both PDE3 and PDE4.
References - Supplemental


