UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Development of a BRET-based Assay to Identify Modulators of Epac Activity : : A Protein Up-regulated in Cancer

Permalink

https://escholarship.org/uc/item/3996r5cn

Author

Brown, Loren Matthew

Publication Date

2014

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Development of a BRET-based Assay to Identify Modulators of Epac Activity: A Protein Up-regulated in Cancer

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Loren Matthew Brown

Committee in Charge:

Professor Paul Insel, Chair
Professor James Feramisco
Professor Chris Glass
Professor Tracy Handel
Professor Mark Kamps

2014

Copyright

Loren Matthew Brown, 2014

All rights reserved

The Dissertation of Loren Matthew Brown is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2014

TABLE OF CONTENTS

SIGNATURE PAGE……..... iii
TABLE OF CONTENTS……..... iv
LIST OF ABBREVIATIONS……..... vii
LIST OF FIGURES……..…..... xi
ACKNOWLEDGMENTS.....…… ……. …..... xiv
VITA……..……..... xvi
ABSTRACT OF THE DISSERTATION……..……..... xvii

Chapter One: Introduction to the cAMP Pathway and the cAMP Effector Epac.. 1
1.1 The Epac Pathway..... 1
1.1.1 G-Protein Coupled Receptors ……..……..... 1
1.1.2 The cAMP Pathway……..……..... 3
1.1.3 Rap Signaling…….....……..……..... 6
1.2 Regulation of Epac..... 7
1.2.1 Epac Activation……..... 7
1.2.2 Pharmacological Tools to Study Epac Signaling..... 8
1.2.3 Expression of Epac…..……..... 12
1.3 Epac Signaling..... 15
1.3.1 Epac in the Brain …..... 16
1.3.2 Epac in the Pancreas..... 17
1.3.3 Epac in the Heart..... 17
1.4 The cAMP Pathway and Cancer…..……..... 19
1.4.1 Chronic Lymphocytic Leukemia..... 20
1.4.2 Melanoma..... 22
1.4.3 Prostate Cancer..... 23
1.5 Summary……..……..... 25
1.6 Figures…….....…….....… 26
1.7 References..... 37

Chapter Two: Lef1-mediated up-regulation of Epac1 expression in Chronic Lymphocytic Leukemic Cells Contributes to the Anti-Apoptotic Phenotype..... 48

2.1 Abstract..... 48

2.2 Introduction..... 49

2.3 Experimental Procedures..... 53

2.4 Results..... 56

2.5 Discussion..... 61

2.6 Figures..... 63

2.7 References..... 76

Chapter Three: Identification and Validation of Modulators of Epac Activity: Structure-function Implications for Epac Activation and Inhibition..... 81

3.1 Abstract..... 81

3.2 Introduction..... 82

3.3 Experimental Procedures..... 84

3.4 Results..... 91

3.5 Discussion..... 101

3.6 Acknowledgments..... 106

3.7 Figures and Table..... 108

3.8 References..... 121

Chapter Four: The Hinge Region of the Cyclic Nucleotide Binding Domain of Epac as a Target for Allosteric Inhibitors 126

4.1 Abstract..... 126

4.2 Introduction..... 127

4.3 Experimental Procedures..... 129

4.4 Results..... 136

4.5 Discussion..... 142

4.6 Acknowledgments..... 144

4.7 Figures..... 146

4.8 References……..... 155

Chapter Five: Conclusions and Discussions……...…… 159

5.1 Regulation of Epac Expression by Lef-1..... 160

5.2 Development of a High-Throughput Assay for Epac Inhibitors.... 162

5.3 Identification of the Hinge of Epac as a Druggable Region..... 164

5.4 Summary..... 166

5.5 Figures..... 167

5.6 References..... 168

LIST OF ABBREVIATIONS

2',5'ddAdo	2',5'dideoxyadenosine
8-Me	8-CPT-2'O-Me-cAMP
8-Me-N6	8-CPT-2'O-Me-N^6-Phe-cAMP
AC	adenylyl cyclase
AE	aminoethyl
ATP	adenosine triphosphate
AU	arbitrary units
Bn	benzene
Bnz	benzoyl
Br	bromine
C3G	Crk SH3-binding guanine nucleotide-releasing factor
CAMYEL	cAMP sensor using yellow fluresecent protein-Epac-luciferase
cAMP	cyclic adenosine monophosphate
cAMPS	cyclic adenosine monophosphorothiorate
CDC25HD	cell division cycle25 homology domain
cDNA	complementary deoxyribonucleic acid
CF	cardiac fibroblasts
CFP	cyan fluorescent protein
cGMP	cyclic guanine monophophate
CNDB	cyclic-nucleotide binding domain
CPT	chlorophenylthio

CPT-N6	8-CPT-N^6-Phenyl-cAMP
DEP	dishevelled egl-10 pleckstrin
EMSA	electrophoretic mobility shift assay
Epac	exchange protein activated by cyclic adenosine monophosphate
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinases
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HCN	hyperpolarization-activated cyclic nucleotide-gated channel
HEK	human embryonic kidney
HIF	hypoxia inducible factor
HMG	high mobility group
HPT	hydroxylphenylthio
HTS	high throughput screen
IFD	induced fit docking
IgHV	immunoglobulin G heavy chain variable region
Lef-1	lymphoid enhancing factor
MB	monobutyrl
MD	molecular docking

Me	methyl
MeOPT	methoxyphenylthio
mRNA	messenger ribonucleic acid
NE	nuclear extracts
PBC	phosphate binding cassette
PBMC	peripheral blood mononuclear cells
PDB	protein data bank
PDGF	platelet-derived growth factor
PDE	phosphodiesterase
PGE	prostaglandin E
Phe	phenyl
PIP	piperdine
PKA	protein kinase A
pVASP	phosphorylated vasodilator stimulated phosphoprotein
QT-PCR	quantitative real-time polymerase chain reaction
RA	Ras assoication
Rap	Ras associated protein
REM	Ras exchange motif
RBD	Rap1-GTP-binding domain
Rp-CPT	(R_p)-8-CPT-cAMPS
RLU	relative luciferase units
RMSD	root-mean-square deviation

sAC	soluble adenylyl cyclases
siRNA	small interfering ribonucleic acid
SOX	sex determining region Y box
SRY	sex determining region Y
TCF	transcription factor
TCL1	T-cell leukemia/lymphoma protein 1
TGF	transforming growth factor
VASP	vasodilator stimulated phosphoprotein

LIST OF FIGURES AND TABLES

Figure 1.1 The cAMP Pathway..... 26

Figure 1.2 Domain Structures of Epacs..... 27

Figure 1.3 Mechanism of Activation of Epac by cAMP..... 28

Figure 1.4 cAMP analogs used to probe the cAMP pathway..... 29

Figure 1.5 Compounds that directly target adenylyl cyclases..... 30

Figure 1.6 Competitive Inhibition..... 31

Figure 1.7 Noncompetitive Inhibition..... 32

Figure 1.8 Uncompetitive Inhibition..... 33

Figure 1.9 Epac Inhibitors..... 34

Figure 1.10 Potential Roles for Epac in CLL..... 35

Figure 1.11 A Proliferative Positive Feedback Loop of Epac in Prostate Cancer..... 36

Figure 2.1 Epac1 mRNA is increased in CLL cells..... 63

Figure 2.2 Epac1 protein is increased in CLL..... 64

Figure 2.3 Epac1 contributes to the anti-apoptotic phenotype of CLL cells..... 65

Figure 2.4 The Epac1 promoter is highly conserved in the rat, mouse, and human genome..... 66

Figure 2.5 Deletional analysis of the Epac1 promoter in WaC3 cells..... 67

Figure 2.6 Mutation of a SRY binding site decreases activity and binding. 68

Figure 2.7 Lef-1 increases activity of the Epac1 promoter in WaC3 cells... 69

Figure 2.8 Lef-1 does not increase Epac1 promoter activity in non-CLL

cells..... 70

Figure 2.9 Lef-1 and Epac1 mRNA expression correlate in CLL PBMC.... 71

Figure 2.10 Ethacrynic acid inhibits Lef-1 activity in WaC3 and HEK293 cells..... 72

Figure 2.11 Ethacrynic acid decreases Epac1 mRNA in CLL cells..... 73

Figure 2.12 Lef-1 binds to the Epac1 promoter..... 74

Figure 2.12 The cAMP pathway in CLL..... 75

Table 3.1 Binding affinity of cAMP analogs, as assessed by CAMYEL.... 108

Figure 3.1 The CAMYEL Assay..... 109

Figure 3.2 CAMYEL activation is induced by Epac agonists in a concentration-dependent manner..... 110

Figure 3.3 CAMYEL can assess if cAMP analogs are agonists, super agonists, or partial agonists..... 111

Figure 3.4 Rp-substituted cAMP analogs are competitive inhibitors and addition of 8-CPT enhances binding affinity..... 112

Figure 3.5 CE3F4, an Epac-specific inhibitor, inhibits CAMYEL activation..... 113

Figure 3.6 N6-modified cAMP analogs are competitive inhibitors of Epac1, as assessed in the CAMYEL assay..... 114

Figure 3.7 cGMP analogs as inhibitors of Epac1, as assessed in the CAMYEL assay. 115

Figure 3.8 Comparison of inhibitors of Epac, as assessed using Epac2-cAMPS or the CAMYEL assay..... 116

Figure 3.9 Epac1 activates Rap1 while PKA activation mediates VASP phosphorylation in Swiss 3T3 cells..... 117

Figure 3.10 CPT-N6 and Rp-CPT inhibit forskolin-promoted Epac activation in Swiss 3T3 cells..... 118

Figure 3.11 Molecular Model of CPT-N6 bound to Epac1..... 119

Figure 3.12 Chembridge compound 5225554 is an allosteric inhibitor of Epac..... 120

Figure 4.1 Domain structure and activation of Epac..... 146

Figure 4.2 Hot Spot near Epac Hinge..... 147

Figure 4.3 Screening of 133 compounds identified 5225554 as an Epac1 inhibitor..... 148

Figure 4.4 A thiobarbituric acidic head group enhances the ability of the compound to inhibit Epac in Swiss 3T3 cells..... 149

Figure 4.5 Ligand Interactions..... 150

Figure 4.6 5376753 and 5225554 are allosteric inhibitors of Epac..... 151

Figure 4.7 Effect of 5225554 and 5376753 on viability of Swiss 3T3 cells. 152

Figure 4.8 5376753 can inhibit Epac1 and Epac2 in HEK293 cells..... 153

Figure 4.9 5376753 inhibits Epac-mediated migration of rat cardiac fibroblasts..... 154

Figure 5.1 8-CPT-2’O-Me-N^6-Phe-cAMP..... 167

ACKNOWLEDGEMENTS

I would like to acknowledge everyone in the Insel laboratory. I would like to thank Fiona Murray for her assistance in helping me get started in the lab. I would like to thank Nakon Aroonsakool for his dedication and help throughout the years. His ability to provide the necessary resources on time and insight into fibroblasts biology was a huge help to my efforts. I would especially like to thank my thesis advisor Paul Insel. His guidance over the years has helped shape me into a better more independent scientist. Without his support I could not have accomplished what I did.

I would like to thank all of my committee members including Dr. Christopher Glass, Dr. James Feramisco, Dr. Tracy Handel, and Dr. Mark Kamps. Their guidance and suggestions were most useful. I thank them for taking the time to help train me as a graduate student.

I would like to thank my collaborators Dr. Kathleen Rogers and Dr. Andy McCammon. Without their work I would not have known where to begin. I would also like to thank them for their patience, financial support, and dedication to this project.

Most importantly, I would like to acknowledge my family and friends for the support they provided. Without their encouragement this would have been an impossible journey. I would like to especially thank my parents for all of their love and motivation over the years.

The contents of Chapter 3, in full, have been published: **Brown, L.M.,** Rogers K.E., McCammon J.A., and Insel, P.A. (2014) “Identification and Validation of Modulators of Epac Activity: Structure-function Implications for Epac Activation and

Inhibition." *Journal of Biological Chemistry*, 289: 8217-8230. The dissertation author was the primary investigator and author of this paper. All co-authors have given written permission for its use and the reproduction of all associated data in this dissertation.

The contents of Chapter 4, in full, is in preparation: **Brown, L.M.,** Rogers K.E., Aroonsakool, N, McCammon J.A., and Insel, P.A. (2014) "The hinge region of the cyclic nucleotide binding domain of Epac as a target for allosteric inhibitors." *Journal TBD.* The dissertation author was the primary investigator and author of this paper. All co-authors have given written permission for its use and the reproduction of all associated data in this dissertation.

This work was supported by National Institutes of Health Molecular Biological Approaches to Endocrinology Training Grant (5T32DK007541-27) and a T32 Pharmacological Sciences Training Grant.

VITA

1999-2001	Undergraduate Clackamas Community College
2001-2003	Undergraduate Oregon State University
2003	Bachelor of Sciences Oregon State University
2004-2006	Research Assistant 2 in Dr. Lundblads Laboratory OHSU
2006-2007	Research Assistant 2 in Dr. Farrens Laboratory OHSU
2007-2014	Graduate Student University of California San Diego
2014	Doctor of Philosophy University of California San Diego

PUBLICATIONS

Peiró AM, Tang CM, Murray F, Zhang L, **Brown LM**, Chou D, Rassenti L, Kipps TJ, Insel PA. "Genetic variation in phosphodiesterase (PDE) 7B in chronic lymphocytic leukemia: overview of genetic variants of cyclic nucleotide PDEs in human disease." *Journal of Human Genetics* 2011 Sep;56(9):676-81.

Insel PA, Murray F, Yokoyama U, Romano S, Yun H, **Brown L**, Snead A, Lu D, Aroonsakool N. "Cyclic AMP and Epac in the regulation of tissue fibrosis" *British Journal of Pharmacology* 2012 May;166(2):447-56

Brown LM, Rogers KE, McCammon JA, Insel PA. "Identification and Validation of Modulators of Epac Activity: Structure-function Implications for Epac Activation and Inhibition" Journal of Biological Chemistry 2014.

ABSTRACT OF THE DISSERTATION

Development of a BRET-based Assay to Identify Modulators of Epac Activity:

A Protein Up-regulated in Cancer

by

Loren Matthew Brown

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2014

Professor Paul Insel, Chair

Cyclic AMP (cAMP) is an important second messenger signaling molecule that is involved in wide variety of physiological processes. The canonical cAMP pathway is initiated when hormones or neurotransmitters bind to G-protein coupled receptors (GPCRs) that are coupled to $G\alpha_s$. This leads to activation of adenylyl cyclases (ACs) that convert ATP to cAMP. There are two main intracellular effectors of cAMP-mediated signaling: Protein Kinase A (PKA) and Exchange Protein

Activated by cAMP (Epac). Dysregulation of the cAMP pathway is implicated in a variety of diseases including diabetes mellitus, heart failure, and cancer. While much work has been done to develop pharmacological tools to study the cAMP signaling system, most of these efforts have focused on PKA, GPCRs, and ACs. Studies on Epac are more limited. The work to be described in this dissertation analyzes the dysregulation of the cAMP effector Epac in chronic lymphocytic leukemia (CLL, Chapter 2). I show that Epac is up-regulated at the mRNA and protein level and this contributes to the anti-apoptotic phenotype of the disease. I further show that Lef-1, a transcription factor up-regulated in leukemia, mediates the increased expression of Epac1. Understanding the dysregulation of Epac signaling, such as occurs in CLL, would be enhanced with new pharmacological tools to study Epac. To assist in the development of new tools I developed a BRET-based assay that takes advantage of the conformational changes that Epac undergoes when activated to analyze modulators of Epac activity *in vitro* (Chapter 3). I demonstrated that this assay can identify competitive inhibitors, such as the cAMP analogs CPT-N6-cAMP and (R_p)-CPT-cAMPS, and can identify allosteric inhibitors such as CE3F4. I further verified these findings in cells by measuring Rap1 activation. I used this assay to experimentally test compounds that were predicted to inhibit Epac activity allosterically (Chapter 4). I identified a novel thiobarbituric acid derivative that binds to the hinge region of the cyclic nucleotide binding domain of Epac1 and Epac2 and inhibits their activity. Together these data implicate Epac as an important signaling protein that is increased in cancer and identify a new approach to identify inhibitors of Epac.

Chapter One: Introduction to the cAMP Pathway and the cAMP Effector Epac

1.1 The Epac Pathway

1.1.1 G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are a large family of seven-transmembrane proteins. There are over 800 GPCRs in the human genome and they comprise approximately 4% of the human genome. GPCRs are transducers of "first messengers" (i. e. hormones and neurotransmitters) that regulate physiological systems, such as cardiac function, immune responses, memory formation, metabolism, and sensory function(1). In addition to their roles in normal physiology GPCRs are also implicated to be important in a wide variety of diseases. Due to their important roles in physiology, their accessibility to the outside environment and their binding pockets GPCRs are considered "classical" drug targets(2). It is estimated that approximately 25% of current drugs on the market target GPCRs, illustrating their importance in physiology and disease(3).

GPCRs are important signaling molecules that translate outside signals to inside signals. The downstream signaling of GPCRs is determined in part by their G-protein coupling. GPCRs are coupled to a heterotrimeric G-protein complex composed

of a Gα, Gβ, and Gγ subunit. Ligand binding to GPCRs activates the GPCR, inducing intramolecular movements that allow it act as a guanine-nucleotide exchange factor (GEF) for the Gα subunit. This causes the displacement of GDP bound to Gα allowing GTP to bind and activate the Gα. The Gα subunit then disassociates from the Gβγ subunits and the Gα and Gβγ subunits activate a variety of downstream signaling pathways. In addition to G-protein-dependent signaling, GPCRs can also signal through β-arrestins in a G-protein-independent manner(4).

The activity of GPCRs is decreased by desensitization, internalization, and down-regulation. Agonist binding to GPCRs induces the recruitment of G-protein coupled receptor kinases (GRKs) to the receptor resulting in the phosphorylation of serine or threonine residues on the intracellular side of the receptor(5). This phosphorylation enhances the binding of β-arrestins to the receptor. β-arrestins are involved in G-protein-independent signaling pathways but were originally discovered to be involved in the desensitization of GPCR signaling. β-arrestin binding to the receptor can result in the internalization of the GPCR by a clathrin-mediated pathway. Once internalized the receptor is targeted either for dephosphorylation and recycling back to the cell membrane or for degradation(6).

There are four main classes of Gα: G_s, G_i, G_q, and $G_{12/13}$. The downstream signaling from GPCRs is in part determined by the class of Gα protein to which they couple. $G\alpha_s$ and $G\alpha_i$ bind to adenylyl cyclases (AC) and stimulate or inhibit, respectively, the conversion of ATP to cyclic adenosine monophosphate (cAMP). $G\alpha_q$ leads to increases in cellular calcium while $G\alpha_{12/13}$ activates the small GTPase Rho. While $G\alpha_s$ and $G\alpha_i$ are the main G-proteins that regulate cAMP levels $G\alpha_q$ and $G\alpha_{12/13}$

activation can also alter cAMP signaling in certain cell types (especially immune cells) through their modulation of AC7(7)(8). The activity of the G-proteins is turned off by regulators of G-protein signaling (RGS). These are GTPase activating proteins (GAPs) which increase the intrinsic GTPase activity of the Gα subunit. This results in hydrolysis of GTP to GDP and the inactivation of Gα signaling(9). The inactive Gα subunit will bind the Gβγ subunits resulting in the inactivation of Gβγ signaling(10).

1.1.2 The cAMP Pathway

Cyclic AMP (cAMP) was the first "second messenger" discovered and is an important signaling molecule involved in a wide variety of cellular processes, including proliferation, differentiation, secretion, migration, memory, and apoptosis(11)(12)(13)(14)(15). The canonical cAMP pathway is initiated when agonists bind to $G\alpha_s$ coupled GPCRs. Binding of the agonist causes the disassociation of the $G\alpha_s$ subunit from the Gβγ subunit. $G\alpha_s$ binds to and activates transmembrane ACs that catalyze the conversion of ATP to cAMP. cAMP will bind to and activate downstream effectors: protein kinase A (PKA), exchange protein directly activated by cAMP (Epac), and cyclic nucleotide-gated channels (CNGs) (Fig. 1.1). There are nine transmembrane ACs that have different expression patterns and are regulated by different mechanisms. There is also a soluble AC (sAC) that is expressed ubiquitously throughout the body, and appears to have important roles in controlling cAMP signaling in the mitochondria and the nucleus(16). Unlike the transmembrane ACs,

sAC is not activated by G-proteins but is instead activated by bicarbonate anions and thus acts as a novel pH- sensing AC(17)(18).

The cAMP pathway is turned off by the activity of G_i-coupled GPCRs and phosphodiesterases (PDEs). Agonist binding to G_i-coupled GPCRs activates $G\alpha_{i,}$ allowing it to bind to ACs and inhibit their activity, thus decreasing the formation of new cAMP molecules. PDEs hydrolyze cyclic nucleotides to 5'derivatives (AMP in the case of cAMP), thereby lowering the intracellular cyclic nucleotide concentrations. There are 11 different PDE families which can act selectively to hydrolyze cAMP, cGMP, or hydrolyze both(19). These PDEs are regulated by different mechanisms, have multiple splice variants, and different tissue and subcellular expression patterns. The combined actions of Gs/Gi proteins, ACs, PDEs, β-arrestins, RGSs and downstream effectors creates a complex network of signaling downstream from GPCRs activated by hormones that can lead to a wide variety of different outcomes depending on the specific tissue.

For many years the effects of cAMP were thought to be mediated solely through the activation of PKA. Two laboratories simultaneously discovered Epac in 1998. The laboratory of Johannes Bos discovered Epac through a search for the mechanism by which cAMP activated Rap1 in a PKA-independent manner and by using a genomic analysis of proteins that share sequence similarity with GEFs for Ras or Rap1 and that have putative cAMP binding domains(20). The laboratory of Ann Graybiel independently identified Epac as a GEF for Rap1 through a search for brain-enriched proteins that have second messenger motifs(21). This search also lead to the discovery of the Ca^{2+} activated Rap GEF, CalDAG-GEF(22). The discovery of Epac

has led to a reassessment of the older literature to determine if the effects of cAMP on cells resulted from activation of PKA, Epac, or both effectors.

There are two isoforms of Epac in mammalian genomes, Epac1 and Epac2. Both isoforms have similar biochemical activity and domains, though Epac2 has an additional cyclic nucleotide binding domain (CNBD) on its N-terminus. Epac has a catalytic region that is responsible for the activation of Rap1/2 and a regulatory region that is responsible for its activation by cAMP and its subcellular localization. The catalytic region is composed of a Ras exchange motif (REM), a Ras association domain (RA), and a CDC25 homology domain that mediates the interaction with Rap1/2. The regulatory region is composed of a CNBD that is responsible for the activation of Epac by cAMP and a Dishevelled, Egl-10, Pleckstrin (DEP) domain that determines the subcellular localization of Epac through its binding to phosphatidic acid (Fig 1.2) (23). Epac2 has two CNBDs. The N-terminal domain (CNDB-A) has lower affinity for cAMP and is involved in the subcellular localization of Epac2 while the CNDB-B has a higher affinity for cAMP and is involved in the activation of Epac2 by cAMP. Activation of Epac is associated with the activation of different signaling pathways that depend on the cell type and include the ERK, Akt, DNA-PK, and NF-κB pathways(24)(25)(26)(27).

1.1.3 Rap Signaling

Rap proteins are low molecular weight GTPases that belong to the Ras superfamily(28). There are five isoforms of Rap in the human genome: Rap1a, Rap1b,

Rap2a, Rap2b, and Rap2c. Rap activity was initially thought to counteract the transformative effects for Ras(29)(30). These effects were later found to be an artifact of overexpression of Rap1 which causes it to inappropriately bind to and sequester the Ras effector c-Raf(31). Newer evidence has indicated a wide variety of functions for Rap1, including the activation of integrins, regulation of proliferation, migration, and gene activation.

The Rap1 pathway is activated by a family of GEFs that include Epac1, Epac2, C3G, CalDAG-GEFI, PDZ-GEFI, and MR-GEF, all of which promote the disassociation of GDP from Rap1. GTP then replaces GDP due to its higher abundance in cells. Activation of Rap signaling is linked to a wide arrange of intracellular signaling events through its GEFs. C3G is activated downstream of receptor tyrosine kinases such as EGFR and the insulin receptor. Epac1 and Epac2 are directly activated by cAMP. CalDAG-GEF1 is regulated by increases in intracellular Ca^{2+} and diacylglycerols. MR-GEF is a constitutively active GEF that is inhibited by the activity of M-Ras(32).

The Rap pathway is inhibited by GAPs that enhance the intrinsic GTPase activity of Rap1 that converts GTP to GDP. There are at least two families of RapGAPs in the human genome. The Rap1GAP family, made up of Rap1GAPI and Rap1GAPII, are the most ubiquitously expressed GAPs for Rap1(33). Rap1GAPI can bind to Gα proteins at the cell membrane and regulate the membrane activation of Rap1. Rap1GAPII localizes with the scaffolding protein 14-3-3 and regulates intracellular activation of Rap1. The second family of RapGAPs is the Sipa1 family, which is comprised of Spa-1, Spar1, Spar2, and Spar3(34). These GAPs have more

tissue-specific expression. Spa-1 is the major RapGAP in hematopoietic cells while the Spar proteins are expressed primarily in neurons.

1.2 Regulation of Epac

1.2.1 Epac Activation

In its unbound state, Epac is auto-inhibited, such that the CNBD interacts directly with the CDC25HD by an ionic "latch" and indirectly by a central "switchboard". This results in the regulatory region sterically preventing the catalytic region from interacting with Rap (Fig 1.3A). In the inactive state the phosphate binding cassette (PBC) of the CNBD blocks conformational changes in the hinge of the CNBD. The inactive state is stabilized by the interactions of a leucine side chain in the PBC (L408 in Epac2) and a phenylalanine side chain of the hinge of the CNBD(F435 in Epac2)(35). Additional interactions of the C-terminus of the hinge helix are also important for stabilizing the inactive state(36). Rotation of the hinge of the CNBD is a conserved step in the activation of either PKA or Epac(37). The hinge of Epac1 and Epac2 is highly conserved between both isoforms but has key differences with the hinge region of the CNBD of the regulatory subunit of PKA (Fig 1.3B)(36).

The first step in the activation of Epac by cAMP is the interaction of cAMP with the PBC and resultant conformational changes that relieve the steric hindrance between the PBC and the hinge allowing the hinge to rotate(38)(39). This results in the release of the ionic latch, which is thought to be due mostly to an increase in the

entropic penalty associated with preserving this latch as opposed to structural changes in this region(40). Thus, upon binding of cAMP, Epac undergoes a conformational change in which the regulatory region moves away from the CDC25HD towards the REM, allowing the CDC25HD to interact with Rap and facilitate the exchange of GDP for GTP(41). The DEP domain also undergoes conformational changes, as assessed by amide hydrogen exchange upon activation by cAMP(42). These conformational changes allow Epac to translocate to the plasma membrane where it can interact with phospholipids.

The exact mechanism of Epac1 activation is not known and understanding would be greatly enhanced by crystal structures of either the active or inactive state of Epac1 as thus far, only Epac2 has been crystallized. No post-translational modification of Epac has been demonstrated, but interaction with the light chain of the microtubule-associated protein increases the affinity of Epac for cAMP by five-fold, suggesting that allosteric regulation of Epac occurs in cells(43).

1.2.2 Pharmacological Tools to Study Epac signaling

Due to the importance that the cAMP pathway has in normal physiology and different diseases much attention has been given to develop tools to study this pathway as well as to treat different diseases. Most of the focus of these tools has been at the level of GPCRs, ACs, PDEs, or PKA. The tools to manipulate Epac's activity are more limited and are just now being discovered(44)(45). cAMP analogs that selectively activate PKA or Epac have provided a commonly used means to determine if signaling is mediated by PKA or Epac (Fig 1.4).

One such Epac agonist is the cAMP analog 8-CPT-2'O-Me-cAMP (8-Me). This analog specifically activates Epac but not PKA at low micromolar concentrations. A conserved glutamic acid in the CNBD of PKA, CNG, and hyperpolarization cyclic nucleotide-gated channel (HCN) is not found in either isoform of Epac. This glutamic acid residue is important for hydrogen bonding to the 2'-hyrodxyl group of cAMP. Substitution of a methoxy group for the hydroxyl group greatly decreases binding to PKA, CNG, and HCN but maintains binding to Epac(46). Interestingly, 8-Me is a "super-agonist" for Epac1 and partial agonist for Epac2, suggesting that it may be possible to develop activators of Epac that are specific for Epac1 or Epac2(47).

N6-substituted cAMP analogs with a phenyl, benzene, or benzoyl substitution are PKA specific activators. These analogs bind to Epac but do not induce activation. The reason for this is not well understood. One reason may be that the 'lid' of the CNBD that interacts with this portion of cAMP is an α-helix in PKA while it is a β-sheet in Epac, implying that cAMP forms different interactions with the lid of PKA than it does with that of Epac.

While 8-Me will not directly alter PKA activity it does have off-target effects. 8-Me inhibits GPCR-mediated P-selectin surface expression in platelets from Epac1-knockout mice(48). The same effect was not seen with another Epac-selective cAMP analog, 8-Br-2'O-Me-cAMP where the chlorophenylthio moiety on the 8 position of the adenine ring is replaced with bromine. This analog binds Epac with a slightly lower affinity than 8-CPT- modified analogs but appears to be slightly more specific. The off-target effects of 8-Me was attributed to antagonistic activity at the P2Y12

receptor based on its similarity in docking to that of the P2Y12 antagonist 2MeSAMP, but more experimental studies are needed to verify this. 8-Me can also inhibit PDEs which will lead to an increase in cAMP or cGMP concentrations (depending on the PDE isoform that is inhibited)(49).

Besides targeting the cAMP effectors the cAMP pathway may also be manipulated at the level of ACs (Fig. 1.5). The most commonly used AC activator is the diterpene forskolin from the Indian Coleus plant(50). This natural product directly activates membrane ACs. No direct pharmacological activator of sAC is currently available. Multiple AC inhibitors have been identified and characterized in cells. The most commonly used ones are the membrane AC-specific inhibitor 2',5'-ddAdo, the nonspecific inhibitor SQ22,536, and the sAC-specific inhibitor KH7(51)(52). Another commonly used AC inhibitor is MDL-12,330A , but this compound will also inhibit PDEs at a lower concentration than that at which it inhibits ACs, making the interpretation of the experiments difficult(53). Despite this, it is still often considered to be a 'specific' AC inhibitor in the literature with its effects being attributed to inhibition of ACs and not PDEs. While these modulators of AC activity along with PKA- or Epac-specific activators have revealed roles for Epac signaling, the development and availability of Epac-specific inhibitors would greatly benefit the field by allowing for the direct targeting of Epac which would be useful to confirm if the effects observed with 8-Me are due to Epac activation or are off-target effects. Additionally, due to the dysregulation of Epac in various diseases inhibitors of its signaling may have therapeutic potential.

The first attempts to identify Epac inhibitors measured the displacement of radiolabeled cAMP from bacterially expressed Epac1(54). These attempts resulted in the identification of compounds were too insoluble and toxic to pursue. The next attempt to identify Epac inhibitors measured the displacement of a fluorescent cAMP analog from bacterially expressed Epac1(55). These studies resulted in the identification of a few putative competitive inhibitors. The key problem with these approaches is that they will only identify competitive inhibitors that displace the ligand (Fig 1.6). Such approaches will potentially have a high false negative rate for the identification of allosteric inhibitors that do not cause the displacement of cAMP or its analogs, for example, noncompetitive and uncompetitive inhibitors (Fig 1.7 and 1.7, respectively). The measurement of the displacement of a fluorescent analog is also problematic since it does not assess the displacement of the endogenous ligand and fluorescent quenchers will be detected as false positives. However, despite such flaws this approach did succeed in identifying the first putative Epac inhibitors (Fig. 1.9).

While these putative Epac inhibitors seemed to successfully inhibit Epac-mediated Rap1 activation *in vitro* their selectivity in cells has been questioned(56). The Epac1 inhibitors ESI-09 and HJC-0197 were shown to inhibit non-Epac mediated Rap1 activation and that this may be due to denaturing of proteins. The Epac2-specific inhibitor ESI-05 was confirmed as an inhibitor of Epac2 *in vitro* but did not completely block its activation at the concentrations first reported.

A third attempt to identify Epac inhibitors measured the displacement of fluorescent GDP from purified Rap1, as occurs with activation of Epac1 by cAMP. This is a direct and robust way to measure Epac inhibition and succeeded in

identifying the uncompetitive inhibitor CE3F4(57). This inhibitor is selective for Epac1 relative to Epac2 and does not affect PKA activity in cells(58). This approach to detect Epac inhibitors has the advantage that although it uses fluorescence as readout, it will not have a high false positive rate by fluorescent quenchers since activated Epac will decrease fluorescence while inhibition of Epac will maintain the fluorescence. However, this approach can be a time-consuming and resource-demanding effort due to its requirement for purified, functional Rap1 and Epac.

1.2.3 Expression of Epac

Epac1 is expressed ubiquitously throughout the body while Epac2's expression is more restricted to the brain and endocrine tissues. Epac1 mRNA can be detected at high levels in the heart, kidneys, ovaries, and thyroid while Epac2 mRNA can be detected at high levels in the brain, pituitary gland, and the adrenal gland. Analysis of Epac1 and Epac2 mRNA expression during development in mice revealed important changes in their expression during the 12 weeks following birth(59). In the kidney Epac1 expression increases during development while Epac2 expression greatly decreases. Epac1 and Epac2 have increased mRNA expression in the heart and brain in 3 and 12 week old mice compared to neonatal expression. Comparison of their mRNA levels indicated that Epac2 is more abundant in 12 week old mice in the heart and brain. However, this result disagrees with analysis of protein levels which showed that Epac1 is more abundant in the heart(60).

Epac expression is altered in a variety of different pathophysiological conditions. Epac1 expression is up-regulated during cardiac hypertrophy in the left

ventricle of patients and mice with heart failure(60). This up-regulation of Epac protein is due to its increased expression in cardiomyocytes as Epac1 protein is down-regulated in cardiac fibroblasts after myocardial infarction(61) Interestingly, patients who recovered from heart failure with left ventricular assists devices had increased Epac2 expression compared to patients that do not recover. It is not known if the increased Epac2 expression is a cause or effect of the recovery but does implicate the cAMP pathway to be important for remodeling of the failing heart (62).

Epac is down-regulated in the lung in different diseases. Both isoforms of Epac are down-regulated in pulmonary arterial smooth cells of patients with pulmonary arterial hypertension and rat models of pulmonary arterial hypertension. Additionally Epac1 protein is down-regulated in lung samples from patients with chronic obstructive pulmonary disease(63).

Epac also has altered expression in brain disorders. Analysis of patients with Alzheimers disease revealed that Epac1 protein is up-regulated in the frontal cortex and hippocampus while Epac2 protein is down-regulated(54). Expression of Epac1 is also altered in dorsal root ganglia during neuropathic pain(64): Epac1 mRNA expression increases and its increased activity is associated with an increase in mechanical allodynia.

Despite the increases or decreases in Epac expression during development and in various diseases, the mechanism of its altered expression is not understood. Only two studies have assessed mechanisms for the regulation of Epac1 expression. Epac1 expression is increased in the cortical tubules of the kidneys in diabetic mice. Analysis of the Epac1 promoter indicated that it has a glucose-response-element whose activity

is increased under high glucose conditions but the transcription factors that bind to the response element were not identified(65). The up-regulation of Epac1 was associated with an increase in hypertrophy. Rap1b, has also been shown to be up-regulated in the kidney during hyperglycemia, suggesting that Epac1 may signal through Rap1b(66).

Epac1 expression is up-regulated in brain slices from patients who died from a fatal ischemic stroke(67). The up-regulation of Epac1 protein in neurons and hematopoietic stem cells was induced by hypoxia. A HIF-1α response element was identified ~1200bp upstream from the start codon in the Epac1 promoter. HIF-1 was shown to bind to this region and induce the up-regulation of Epac1 protein, which peaked 4 hours after hypoxia. The up-regulation of Epac1 was found to be neuroprotective and correlated with a decrease in infarct size in animal models.

Three different isoforms of Epac2 are expressed in mammals due to different promoter usage (Fig 1.2). These isoforms (known as Epac2A, Epac2B, and Epac2C) differ in their expression patterns and in their subcellular localization(68). Epac2A is the longest isoform with an additional CNBD on the N-terminus important for subcellular localization(69). Epac2A is expressed in the brain, pituitary, and pancreatic cells(70). Epac2B has a similar domain structure to Epac1 and lacks the additional CNBD. Epac2B is expressed in the adrenal glands and is diffusely expressed in the cytoplasm and at the nuclear envelope. Epac2C lacks the DEP-domain and is found exclusively in the liver. No functional differences are known between the three Epac 2 isoforms. Subcellular localization can determine the downstream signaling pathways activated, implying that the different Epac2 isoforms may regulate different pathways due to their different subcellular localization(71).

The expression of Epac is altered in a variety of disease states, implicating Epac signaling as important in these diseases. The exact mechanism for its change in expression and the downstream signaling consequences still needs to be determined in the majority of cases. The data implicate Epac signaling and the dysregulation of Rap1 signaling as important in a variety of diseases. A better understanding of whether the changes in Epac expression are a cause or effect of the pathological conditions and the mechanisms for these changes mayl potentially lead to new therapeutic targets.

1.3 Epac Signaling

In the 15 years since its discovery many different physiological responses have been attributed to signaling by Epac. Epac has important roles in cardiac function and hypertrophy, insulin release from pancreatic β-cells, neurotransmitter release, memory formation and synaptic plasticity in the brain, and modulation of the immune system. Knockout mice for Epac1, Epac2, or both isoforms have been developed and are being studying to elucidate more roles for Epac and for its isoforms. The original knockout mice have global deletions of Epac. The creation of tissue-specific knockouts should further understanding of Epac signaling.

1.3.1 Epac in the Brain

cAMP regulates many functions in the brain including growth, differentiation, neurotransmitter release, learning, and memory(72)(73). Since cAMP plays an important role in the brain and Epac2 is highly expressed in the brain, Epac may be involved in such functions. Epac is neuroprotective in the brain through its ability to

activate the Akt pathway and has been suggested to enhance neurite regeneration in adult spinal cords(25)(74). Epac can enhance neurotransmitter release and the remodeling of spiny synapses(75)(76). A role for Epac in learning was revealed in the double knockout mice that display defects in long-term potentiation, spatial learning, and social interactions; this was shown to be via Epac2-mediated suppression of miR-124 in a Rap1-dependent manner(77)(78). Epac may also play a role in brain disorders as a small subset of autistic patients have a genetic mutation in Epac2 that alters its ability to interact with Ras, leading to alterations in dendritic morphology(79). While Epac2 is the predominant isoform in the brain SNPs in the Epac1 gene are associated with anxiety, depression, and nicotine addiction(80)(81). More research is needed to understand how these SNPs alter Epac expression or function and whether they are merely associations or are causal for different brain disorders.

1.3.2 Epac in the Pancreas

The pancreas is another organ that is highly regulated by the cAMP pathway. The hormone glucagon-like peptide-1 (GLP-1) binds to GPCRs and activates the cAMP pathway in pancreatic β-cells, enhancing glucose-induced insulin secretion (GSIS). Epac2 has been implicated as the primary cAMP effector involved in GSIS(82). The anti-diabetic drugs, sulfonylureas, increase insulin secretion mainly through their actions on ATP-sensitive K^+-channels. Some sulfonylureas may also act on Epac2 by binding to the CNBD-A, resulting in cooperative activation with cAMP, though these data are controversial(83).

Most research in the pancreas has focused on Epac2 but Epac1-knockout mice have also revealed important and unexpected roles for Epac1(84). The Epac1-knockout mice have impaired glucose tolerance with decreased GSIS. The reason is not understood, but those mice have decreased expression of Glut2, which is the major glucose transporter, and GLP-1 receptors. They also show higher susceptibility to hyperglycemia and β-cell death when stressed and increased susceptibility to obesity and insulin resistance when placed on a high-fat diet. These data identify important roles for both Epac1 and Epac2 in the pancreas and suggest that modulation of Epac activity may be a possible therapeutic approach.

1.3.3 Epac in the Heart

cAMP regulates many important physiological processes in the heart including contractility, relaxation, and automaticity(85)(86). cAMP has also been shown to play roles in cardiovascular diseases such as cardiac hypertrophy and heart failure(87). The effects of cAMP on the heart were previously thought to be mediated by PKA and by hyperpolarization-activated CNGs. Cardiac contraction is initiated by transient increases in intracellular Ca^{2+}. Epac has been shown to regulate Ca^{2+}-induced Ca^{2+} release in the heart and in pancreatic β cells. Epac can specifically raise the Ca^{2+} levels in the nucleus (25). Epac can also alter cardiac function by modulating gap junction formation, which contributes to intercellular communication between myocytes and fibroblasts. Gap junctions are formed by connexins such as connexin-43 and connexin-45. cAMP increases the expression of connexin-43 and connexin-45 in cardiac myocytes in a PKA- and Epac dependent manner(88).

In addition to its roles in normal physiology, Epac contributes to cardiac hypertrophy. Activation of Epac increases cardiac protein synthesis and markers of hypertrophy; the latter effect is mediated by the nuclear export of histone deacetylases, which increase the activity of the pro-hypertrophic transcription factor MEF2. It is unclear if such effects are mediated by Epac1 or Epac2 although Epac1 expression is up-regulated in cardiac hypertrophy(59).

Epac also has important roles in the regulation of cardiac fibroblasts. Cardiac fibroblasts control the regulation of the extracellular matrix through their synthesis and secretion of collagens and by their expression of proteases that degrade extracellular matrix(89). Excessive deposition of collagen occurs during fibrosis and impairs cardiac function. Transforming growth factor-β (TGFβ) is a main driver of fibrosis and stimulation with TGFβ decreases Epac1 expression through a Smad and JNK pathway(90). The decrease in Epac1 expression is necessary for increased collagen synthesis. Epac has pro- and anti- fibrotic functions, such that Epac will induce the migration of cardiac fibroblasts but will inhibit collagen synthesis(61). In dermal fibroblasts Epac1 knockdown increases collagen synthesis induced by the adenosine A_{2A} receptor whereas Epac2 knockdown led to a dramatic decrease in collagen synthesis indicating that the two isoforms may have competing functions(91). Epac activation by prostaglandin E_2 acting at EP_2 receptors decreases proliferation of lung and gingival fibroblasts(92)(93). Thus Epac helps regulate the function of fibroblasts in numerous tissues. The further development of isoform-specific agonists/antagonists and tissue specific knockouts of either isoform will assist in determining the specific roles of the Epac isoforms in fibroblasts in normal and pathophysiological conditions.

1.4 The cAMP Pathway and Cancer

Cancer is the second leading cause of death in the United States accounting for approximately 25% of all reported deaths(94). Over 13.7 million people living in the United States have been previously diagnosed with cancer. It is estimated that there will be ~1.6 million new diagnoses of cancer in the United States in 2014 and about 585,000 deaths due to cancer. Besides the personal toll this takes on those diagnosed with cancer and their families the economic costs are estimated to be >$200 billion per year. GPCRs are involved in the development and progression of cancer and may be drug targets for some cancers(95). GPCRs and their G-proteins, including G_s, are mutated in a variety of cancers(96). Depending on the type of cancer, different G_s-GPCRs can influence the growth, metastasis, and drug-resistance of the tumors(97). Epac has been shown to be involved in a variety of cancers, including chronic lymphocytic leukemia, melanoma, pancreatic cancer, and prostate cancer.

1.4.1 Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the Western world(98). CLL is characterized by an accumulation of $CD5^+$ B-cells that have a defect in apoptosis and proliferate in various sites, including lymph nodes. CLL is divided into an indolent and aggressive form of disease, which are treated differently. While current treatment of CLL will slow the disease it is not curative. CLL was the first type of cancer in which Epac was implicated to play a role.

The first indication that Epac has a role in cancer came from microarray analysis of CLL cells compared to other lymphomas and normal B-cells. Epac1 mRNA was uniquely up-regulated in all CLL patients compared to normal B-cells or other lymphomas. This was interesting since sustained increases in cAMP by PDE inhibition or by long acting agonists of G_s-GPCRs induce apoptosis in CLL cells(99). Further analysis indicated that the apoptosis in CLL promoted by PDE inhibition is mediated by PKA activating the intrinsic apoptotic pathway, in particular by facilitating the dephosphorylation of the pro-apoptotic protein Bad(100). By contrast, Epac activation decreases apoptosis whether cells are untreated or treated with PDE inhibitors, indicating opposing roles for Epac and PKA in CLL(101). The downstream signaling from Epac is unknown, though adhesion to the ECM is anti-apoptotic in CLL, suggesting that Epac may signal through activation of a Rap1/integrin pathway leading to increased adhesion and a reduction in apoptosis(102)(103).

CLL cells have increased expression of G_s-coupled adenosine A_{2A} receptors(104). Activation of this receptor increases cAMP and reduces spontaneous and drug-induced apoptosis. While a role for Epac was not directly shown it is likely that Epac was the downstream effector that reduced apoptosis since Epac1 is anti-apoptotic and overexpression of Rap1GAP in leukemia cells sensitizes leukemic cells to apoptosis. Adenosine can be generated from the hydrolysis of ATP or ADP to AMP by CD39 which can then be degraded to adenosine by CD73. CLL cells in proliferation nodes expressed higher amounts of CD73 than CLL cells with low proliferation markers. This suggests a model where local adenosine production in

proliferation nodes stimulates A_{2A} receptors leading to activation of Epac followed by a decrease in apoptosis and an increase in proliferation.

Besides its role in reducing apoptosis Epac may also play a role in the progression of CLL. Deletion of Spa-1, the main Rap GAP in hematopoietic cells, can lead to the development of different leukemias in mice(105). While the majority of mice develop leukemia resembling chronic myelogenous leukemia a subset of the mice develop leukemia that resembles CLL. The development of leukemia was preceded by an increase in c-Myc expression due to a decrease in c-Myc protein degradation(106). Epac can increase proliferation through an Akt-dependent pathway that leads to increased expression of c-Myc(107).

Overexpression of TCL-1, a protein that enhances Akt activity, in B-cells also leads to the development of leukemia in mice that resembles CLL(108)(109). In addition to activating Akt, TCL-1 was also found to enhance NF-κB signaling in CLL cells leading to a decrease in apoptosis. Sequencing of the TCL-1 gene found two mutations associated with CLL patients that were shown to be gain-of-function mutations that cause TCL-1 to inhibit AP-1 transcriptional activity leading to a decrease in pro-apoptotic genes. Epac can interact with TCL-1 and enhance its ability to activate Akt(110). While Epac1's interaction with TCL-1 and its effect on NF-κB and AP-1 activity have not been studied, Epac1 was shown to colocalize with TCL-1 in the nucleus and Epac1 activation can increase NF-kB activity (110)(111). Thus, up-regulation of Epac in B-cells may participate in the progression of CLL through its ability to interact with TCL-1 and enhance its activity, leading to a decrease in apoptosis (Fig 1.9).

1.4.2 Melanoma

Melanoma accounts for the majority of deaths due to skin cancer. The incidence of malignant melanoma is one of the few types of cancer that is on the rise, though this may be due to better detection methods(112). In melanoma Epac expression is associated with an increase in migration and an invasive phenotype. Epac induces migration through activation of inositol triphosphate receptors which lead to Ca^{2+} release from the ER that results in an increase in migration through modifications to heparan sulfate. Activation of $G_{\beta\gamma}$ proteins inhibited Epac-mediated migration of melanoma cells(113). Additionally, Rap1GAPI is down-regulated in melanoma patient samples and this is associated with an increase in an invasive phenotype(114). Epac may also contribute to resistance to chemotherapy in melanoma. A recent study analyzing melanoma cells dependent on the RAF-MEK-ERK pathway for tumor growth identified overexpression of GPCRs and ACs as mechanisms by which the cells overcome resistance to MAP kinase inhibitors although the exact downstream signaling pathways were not elucidated(97). Thus, selective activation or inhibition of GPCRs (and perhaps effects on Epac) may be a potential therapeutic target in melanoma to decrease metastasis or drug-resistance.

Epac is also reported it enhance migration of pancreatic cancer cells(115). The data for Epac-induced migration of pancreatic cancer cells is more controversial since it required high concentrations of cAMP analogs and was blocked by Epac inhibitors that may not be specific for Epac(116)(56). While the role of Epac in migration of pancreatic cancer is unclear, Epac is highly up-regulated in patient samples, implying that it may have a role in this disease(117).

1.4.3 Prostate Cancer

Epac may also contribute to the pathophysiology of prostate cancer, one of the most frequently diagnosed cancers in men(118). Activation of Epac1 in prostate cancer cell lines increases proliferation by activating the B-Raf/Erk/mTOR and the PI3K/Akt/mTOR pathways(119)(120). Activation of Epac also leads to an increase in COX-2 expression and activity which in turn increases conversion of arachidonic acid to prostaglandins such as PGE_2. PGE_2 can activate the G_s-coupled GPCRs EP2 and EP4 which have increased expression during prostate cancer progression(121). In addition to leading to increased cAMP to activate Epac PGE_2 can also increase Epac signaling by increasing Epac expression in certain cell types(122). This leads to a positive feedback loop whereby PGE_2 will lead to increased Epac expression, increased cAMP accumulation, and increased Epac activation which then increases PGE_2 production through increased COX-2 activation which leads to further increased cAMP signaling and Epac activation (Fig 1.11).

There is a strong link between androgen receptor signaling and prostate cancer development and between androgen-independent growth and an increased likelihood of metastasis(123). Previous studies have shown crosstalk between androgen receptors and cAMP signaling in prostate cancer development, though these studies implicated PKA signaling as the main mediator of cAMP signaling downstream from GPCRs(124). Recent evidence indicated that sAC expression is up-regulated in prostate cancer that inhibition of sAC decreases proliferation and induces apoptosis and that this effect was androgen-receptor independent(125). Epac1 was implicated in the proliferation induced by sAC. Thus, while PKA signaling may be involved in the

crosstalk of cAMP with androgen receptor signaling-induced proliferation, this may switch to an Epac-mediated proliferation in androgen-independent proliferation and perhaps an increase in metastasis.

While most research has indicated that Epac is involved in the induction of proliferation in prostate cancer there are also reports that Epac inhibits migration and proliferation of prostate cancer cells(126). These studies, however, used high concentrations of the Epac-specific cAMP analog so off-target effects cannot be ruled out. Generating Epac knockouts or overexpression of Epac in murine cancer models and the further development of Epac-specific inhibitors would greatly enhance understanding of Epac's role in a variety of cancers.

1.5 Summary

The cAMP pathway is an important signaling pathway that is involved in a wide variety of normal and pathophysiological functions. The discovery of Epac1 and Epac2 and their downstream activation of Rap proteins has greatly expanded understanding of how cAMP mediates its different functions. This has added a further layer of complexity to the already complex cAMP pathway. While Epac and Rap have been implicated to be dysregulated in a variety of diseases, the exact mechanisms and consequences of this dysregulation are active areas of research.

The up-regulation of Epac1 in CLL has been reported but the research was limited and no mechanism for the up-regulation has been shown. This thesis research thus set out to identify mechanisms that regulate the expression of Epac1 and to

develop a cost-effective method to characterize different types of Epac1 activators and inhibitors and to verify the inhibitors in cells. After the development of this assay I went on to identify novel allosteric inhibitors in collaboration with Kate Rogers from the McCammon lab at UCSD. The latter inhibitors potentially bind to the hinge region of Epac1 or Epac2 and their action was verified through both *in vitro* studies and experiments with multiple cell types. Elucidating the mechanism by which Epac expression is regulated and the development of new pharmacological tools for Epac will assist in advancing understanding of the roles of Epac and consequences of its activation in cancer and other diseases.

1.6 Figures

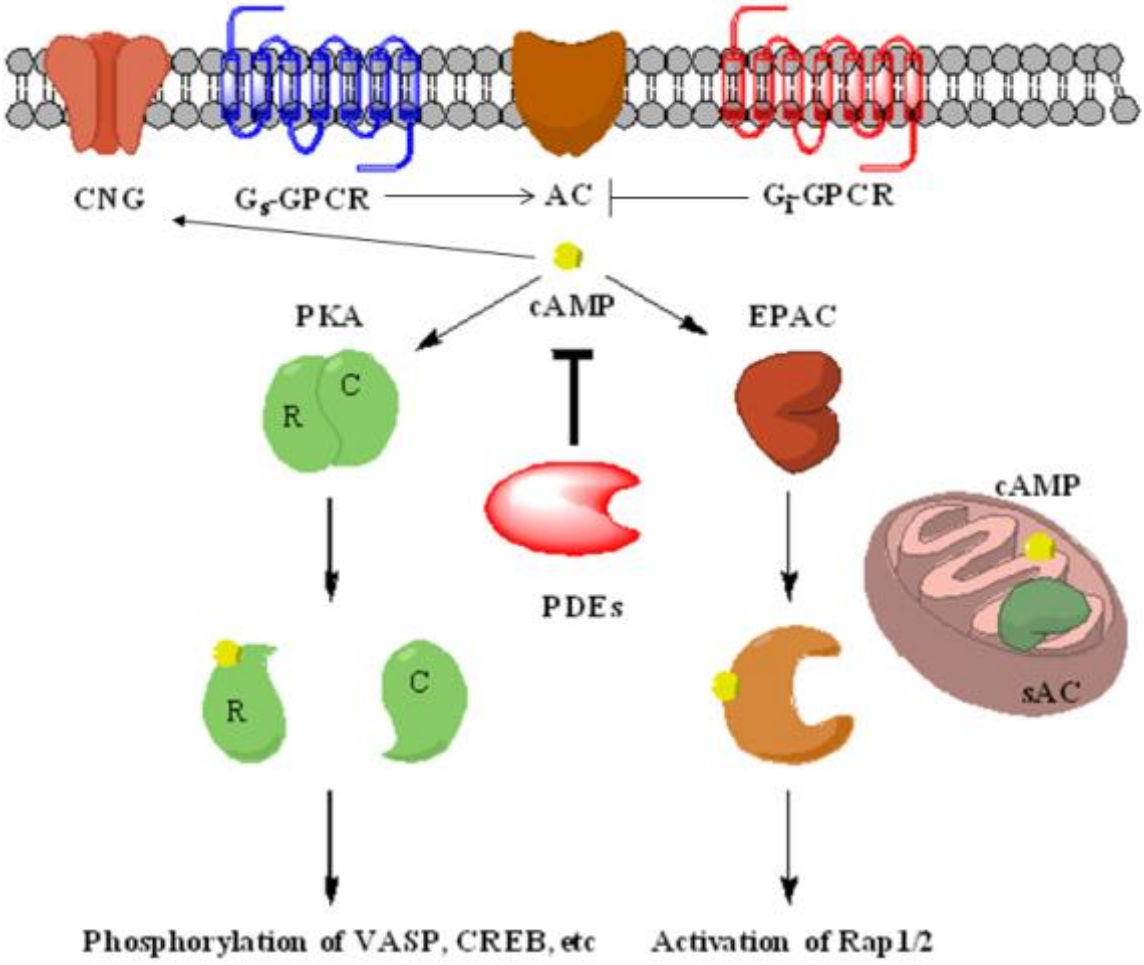


Figure 1.1 The cAMP Pathway. Schematic of the cAMP signaling pathway in cells. Activation of G_s-coupled GPCRs leads to activation of adenylyl cyclases (AC) which convert ATP to cAMP. cAMP activates protein kinase A (PKA), exchange protein activated by cAMP (Epac), and cyclic-nucleotide gated channels (CNG) leading to downstream signaling. Alternatively cAMP can also be generated by soluble adenylyl cyclases (sAC). The pathway is turned off by the activity of phosphodiesterases (PDEs) which hydrolyze cAMP to 5'AMP and G_i-coupled GPCRs which inhibit ACs.

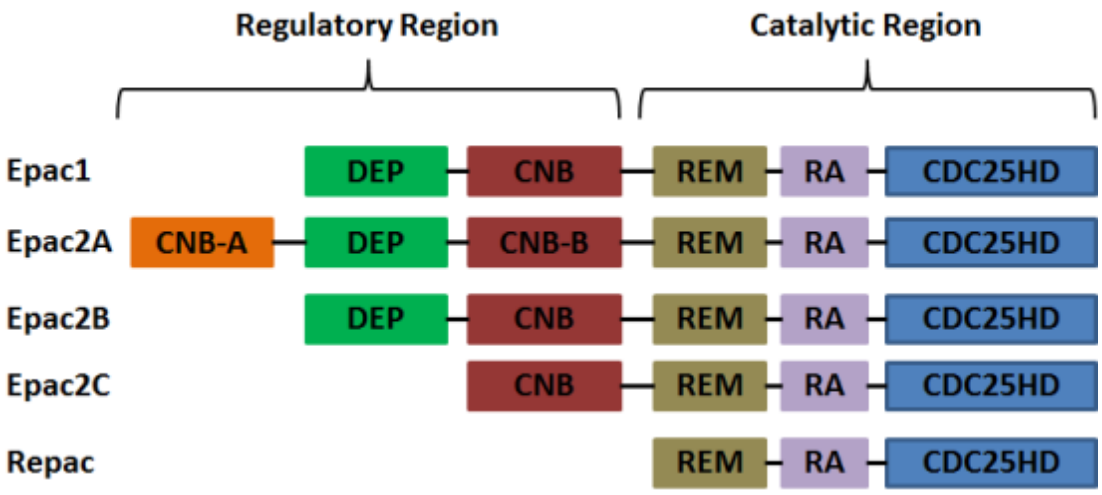


Figure 1.2 Domain Structures of Epacs. Epac has a N-terminal regulatory region and a C-terminal catalytic region. Epac1 and 2 have similar functional domains. Epac2A has an additional CNBD involved in subcellular localization. Epac2B has similar domain organization compared to Epac1. Epac2C is lacking the DEP domain. Repac is a constitutively active GEF that lacks the regulatory region.

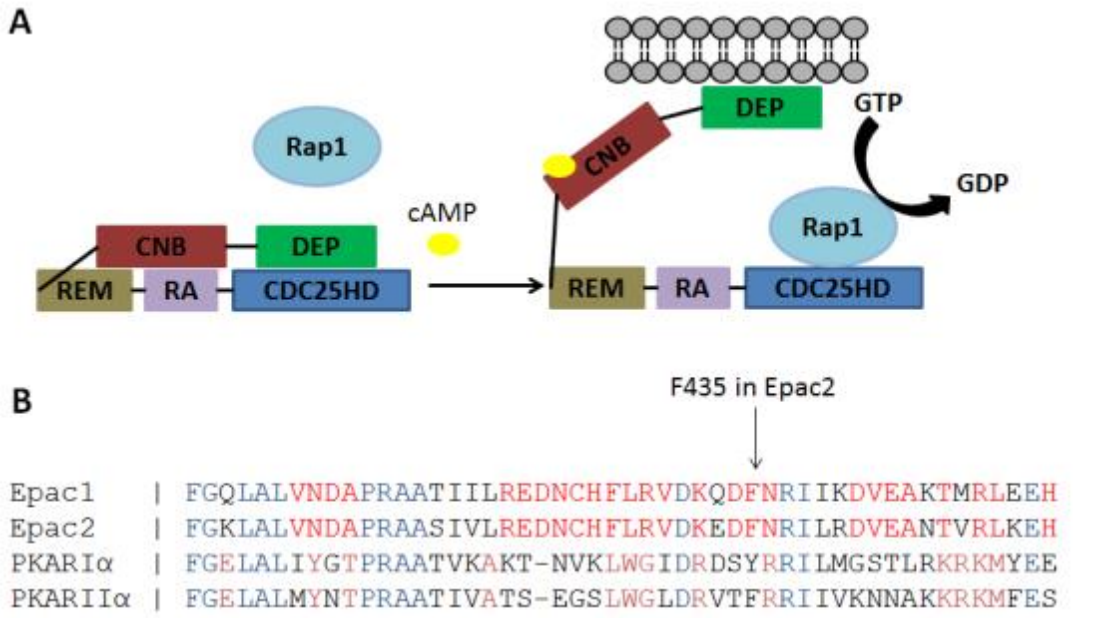


Figure 1.3 Mechanism of activation of Epac by cAMP. (A) In the inactive unbound state the regulatory region of Epac sterically blocks the catalytic region. cAMP binding to the CNB induces conformational changes at the hinge of the CNB domain that moves the regulatory region away from the catalytic region. This allows Rap1 to interact with the CDC25HD and facilitate the exchange of GTP for GDP. Binding of cAMP induces conformational changes in the DEP domain allowing it to interact with phospholipids at the cell membrane. The domains are: cyclic-nucleotide binding domain (CNB), Dishevelled, Egl-10, Pleckstrin domain (DEP), CDC25 Homology Domain (CDC25HD), Ras association domain (RA), Ras Exchange Motif (REM). **(B)** Sequence alignment of the hinge region of the CNB domain of Epac1, Epac2, PKARIα, and PKARIIα. Amino acid residues in blue are conserved between all four proteins. Amino acid residues in red are conserved in Epac but not PKA. Amino acid residues in purple are conserved in PKA but not Epac.

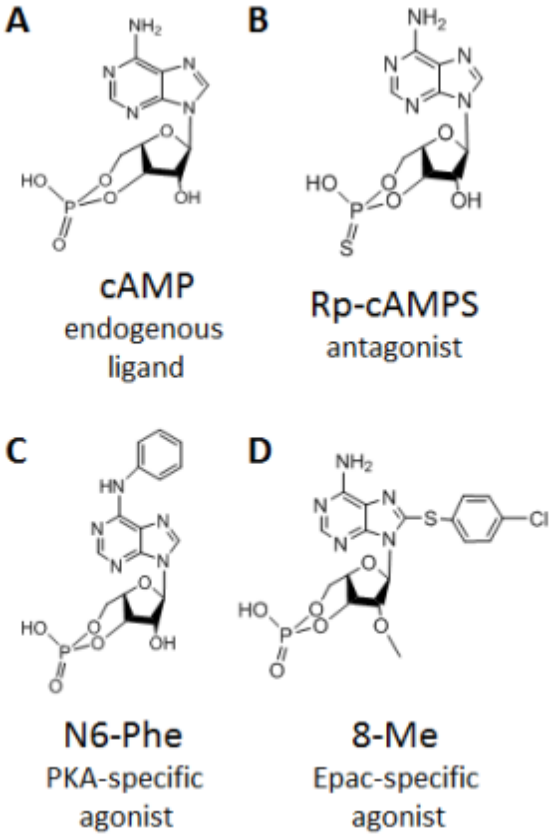


Figure 1.4 cAMP analogs used to probe the cAMP pathway. The structures for the **(A)** cAMP, **(B)** the CNBD antagonist (R_p)-cAMPS, **(C)** the PKA activator N6-Phe, and **(D)** the Epac activator 8-Me are shown.

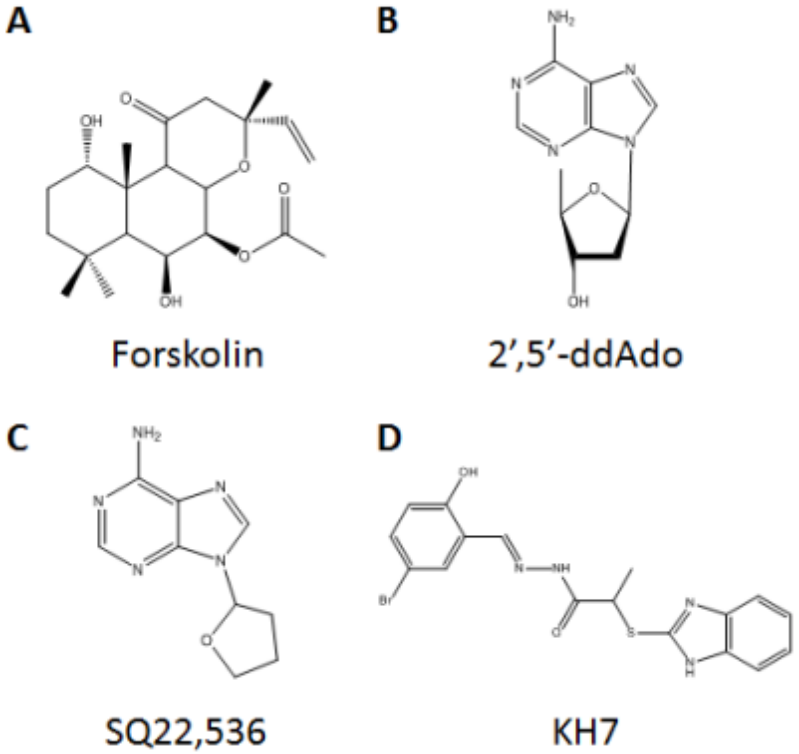


Figure 1.5 Compounds that directly target adenylyl cyclases. (A) Forskolin is a direct activator of membrane adenylyl cyclases. **(B)** 2,5,-dideoxyadenosie is a competitive membrane adenylyl cyclase inhibitor. **(C)** SQ22.536 inhibits membrane and soluble adenylyl cyclase. **(D)** KH7 inhibits soluble adenylyl cyclase

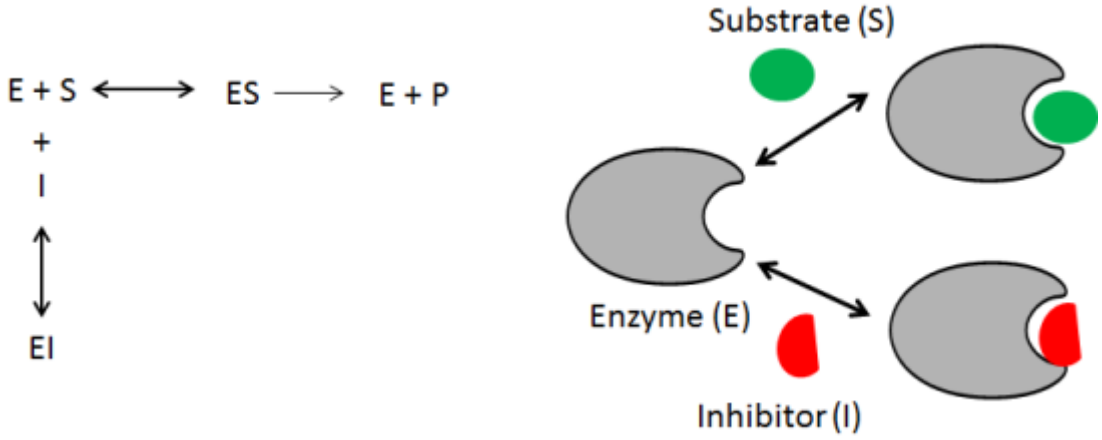


Figure 1.6 Competitive Inhibition. A competitive inhibitor (I) will directly compete with the substrate (S) for binding to the enzyme (E). The enzyme is only active when the substrate is bound and not the inhibitor.

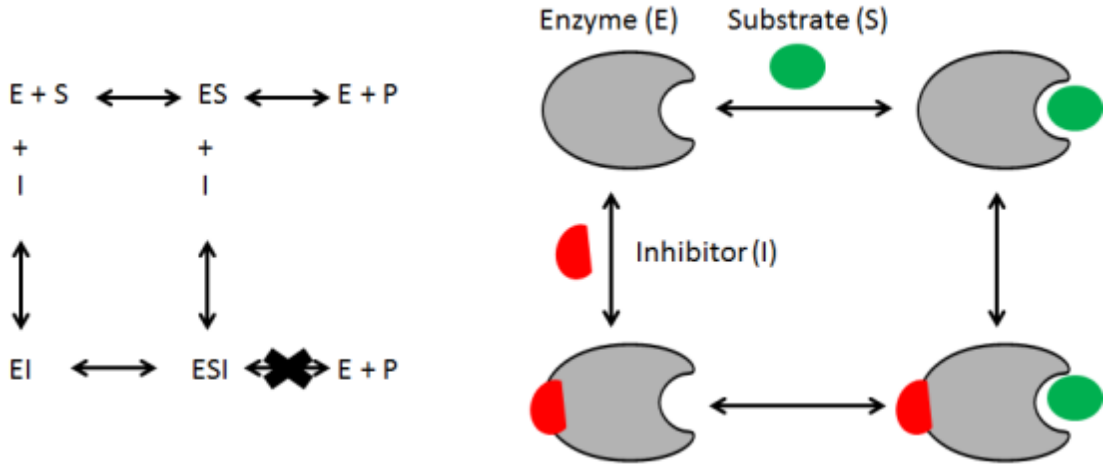


Figure 1.7 Noncompetitive Inhibition. A noncompetitive inhibitor (I) binds to an enzyme (E) in a different place than the substrate (S). A noncompetitive inhibitor will bind to the enzyme in the inactive or active state. A noncompetitive inhibitor will not displace the substrate but will render the enzyme catalytically inactive.

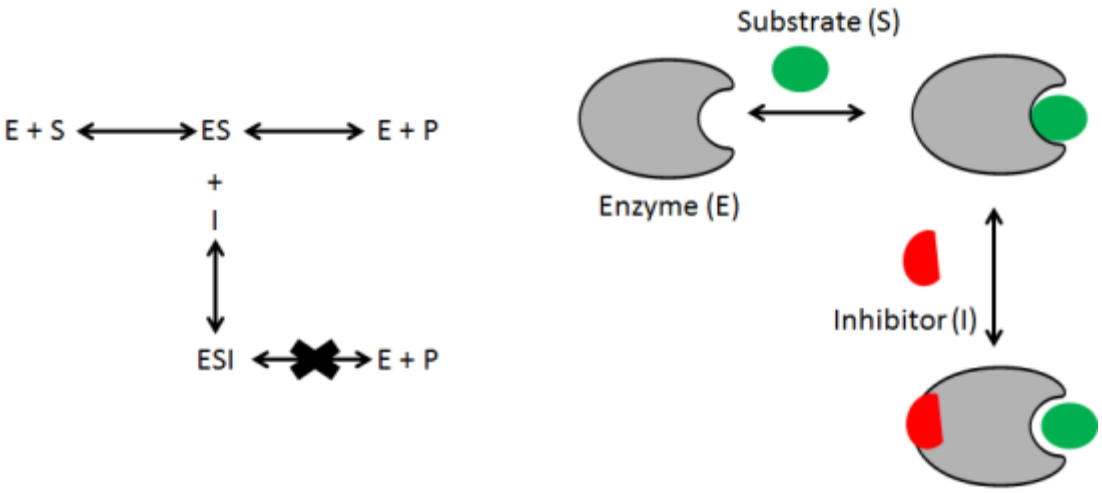


Figure 1.8 Uncompetitive Inhibition. An uncompetitive inhibitor (I) will only bind to the activated state of the enzyme (E) bound by the substrate (S). The inhibitor will not displace the substrate but does render the enzyme catalytically inactive.

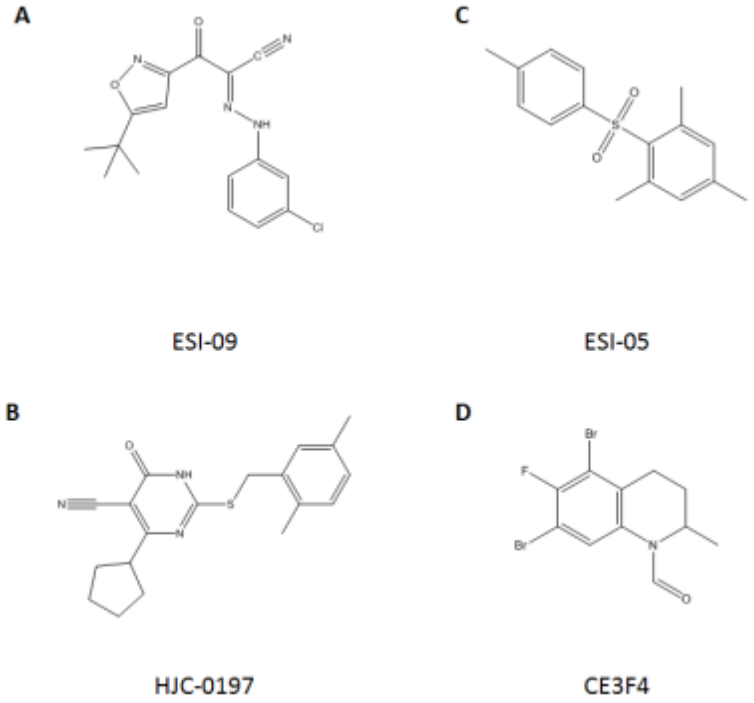


Figure 1.9 Epac Inhibitors. The chemical structures for the competitive Epac1 **(A)**, Epac2 **(B)**, Epac1 and 2 **(C)**, and the uncompetitive Epac1 inhibitor **(D)** are shown.

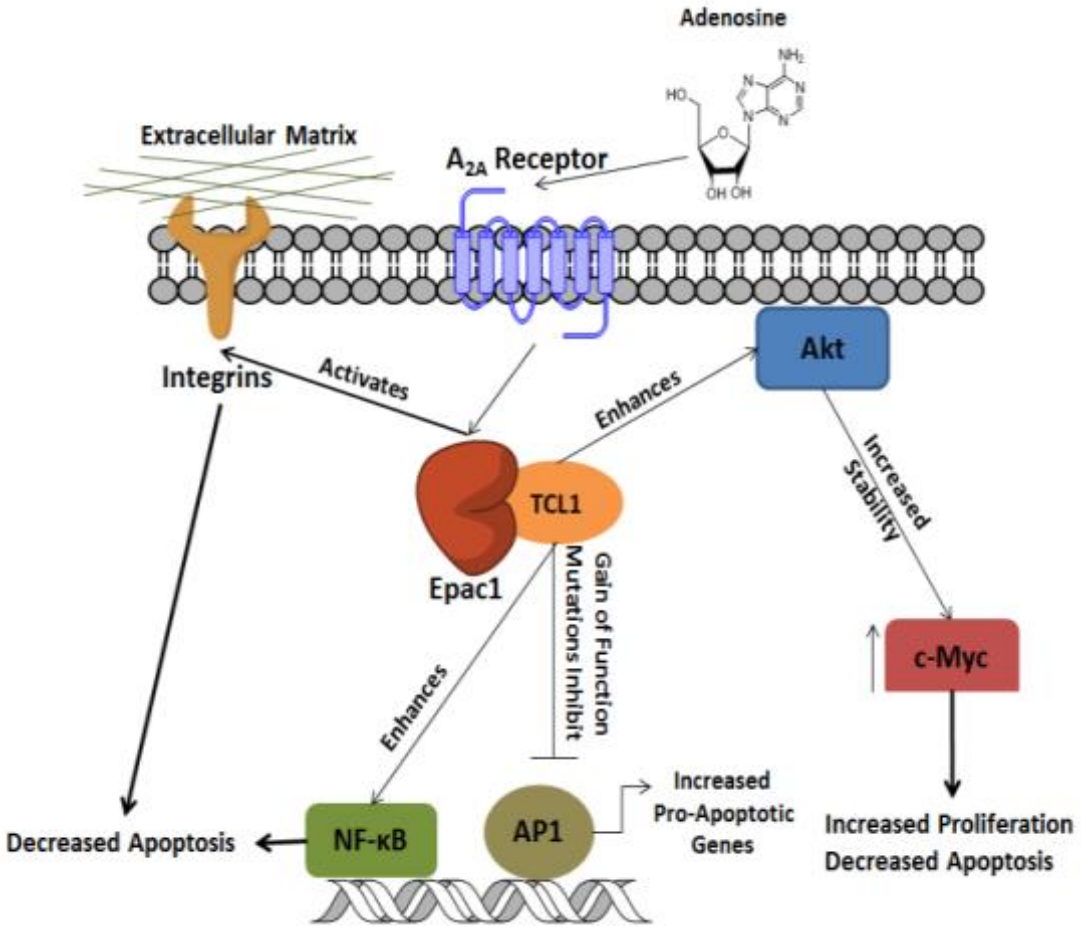


Figure 1.10 Potential Roles for Epac in CLL. Adenosine acting through A_{2A} receptors activates Epac1 by increasing cAMP. Epac1 can activate integrins through a Rap1 dependent pathway. Integrins interact with the extracellular matrix (such as fibronectin) and this activates intracellular pathways that decrease apoptosis. Epac1 can interact with TCL1 which enhances Akt activation leading to increased stability of c-Myc protein which can result in increased proliferation and decreased apoptosis. TCL1 can also affect transcription by inhibiting AP1 which controls the expression of pro-apoptotic genes or by enhancing the activity of NF-κB which can control the expression of anti-apoptotic genes.

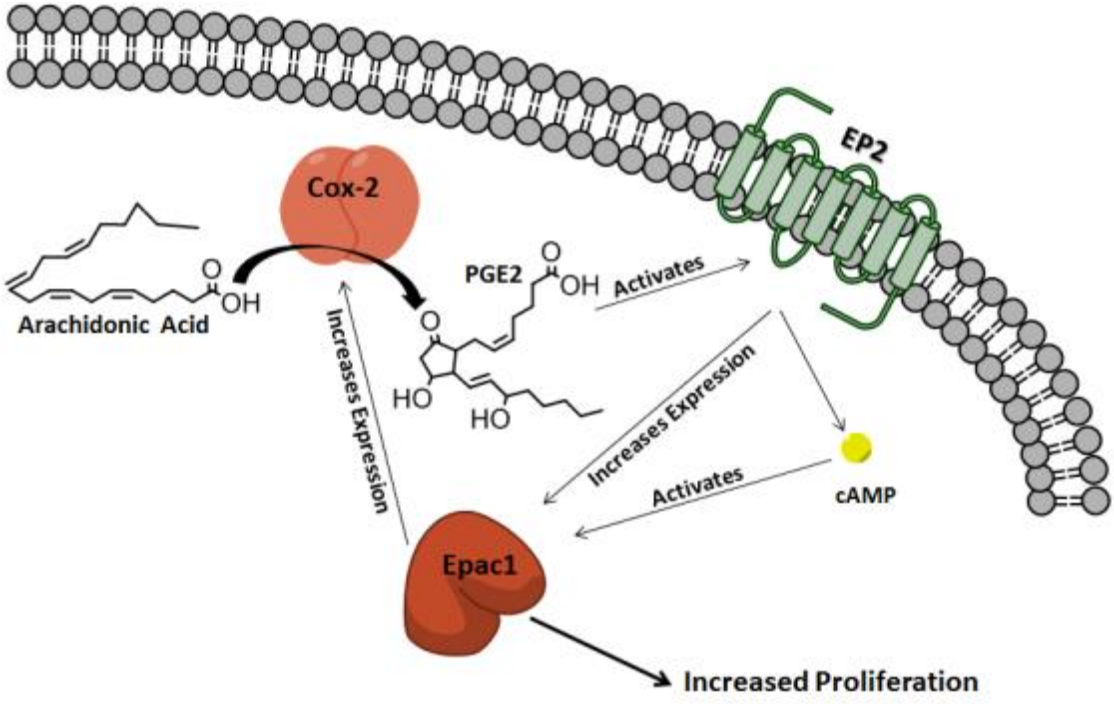


Figure 1.11 A Proliferative Positive Feedback Loop of Epac in Prostate Cancer. Activation of the G_s-coupled prostaglandin E2 receptors (EP2) increases cAMP levels which activate Epac1 and increases Epac1 expression. Epac1 activation results in an increase in Cox-2 expression. Cox-2 catalyzes the formation of arachidonic acid to prostaglandins resulting in an increase in prostaglandin E2 (PGE2) levels. PGE2 activates EP2 receptors and results in a positive feedback loop that enhances the increased proliferation of prostate cancer cells induced by Epac activation.

1.7 References

1. Latek D, Modzelewska A, Trzaskowski B, Palczewski K, Filipek S. G protein-coupled receptors--recent advances. Acta Biochim Pol. 2012;59(4):515–29.

2. Hopkins AL, Groom CR. The druggable genome. Nat Rev Drug Discov. 2002 Sep;1(9):727–30.

3. Overington JP, Al-Lazikani B, Hopkins AL. How many drug targets are there? Nat Rev Drug Discov. 2006 Dec;5(12):993–6.

4. Lymperopoulos A. Beta-arrestin biased agonism/antagonism at cardiovascular seven transmembrane-spanning receptors. Curr Pharm Des. 2012;18(2):192–8.

5. Claing A, Laporte SA, Caron MG, Lefkowitz RJ. Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins. Prog Neurobiol. 2002 Feb;66(2):61–79.

6. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci. 2002 Feb 1;115(Pt 3):455–65.

7. Natarajan M, Lin K-M, Hsueh RC, Sternweis PC, Ranganathan R. A global analysis of cross-talk in a mammalian cellular signalling network. Nat Cell Biol. 2006 Jun;8(6):571–80.

8. Jiang LI, Wang JE, Sternweis PC. Regions on adenylyl cyclase VII required for selective regulation by the G13 pathway. Mol Pharmacol. 2013 Mar;83(3):587–93.

9. Kimple AJ, Bosch DE, Giguère PM, Siderovski DP. Regulators of G-protein signaling and their Gα substrates: promises and challenges in their use as drug discovery targets. Pharmacol Rev. 2011 Sep;63(3):728–49.

10. Xie K, Masuho I, Brand C, Dessauer CW, Martemyanov KA. The complex of G protein regulator RGS9-2 and Gβ(5) controls sensitization and signaling kinetics of type 5 adenylyl cyclase in the striatum. Sci Signal. 2012 Aug 28;5(239):ra63.

11. Insel PA, Zhang L, Murray F, Yokouchi H, Zambon AC. Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger. Acta Physiol Oxf Engl. 2012 Feb;204(2):277–87.

12. Billington CK, Ojo OO, Penn RB, Ito S. cAMP regulation of airway smooth muscle function. Pulm Pharmacol Ther. 2013 Feb;26(1):112–20.

13. Beavo JA, Brunton LL. Cyclic nucleotide research -- still expanding after half a century. Nat Rev Mol Cell Biol. 2002 Sep;3(9):710–8.

14. Ozaki N, Shibasaki T, Kashima Y, Miki T, Takahashi K, Ueno H. cAMP-GEFII is a direct target of cAMP in regulated exocytosis. Nat Cell Biol. 2000 Nov;2(11):805–11.

15. Blumenthal SA. Earl Sutherland (1915-1974) [corrected] and the discovery of cyclic AMP. Perspect Biol Med. 2012;55(2):236–49.

16. Valsecchi F, Ramos-Espiritu LS, Buck J, Levin LR, Manfredi G. cAMP and mitochondria. Physiol Bethesda Md. 2013 May;28(3):199–209.

17. Rahman N, Buck J, Levin LR. pH sensing via bicarbonate-regulated "soluble" adenylyl cyclase (sAC). Front Physiol. 2013;4:343.

18. Chen Y, Cann MJ, Litvin TN, Iourgenko V, Sinclair ML, Levin LR. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science. 2000 Jul 28;289(5479):625–8.

19. Bender AT, Beavo JA. Cyclic nucleotide phosphodiesterases: molecular regulation to clinical use. Pharmacol Rev. 2006 Sep;58(3):488–520.

20. De Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature. 1998 Dec 3;396(6710):474–7.

21. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M. A family of cAMP-binding proteins that directly activate Rap1. Science. 1998 Dec 18;282(5397):2275–9.

22. Kawasaki H, Springett GM, Toki S, Canales JJ, Harlan P, Blumenstiel JP. A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. Proc Natl Acad Sci U S A. 1998 Oct 27;95(22):13278–83.

23. Consonni SV, Gloerich M, Spanjaard E, Bos JL. cAMP regulates DEP domain-mediated binding of the guanine nucleotide exchange factor Epac1 to phosphatidic acid at the plasma membrane. Proc Natl Acad Sci U S A. 2012 Mar 6;109(10):3814–9.

24. Lotfi S, Li Z, Sun J, Zuo Y, Lam PPL, Kang Y. Role of the exchange protein directly activated by cyclic adenosine 5'-monophosphate (Epac) pathway in regulating proglucagon gene expression in intestinal endocrine L cells. Endocrinology. 2006 Aug;147(8):3727–36.

25. Nijholt IM, Dolga AM, Ostroveanu A, Luiten PGM, Schmidt M, Eisel ULM. Neuronal AKAP150 coordinates PKA and Epac-mediated PKB/Akt phosphorylation. Cell Signal. 2008 Oct;20(10):1715–24.

26. Huston E, Lynch MJ, Mohamed A, Collins DM, Hill EV, MacLeod R. EPAC and PKA allow cAMP dual control over DNA-PK nuclear translocation. Proc Natl Acad Sci U S A. 2008 Sep 2;105(35):12791–6.

27. Moon E-Y, Pyo S. Lipopolysaccharide stimulates Epac1-mediated Rap1/NF-kappaB pathway in Raw 264.7 murine macrophages. Immunol Lett. 2007 Jun 15;110(2):121–5.

28. Minato N. Rap G protein signal in normal and disordered lymphohematopoiesis. Exp Cell Res. 2013 Sep 10;319(15):2323–8.

29. Kitayama H, Sugimoto Y, Matsuzaki T, Ikawa Y, Noda M. A ras-related gene with transformation suppressor activity. Cell. 1989 Jan 13;56(1):77–84.

30. Cook SJ, Rubinfeld B, Albert I, McCormick F. RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. EMBO J. 1993 Sep;12(9):3475–85.

31. Bos JL, de Bruyn K, Enserink J, Kuiperij B, Rangarajan S, Rehmann H. The role of Rap1 in integrin-mediated cell adhesion. Biochem Soc Trans. 2003 Feb;31(Pt 1):83–6.

32. Rebhun JF, Castro AF, Quilliam LA. Identification of guanine nucleotide exchange factors (GEFs) for the Rap1 GTPase. Regulation of MR-GEF by M-Ras-GTP interaction. J Biol Chem. 2000 Nov 10;275(45):34901–8.

33. Hattori M, Minato N. Rap1 GTPase: functions, regulation, and malignancy. J Biochem (Tokyo). 2003 Oct;134(4):479–84.

34. Spilker C, Kreutz MR. RapGAPs in brain: multipurpose players in neuronal Rap signalling. Eur J Neurosci. 2010 Jul;32(1):1–9.

35. Rehmann H, Das J, Knipscheer P, Wittinghofer A, Bos JL. Structure of the cyclic-AMP-responsive exchange factor Epac2 in its auto-inhibited state. Nature. 2006 Feb 2;439(7076):625–8.

36. Selvaratnam R, Mazhab-Jafari MT, Das R, Melacini G. The auto-inhibitory role of the EPAC hinge helix as mapped by NMR. PloS One. 2012;7(11):e48707.

37. Tsalkova T, Blumenthal DK, Mei FC, White MA, Cheng X. Mechanism of Epac activation: structural and functional analyses of Epac2 hinge mutants with constitutive and reduced activities. J Biol Chem. 2009 Aug 28;284(35):23644–51.

38. Harper SM, Wienk H, Wechselberger RW, Bos JL, Boelens R, Rehmann H. Structural dynamics in the activation of Epac. J Biol Chem. 2008 Mar 7;283(10):6501–8.

39. Rehmann H, Arias-Palomo E, Hadders MA, Schwede F, Llorca O, Bos JL. Structure of Epac2 in complex with a cyclic AMP analogue and RAP1B. Nature. 2008 Sep 4;455(7209):124–7.

40. Das R, Mazhab-Jafari MT, Chowdhury S, SilDas S, Selvaratnam R, Melacini G. Entropy-driven cAMP-dependent allosteric control of inhibitory interactions in exchange proteins directly activated by cAMP. J Biol Chem. 2008 Jul 11;283(28):19691–703.

41. De Rooij J, Rehmann H, van Triest M, Cool RH, Wittinghofer A, Bos JL. Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. J Biol Chem. 2000 Jul 7;275(27):20829–36.

42. Li S, Tsalkova T, White MA, Mei FC, Liu T, Wang D. Mechanism of intracellular cAMP sensor Epac2 activation: cAMP-induced conformational changes identified by amide hydrogen/deuterium exchange mass spectrometry (DXMS). J Biol Chem. 2011 May 20;286(20):17889–97.

43. Borland G, Gupta M, Magiera MM, Rundell CJ, Fuld S, Yarwood SJ. Microtubule-associated protein 1B-light chain 1 enhances activation of Rap1 by exchange protein activated by cyclic AMP but not intracellular targeting. Mol Pharmacol. 2006 Jan;69(1):374–84.

44. Schwede F, Maronde E, Genieser H, Jastorff B. Cyclic nucleotide analogs as biochemical tools and prospective drugs. Pharmacol Ther. 2000 Sep;87(2-3):199–226.

45. Chen H, Wild C, Zhou X, Ye N, Cheng X, Zhou J. Recent Advances in the Discovery of Small Molecules Targeting Exchange Proteins Directly Activated by cAMP (EPAC). J Med Chem. 2013 Nov 27;

46. Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol. 2002 Nov;4(11):901–6.

47. Courilleau D, Bouyssou P, Fischmeister R, Lezoualc'h F, Blondeau J-P. The (R)-enantiomer of CE3F4 is a preferential inhibitor of human exchange protein directly activated by cyclic AMP isoform 1 (Epac1). Biochem Biophys Res Commun. 2013 Oct 4;

48. Herfindal L, Nygaard G, Kopperud R, Krakstad C, Døskeland SO, Selheim F. Off-target effect of the Epac agonist 8-pCPT-2'-O-Me-cAMP on P2Y12

receptors in blood platelets. Biochem Biophys Res Commun. 2013 Aug 9;437(4):603–8.

49. Poppe H, Rybalkin SD, Rehmann H, Hinds TR, Tang X-B, Christensen AE. Cyclic nucleotide analogs as probes of signaling pathways. Nat Methods. 2008 Apr;5(4):277–8.

50. Seamon KB, Padgett W, Daly JW. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc Natl Acad Sci U S A. 1981 Jun;78(6):3363–7.

51. Seifert R, Lushington GH, Mou T-C, Gille A, Sprang SR. Inhibitors of membranous adenylyl cyclases. Trends Pharmacol Sci. 2012 Feb;33(2):64–78.

52. Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M. The "soluble" adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. Dev Cell. 2005 Aug;9(2):249–59.

53. Hunt NH, Evans T. RMI 12330A, an inhibitor of cyclic nucleotide phosphodiesterases and adenylate cyclase in kidney preparations. Biochim Biophys Acta. 1980 Jun 13;613(2):499–506.

54. McPhee I, Gibson LCD, Kewney J, Darroch C, Stevens PA, Spinks D. Cyclic nucleotide signalling: a molecular approach to drug discovery for Alzheimer's disease. Biochem Soc Trans. 2005 Dec;33(Pt 6):1330–2.

55. Tsalkova T, Mei FC, Cheng X. A fluorescence-based high-throughput assay for the discovery of exchange protein directly activated by cyclic AMP (EPAC) antagonists. PloS One. 2012;7(1):e30441.

56. Rehmann H. Epac-inhibitors: facts and artefacts. Sci Rep. 2013;3:3032.

57. Courilleau D, Bisserier M, Jullian J-C, Lucas A, Bouyssou P, Fischmeister R. Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac. J Biol Chem. 2012 Dec 28;287(53):44192–202.

58. Brown LM, Rogers KE, McCammon JA, Insel PA. Identification and Validation of Modulators of Epac Activity: Structure-function Implications for Epac Activation and Inhibition. J Biol Chem. 2014 Feb 4;

59. Ulucan C, Wang X, Baljinnyam E, Bai Y, Okumura S, Sato M. Developmental changes in gene expression of Epac and its upregulation in myocardial hypertrophy. Am J Physiol Heart Circ Physiol. 2007 Sep;293(3):H1662–1672.

60. Métrich M, Lucas A, Gastineau M, Samuel J-L, Heymes C, Morel E. Epac mediates beta-adrenergic receptor-induced cardiomyocyte hypertrophy. Circ Res. 2008 Apr 25;102(8):959–65.

61. Yokoyama U, Patel HH, Lai NC, Aroonsakool N, Roth DM, Insel PA. The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals. Proc Natl Acad Sci U S A. 2008 Apr 29;105(17):6386–91.

62. Felkin LE, Lara-Pezzi EA, Hall JL, Birks EJ, Barton PJR. Reverse remodelling and recovery from heart failure are associated with complex patterns of gene expression. J Cardiovasc Transl Res. 2011 Jun;4(3):321–31.

63. Oldenburger A, Roscioni SS, Jansen E, Menzen MH, Halayko AJ, Timens W. Anti-inflammatory role of the cAMP effectors Epac and PKA: implications in chronic obstructive pulmonary disease. PloS One. 2012;7(2):e31574.

64. Eijkelkamp N, Linley JE, Torres JM, Bee L, Dickenson AH, Gringhuis M. A role for Piezo2 in EPAC1-dependent mechanical allodynia. Nat Commun. 2013;4:1682.

65. Sun L, Kondeti VK, Xie P, Raparia K, Kanwar YS. Epac1-mediated, high glucose-induced renal proximal tubular cells hypertrophy via the Akt/p21 pathway. Am J Pathol. 2011 Oct;179(4):1706–18.

66. Lin S, Chugh S, Pan X, Wallner EI, Wada J, Kanwar YS. Identification of up-regulated Ras-like GTPase, Rap1b, by suppression subtractive hybridization. Kidney Int. 2001 Dec;60(6):2129–41.

67. Lai TW, Lin S-Z, Lee H-T, Fan J-R, Hsu Y-H, Wang H-J. HIF-1α binding to the Epac1 promoter recruits hematopoietic stem cells to the ischemic brain following stroke. J Mol Cell Biol. 2012 Jun;4(3):184–7.

68. Hoivik EA, Witsoe SL, Bergheim IR, Xu Y, Jakobsson I, Tengholm A. DNA methylation of alternative promoters directs tissue specific expression of Epac2 isoforms. PloS One. 2013;8(7):e67925.

69. Niimura M, Miki T, Shibasaki T, Fujimoto W, Iwanaga T, Seino S. Critical role of the N-terminal cyclic AMP-binding domain of Epac2 in its subcellular localization and function. J Cell Physiol. 2009 Jun;219(3):652–8.

70. Shibasaki T, Takahashi H, Miki T, Sunaga Y, Matsumura K, Yamanaka M. Essential role of Epac2/Rap1 signaling in regulation of insulin granule dynamics by cAMP. Proc Natl Acad Sci U S A. 2007 Dec 4;104(49):19333–8.

71. Wang Z, Dillon TJ, Pokala V, Mishra S, Labudda K, Hunter B. Rap1-mediated activation of extracellular signal-regulated kinases by cyclic AMP is dependent on the mode of Rap1 activation. Mol Cell Biol. 2006 Mar;26(6):2130–45.

72. Lee Y-S, Silva AJ. The molecular and cellular biology of enhanced cognition. Nat Rev Neurosci. 2009 Feb;10(2):126–40.

73. Abel T, Nguyen PV. Regulation of hippocampus-dependent memory by cyclic AMP-dependent protein kinase. Prog Brain Res. 2008;169:97–115.

74. Murray AJ, Shewan DA. Epac mediates cyclic AMP-dependent axon growth, guidance and regeneration. Mol Cell Neurosci. 2008 Aug;38(4):578–88.

75. Sakaba T, Neher E. Direct modulation of synaptic vesicle priming by GABA(B) receptor activation at a glutamatergic synapse. Nature. 2003 Aug 14;424(6950):775–8.

76. Woolfrey KM, Srivastava DP, Photowala H, Yamashita M, Barbolina MV, Cahill ME. Epac2 induces synapse remodeling and depression and its disease-associated forms alter spines. Nat Neurosci. 2009 Oct;12(10):1275–84.

77. Yang Y, Shu X, Liu D, Shang Y, Wu Y, Pei L. EPAC null mutation impairs learning and social interactions via aberrant regulation of miR-124 and Zif268 translation. Neuron. 2012 Feb 23;73(4):774–88.

78. Srivastava DP, Jones KA, Woolfrey KM, Burgdorf J, Russell TA, Kalmbach A. Social, communication, and cortical structural impairments in Epac2-deficient mice. J Neurosci Off J Soc Neurosci. 2012 Aug 22;32(34):11864–78.

79. Srivastava DP, Woolfrey KM, Jones KA, Anderson CT, Smith KR, Russell TA. An autism-associated variant of Epac2 reveals a role for Ras/Epac2 signaling in controlling basal dendrite maintenance in mice. PLoS Biol. 2012;10(6):e1001350.

80. Chen X, Wu B, Kendler KS. Association study of the Epac gene and tobacco smoking and nicotine dependence. Am J Med Genet Part B Neuropsychiatr Genet Off Publ Int Soc Psychiatr Genet. 2004 Aug 15;129B(1):116–9.

81. Middeldorp CM, Vink JM, Hettema JM, de Geus EJC, Kendler KS, Willemsen G. An association between Epac-1 gene variants and anxiety and depression in two independent samples. Am J Med Genet Part B Neuropsychiatr Genet Off Publ Int Soc Psychiatr Genet. 2010 Jan 5;153B(1):214–9.

82. Idevall-Hagren O, Barg S, Gylfe E, Tengholm A. cAMP mediators of pulsatile insulin secretion from glucose-stimulated single beta-cells. J Biol Chem. 2010 Jul 23;285(30):23007–18.

83. Takahashi T, Shibasaki T, Takahashi H, Sugawara K, Ono A, Inoue N. Antidiabetic sulfonylureas and cAMP cooperatively activate Epac2A. Sci Signal. 2013 Oct 22;6(298):ra94.

84. Kai AKL, Lam AKM, Chen Y, Tai ACP, Zhang X, Lai AKW. Exchange protein activated by cAMP 1 (Epac1)-deficient mice develop β-cell dysfunction and

metabolic syndrome. FASEB J Off Publ Fed Am Soc Exp Biol. 2013 Oct;27(10):4122–35.

85. Bers DM. Cardiac excitation-contraction coupling. Nature. 2002 Jan 10;415(6868):198–205.

86. Métrich M, Berthouze M, Morel E, Crozatier B, Gomez AM, Lezoualc'h F. Role of the cAMP-binding protein Epac in cardiovascular physiology and pathophysiology. Pflüg Arch Eur J Physiol. 2010 Mar;459(4):535–46.

87. Movsesian MA, Bristow MR. Alterations in cAMP-mediated signaling and their role in the pathophysiology of dilated cardiomyopathy. Curr Top Dev Biol. 2005;68:25–48.

88. Somekawa S, Fukuhara S, Nakaoka Y, Fujita H, Saito Y, Mochizuki N. Enhanced functional gap junction neoformation by protein kinase A-dependent and Epac-dependent signals downstream of cAMP in cardiac myocytes. Circ Res. 2005 Sep 30;97(7):655–62.

89. Bujak M, Frangogiannis NG. The role of TGF-beta signaling in myocardial infarction and cardiac remodeling. Cardiovasc Res. 2007 May 1;74(2):184–95.

90. Olmedo I, Muñoz C, Guzmán N, Catalán M, Vivar R, Ayala P. EPAC expression and function in cardiac fibroblasts and myofibroblasts. Toxicol Appl Pharmacol. 2013 Oct 15;272(2):414–22.

91. Perez-Aso M, Mediero A, Cronstein BN. Adenosine A2A receptor (A2AR) is a fine-tune regulator of the collagen1:collagen3 balance. Purinergic Signal. 2013 Dec;9(4):573–83.

92. Weinberg E, Zeldich E, Weinreb MM, Moses O, Nemcovsky C, Weinreb M. Prostaglandin E2 inhibits the proliferation of human gingival fibroblasts via the EP2 receptor and Epac. J Cell Biochem. 2009 Sep 1;108(1):207–15.

93. Haag S, Warnken M, Juergens UR, Racké K. Role of Epac1 in mediating anti-proliferative effects of prostanoid EP(2) receptors and cAMP in human lung fibroblasts. Naunyn Schmiedebergs Arch Pharmacol. 2008 Dec;378(6):617–30.

94. American Cancer Society. Cancer Facts & Figures 2013. Atlanta: American Cancer Society; 2013.

95. Dorsam RT, Gutkind JS. G-protein-coupled receptors and cancer. Nat Rev Cancer. 2007 Feb;7(2):79–94.

96. O'Hayre M, Vázquez-Prado J, Kufareva I, Stawiski EW, Handel TM, Seshagiri S. The emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. Nat Rev Cancer. 2013 Jun;13(6):412–24.

97. Johannessen CM, Johnson LA, Piccioni F, Townes A, Frederick DT, Donahue MK. A melanocyte lineage program confers resistance to MAP kinase pathway inhibition. Nature. 2013 Dec 5;504(7478):138–42.

98. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med. 2005 Feb 24;352(8):804–15.

99. Zhang L, Murray F, Zahno A, Kanter JR, Chou D, Suda R. Cyclic nucleotide phosphodiesterase profiling reveals increased expression of phosphodiesterase 7B in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2008 Dec 9;105(49):19532–7.

100. Moon E-Y, Lerner A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. Blood. 2003 May 15;101(10):4122–30.

101. Tiwari S, Felekkis K, Moon E-Y, Flies A, Sherr DH, Lerner A. Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1, but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. Blood. 2004 Apr 1;103(7):2661–7.

102. De la Fuente MT, Casanova B, Moyano JV, Garcia-Gila M, Sanz L, Garcia-Marco J. Engagement of alpha4beta1 integrin by fibronectin induces in vitro resistance of B chronic lymphocytic leukemia cells to fludarabine. J Leukoc Biol. 2002 Mar;71(3):495–502.

103. Buchner M, Baer C, Prinz G, Dierks C, Burger M, Zenz T. Spleen tyrosine kinase inhibition prevents chemokine- and integrin-mediated stromal protective effects in chronic lymphocytic leukemia. Blood. 2010 Jun 3;115(22):4497–506.

104. Serra S, Horenstein AL, Vaisitti T, Brusa D, Rossi D, Laurenti L. CD73-generated extracellular adenosine in chronic lymphocytic leukemia creates local conditions counteracting drug-induced cell death. Blood. 2011 Dec 1;118(23):6141–52.

105. Ishida D, Su L, Tamura A, Katayama Y, Kawai Y, Wang S-F. Rap1 signal controls B cell receptor repertoire and generation of self-reactive B1a cells. Immunity. 2006 Apr;24(4):417–27.

106. Tanaka H, Tamura A, Sekai M, Hamazaki Y, Minato N. Increased c-Myc activity and DNA damage in hematopoietic progenitors precede myeloproliferative disease in Spa-1-deficiency. Cancer Sci. 2011 Apr;102(4):784–91.

107. Jang MW, Yun SP, Park JH, Ryu JM, Lee JH, Han HJ. Cooperation of Epac1/Rap1/Akt and PKA in prostaglandin E(2) -induced proliferation of human

umbilical cord blood derived mesenchymal stem cells: involvement of c-Myc and VEGF expression. J Cell Physiol. 2012 Dec;227(12):3756–67.

108. Bichi R, Shinton SA, Martin ES, Koval A, Calin GA, Cesari R. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. Proc Natl Acad Sci U S A. 2002 May 14;99(10):6955–60.

109. Johnson AJ, Lucas DM, Muthusamy N, Smith LL, Edwards RB, De Lay MD. Characterization of the TCL-1 transgenic mouse as a preclinical drug development tool for human chronic lymphocytic leukemia. Blood. 2006 Aug 15;108(4):1334–8.

110. Misra UK, Kaczowka SJ, Pizzo SV. Interaction between TCL1 and Epac1 in the activation of Akt kinases in plasma membranes and nuclei of 8-CPT-2-O-Me-cAMP-stimulated macrophages. Cell Signal. 2008 Jan;20(1):130–8.

111. Moon E-Y, Lee J-H, Lee J-W, Song J-H, Pyo S. ROS/Epac1-mediated Rap1/NF-kappaB activation is required for the expression of BAFF in Raw264.7 murine macrophages. Cell Signal. 2011 Sep;23(9):1479–88.

112. Erdmann F, Lortet-Tieulent J, Schüz J, Zeeb H, Greinert R, Breitbart EW. International trends in the incidence of malignant melanoma 1953-2008--are recent generations at higher or lower risk? Int J Cancer J Int Cancer. 2013 Jan 15;132(2):385–400.

113. Baljinnyam E, Umemura M, De Lorenzo MS, Xie L-H, Nowycky M, Iwatsubo M. Gβγ subunits inhibit Epac-induced melanoma cell migration. BMC Cancer. 2011;11:256.

114. Zheng H, Gao L, Feng Y, Yuan L, Zhao H, Cornelius LA. Down-regulation of Rap1GAP via promoter hypermethylation promotes melanoma cell proliferation, survival, and migration. Cancer Res. 2009 Jan 15;69(2):449–57.

115. Burdyga A, Conant A, Haynes L, Zhang J, Jalink K, Sutton R. cAMP inhibits migration, ruffling and paxillin accumulation in focal adhesions of pancreatic ductal adenocarcinoma cells: effects of PKA and EPAC. Biochim Biophys Acta. 2013 Dec;1833(12):2664–72.

116. Almahariq M, Tsalkova T, Mei FC, Chen H, Zhou J, Sastry SK. A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion. Mol Pharmacol. 2013 Jan;83(1):122–8.

117. Lorenz R, Aleksic T, Wagner M, Adler G, Weber CK. The cAMP/Epac1/Rap1 pathway in pancreatic carcinoma. Pancreas. 2008 Jul;37(1):102–3.

118. Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. CA Cancer J Clin. 2002 Feb;52(1):23–47.

119. Misra UK, Pizzo SV. Epac1-induced cellular proliferation in prostate cancer cells is mediated by B-Raf/ERK and mTOR signaling cascades. J Cell Biochem. 2009 Nov 1;108(4):998–1011.

120. Misra UK, Pizzo SV. Upregulation of mTORC2 activation by the selective agonist of EPAC, 8-CPT-2Me-cAMP, in prostate cancer cells: assembly of a multiprotein signaling complex. J Cell Biochem. 2012 May;113(5):1488–500.

121. Terada N, Shimizu Y, Kamba T, Inoue T, Maeno A, Kobayashi T. Identification of EP4 as a potential target for the treatment of castration-resistant prostate cancer using a novel xenograft model. Cancer Res. 2010 Feb 15;70(4):1606–15.

122. Jang MW, Yun SP, Park JH, Ryu JM, Lee JH, Han HJ. Cooperation of Epac1/Rap1/Akt and PKA in prostaglandin E(2) -induced proliferation of human umbilical cord blood derived mesenchymal stem cells: involvement of c-Myc and VEGF expression. J Cell Physiol. 2012 Dec;227(12):3756–67.

123. Heinlein CA, Chang C. Androgen receptor in prostate cancer. Endocr Rev. 2004 Apr;25(2):276–308.

124. Merkle D, Hoffmann R. Roles of cAMP and cAMP-dependent protein kinase in the progression of prostate cancer: cross-talk with the androgen receptor. Cell Signal. 2011 Mar;23(3):507–15.

125. Flacke J-P, Flacke H, Appukuttan A, Palisaar R-J, Noldus J, Robinson BD. Type 10 soluble adenylyl cyclase is overexpressed in prostate carcinoma and controls proliferation of prostate cancer cells. J Biol Chem. 2013 Feb 1;288(5):3126–35.

126. Grandoch M, Rose A, ter Braak M, Jendrossek V, Rübben H, Fischer JW. Epac inhibits migration and proliferation of human prostate carcinoma cells. Br J Cancer. 2009 Dec 15;101(12):2038–42.

Chapter Two: Lef-1-mediated Epac1 Expression in Chronic Lymphocytic Leukemic Contributes to the Anti-Apoptotic Phenotype

2.1 Abstract

Exchange protein activated by cAMP 1 (Epac1), a guanine-nucleotide exchange factor for the small G-protein Rap1, contributes to cAMP-mediated actions. Here we show that inhibition or siRNA knockdown of Epac1 increases apoptosis and sensitizes chronic lymphocytic leukemia (CLL) cells to drug-induced apoptosis. Limited previous studies have noted that up-regulation of Epac1 expression in CLL cells. We assessed peripheral blood mononuclear cells (PBMC) and found that Epac1 mRNA (real-time PCR) and protein (immunoblotting) are up-regulated ~70 fold and ~10 fold, respectively, in CLL patients compared to controls. Deletional analysis of the 2kb promoter region directly upstream of the Epac1 start site revealed that the region necessary for basal expression is located within 100 base pairs of the transcriptional start site. Overexpression of Lef-1, a transcription factor up-regulated in CLL, increased activity of an Epac1 promoter luciferase reporter in a CLL cell line but not a normal B-cell cell line. These results, indicate that Epac1 is up-regulated in CLL patients and that Lef-1 regulates Epac1 by a non-canonical pathway

unique to the CLL B cells, thereby defining a transcriptional mechanism for the regulation of Epac expression that contributes to the anti-apoptotic phenotype in this disease.

2.2 Introduction

Chronic lymphocytic leukemia (CLL), the most common leukemia in the Western world, is a clinically heterogeneous disease that is characterized by an accumulation of CD5+ CD19+ B cells, primarily as a consequence of reduction in apoptosis(1). The 5 year survival rate for CLL is 60%; but varies among patients, who are separated into those who have clinical courses that are termed either *indolent* or *aggressive* forms of the disease(2). The diagnosis of these two disease phenotypes is often determined by the mutational status of Ig heavy chain variable region (IgHV). Patients who have mutated IgHV typically have the indolent form of CLL and a better prognosis with one study showing a median survival rate of 293 months and some patients never requiring treatment. By contrast, patients with unmutated IgHV tend to have an aggressive form of CLL and a worse outcome: their median survival rate is 95 months(3). Understanding the mechanisms by which CLL cells continue to proliferate and develop resistance to apoptosis as well as understanding the differences between the indolent and the aggressive form of the disease are crucial to designing new therapies for this disease.

The microenvironment in which CLL cells reside is important for the survival of the cells as they quickly die when cultured *in vitro*(4). This finding indicates that the cells have a predisposition to undergo apoptosis but are prevented from doing so as a consequence of signals *in vivo* that affect cellular signaling(5). One such cellular pathway altered in CLL is the cAMP signaling pathway. This pathway is initiated when adenylyl cyclases (ACs) catalyze the conversion of ATP to cAMP. The effects of cAMP on cells are mediated by two principal effectors: protein kinase A (PKA) and Epac, the exchange protein activated by cAMP. Epac is a guanine nucleotide exchange factor that enhances activity of Rap and certain other low-molecular weight G-proteins and has been implicated in the regulation of many cAMP-dependent events(6)(7)(8). Cyclic nucleotide phosphodiesterases (PDEs) terminate cAMP signaling by catalyzing the conversion of cAMP to 5'-AMP. Regulation of the cAMP pathway is maintained by the expression and localization of ACs, PDEs, PKA, and Epac in distinct subcellular signaling complexes(9).

Increases in cAMP levels can be pro- or anti-apoptotic depending on the cell type(10)(11)(12). The precise mechanisms that determine these alternative effects of cAMP signaling on cell death are unknown. cAMP is pro-apoptotic in some B cell leukemias, including CLL. Inhibition of PDEs in CLL can initiate apoptosis, indicating that the basal activity of ACs in these cells is potentially sufficient to activate the pro-apoptotic pathway(13). Apoptosis promoted by inhibition of PDEs in CLL is mediated by PKA, which activates the intrinsic apoptotic pathway, in particular by facilitating the dephosphorylation of the pro-apoptotic protein Bad(13)(14). By contrast, Epac activation decreases apoptosis whether cells are

untreated or treated with PDE inhibitors(15). The mechanism for this anti-apoptotic effect of Epac activation in CLL is unknown, however in hepatocytes Epac1 can reduce apoptosis through Rap-dependent activation of Src and PI3K, suggesting that Epac signaling through Rap is anti-apoptotic(16),(17). Mouse models where Spa-1 (a GAP for Rap) is knocked out (thereby preventing Rap from being deactivated) develop diseases similar to chronic myelogenous leukemia and a subset of mice develop a disease similar to CLL(18). Overexpression of the oncogene TCL1 in murine B-cells leads to the development of leukemia that resembles the aggressive form of CLL(19). Epac activation can enhance TCL1 activation of its downstream target Akt in immune cells(20). This implies that increased Epac/Rap1 signaling may also contribute to the development of CLL.

Two reports indicate that Epac1 (but not Epac2) is up-regulated in CLL(15),(21). These studies were limited and only analyzed mRNA expression levels, which may not accurately reflect changes in protein expression. In addition to changes in Epac1 expression, a specific PDE isoform, PDE7B, is up-regulated in CLL cells(14). The transcriptional mechanisms responsible for the up-regulation of proteins in the cAMP pathway are unknown. The Epac1 gene is located on chromosome 12, spanning $\geq$ 20kb and comprised of 27 exons and 26 introns. Very little is known about what regulates the expression of either form of Epac, although in addition to increased expression in CLL, Epac1 is up-regulated in prostate cancer, pancreatic cancer, and melanoma(22)(23)(24).

Lef-1 is a member of the Lef-1/TCF family of HMG (high-mobility group) transcription factors and is up-regulated in CLL(25). Lef-1 is an important

transcription factor in Wnt signaling and tumorigenesis(26). Lef-1 is necessary for B-cell development as Lef-$1^{-/-}$ mice have decreased proliferation and increased apoptosis in their pro-B cells(27). Lef-1 binds to DNA but alone, does not have transcriptional activity. Binding of co-activators or co-repressors determines Lef-1-mediated transcriptional activity. Wnt signaling activates β-catenin and drives β-catenin to the nucleus where it interacts with the amino-terminus of Lef-1 and activates Lef-1-mediated transcription. However, Lef-1 also has β-catenin-independent effects and can regulate transcription through interactions with the co-activator ALY, Smad transcription factors, and the Notch-intracellular domain(28)(29)(30). The up-regulation of Lef-1 in CLL contributes to the increased proliferation and decreased apoptosis of CLL cells, though the mechanism is not known(25)(31).

We hypothesized that Epac1 is transcriptionally up-regulated in CLL and that this contributes to the anti-apoptotic phenotype. To test this hypothesis we: 1) determined that the Epac1 promoter has increased activity in a CLL cell line compared to two normal B-cell cell lines; 2) identified a novel Lef-1 binding site in the Epac1 promoter that drives its increased expression; and 3) demonstrate that inhibition of Epac1 through siRNA or small molecule inhibition increases apoptosis in primary CLL cells.

2.3 Experimental Procedures

PBMC Isolation: Blood was collected from healthy donors and CLL patients after informed consent. PBMC were isolated by density-gradient centrifugation using Ficoll-Paque (Amersham Biosciences), washed, suspended in FCS containing 10% DMSO, then stored in liquid N2 for subsequent use.

Isolation of B Cells: B cells were isolated from PBMC by using either Dynabeads CD19 pan B or a Dynal B cell-negative isolation kit (Invitrogen), which resulted in 90% CD19 cells, as assessed by flow cytometry.

Cell Culture: Primary CLL cells, EHEB and WaC3 cells were grown in RPMI supplemented with 10% FBS, penicillin, and streptomycin. HEK293 cells were grown in DMEM with 10% FBS, penicillin, and streptomycin at 5% CO_2 at 37 °C.

Real-time RT-PCR: Total RNA was isolated from PBMC by use of the Versagene RNA Cell Kit (Gentra). SuperScript III First Strand Synthesis System (Invitrogen) was used to synthesize cDNA. Real-time PCR (MJ Research Opticon 2 using Eurogentec QPCR Mastermix Plus SYBR Green Kit) was performed by using 8 ng of RNA per reaction and 100 nM sense/antisense primers. All primers were validated by using human reference RNA (Stratagene). Thermal cycling conditions were as follows: incubation 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C. Samples were compared by using the relative cycle threshold (C_t)

method normalizing to 28S rRNA. The following primers were used: Epac1 Forward: ACTTTATCCCCAACTTGGAT, Epac1 Reverse TCCTCCAGAAACTCTCAGAC, Lef-1 Forward AATGAGAGCGAATGTCGTTGC , Lef-1 Reverse GCTGTCTTTCTTTCCGTGCTA , Sox-4 Forward GACCTGCTCGACCTGAACC, Sox-4 Reverse CCGGGCTCGAAGTTAAAATCC, 28S Forward GCCTAGCAGCCGACTTAGAA, 28S Reverse AAATCACATCGCGTCAACAC.

Western Blot: Western blotting was performed on 5–10 × 10^6 cells per sample. Lysates were prepared as previously described (14), separated by gel electrophoresis, immunoblotted by using the NuPage gel system (Invitrogen), and visualized by horseradish peroxidase and enhanced chemiluminescence (ECL) (Amersham). Antibodies used were directed against Epac1, Epac2 (Santa Cruz Biotechnology) and GAPDH (Abcam)

Luciferase Assays: WaC3 cells were electroporated with the indicated plasmids using a BIORAD Gene Pulser Xcell in a 4mm cuvette at 225V and 900µF. Activity was measured with the DLR Assay System (Promega) on a Turner 20/20 Luminometer 48hrs after transfection

EMSAs: Oligonucleotides were labeled with biotin using the Biotin 3' End DNA Labeling Kit (Pierce). Biotin-labeled oligonucleotides were mixed with 5 µg of nuclear extracts in binding buffer (25mM HEPES, pH7.9, 5mM $MgCl_2$, 80mM NaCl,

5% glycerol) for 30 min at room temperature. The samples were loaded onto a NuPage 6% DNA Retardation gel (Invitrogen) and the gel was visualized by ECL using the Chemiluminescent Nucleic Acid Detection Module (Pierce).

Apoptosis Assay: 1×10^6 cells/ml were cultured in 90% RPMI medium 1640 plus 10% FBS at 37 °C in 5% CO_2 and treated with the indicated treatments or vehicle for 48 h. Apoptosis was assessed by flow cytometry as the percentage of cells stained with annexin V and propidium iodide and analyzed by using CELLQuest software (Becton Dickinson Immunocytometry Systems) (2). B cells were labeled by using a PE-conjugated anti-CD19 antibody (Invitrogen), and apoptosis was measured on the positively stained cells.

Statistical Analyses: All determinations were performed in duplicate or triplicate; each experiment was repeated at least 3 times. Values are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni's post hoc test. Comparison between two groups was based on an unpaired Student *t* test or paired *t* test for comparing combined treatments. A value of $P < 0.05$ was considered statistically significant.

2.4 Results

Epac1 is up-regulated in Chronic Lymphocytic Leukemia Cells

We confirmed that Epac1 mRNA is up-regulated in peripheral blood mononuclear cells (PBMC) isolated from CLL compared to PBMC isolated from healthy controls. B-cells were isolated to verify that the up-regulation of Epac1 mRNA in CLL PBMCs is not due to the enrichment of B-cells in the PBMC of CLL patients (Fig. 2.1A). We found that Epac1 mRNA is up-regulated ~70-fold in CLL compared to healthy B-cells. A comparison of the mRNA expression levels in aggressive patients and indolent patients revealed a trend towards higher Epac1 mRNA in the aggressive patients, though this was not statistically significant (Fig 2.1B). We analyzed the mRNA expression of other cAMP effectors to determine if their expression is altered in CLL. Epac2 expression was unaltered while PKARIIβ expression was decreased in CLL compared to control (Fig 2.1C).

Increases in mRNA expression can be due to increased mRNA stability and/or increased transcription(32)(33). We treated CLL cells with actinomycin D, an inhibitor of mRNA synthesis, and assessed the rate of mRNA degradation as a function of time. While Gapdh and PDE7B mRNA were quickly degraded ($t_{1/2}$ ~2h), Epac1 mRNA was more stable ($t_{1/2}$ ~8h) (Fig 2.1D). Thus, the increase in Epac1 expression may in part be due to decreased mRNA degradation.

In agreement with the mRNA data western blot analysis of CLL cells indicated that Epac1 protein is up-regulated in CLL ~14-fold compared to control PBMC while Epac2 protein expression is unchanged (Fig 2.2). The indolent CLL cell line EHEB

and the aggressive CLL cell line WaC3 also show up-regulated Epac1 protein expression compared to control PBMC (data not shown). The increased expression of Epac1, compared to other cAMP effectors, implies a role for Epac1 signaling in the biology of CLL.

Inhibition of Epac1 by siRNA or with CE3F4 induces apoptosis in CLL cells

To assess the function of Epac1 up-regulation, we used siRNA directed at Epac1 or control siRNA and assessed the viability of patient samples. siRNA knockdown of Epac1 reduced the protein expression of Epac1 (Fig 2.3A). siRNA knockdown of Epac1 induced apoptosis in CLL cells and enhanced the apoptosis induced by inhibition of PDEs (Fig 2.3B). CE3F4 is an Epac1-specific uncompetitive inhibitor(34). Treatment of two patient samples with the Epac1 inhibitor CE3F4 induced apoptosis in both patients (Fig2.3C). These results indicate that the up-regulation of Epac1 contributes to the anti-apoptotic phenotype of CLL cells.

Deletional Analysis of the Epac1Promoter

Analysis of the promoter of Epac1 indicated that it is highly conserved in the human, mouse, and rat genomes (Fig 2.4). We cloned the evolutionarily conserved 2kb upstream from the Epac1 start codon into the pGL4.10 luciferase vector. Since primary CLL patient samples are difficult to transfect and to culture we used WaC3, a cell line derived from a patient with aggressive CLL, to analyze the Epac1 promoter(35). Deletional analysis of the Epac1 promoter in WaC3 indicated that the first 100bp are sufficient for activity (Fig 2.5A). To determine if the activity of the

promoter is changed in CLL, we transfected the promoter into B51 and B67 cells, cell lines derived from normal B-cells that have been immortalized with Epstein Barr virus(36). The Epac1 promoter has higher activity in the CLL WaC3 cell line than in the normal B-cell cell lines, implying that changes at the promoter of Epac1 contribute to its up-regulation (Fig 2.5B). Analysis of the first 100bp indicated that there were 4 putative transcription factor binding sites (Fig 2.5C).

A putative SRY site is important for activity and binding in the Epac1 promoter

To assess which of the four putative binding sites is important for Epac1 transcription, we mutated all four of the binding sites and assessed their effect on the activity of the promoter (data not shown). Mutation of the SRY site decreased the activity of the Epac1 promoter (Fig. 2.6A). Nuclear extracts from WaC3 cells showed binding to the wild-type but not the mutant oligonucleotide, as assessed by EMSA (Fig 2.6B). Nuclear extracts from patient samples bound to the wild-type oligonucleotide similarly to nuclear extracts from WaC3 cells (Fig. 2.6C), indicating that binding to this site occurs in WaC3 cells and in CLL patient cells.

Lef-1 increases activity of the Epac1 promoter in WaC3 cells

SRY belongs to the SOX transcription factor family that are HMG transcription factor proteins involved in development(37). The TCF and SOX proteins can bind to similar DNA sequences and modulate transcription(38). Since Lef-1 is up-regulated in CLL, we sought to determine if it could modulate Epac1 promoter activity(25)(39). TopFlash is a Lef-1 luciferase reporter that has 7 binding sites for

Lef-1 that control the expression of the reporter luciferase(40). Transfection of TopFlash in HEK293 cells or WaC3 cells had minimal activity by itself (Fig 2.7A, B) but overexpression of Lef-1, β-catenin (the Lef-1 co-activator), or both greatly increased the activity of the reporter. Overexpression of Lef-1 increased activity of the Epac1 promoter in WaC3 cells but this activity was reduced when β-catenin was also overexpressed (Fig 2.7C). Lef-1 overexpression did not enhance the activity of the empty vector (Fig 2.7D). These results imply that Lef-1 increases activity of the Epac1 promoter in a β-catenin-independent manner.

We used the cell lines B51 and B67 to determine if this activity is conserved in B-cells or if it is CLL specific. Overexpression of Lef-1 did not increase activity of the Epac1 promoter in either cell line (Fig 2.8A, B). Overexpression of Lef-1 or β-catenin increased activity of the TopFlash reporter in B51 cells, indicating that they are functional when overexpressed in these cells (Fig 2.8C). Transfection of either transcription factor did not increase the Epac1 promoter in HEK293 cells (Fig 2.8D), thus implying a CLL-specific co-activator is necessary for Lef-1 activity.

Inhibition of Lef-1 with ethacrynic acid decreases Epac1 expression in cells from CLL patients

We analyzed the level of expression of Epac1 and Lef-1 mRNA in CLL patients and CLL cell lines to determine if their expression correlates with one another. Using Spearman's correlation coefficient, we found a positive correlation (Spearman's correlation coefficient = 0.61) (Fig. 2.9A). We compared the expression

of Epac1 to that of Sox4, which is a B-cell transcription factor down-regulated in CLL, and found that they did not correlate (Fig 2.9B).

We sought to determine if down-regulating Lef-1 activity decreases Epac1 expression in CLL cells. To accomplish this we used ethacrynic acid, a drug that reportedly inhibits Lef-1 activity by binding to it and inhibiting its transcriptional activity(41). To verify that ethacrynic acid specifically inhibits Lef-1-mediated transcription, we tested its ability to inhibit Lef-1 or NF-kB activity in cells. We found that ethacrynic acid dose-dependently inhibited Lef-1 activity in WaC3 cells, as assessed by Lef-1's ability to activate the TopFlash reporter (Fig 2.10A). The Lef-1 pathway was activated in HEK293 cells transfected with the TopFlash reporter by stimulation with Wnt3a-conditioned media. Ethacrynic acid dose-dependently decreased the activity of the Lef-1 reporter (Fig 2.10B). Ethacrynic acid failed to inhibit the activity of the NF-kB reporter, IgK-Luc transfected into HEK293 cells and stimulated with 10ng/mL TNFα, indicating that the drug specifically inhibits Lef-1-mediated transcription (Fig 2.10C) (42). Treatment of cells from two CLL patients with ethacrynic acid for 48h dose-dependently decreased Epac1 mRNA (Fig 2.11A). To verify that this decrease in Epac1 mRNA is not due to a decrease in global transcription, we analyzed the expression of PDE7A. PDE7A is expressed in B-cells but its expression is not altered in CLL. Ethacrynic acid did not decrease PDE7A expression, consistent with the conclusion that the decrease in Epac1 mRNA is specific to inhibition of Lef-1 activity (Fig 2.11B).

2.5 Discussion

These results confirm prior results indicating that Epac1 is up-regulated at the mRNA level and show that this correlates with an increase at the protein level in CLL cells(21)(15). The up-regulation of Epac1 mRNA is attributable, at least in part, to increased activity at the Epac1 promoter that results from the activity of the transcription factor Lef-1. We found that the increased activity of Lef-1 at the Epac promoter is β-catenin-independent, a result that is in agreement with another report on the Lef-1 pathway in CLL cells which showed increased Lef-1 expression without increased β-catenin nuclear localization(43) (Fig 2.12). Thus, at least part of the oncogenic activity of Lef-1 in CLL is β-catenin-independent. Epac1 mRNA may also be increased due to a decrease in degradation but more experiments are needed to confirm this.

Epac1 expression is increased in several cancers but the mechanism for it has yet to be determined. Our results imply that a Lef-1 binding site within the first 100bp of the Epac1 promoter is important for activity. PDE7B is also uniquely increased in CLL compared to other PDEs and inhibition of PDE7B induces apoptosis in CLL cells, presumably through increased PKA activation(14). A SRY binding site has also been identified in the PDE7B promoter(44). This suggests that increased Lef-1 activity in CLL may be responsible for the increased expression of both PDE7B and Epac1.

Increased Lef-1 expression is found CLL patients before the onset of the disease(31)(45). It would be interesting to determine at what stage of the disease Epac is up-regulated. Higher Lef-1 expression is associated with a worse prognosis(46). We

found that patients with the aggressive form of the disease have a tendency to have higher Epac1 expression. It will thus be of interest to determine if the high expression of Epac correlates with the high Lef-1 expression and is thus “driven” by Lef-1 as its primary mechanism for increased expression in CLL or if other factors contribute to Epac expression in the aggressive form of the disease such as increased mRNA stability.

Our results show that Epac1 is uniquely up-regulated in CLL cells compared to other downstream effectors of cAMP signaling (i. e. Epac2, PKARI, and PKARII). These results imply an important role for Epac1 signaling in the biology of CLL. siRNA knockdown of Epac1 or inhibition of Epac1 with CE3F4 induced an increase in apoptosis, implying a role for Epac in the anti-apoptotic phenotype of CLL. Activation of the adenosine A_{2A} receptor in CLL cells reduces apoptosis in a cAMP-dependent manner(47). Since Epac decreases apoptosis while PKA increases apoptosis, the A_{2A} receptor may signal via Epac. Together with the current data, such results suggest that inhibition of Lef-1 by small molecule inhibitors such as ethacrynic acid in combination with GPCR agonists that activate PKA may synergistically induce apoptosis in CLL cells by switching the balance of cAMP effectors from anti-apoptotic to pro-apoptotic (Fig 2.13).

2.6 Figures

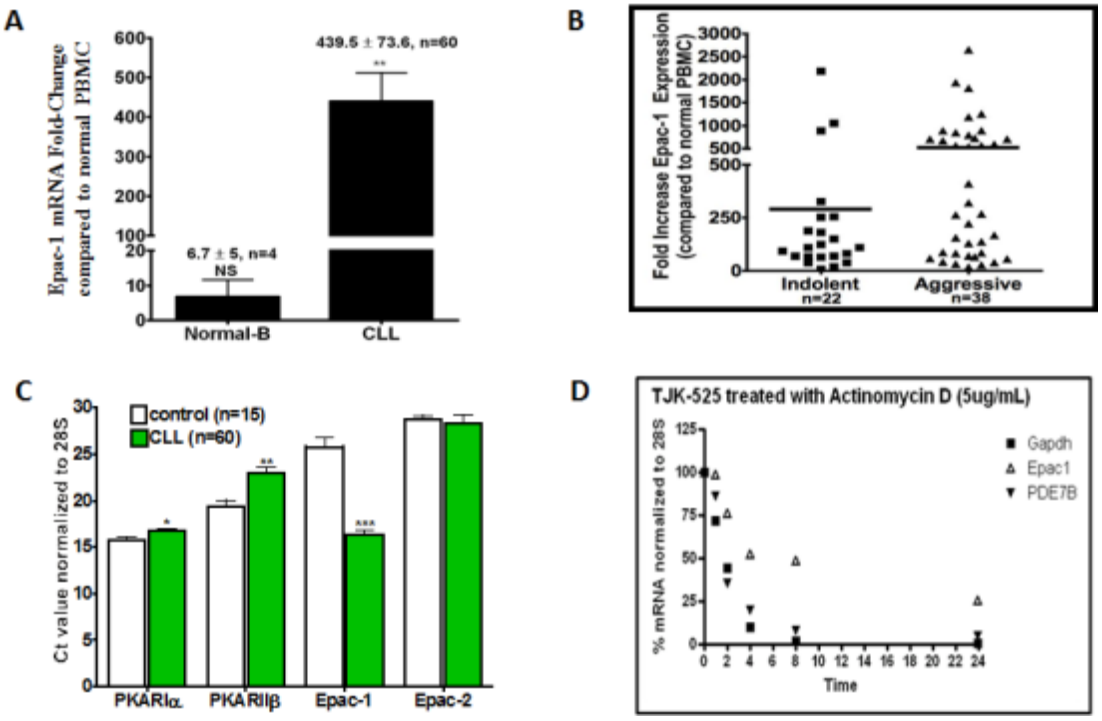


Figure 2.1 Epac1 mRNA is increased in CLL cells. (A) Epac1 mRNA was assessed by QT-PCR in PBMC from healthy people, isolated normal B-cells, or PBMC from CLL patients. **(B)** QT-PCR analysis of Epac1 mRNA separated by patients with the indolent or aggressive form of CLL. **(C)** QT-PCR analysis of mRNA expression of cAMP effectors from PBMC from healthy controls or CLL patients. **(D)** CLL patient cells were treated with actinomycin D (5µg/mL) and mRNA expression was analyzed by QT-PCR. *n=3. * = p<0.05, ** = p<0.01, *** = p<0.001*

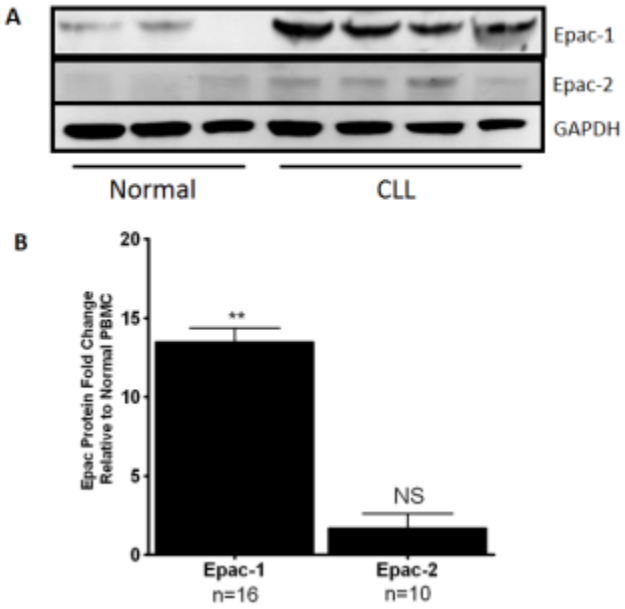


Figure 2.2 Epac1 protein is increased in CLL. (A) Protein extracts from normal PBMC or PBMC from CLL patients was analyzed for Epac1, Epac2, or GAPDH expression and quantified **(B) **=** $p<0.01$.

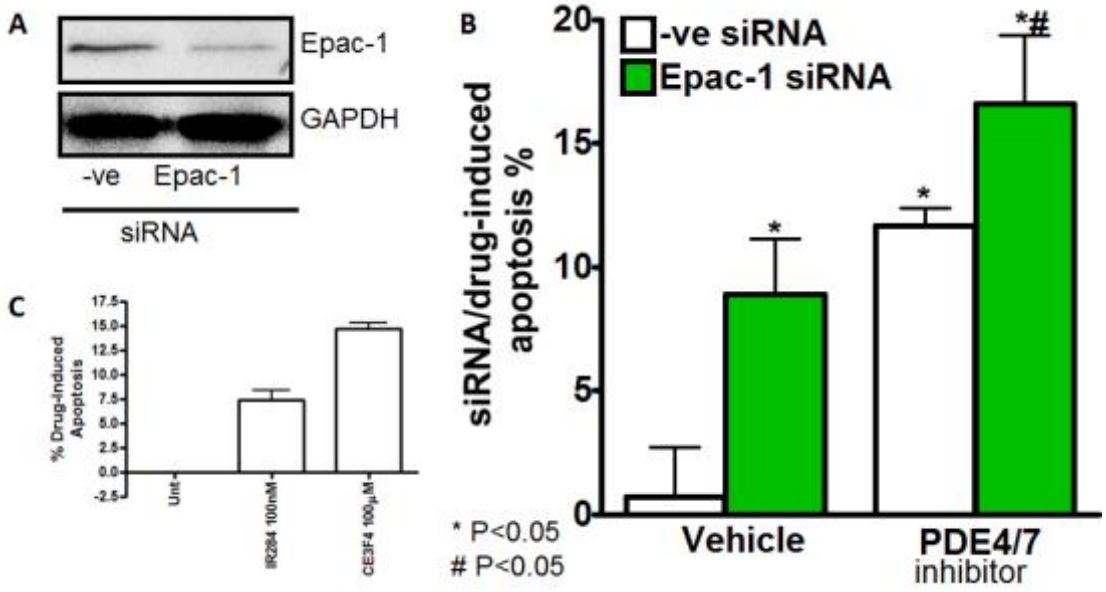


Figure 2.3 Epac1 contributes to the anti-apoptotic phenotype of CLL cells. (A) CLL cells were treated with either control or Epac1 siRNA and the protein expression of Epac1 and GAPDH was analyzed 48hrs later. **(B)** CLL cells were treated with a dual PDE4/7 inhibitor either with control siRNA or Epac1 siRNA and the amount of apoptosis was quantified by Annexin V staining 48hrs later. *n=3.* **(C)** CLL cells were treated with the PDE4/7 inhibitor IR284 (100nM) or the Epac1 inhibitor CE3F4 (100μM) and apoptosis was assessed 48hr later. *n=2.*

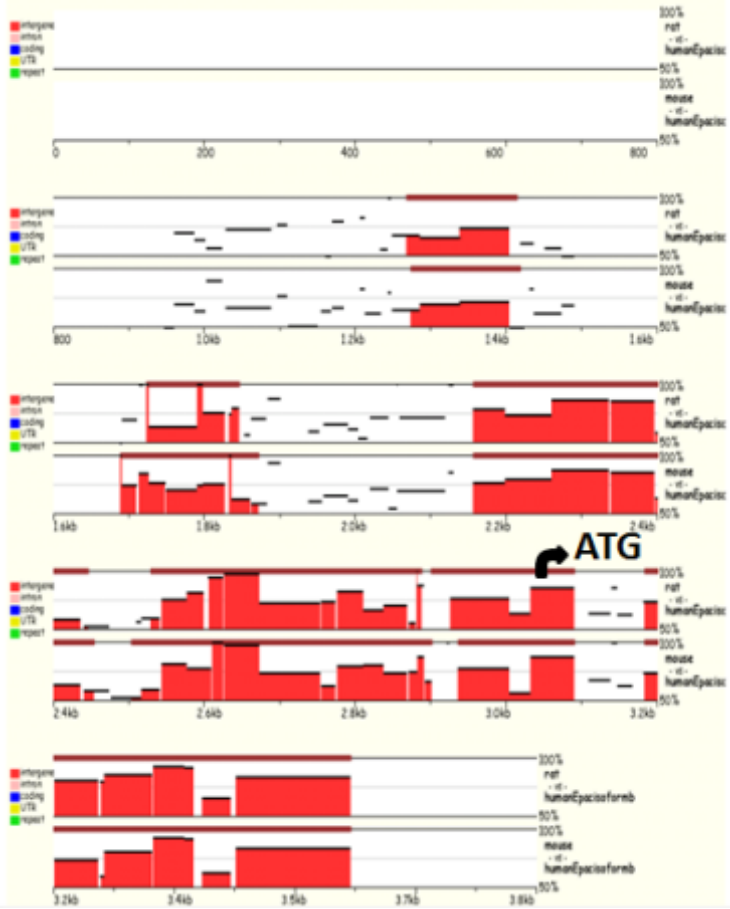


Figure 2.4 The Epac1 promoter is highly conserved in the rat, mouse, and human genome. The DNA sequence from 3kb upstream of the start codon of Epac1 to 800bp downstream of the start codon was compared in mouse, rats, and humans using the mulan alignment tool. Any regions that are more than 50% conserved between rats and humans (top) or mouse and humans (bottom) are shown in red.

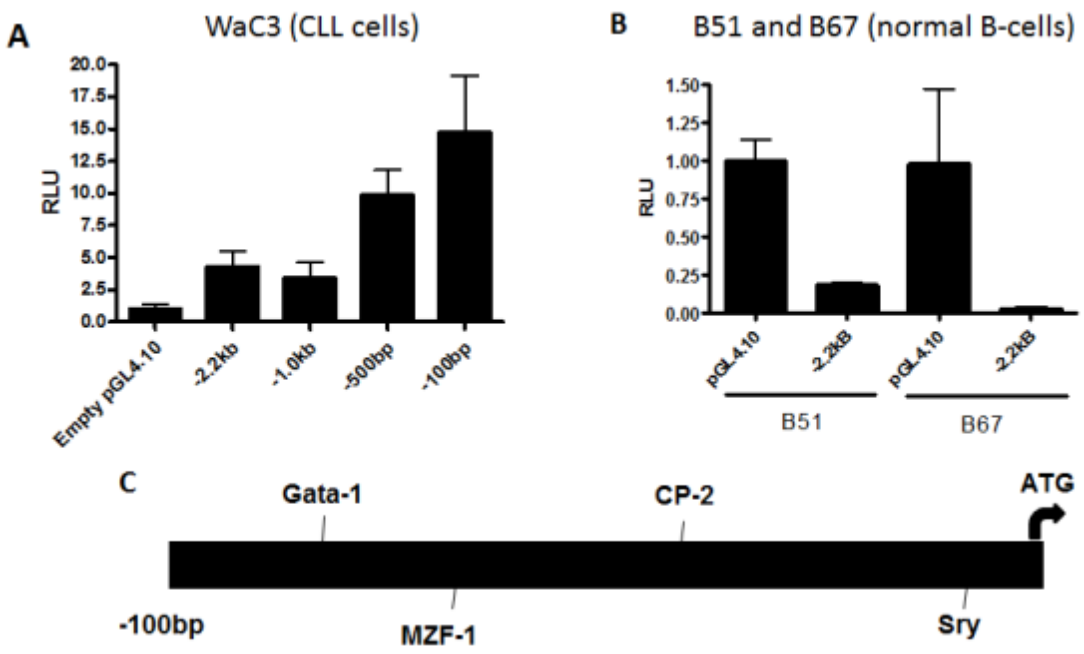


Figure 2.5 Deletional analysis of the Epac1 promoter inWaC3 cells. (A) The full-length and truncated Epac1 promoter was cloned into pGL4.10 and transfected into WaC3 cells. The activity of the constructs was measured 48hr later. **(B)** The full-length Epac1 promoter was transfected into the control B51 and B67 cell lines. **(C)** Diagram of the four putative transcription factor binding sites in the first 100bp of the Epac1 promoter. *n=3.*

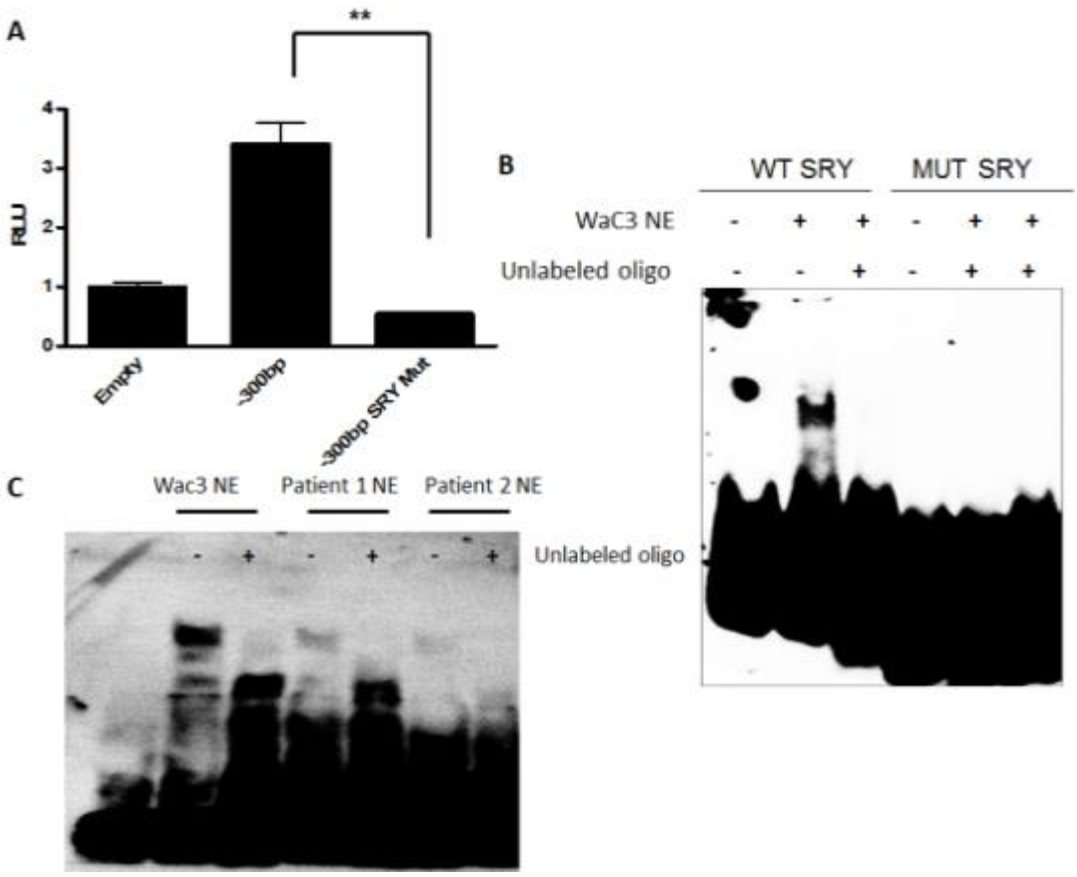


Figure 2.6 Mutation of a SRY binding site decreases activity and binding. (A) Mutation of the SRY site in the truncated Epac1 promoter decreases its overall activity in WaC3 cells measured by luciferase activity **(B)** Nuclear extracts from WaC3 cells were mixed with biotin-labeled oligos containing the SRY site and binding was analyzed by EMSA. NE's bind to the wild-type but not the mutated oligonucleotide. **(C)** Nuclear extracts from CLL patients also show binding to the WT SRY site similarly to WaC3 NEs. *n=3. **=p<0.01*

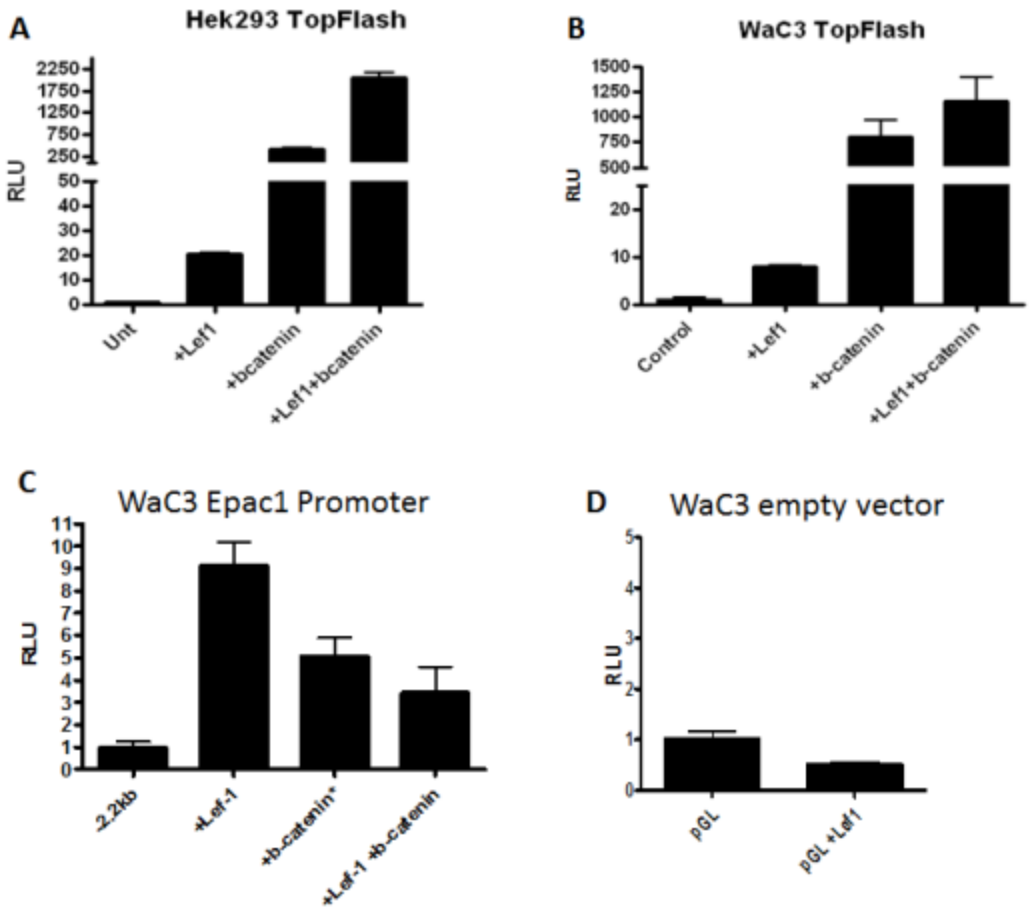


Figure 2.7 Lef-1 increases activity of the Epac1 promoter in WaC3 cells. (A) Hek293 or **(B)** cells were transfected with TopFlash and the indicated transcription factors. Luciferase activity was analyzed 48hr later. **(C)** The full-length Epac1 promoter and the indicated transcription factors were transfected into WaC3 cells and the luciferase activity was measured 48hr later. **(D)** The pG4.10 empty vector was transfected into WaC3 cells +/- Lef-1 and luciferase activity was measured 48hr later. *n=3.*

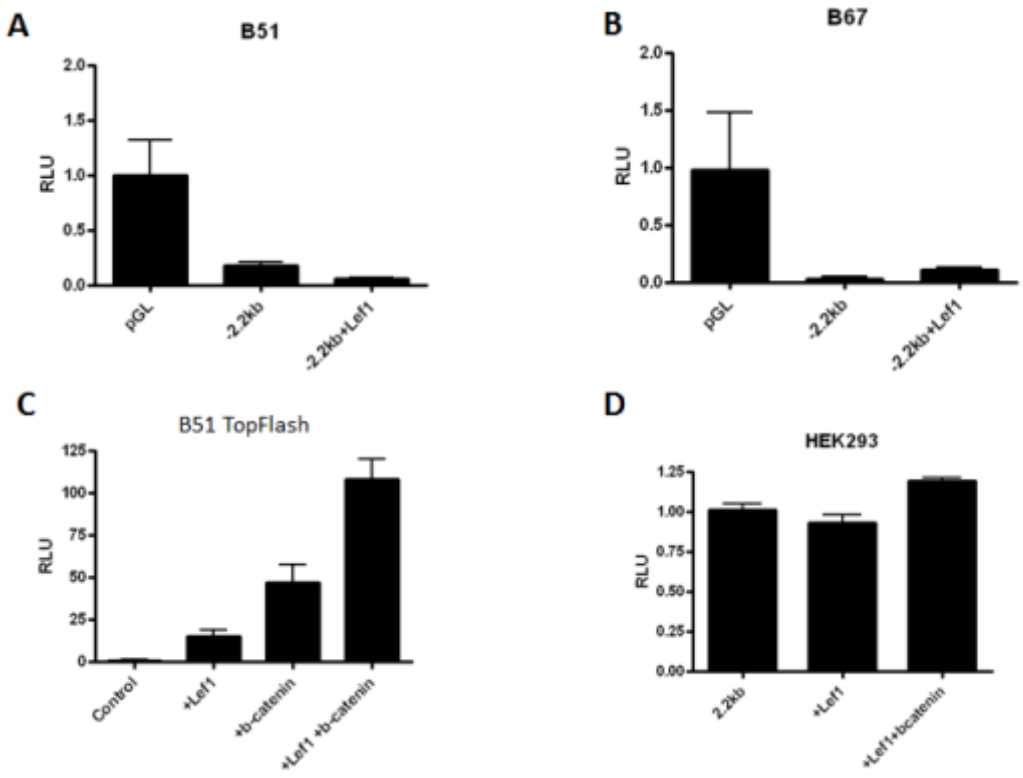


Figure 2.8 Lef-1 does not increase Epac1 promoter activity in non-CLL cells. The Epac1 promoter was transfected into **(A)** B51, **(B)**, B67, and **(D)**, HEKk293 cells +/- or the indicated transcription factors. Luciferase activity was measured 48hr later. **(C)** B51 cells were transfected with TopFlash and the indicated transcription factors and luciferase activity was measured 48hr later. *n=3.*

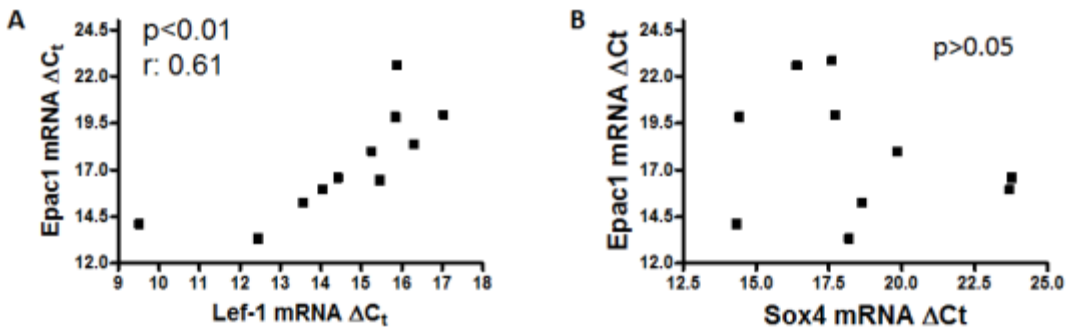


Figure 2.9 Lef-1 and Epac1 mRNA correlate in CLL PBMC The expression of Lef-1, Epac1, and Sox4 mRNA in CLL and CLL cell lines was analyzed by QT-PCR. Epac1 expression was compared versus **(A)** Lef-1 or **(B)** Sox4 expression. R was calculated by using the Spearman correlation.

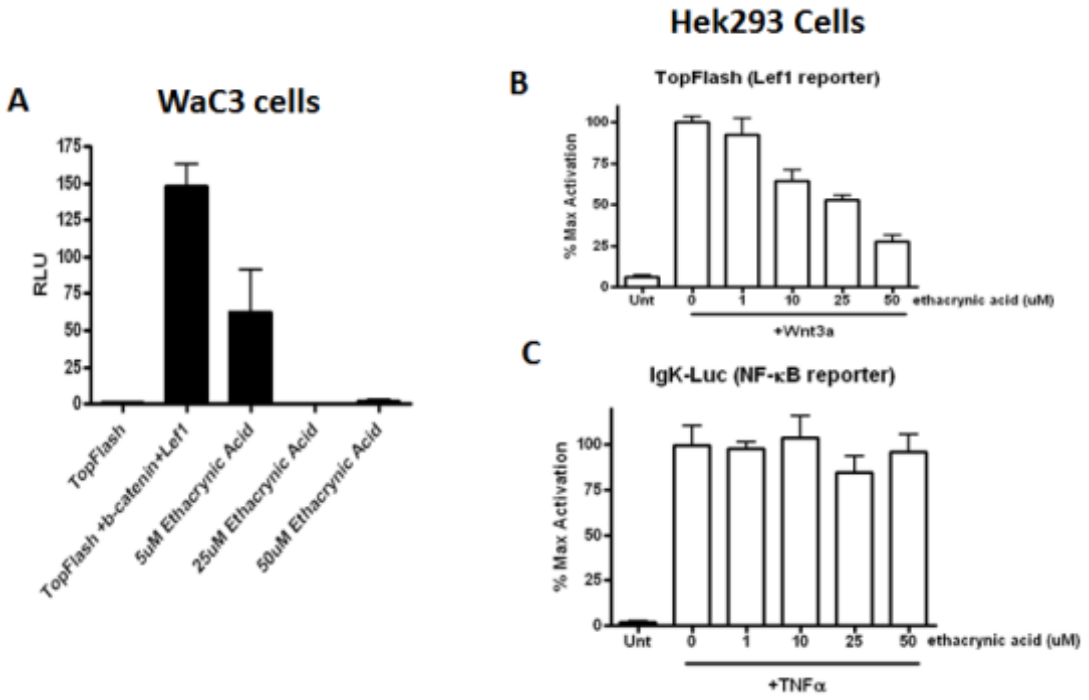


Figure 2.10 Ethacrynic acid inhibits Lef-1 activity in HEK293 and WaC3 cells **(A)** WaC3 cells were transfected with the indicated plasmids and treated with ethacrynic acid 24 hr after transfection. The luciferase activity was determined 24 hr after addition of ethacrynic acid. **(B)** Hek293 cells were transfected with the TopFlash reporter. 24 hr after transfection the media was changed to Wnt3a-conditioned media and ethacrynic acid was added to the media at the indicated concentrations. Luciferase activity was assessed 24hr later. **(C)** Hek293 cells were transfected with the IgK-Luc plasmid and were stimulated with TNFα (10ng/mL) 24 hr after transfection. Ethacrynic acid was added immediately after stimulation with TNFα and luciferase activity was assessed 24hr later. *n=3.*

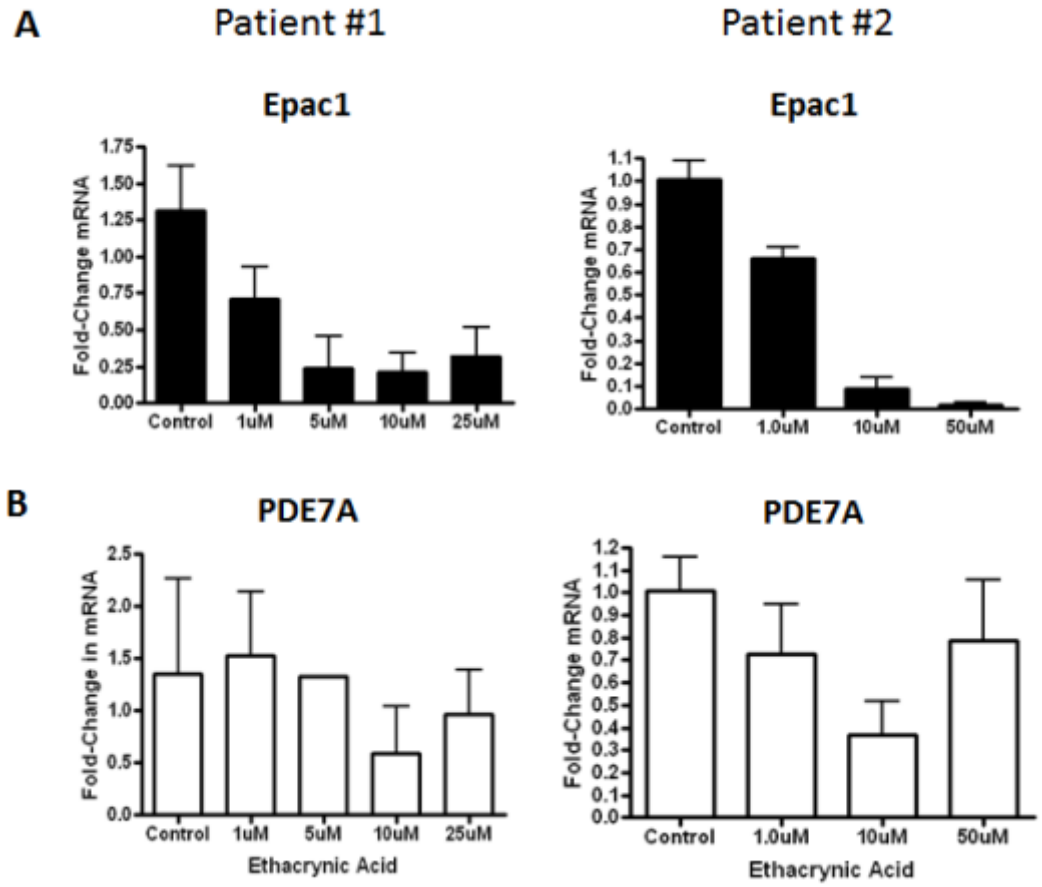


Figure 2.11 Ethacrynic acid decreases Epac1 mRNA in CLL cells. Patient CLL samples were treated with the indicated concentrations of ethacrynic acid for 48hr and then Epac1 **(A)** and PDE7A **(B)** mRNA was assessed by QT-PCR. *n=3*

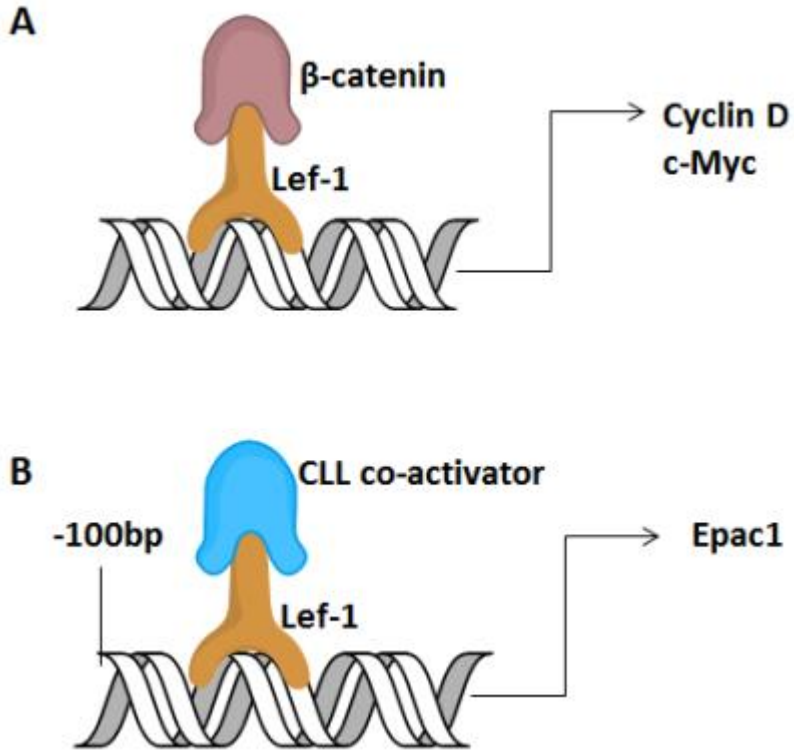


Figure 2.12 Lef-1 binds to the Epac1 promoter. (A) In the canonical Lef-1 pathway Lef-1 binds to the promoter regions of transcriptional targets such as c-Myc and Cyclin D1/2. Acitivty of the promoter is controlled by binding of the co-activator β-catenin. **(B)** In CLL Lef-1 binds to the promoter region directly upstream of the start site of Epac1 and regulates its transcriptional activity independently of β-catenin.

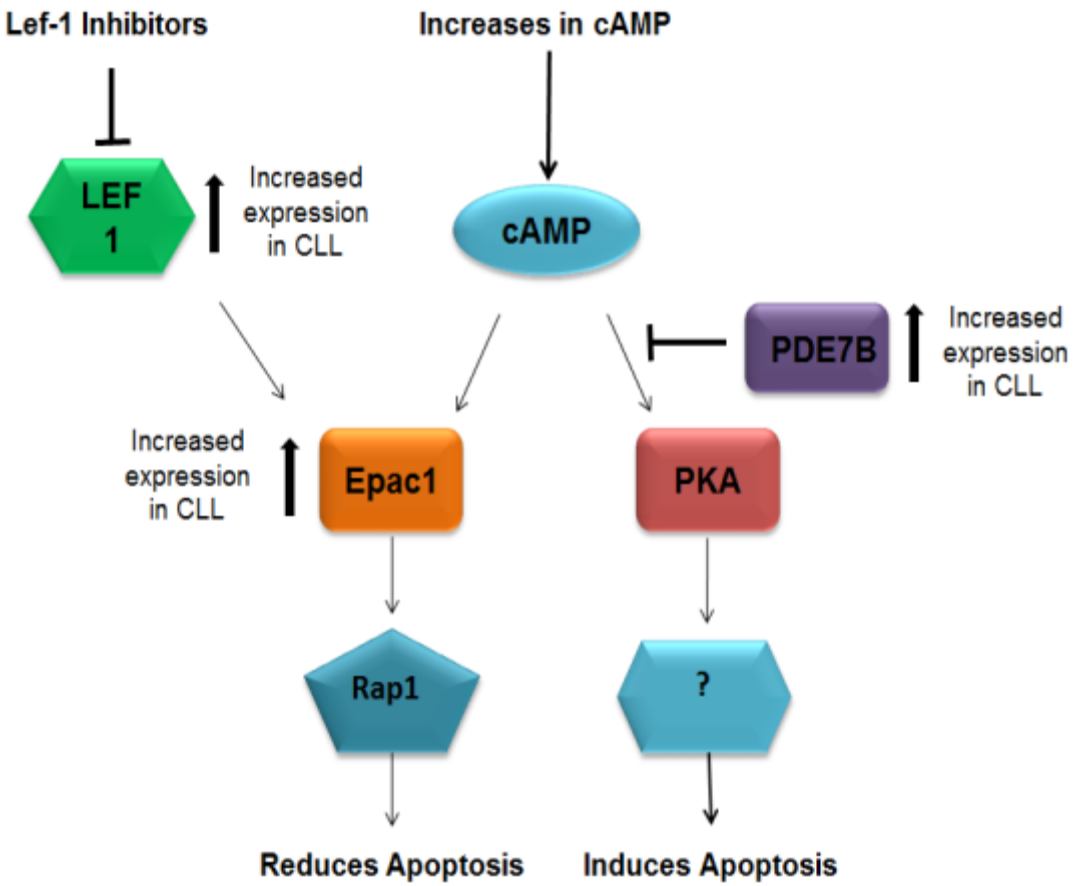


Figure 2.13 The cAMP pathway in CLL. cAMP can bind to two main effectors in CLL: Epac1 and PKA. Activation of Epac1 reduces apoptosis in CLL while activation of PKA induces apoptosis in CLL. Epac1, PDE7B, and Lef-1 have increased expression in CLL. Inhibition of Lef-1 will lead to a decrease in Epac1 expression thus, decreasing the anti-apoptotic effects of Epac1 from reducing the apoptosis induced by PKA activation upon increases in cAMP.

2.7 References

1. Rozman C, Montserrat E. Chronic lymphocytic leukemia. N Engl J Med. 1995 Oct 19;333(16):1052–7.

2. Brenner H, Gondos A, Pulte D. Trends in long-term survival of patients with chronic lymphocytic leukemia from the 1980s to the early 21st century. Blood. 2008 May 15;111(10):4916–21.

3. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999 Sep 15;94(6):1848–54.

4. Collins RJ, Verschuer LA, Harmon BV, Prentice RL, Pope JH, Kerr JF. Spontaneous programmed death (apoptosis) of B-chronic lymphocytic leukaemia cells following their culture in vitro. Br J Haematol. 1989 Mar;71(3):343–50.

5. Munk Pedersen I, Reed J. Microenvironmental interactions and survival of CLL B-cells. Leuk Lymphoma. 2004 Dec;45(12):2365–72.

6. Laurent A-C, Breckler M, Berthouze M, Lezoualc'h F. Role of Epac in brain and heart. Biochem Soc Trans. 2012 Feb;40(1):51–7.

7. Bos JL. Epac: a new cAMP target and new avenues in cAMP research. Nat Rev Mol Cell Biol. 2003 Sep;4(9):733–8.

8. Schmidt M, Dekker FJ, Maarsingh H. Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. Pharmacol Rev. 2013 Apr;65(2):670–709.

9. Dodge-Kafka KL, Soughayer J, Pare GC, Carlisle Michel JJ, Langeberg LK, Kapiloff MS. The protein kinase A anchoring protein mAKAP coordinates two integrated cAMP effector pathways. Nature. 2005 Sep 22;437(7058):574–8.

10. Fujita H, Ogino T, Kobuchi H, Fujiwara T, Yano H, Akiyama J. Cell-permeable cAMP analog suppresses 6-hydroxydopamine-induced apoptosis in PC12 cells through the activation of the Akt pathway. Brain Res. 2006 Oct 3;1113(1):10–23.

11. Ji Z, Mei FC, Miller AL, Thompson EB, Cheng X. Protein kinase A (PKA) isoform RIIbeta mediates the synergistic killing effect of cAMP and glucocorticoid in acute lymphoblastic leukemia cells. J Biol Chem. 2008 Aug 8;283(32):21920–5.

12. Insel PA, Zhang L, Murray F, Yokouchi H, Zambon AC. Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger. Acta Physiol Oxf Engl. 2012 Feb;204(2):277–87.

13. Moon E-Y, Lerner A. PDE4 inhibitors activate a mitochondrial apoptotic pathway in chronic lymphocytic leukemia cells that is regulated by protein phosphatase 2A. Blood. 2003 May 15;101(10):4122–30.

14. Zhang L, Murray F, Zahno A, Kanter JR, Chou D, Suda R. Cyclic nucleotide phosphodiesterase profiling reveals increased expression of phosphodiesterase 7B in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2008 Dec 9;105(49):19532–7.

15. Tiwari S, Felekkis K, Moon E-Y, Flies A, Sherr DH, Lerner A. Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1, but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. Blood. 2004 Apr 1;103(7):2661–7.

16. Gates A, Hohenester S, Anwer MS, Webster CRL. cAMP-GEF cytoprotection by Src tyrosine kinase activation of phosphoinositide-3-kinase p110 beta/alpha in rat hepatocytes. Am J Physiol Gastrointest Liver Physiol. 2009 Apr;296(4):G764–774.

17. Cullen KA, McCool J, Anwer MS, Webster CRL. Activation of cAMP-guanine exchange factor confers PKA-independent protection from hepatocyte apoptosis. Am J Physiol Gastrointest Liver Physiol. 2004 Aug;287(2):G334–343.

18. Ishida D, Su L, Tamura A, Katayama Y, Kawai Y, Wang S-F. Rap1 signal controls B cell receptor repertoire and generation of self-reactive B1a cells. Immunity. 2006 Apr;24(4):417–27.

19. Bichi R, Shinton SA, Martin ES, Koval A, Calin GA, Cesari R. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. Proc Natl Acad Sci U S A. 2002 May 14;99(10):6955–60.

20. Misra UK, Kaczowka SJ, Pizzo SV. Interaction between TCL1 and Epac1 in the activation of Akt kinases in plasma membranes and nuclei of 8-CPT-2-O-Me-cAMP-stimulated macrophages. Cell Signal. 2008 Jan;20(1):130–8.

21. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med. 2001 Dec 3;194(11):1625–38.

22. Misra UK, Pizzo SV. Evidence for a pro-proliferative feedback loop in prostate cancer: the role of Epac1 and COX-2-dependent pathways. PloS One. 2013;8(4):e63150.

23. Lorenz R, Aleksic T, Wagner M, Adler G, Weber CK. The cAMP/Epac1/Rap1 pathway in pancreatic carcinoma. Pancreas. 2008 Jul;37(1):102–3.

24. Baljinnyam E, Iwatsubo K, Kurotani R, Wang X, Ulucan C, Iwatsubo M. Epac increases melanoma cell migration by a heparan sulfate-related mechanism. Am J Physiol Cell Physiol. 2009 Oct;297(4):C802–813.

25. Lu D, Zhao Y, Tawatao R, Cottam HB, Sen M, Leoni LM. Activation of the Wnt signaling pathway in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2004 Mar 2;101(9):3118–23.

26. Novak A, Dedhar S. Signaling through beta-catenin and Lef/Tcf. Cell Mol Life Sci CMLS. 1999 Oct 30;56(5-6):523–37.

27. Busslinger M. Transcriptional control of early B cell development. Annu Rev Immunol. 2004;22:55–79.

28. Ross DA, Kadesch T. The notch intracellular domain can function as a coactivator for LEF-1. Mol Cell Biol. 2001 Nov;21(22):7537–44.

29. Bruhn L, Munnerlyn A, Grosschedl R. ALY, a context-dependent coactivator of LEF-1 and AML-1, is required for TCRalpha enhancer function. Genes Dev. 1997 Mar 1;11(5):640–53.

30. Hussein SM, Duff EK, Sirard C. Smad4 and beta-catenin co-activators functionally interact with lymphoid-enhancing factor to regulate graded expression of Msx2. J Biol Chem. 2003 Dec 5;278(49):48805–14.

31. Gutierrez A Jr, Tschumper RC, Wu X, Shanafelt TD, Eckel-Passow J, Huddleston PM 3rd. LEF-1 is a prosurvival factor in chronic lymphocytic leukemia and is expressed in the preleukemic state of monoclonal B-cell lymphocytosis. Blood. 2010 Oct 21;116(16):2975–83.

32. Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev. 2006 Mar 1;20(5):515–24.

33. Moore AE, Chenette DM, Larkin LC, Schneider RJ. Physiological networks and disease functions of RNA-binding protein AUF1. Wiley Interdiscip Rev RNA. 2014 Mar 28;

34. Courilleau D, Bouyssou P, Fischmeister R, Lezoualc'h F, Blondeau J-P. The (R)-enantiomer of CE3F4 is a preferential inhibitor of human exchange protein directly activated by cyclic AMP isoform 1 (Epac1). Biochem Biophys Res Commun. 2013 Oct 4;

35. Lanemo Myhrinder A, Hellqvist E, Sidorova E, Söderberg A, Baxendale H, Dahle C. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. Blood. 2008 Apr 1;111(7):3838–48.

36. Vacic V, McCarthy S, Malhotra D, Murray F, Chou H-H, Peoples A. Duplications of the neuropeptide receptor gene VIPR2 confer significant risk for schizophrenia. Nature. 2011 Mar 24;471(7339):499–503.

37. Vervoort SJ, van Boxtel R, Coffer PJ. The role of SRY-related HMG box transcription factor 4 (SOX4) in tumorigenesis and metastasis: friend or foe? Oncogene. 2013 Jul 18;32(29):3397–409.

38. Van de Wetering M, Clevers H. Sequence-specific interaction of the HMG box proteins TCF-1 and SRY occurs within the minor groove of a Watson-Crick double helix. EMBO J. 1992 Aug;11(8):3039–44.

39. Gandhirajan RK, Staib PA, Minke K, Gehrke I, Plickert G, Schlösser A. Small molecule inhibitors of Wnt/beta-catenin/lef-1 signaling induces apoptosis in chronic lymphocytic leukemia cells in vitro and in vivo. Neoplasia N Y N. 2010 Apr;12(4):326–35.

40. Kim K, Pang KM, Evans M, Hay ED. Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. Mol Biol Cell. 2000 Oct;11(10):3509–23.

41. Lu D, Liu JX, Endo T, Zhou H, Yao S, Willert K. Ethacrynic acid exhibits selective toxicity to chronic lymphocytic leukemia cells by inhibition of the Wnt/beta-catenin pathway. PloS One. 2009;4(12):e8294.

42. Pomerantz JL, Denny EM, Baltimore D. CARD11 mediates factor-specific activation of NF-kappaB by the T cell receptor complex. EMBO J. 2002 Oct 1;21(19):5184–94.

43. Tandon B, Peterson L, Gao J, Nelson B, Ma S, Rosen S. Nuclear overexpression of lymphoid-enhancer-binding factor 1 identifies chronic lymphocytic leukemia/small lymphocytic lymphoma in small B-cell lymphomas. Mod Pathol Off J U S Can Acad Pathol Inc. 2011 Nov;24(11):1433–43.

44. Peiró AM, Tang C-M, Murray F, Zhang L, Brown LM, Chou D. Genetic variation in phosphodiesterase (PDE) 7B in chronic lymphocytic leukemia: overview of genetic variants of cyclic nucleotide PDEs in human disease. J Hum Genet. 2011 Sep;56(9):676–81.

45. Chadeau-Hyam M, Vermeulen RCH, Hebels DGAJ, Castagné R, Campanella G, Portengen L. Pre-diagnostic Transcriptomic Markers of Chronic Lymphocytic Leukemia Reveal Perturbations 10 years before diagnosis. Ann Oncol Off J Eur Soc Med Oncol ESMO. 2014 Feb 22;

46. Erdfelder F, Hertweck M, Filipovich A, Uhrmacher S, Kreuzer K-A. High lymphoid enhancer-binding factor-1 expression is associated with disease progression and poor prognosis in chronic lymphocytic leukemia. Hematol Rep. 2010 Jan 26;2(1):e3.

47. Serra S, Horenstein AL, Vaisitti T, Brusa D, Rossi D, Laurenti L. CD73-generated extracellular adenosine in chronic lymphocytic leukemia creates local conditions counteracting drug-induced cell death. Blood. 2011 Dec 1;118(23):6141–52.

Chapter 3: Identification and Validation of Modulators of Epac Activity: Structure-function Implications for Epac Activation and Inhibition

3.1 Abstract

The signaling molecule cAMP primarily mediates its effects by activating PKA and/or Epac. Epac has been implicated in many responses in cells but its precise roles have been difficult to define in the absence of Epac inhibitors. Epac, a guanine nucleotide exchange factor for the low molecular weight G protein Rap, is directly activated by cAMP. Using a BRET-based assay (CAMYEL) to examine modulators of Epac activity, we took advantage of its intramolecular movement that occurs upon cAMP binding to assess Epac activation. We found that the use of CAMYEL can detect the binding of cAMP analogs to Epac and their modulation of its activity and can distinguish between agonists (cAMP), partial agonists (8-CPT-cAMP), and super-agonists (8-CPT-2'-O-Me-cAMP). The CAMYEL assay can also identify competitive and uncompetitive Epac inhibitors, e.g., Rp-cAMPS and CE3F4, respectively. To confirm the results with the CAMYEL assay, we used Swiss 3T3 cells and assessed the ability of cyclic nucleotide analogs to modulate the activity of Epac or PKA, determined by Rap1 activity or VASP phosphorylation, respectively. We used computational molecular modeling to analyze the interaction of analogs with Epac1.

The results reveal a rapid means to identify modulators (potentially including allosteric inhibitors) of Epac activity that also provides insight into the mechanisms of Epac activation and inhibition.

3.2 Introduction

The second messenger cyclic AMP (cAMP) is an important signaling molecule involved in a wide variety of cellular processes including proliferation, differentiation, secretion, migration, and apoptosis(1)(2)(3)(4). Increases in cAMP occur following activation of adenylyl cyclases, which catalyze the conversion of ATP to cAMP. The effects of cAMP in mammalian cells are mediated by three effectors: protein kinase A (PKA[1]), the Exchange protein activated by cAMP (Epac), and cyclic nucleotide-gated channels. Epac is a guanine nucleotide exchange factor (GEF) that enhances activity of Rap1 and certain other low-molecular weight G-proteins and has been implicated in the regulation of numerous cAMP-mediated events(5)(6). Cyclic nucleotide phosphodiesterases (PDEs) terminate cAMP signaling by catalyzing the conversion of cAMP to 5'-AMP. Regulation of the cAMP pathway is maintained by the expression and localization of adenylyl cyclases, PDEs, PKA, and Epac in distinct subcellular signaling complexes

Due to the important roles of the cAMP signaling pathway in normal cells and in disease settings, considerable effort has been dedicated to developing tools to manipulate and study this pathway. While much work has been done on understanding

how cAMP and various cAMP analogs bind to and regulate PKA activity, the work on Epac is more limited(7).

There are two isoforms of Epac, Epac1 and Epac2, which have similar domain structures and mechanisms of activation. Epac possesses a regulatory region that sterically blocks Rap1 from interacting with the catalytic region. The regulatory region is composed of a Dishevelled, Egl-10, Pleckstrin (DEP) domain, which is responsible for the subcellular localization of Epac through its binding to phosphatidic acid and a cyclic nucleotide binding domain (CNBD)(8)(9). Epac2 has a second lower affinity CNBD that is involved in the subcellular localization but not in Epac2 activation. The catalytic region is composed of a CDC25 homology domain (CDC25HD), which interacts with Rap1 and facilitates the exchange of GTP for GDP, a Ras exchange motif (REM), and a Ras association motif(10). In its unbound state, Epac is auto-inhibited, such that the CNBD interacts directly with the CDC25HD by an ionic "latch" and indirectly by a central "switchboard", thereby sterically preventing interaction with Rap. Upon binding of cAMP, Epac undergoes a conformational change in which the regulatory domain moves away from the CDC25HD towards the REM, thus allowing the CDC25HD to interact with Rap and facilitate the exchange of GDP for GTP(11).

In the current studies, we set out to develop a rapid and inexpensive method to identify Epac1 inhibitors and examine their interaction with Epac. We took advantage of the conformational changes in Epac that are induced upon binding of cAMP as a means to assess Epac activation. Although first generation Epac-based probes of cAMP utilized FRET, constructs using BRET produce a higher signal-to-noise ratio

and are better suited for the adaptation to high-throughput screening(12). One such BRET-based sensor, named CAMYEL, was designed to monitor cAMP levels in living cells(13). In this sensor, human Epac1 (amino acids 149-881) is sandwiched between Renilla luciferase and Citrine, a modified variant of YFP. The auto-inhibited state yields a strong BRET signal that results from the transfer of energy from luciferase to Citrine. Conformational changes that occur upon activation result in a decrease in the BRET signal as the luciferase moves away from Citrine. This construct lacks the DEP domain of Epac and has two mutations (T781A and F782A) that render it catalytically inactive. As a result, the construct has a better signal-to-noise ratio than do similar constructs of full-length wild-type Epac(14).

To verify that this assay measures Epac activation, we compared our findings with previous measurements of ligand binding to Epac and the activation or inhibition of Epac. Here we show the ability of CAMYEL to identify Epac inhibitors and to predict agonism, partial agonism, and super agonism of cAMP analogs. The results provide new information regarding the binding of such analogs and the conformational changes that occur upon their interaction with Epac1.

3.3 Experimental Procedures

Cell Lines and Transfections: Swiss 3T3 and HEK293 cells were grown in 10-cm culture dishes at 37°C, 5% CO_2 in DMEM supplemented with 5% FBS, 1% penicillin, and 1% streptomycin. Transient transfection of pcDNA3 CAMYEL into HEK293 cells was carried out using TransIT LT-1 (Mirus) according to the manufacturer's

instructions. Lysis and BRET measurements were performed 48-72 h after transfection. For Rap1 pull-down assays, Swiss 3T3 cells were split in 10-cm culture dishes, allowed to adhere overnight, and then serum-starved in DMEM for 24 h before subsequent assays.

BRET/FRET- Assay: HEK293 cells expressing CAMYEL were harvested and lysed in CAMYEL Assay Buffer (40mM Hepes, pH7.2, 140mM KCl, 10mM NaCl, 1.5mM $MgCl_2$) with 0.5% Triton X100 and 1% Complete protease inhibitor cocktail (Roche) as described(15). After centrifugation at 20,000× *g* for 10 min, the supernatant was removed and diluted to the desired volume. 100µL was added to 96-well white plates and treated for 5 min at room temperature with the indicated treatments. Inhibitors were added 5 min before the indicated treatments. Coelenterazine was added to a final concentration of 2 µM immediately before measuring BRET. Emission from RLuc and Citrine was measured simultaneously at 465 nm and 535 nm in a DTX-800 Plate Reader (Beckman Coulter). Apparent activation and inhibition constants were determined by fitting the data to a sigmoidal dose-response curve. Apparent K_i for CE3F4 was determined by fitting the data to that for an uncompetitive inhibitor (GraphPad Prism 6). HEK293 cells expressing Epac2-camps were harvested and lysed in CAMYEL Assay Buffer. After centrifugation at 20,000 *g* for 10min, the supernatant was removed and diluted to the desired volume with CAMYEL Assay Buffer. 100µL was added to 96-well black plates and treated for 5 min at room temperature with the indicated treatments. Emission from YFP and CFP was measured simultaneously at

465m and 535nm after being excited at 430nm in an Infinite M200 plate reader (Tecan)

Quantitative Real-time PCR (QT-PCR): Total RNA was isolated by TRIzol extraction (Invitrogen), and cDNA was generated using the High Capacity mRNA-cDNA system (Applied Biosystems) according to the manufacturer's instructions. QT-PCR analysis was performed on a DNA Engine Opticon 2 (Bio-Rad, Hercules, CA) using the qPCR Mastermix Plus for SYBR Green I kit (Eurogentec, Fremont, CA). Primers for PCR amplification were designed based on the nucleotide sequences of the respective gene target using Primer3Plus software. The primer sequences are:

18S Forward (GTAACCCGTTGAACCCCATT),

18S Reverse (CCATCCAATCGGTAGTAGCG),

Epac1 Forward (CTGGACACCACTTACCAACA),

Epac1 Reverse (ATTTTTGTGTCTCGGATGAGG),

Epac2 Forward (GGCAGGGTCTTTGGATGTTA),

Epac2 Reverse (GTGCCTTGAAGTCCTTCTGC).

When possible, each forward and reverse primer set was designed between multiple exons. Amplification efficiency of each primer pair was tested before analysis. Relative gene expression levels were determined using the $\Delta\Delta C_T$ method with 18S as the reference gene(16).

Immunoblot Analysis: Whole-cell lysates were prepared in 150 mM Na_2CO_3 buffer (pH 11) and homogenized by sonication. Equal amounts of protein (assayed using a dye-binding reagent; Bio-Rad) were separated by SDS/PAGE using 10%

polyacrylamide precast gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane with the X-Cell II Blot Module (Invitrogen). Membranes were blocked in PBS Tween (1%) containing 5% nonfat dry milk and incubated with primary antibody 18 h at 4°C. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL reagent (Amersham Pharmacia, Pittsburgh, PA). Bands were compared with molecular weight standards to confirm migration of proteins at the appropriate size. Quantification of protein expression densitometry was performed using ImageJ software (NIH, Bethesda, MD). Epac1 (5D3), pVASP S157, and Rap1A/Rap1B antibodies were purchased from Cell Signaling (Danvers, MA).

Rap1 Activation Assay: The Rap1-GTP–binding domain (RBD) of mammalian RalGDS was expressed in *E. coli* as a GST fusion protein as described previously(17). The purified GST-RalGDS-RBD was used for the detection of activated Rap1. 10-cm plates of Swiss 3T3 cells were washed twice with ice-cold PBS and then lysed with 1mL Tris-Lysis Buffer (100 mM Tris, pH 7.5, 300 mM NaCl, 50 mM $MgCl_2$, 20% glycerol, 1% NP-40, 2 mM DTT, 2 mM vandate, aprotinin, and 5 μg/ml leupeptin) at the indicated times. The lysates were centrifuged for 10 min, and the supernatants were incubated with 10 μg GST-RBD on glutathione–Sepharose beads at 4°C for 60 min. The beads were washed three times and subjected to SDS-PAGE and Western blot analysis with either an anti-pVASP mAb or an anti-Rap1 pAb. For control of the input amount of Rap1 protein, we analyzed 20 μl of the cell lysates.

Reagents: cAMP and cAMP analogs were purchased from Biolog (Bremen, Germany). Chembridge compounds were purchased from Chembridge (San Diego, CA). Coelenterazine was purchased from Nanolight (Pinetop, AZ). Forskolin (Fsk) was purchased from Abcam Biochemicals (Cambridge, MA). Complete protease inhibitors were purchased from Roche (Indianapolis, IN). Phosphatase inhibitor Cocktail 3 was purchased from Sigma-Aldrich (St. Louis, MO). Mouse reference cDNA was purchased from Zyagen (San Diego, CA). pcDNA3 CAMYEL was a kind gift from Dr. Paul Sternweis (UT Southwestern). CE3F4 was kindly provided by Frank Lezoualc'h (INSERM UMR-1048, Toulouse, France).

Molecular Modeling and Virtual Docking: The Schrödinger LigPrep Program was used to generate 3D molecular models of the cAMP analog 8-CPT-N6-Phe-cAMP (CPT-N6)(18). Using I-TASSER(19) , a 3D model of the Epac1 structure was produced, based on the 3CF6 PDB of Epac2 in complex with cAMP and Rap(20) ((19). The Schrödinger Protein Preparation Wizard was then used for the model to yield a more accurate structure. The 3D models of CPT-N6 were then screened virtually with the model of Epac1 via Induced Fit Docking (IFD)(19)(19) centered in the CNBD.

Epac1-CPT-N6 System Preparation: The top IFD pose with the phosphate-sugar moiety interacting with the phosphate binding cassette was saved as a pdb and the protein-ligand system was prepared for molecular dynamics (MD). First, the topology and coordinate files were created using Amber Antechamber tools (22) and Xleap

(23)(24). Next, the system was solvated in a water box of TIP3P, with the minimum distance of 10 Å between any atom of the protein-ligand system and the edge of the box. The solvated system was then neutralized before the parameter and coordinate files were saved and prepared for AMBER MD simulations.

Amber Molecular Dynamics: The solvated system was minimized via a two-step process with the sander program. First, the protein was kept fixed while the water positions were minimized. Second, the energy of the whole system was minimized together. Next, it was allowed to heat from 0K to 300K during 50 ps of MD at constant volume using the Langevin thermostat. The system was then equilibrated during 500 ps of MD at constant pressure before production MD. This equilibrated system was then used to produce two independent MD trajectories of about 30 ns each. All MD simulations were performed with isotropic position scaling under constant pressure periodic boundary conditions, the reference pressure of 1 atm, and a pressure relaxation time of 2 ps. The system was maintained at a reference temperature of 300K using the Langevin thermostat with a collision frequency of 2 ps^{-1}(25). A simple Leapfrog integrator was used to solve Newton's equations of motion and to propagate the simulation with a time step of 0.002 ps. Utilizing the sander Amber module, the long-range electrostatics interactions were handled with the particle-mesh Ewald procedure; the long-range van der Waals interactions were estimated by a continuum model. See reference 23 for further computational details.

Root-Mean Square Deviation (RMSD) Trajectory Clustering: The independent MD trajectories were further analyzed to determine the stability of the docking pose. An RMSD-based *gromos* clustering algorithm was used to group or "cluster" the different conformations sampled by the trajectories depending on structural similarity within the CNBD pocket(26)(27). Frames were taken from the simulation every 2 ps and aligned to the starting structure. The trajectories were then clustered based on the residues within 10 Å of the CPT-N6 ligand using an RMSD cutoff of 0.14 Å. The largest cluster was considered most representative of the trajectory.

Virtual Screening for Allosteric Epac Modulators: I-TASSER(19) was used to generate a 3D model of the Epac1 structure in its inactive, unbound state. This model and a library of compounds from Chembridge were prepared with the Schrödinger Protein Preparation Wizard and the LigPrep Program, respectively. A preliminary virtual screen was performed, centered on the Epac hinge, and docking poses were generated using Schrödinger Induced Fit Docking.

Statistical Analysis: Calculations and statistics were performed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA). Numerical values are presented as means ± se. All experiments were performed in triplicate or quadruplicate. Analysis of data from experiments with multiple comparisons was done using ANOVA with Bonferroni's correction. Values of $p < 0.05$ were considered significant.

3.4 Results

Use of CAMYEL to measure Epac activity in cell lysates

CAMYEL is a BRET-based cAMP construct composed of Epac1 sandwiched between Renilla luciferase and Citrine. Upon binding of cAMP, Epac1 undergoes conformational changes that result in a decrease in BRET due to luciferase moving away from Citrine (Fig. 3.1A). We initially assessed whether CAMYEL could be used to detect Epac modulators. CAMYEL was originally designed for detection of cAMP in cells but we tested it with cell lysates in order to eliminate the contribution of cell permeability. We prepared cell lysates from HEK293 cells transiently transfected with CAMYEL and then assessed BRET activity in white 96-well plates in a DTX-800 plate reader. We found that addition of cAMP decreased BRET, measured as the 485nm/535nm ratio (Fig. 3.1B). The time course for CAMYEL activation indicated that cAMP activates the construct in <1min and that the activated state is sustained for >60min at room temperature. Incubation with cAMP at 37° for >30min resulted in a decreased change in BRET due to decreased concentration of cAMP, a result of its hydrolysis by phosphodiesterases (PDE) which could be blocked with the PDE4 inhibitor, rolipram (data not shown). Because the hydrolysis of cAMP by PDEs is temperature-dependent, we conducted all subsequent assays at room temperature and measured the activation of CAMYEL 5 min after treatments.

To verify that CAMYEL can be used to assess modulators of Epac activity, we first determined the response to cAMP analogs with known activity towards Epac (Fig. 3.2B). cAMP produced a concentration-dependent reduction in BRET of

CAMYEL and yielded an EC_{50} of 15μM (Fig. 3.2A), a value similar to the 30 μM EC_{50} obtained for the *in vitro* activation of Rap1 by Epac1 (28). We normalized the ability of cAMP analogs to activate Epac, as assessed by a reduction in BRET of CAMYEL, to the reduction of BRET produced by cAMP. The Epac-specific agonist 8-CPT-2'-O-Me-cAMP (8-Me) activated CAMYEL with an EC_{50} of 0.5μM and produced a larger decrease in BRET than cAMP, suggesting that 8-Me produces a more activated state of Epac than does cAMP. The larger decrease in BRET produced by 8-Me compared to cAMP is consistent with its ability to act as an agonist of Epac with greater efficacy than cAMP(29). N6-Phe-cAMP (N6-Phe), a PKA-specific analog, bound with an EC_{50} of 15μM and produced a decrease in BRET that was only 10% of that promoted by cAMP. Rp-cAMPS and Sp-cAMPS are hydrolysis-resistant analogs of cAMP with a sulfur-substituted for the oxygen in the axial or equatorial position and act as an antagonist or agonist, respectively. Rp-cAMPS bound to CAMYEL with an EC_{50} of 68μM but similar to N6-Phe, did not stabilize the open conformation and thus, produced minimal activation relative to that of cAMP (Fig. 3.2A). Sp-cAMPS activated CAMYEL to a similar maximal extent but with a slightly decreased affinity compared to cAMP.

CAMYEL can distinguish between partial agonists and agonists of Epac

To determine if the increased binding affinity and activation by 8-Me derive from the 8-CPT or 2'-O-Me modification, we assessed the ability of CPT, 8-Me, or 2'-O-Me-cAMP (2'-O-Me) to activate CAMYEL. Relative to cAMP, 2'-O-Me had an increased ability to activate CAMYEL similar to that of 8-Me but with decreased

binding affinity (Fig. 3.3A). The binding affinity of CPT for CAMYEL was increased relative to that of cAMP but CPT caused a smaller decrease in BRET. This smaller decrease in BRET is consistent with evidence that the analog acts as a partial agonist and implies that the 2'-O-Me group is responsible for the increased activation while the 8-CPT produces increased binding affinity. By monitoring the total change in BRET (Fig. 3.3B) at saturating conditions, we were thus able to use the CAMYEL assay to detect whether cAMP analogs are agonists, "super agonists", or partial agonists.

Modifications at the 8-position of the adenine ring modulate binding affinity and agonism

Because analogs modified at the 8-position of the adenine ring on cAMP have altered binding affinity for Epac, we sought to determine the optimal modification to enhance binding by testing the ability of 8-CPT-cAMP, 8-Br-cAMP, and 8-PIP-cAMP to bind and activate CAMYEL (Fig. 3.3C). Relative to cAMP, 8-CPT and 8-Br-cAMP had increased affinity in binding CAMYEL and both acted as partial agonists. 8-PIP-cAMP had a decreased binding affinity to CAMYEL and, although a partial agonist (up to 300μM), decreased BRET to an extent similar to that produced by cAMP. Since the highest binding affinity occurred with the 8-CPT modification, we tested if the functional group on the *para* position of the phenol affects binding by assessing the ability of three cAMP analogs (8-CPT-2'-O-Me-cAMP, 8-HPT-2'-O-Me-cAMP and 8-MeOPT-2'-O-Me-cAMP) to bind and activate Epac. These analogs also have the 2'-O-Me modification that provides super agonism and were used because the

corresponding analogs without 2'-O-Me were not available to us. These analogs have, respectively, chlorine, hydroxyl, or a methoxy group in the *para* position of the phenyl group. All had increased binding to CAMYEL, both in terms of affinity and maximal activity, compared to cAMP (Fig. 3.3D). 8-HPT-2'-O-Me-cAMP, which bound with an EC_{50} of 240nM, had the highest affinity of any cAMP analog tested (Fig. 3.3E). All three analogs had a similar extent of super agonism (Fig. 3.3F). These results indicate that substitutions at the 8 position of cAMP modulate binding affinity and can also result in partial agonism.

Use of CAMYEL to identify inhibitors of Epac

We next set out to determine if the CAMYEL assay can be used to identify Epac inhibitors. Rp-cAMPS is an antagonist of CNBDs in proteins. We tested its ability to act as a competitive inhibitor on Epac by assessing a range of concentrations for inhibition of CAMYEL pre-activated with 100µM cAMP for 5 min and found that Rp-cAMPS acts as a weak competitive inhibitor (i.e. in the high micromolar range (Fig. 3.4A)). Rp-8-CPT-cAMPS (Rp-CPT), an Rp-cAMPS analog with a chlorophenylthio substitution at the 8 position of the adenine ring of cAMP, inhibited CAMYEL with an IC_{50} of 39µM. Increasing concentrations of Rp-CPT shifted the concentration curve for activation of CAMYEL by cAMP to the right without changing the maximal activation, results indicative of a competitive inhibitor (Fig. 3.4B). To determine the disassociation constant for 8-CPT-Rp-cAMPS, we conducted Schild analysis (Fig. 3.4C). The slope of the Schild plot was 1.0, confirming that Rp-cAMPS is a competitive inhibitor. The intercept on the abscissa was -5.5,

corresponding to a K_i of 3.2μM. Thus, the use of CAMYEL can detect competitive inhibitors of Epac and their disassociation constant can be determined by Schild analysis.

We used the recently identified uncompetitive inhibitor CE3F4 to test the ability of CAMYEL to identify allosteric inhibitors of Epac(30). Pre-incubation with 3, 10, or 20μM CE3F4 reduced the maximal decrease in BRET produced by cAMP in a concentration-dependent manner and yielded a K_i of 5.2μM (Fig. 3.5A). CE3F4 was also able to inhibit the activation of CAMYEL by 8-Me or CPT in a concentration-dependent manner with K_i values of 16.1μM and 2.3μM, respectively (Fig. 3.5B, 3.5C). In combination with the data obtained for Rp-CPT, these results demonstrate that the use of CAMYEL can identify competitive and uncompetitive (allosteric) inhibitors of Epac.

N6-modified analogs are competitive inhibitors of Epac

The amino group of the adenine ring of cAMP is important for interaction with the "lid" of Epac protein and for stabilizing the active conformation (20). Since N6-Phe-cAMP did not fully activate CAMYEL but bound to CAMYEL with an affinity similar to that of cAMP, we tested if a similar interaction occurred for other N6-modified cAMP analogs (Fig. 3.6A). Several such analogs (up to 300μM) did not show significant activation (Fig. 3.6B). However, the compounds bound to some extent, competing with 100μM cAMP with affinities in the following rank-order: Phe>MB>Bn=Bnz>AE (Fig. 3.6C). While both N6-MB-cAMP and N6-Phe-cAMP bound and inhibited Epac, their binding affinities were similar to that of cAMP. Thus,

these would not be suitable as competitive inhibitors for Epac *in vivo*. To determine if modifications at the 8 position would increase their binding affinity, we tested the ability of 8-CPT-N6-Phe-cAMP (CPT-N6) to inhibit CAMYEL. CPT-N6 inhibited CAMYEL with an IC_{50} of 2.2µM while N6-Phe had an IC_{50} of ~100µM (Fig. 3.6D). Activation of CAMYEL by cAMP in the presence of increasing amounts of CPT-N6 shifted the concentration-response curve to the right without changing the maximal activation, consistent with competitive inhibition (Fig. 3.6E). Schild analysis confirmed that CPT-N6 acts as a competitive inhibitor (K_i = 0.5µM [Fig. 3.6F]). Thus N6-modified analogs can inhibit Epac activity, especially if such analogs contain modifications at the 8 position that enhance binding.

cGMP has a carbonyl group in place of the N6 amino group on cAMP and is a competitive inhibitor for Epac at high concentrations. To determine if cGMP derivatives might be leads for Epac inhibitors, we tested cGMP, CPT-cGMP, and CPT-2'-O-Me-cGMP for their ability to activate (Fig. 3.7A) or inhibit (Fig. 3.7B) Epac. cGMP and CPT-cGMP were unable to activate Epac at any concentration tested. CPT-2'-O-Me-cGMP may be a weak partial agonist at >300µM. CPT-cGMP and CPT-2'-O-Me-cGMP were able to inhibit Epac (IC_{50} = 75µM and 360µM, respectively) but at concentrations significantly higher than that of CPT-N6. For this reason we did not pursue cGMP analogs as Epac inhibitors.

Comparison of inhibitors

Rp-cAMPS can bind CNBDs but does not activate them, due to the inability of the analog to properly interact with the phosphate binding cassette. N6-Phe-cAMP can

bind to CNBDs and activate them but cannot stabilize the active conformation of Epac as a consequence of decreased interaction with the 'lid'. We sought to verify that Rp-cAMPS is an antagonist that does not activate the CNBD while N6-Phe would be able to interact with the CNBD of Epac and activate the isolated domain. To test this, we used Epac2-cAMPS, a FRET-based sensor that has the CNBD-B of Epac2 sandwiched between CFP and YFP. Activation of the CNBD is detected by a decrease in FRET between CFP and YFP. cAMP induced a concentration-dependent decrease in FRET (EC_{50} = 1.9µM) (Fig. 3.8A). N6-Phe-cAMP activated the CNBD to the same extent as cAMP but with higher apparent affinity (EC_{50} = 0.2µM). Rp-cAMPS did not activate the CNBD but instead increased the FRET signal (EC_{50}= 176µM), implying that it stabilizes the closed, inhibited state of the CNBD. Pretreatment of Epac2-cAMPS with Rp-CPT (100µM) increased the apparent affinity of cAMP, indicating that Rp-cAMPS analogs can be competitive inhibitors for Epac2 as well as Epac1. Comparison of cAMP activation of CAMYEL in the presence of 10µM CPT-N6 or 10µM Rp-CPT revealed that CPT-N6 has a greater ability than Rp-CPT to shift the curve to the right and increase the apparent affinity of cAMP (Fig. 3.8B). To determine the affinity of the different inhibitors, we compared their ability to inhibit CAMYEL activated by 100µM cAMP. CPT-N6 and CE3F4 had similar affinities with IC_{50} values of 2.2µM and 7.3µM, respectively (Fig. 3.8C), while Rp-CPT had a lower affinity (IC_{50} = 39µM).

Verification of inhibition of Epac by cAMP analogs in cells

Rp-CPT and CPT-N6 were able to inhibit Epac *in vitro* but we thought it was important to test their ability to inhibit endogenous cAMP signaling in cells. To test this, we employed Swiss 3T3 cells, which express mRNA (assessed by QT-PCR) of Epac1 and Epac2 (Fig. 3.9A). Epac1 was the main isoform expressed (ΔCt = 23 vs. Epac2 with ΔC_t = 35), corresponding to ~4000-fold more Epac1 mRNA than Epac2 mRNA. Analysis of protein expression showed abundant Epac1 protein but no detectable Epac2 protein (data not shown). Swiss 3T3 cells express Rap1, which can be activated by stimulants that increase cAMP(31). Incubation of Swiss 3T3 cells with the adenylyl cyclase activator, forskolin, increased Rap1 activation and the phosphorylation of VASP at Serine 157 (Fig. 3.9B). Treatment of cells with 50µM N6-Phe (a PKA-specific analog) increased VASP phosphorylation but did not change Rap1 activation, indicating that PKA mediates VASP phosphorylation (32). Treatment of the cells with 50µM 8-Me (an Epac-specific analog), increased Rap1 activation but did not promote VASP phosphorylation, confirming that Epac mediates Rap1 activation. Treatment of the cells with 50µM of CE3F4 for 30min prevented the activation of Rap1 by forskolin without changing VASP phosphorylation, further indicating that Epac activates Rap and PKA phosphorylates VASP (Fig. 3.9E). These results led us to test the activation or inhibition of Epac1 and PKA activity by simultaneously monitoring Rap1 activation or VASP phosphorylation, respectively, via activation of the cAMP pathway by forskolin alone or in the presence of putative inhibitors. We tested the ability of Rp-CPT or CPT-N6 to inhibit Epac1 activation. Treatment of the cells with either 50µM CPT-N6 (Fig. 3.10A) or 100µM Rp-CPT

(Fig. 3.10B) prevented the activation of Rap1 by forskolin. Thus, the findings obtained from the CAMYEL assay *in vitro* predict Epac activation and inhibition in cells.

Molecular model of CPT-N6 docking pose

To obtain further insight regarding the binding of the CPT-N6 analog to Epac1 and because a structure of Epac1 has not been solved, we undertook a computational molecular modeling approach based on a solved structure for activated Epac2. The 3D model of active Epac1 was generated with I-TASSER and had an estimated accuracy of 0.52±0.15 (TM-score, where a TM-score <0.17 suggests a random similarity and a TM-score >0.5 typically corresponds to the same fold or domain in SCOP/CATH). (33) The Protein Prep Wizard and LigPrep Program of the Maestro 9.2 (Schrödinger, LLC) module were used to prepare the Epac1 model and CPT-N6, respectively. The Schrödinger Induced Fit Docking procedure was used to generate predicted binding poses and scores(34). Among the results where the phosphate-sugar moiety interacted with the phosphate binding cassette (PBC), the highest Glide XP score for CPT-N6 docked into the CNBD of the Epac1 model was -10.1kcal/mol (35). Taking this top pose, the protein-ligand system was prepared for molecular dynamics (MD). The two independent 30ns trajectories were then clustered by RMSD near the CNBD to identify the most representative structures of the simulation. These structures are also the most stable conformations during these MD runs, suggesting that they may be possible binding poses for the protein-ligand complex. These two poses were also very similar, even though the trajectories were run independently (Fig. 3.11B). As shown in Figure 3.11C and 3.11D, residues around the PBC that appear to form hydrogen bonds

with the CPT-N6 analog include G311, Q312, L313, A314, R321; there is a possibility for interaction between the CPT moiety and L356. The structure most representative of trajectory 1 (Fig. 3.11C) includes two waters able to hydrogen bond with the ligand phosphate group, where that of trajectory 2 (Fig. 3.11D) shows only one water molecule hydrogen bonded to it. Both appear to be very stably bound to the PBC but lack any bonds to the "lid" akin to what is seen between Sp-cAMPS and Epac2 (Fig.3.11A).

Virtual screening of compounds that interact at the Epac hinge identifies a possible allosteric inhibitor

We undertook a similar approach to generate a model of Epac1 in its inactive state. A library of compounds from Chembridge was then virtually docked to the hinge region of this Epac1 model so as to identify compounds that might be allosteric inhibitors. The 133 compounds that had the highest score were tested experimentally.

Compound 5225554, a barbituric acid derivative (Fig 3.12A), had the best activity as an inhibitor in the CAMYEL assay. Further studies using this assay revealed that compound 5225554 bound in a non-competitive manner (Fig 3.12B). Experiments conducted with Swiss 3T3 cells indicated that 5225554 inhibits forskolin-stimulated Rap1 activation without altering VASP phosphorylation (Figs 3.12C and 3.12D). The Schrödinger Induced Fit Docking generated high scoring poses suggesting that compound 5225554 fits nicely into a pocket near the Epac hinge and residues L315 and F342 (L408 and F435 in Epac 2). Fig.3.12E shows possible hydrogen bonding between the compound and residues S282 and V337. Fig.3.12F

shows a slightly different possible pose where 5225554 may form bonds with residues H248, D309, and Q312. The CAMYEL assay can thus identify novel inhibitors of Epac1, whose site of action is not in the cAMP binding site but likely in the hinge region of the protein.

3.5 Discussion

These results show that the use of the CAMYEL assay with cell lysates provides a means to assess conformational changes that occur upon ligand binding and to identify modulators of Epac activity. While Epac-cAMP sensors, such as CAMYEL, are used to assess Epac activation in cells, we tested whether it can provide a method to detect different modes of inhibition and of agonism. The EC_{50} values for cAMP analogs obtained for activation of CAMYEL are similar to those obtained for their activation of Rap1 in an *in vitro* Epac GEF assay(36). The absolute affinities are somewhat higher than those obtained by displacement of cAMP but the relative affinities compared to cAMP are similar (**Table 3.1**) (37)(38).

Previous methods to identify Epac inhibitors have involved analysis of the displacement of fluorescent or radioactive cAMP analogs from purified Epac protein(39)(40). While this approach can identify competitive inhibitors, it only measures binding interactions and does not provide information about the mechanism of inhibition of the competitor and thus, is likely unable to detect allosteric inhibitors that do not displace the ligand. Those prior studies involved the use of purified protein, which is expensive (in terms of time and effort) to obtain. Our method is a

novel, rapid, and cost-effective way to identify modulators of Epac activity in cell lysates. Thus, the CAMYEL assay provides a means to reliably measure the binding of analogs to Epac and simultaneously measure their ability to activate it.

Our results show that in addition to the ability of the CAMYEL assay to measure binding affinity, it can also be used to predict the efficacy of activators based on differences in the maximal decrease in BRET. We found that 8-CPT produces a 50% smaller decrease while 8-Me shows a 125% larger decrease in BRET than does cAMP. The overall changes in BRET correlate with the decreased (50%) and increased (300%) GEF activity induced by 8-CPT and 8-Me, respectively, indicating that the CAMYEL assay can predict if analogs are agonists, partial agonists, or super agonists. The differing maximal decreases in BRET may result from ability of the compounds to produce a smaller or greater open state of the protein or perhaps from a shift in the equilibrium between the activated and inactivated states by the compounds. While this assay cannot distinguish between those two possibilities, nor can it determine the exact extent of change in enzymatic activity, CAMYEL provides a means to assess modulators of Epac activity and to infer potential effects on the conformation and activity of the protein.

We found that the CAMYEL assay is particularly well-suited to identify competitive inhibitors of Epac. Rp-cAMPS, a phosphothiorate analog of cAMP with a sulfur in the equatorial position of the phosphate, is an antagonist of CNBDs, based on its inability to interact properly with the phosphate binding cassette (PBC) of CNBDs, which is necessary for their activation(41). The addition of 8-CPT to Rp-cAMPS (Rp-CPT) enhances its binding to Epac and its ability to act as a competitive inhibitor *in*

vitro (Fig. 3.4) and *in vivo* (Fig. 3.10). Rp-CPT also antagonizes PKA and cyclic nucleotide-gated channels and thus is a general antagonist of CNBDs(42).

Our results show that CAMYEL can also identify allosteric modulators of Epac. The uncompetitive inhibitor CE3F4 reduced the maximal activation of CAMYEL and slightly shifted the EC_{50} of cAMP to the left in a concentration-dependent manner, results indicative of an uncompetitive inhibitor. We further showed that the apparent K_i of CE3F4 depends on the mode of activation of Epac. This result suggests that either the binding affinity changes between the different activated states or perhaps that 8-Me induces a more stable active open form that is more difficult to close.

We verified our *in vitro* results using Swiss 3T3 cells, which express Rap1 and Epac (Fig. 3.9). We chose these cells because we found that they have a more dynamic range of Rap1 activation upon cAMP stimulation than do several other cell types we tested (data not shown). As a result, we were able to assess activation or inhibition of PKA or Epac1 in parallel by monitoring phosphorylation of VASP or activation of Rap1, respectively, in response to forskolin. CE3F4, CPT-N6, and Rp-CPT all inhibited Rap1 activation induced by forskolin (Fig. 3.9, 3.10). The concentrations necessary for inhibition by cAMP analogs is higher than in our *in vitro* assays, perhaps reflecting the contribution of cell-permeability for the cAMP analogs.

In the crystal structure of Epac2 activated by Sp-cAMPS, the amino group of cAMP interacts with a conserved VLVLE motif in the CNBD (termed the ‘lid’) that is crucial for Epac activation. N6-substituted cAMP analogs can activate the CNBD of Epac but, due to an inability to interact with the lid and stabilize the activated state,

they cannot overcome auto-inhibition constraints(43). N6-Phe activated Epac2-camps, in which the CNBD-B is sandwiched between YFP and CFP, lacks the residues necessary to stabilize the auto-inhibited state. N6-Phe and cAMP activated this construct similarly while Rp-cAMPS could not and increased FRET, consistent with the idea that it stabilizes the inactive form of Epac. Because this increase in energy transfer did not occur in the CAMYEL assay, we infer that interactions between the regulatory and catalytic domains contribute to stabilizing the auto-inhibited state, causing full-length Epac protein to be predominantly in the closed state when not bound to cAMP. This suggests that the two analogs work via two different mechanisms to inhibit Epac: Rp-cAMPS inhibits by displacing cAMP and not activating the CNBD, while N6-Phe activates the CNBD but cannot interact with the lid properly to overcome the auto-inhibition necessary for Epac activation.

Since CPT-N6 was a more efficient inhibitor of Epac than Rp-CPT, we sought to determine how it binds to Epac. Figure 3.11B shows a potential binding mode for CPT-N6 to a model of activated Epac1. From the MD trajectories, the conformations of the protein-ligand system were clustered based on RMSD at the CNBD of Epac1 in order to identify the most stable states. The top cluster from each 30 ns trajectory shows CPT-N6 with the phosphate sugar extensively interacting with the PBC and surrounding residues. As in the PDB of Epac2 with Sp-cAMPS, both top clusters show CPT-N6 with possible water interactions. However, the CPT-N6 cAMP analog does not appear to interact with the Epac1 lid, which is necessary to stabilize the active conformation. A possible explanation for the activity of CPT-N6, which binds well to Epac but inhibits its activation, may be that it stably interacts with the PBC,

blocking cAMP, but not with the lid and therefore cannot stabilize the active conformation. This idea is consistent with the experimental results because the 8-CPT moiety enhances binding while N6-Phe inhibits Epac activation. The interaction of L356 with the 8-CPT moiety instead of the adenine base may explain the partial agonism of 8-CPT-cAMP as this interaction is crucial for the full activation of Epac. Even if the L356 residue (part of the CNBD lid) is stabilized by the CPT moiety, the REM side of the lid was not able to securely interact with the analog in our MD simulations. There may be an intermediate state, between the inactive and active structures, in which CPT-N6 binds but the full active conformation is not achieved and/or stabilized.

Previous studies show that bending of the hinge in the CNBD of Epac induced by cAMP binding is important for Epac activation and is maintained by hydrophobic interactions formed among L408, F435, and L439 (which are conserved in Epac1)(44). A F435G Epac mutant possesses GEF activity close to that of the wild-type protein activated with cAMP and can partially activate Rap1 even without cAMP, suggesting that F435 is restrictive enough to prevent GEF activity without cAMP, but permits full bending of the hinge with cAMP present. However, a F435W mutation, with the bulkier tryptophan residue, inhibits Epac bending, resulting in a decrease in catalytic activity. Hence, this hinge region appears to be an attractive region to target with Epac inhibitors.

Investigation of 3D Epac models indicate that there may be a “druggable” region near these hinge residues, based on preliminary virtual screening and experimental studies (data not shown). The assay described here provides a useful

means to identify such modulators and succeeded in identifying the barbituric acid derivative 5225554 as an allosteric inhibitor of Epac. 5225554 decreased maximal activation of CAMYEL without a changing the EC_{50}, results indicative of a noncompetitive inhibitor. This is in agreement with the predicted binding at the hinge region instead of the cAMP binding pocket. Use of these complementary approaches suggests that targeting this area with small molecules can identify allosteric inhibitors of Epac.

The two cAMP effectors, PKA and Epac, are important intracellular signal transducers involved in signal transduction from G_s-coupled GPCRs. While multiple PKA inhibitors are available, first generation Epac inhibitors are just now being discovered. Compared to receptors and kinases, GEFs and their respective GTPases are not generally considered to be classical “druggable” targets due to the lack of suitable binding pockets(45). The CBND of Epac presents a target for small molecules that will act as competitive inhibitors. The discovery of CE3F4 and 5225554 indicates that Epac activity can also be modulated by allosteric effectors. The CAMYEL assay provides a rapid and cost-effective assay to identify both types of these inhibitors and also contributes information regarding the mechanism for Epac activation. Thus, this assay may prove to be useful for identifying novel inhibitors of Epac.

3.6 Acknowledgments

We thank Dr. Paul Sternweis for the pcDNA3 CAMYEL plasmid. We thank Dr. Frank Lezoualc’h for the CE3F4 compound. This work was supported in part by

the National Institutes of Health Molecular Biological Approaches to Endocrinology Training Grant at the University of California, San Diego CA, USA (5T32DK007541-27). Work in the JAM group was supported in part by National Science Foundation Grant MCB1020765, National Institutes of Health Grant GM31749, the Howard Hughes Medical Institute, the National Biomedical Computation Resource, the Center for Theoretical Biological Physics, and NSF Supercomputer Centers.

The contents of Chapter 3, in full, have been published: **Brown, L.M.,** Rogers K.E., McCammon J.A., and Insel, P.A. (2014) "Identification and Validation of Modulators of Epac Activity: Structure-function Implications for Epac Activation and Inhibition." *Journal of Biological Chemistry,* 289: 8217-8230. The dissertation author was the primary investigator and author of this paper. All co-authors have given written permission for its use and the reproduction of all associated data in this dissertation.

3.7 Figures and Table

Table 3.1 Binding affinity of cAMP analogs, as assessed by CAMYEL. The table shows the affinity of cAMP analogs compared to cAMP as measured by the CAMYEL assay (described in Experimental Procedures). The ability of the analogs to activate or inhibit CAMYEL compared to cAMP is also shown. The relative affinity of the analogs as measured by displacement of radioactive cAMP as reported in references 37 and 38 is shown for comparison to the values obtained by the CAMYEL assay.

Compounds	Abbr.	EC_{50} (uM)	IC_{50} (uM)	Maximal Activation	Change in affinity compared to cAMP	Reported affinity compared to cAMP[1,2]
cAMP	cAMP	15uM	NA	100	1	2.9uM[1]
Compounds with 8 Substitions						
8-Bromine-cAMP	8-Br	3.5		60	3.71	8.10[1]
8-Piperidino-cAMP	8-PIP	78		89	0.17	0.21[1]
8-Chlorophenylthio-cAMP	CPT	0.59		54	22.03	65.00[1]
8-Chlorophenylthio-2'-O-Methyl-cAMP	8-Me	0.43	NA	130	30.23	4.60[1]
8-Hydroxyphenylthio-2'-O-Methyl-cAMP	8-HPT-Me	0.23	NA	132	56.52	6.90[2]
8-Methoxyphenylthio-2'-O-Methyl-cAMP	8-MeOPT-Me	0.46	NA	126	28.26	7.10[2]
Compounds N6 Substitions						
N6-Phenol-cAMP	N6-Phe	13	100	8	1.00	2.80[1]
N6-Benzene-cAMP	N6-Bn	9.9	100	22	1.31	
N6-Benzoyl-cAMP	N6-Bnz	9.6	300	NA	1.35	1.30[2]
N6-Aminoethyl-cAMP	AE-cAMP		>300			
2-Aza-ε-cAMP	Aza-cAMP		300			
8-Chlorophenylthio-N6-Phenol-cAMP	CPT-N6	0.27	3	12	48.15	110.00[2]
N6-monobutyrl-cAMP	N6-MB	64	>300	6		
Compounds with thiophosphates						
Rp-cAMPS	Rp-cAMPS	68	>300	6	0.19	
Sp-cAMPS	Sp-cAMPS	41		105		
Rp-CPT-cAMPS	Rp-CPT		39			
Compounds with 2' Substitutions						
2'O-Me-cAMP	2'-Me-cAMP	50		125	0.26	0.12[1]
2'-dcAMP	2-dcAMP	600		92	0.02	0.003[1]
2'-F-cAMP	2-F-cAMP	400		88	0.03	
Compounds based on cGMP						
cGMP		250			0.05	0.08[1]
8-CPT-cGMP	CPT-cGMP	4.9	30	14	2.65	
8-CPT-2'-O-Me-cGMP	8-Me-cGMP	33	300	29	0.39	

1:Reference 38
2 Reference 37

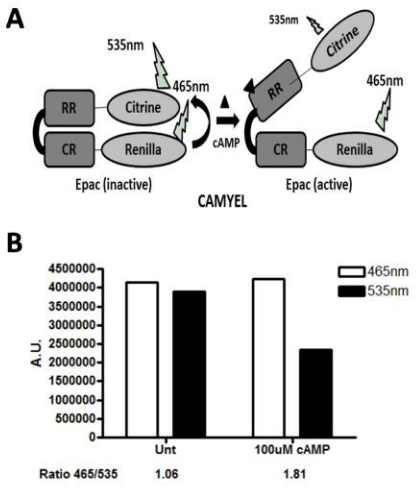


Figure 3.1 The CAMYEL Assay. (A) Schematic diagram of the activation of the CAMYEL construct: the catalytic region (CR) and regulatory region (RR) (Epac1 a.a. 149-880) with Renilla Luciferase attached to the CR and Citrine attached to the RR. **(B)** cAMP produces a decrease in the BRET signal upon binding to CAMYEL, as measured by the decrease of 465/535nm.

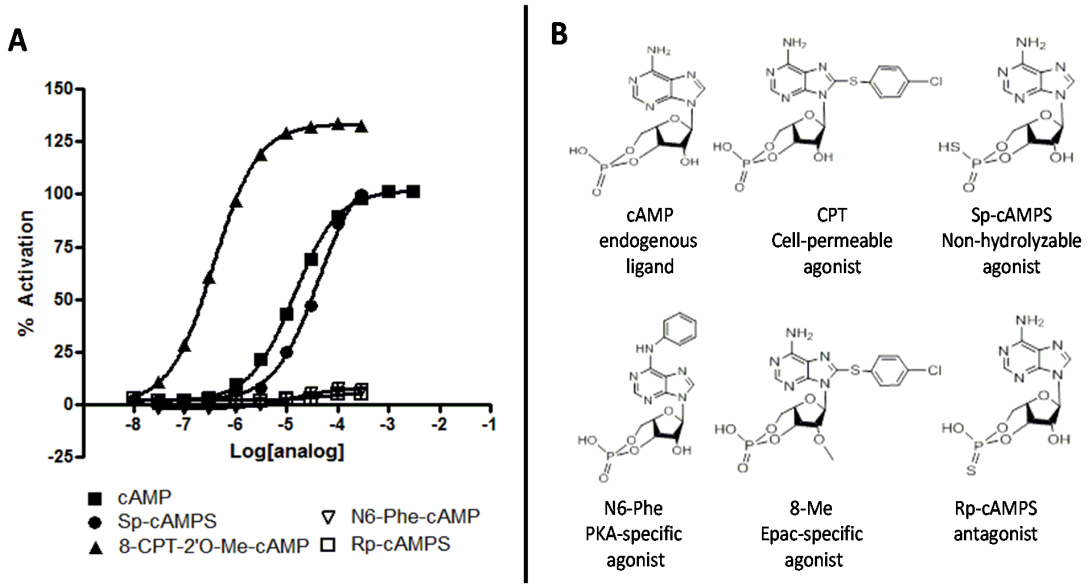


Figure 3.2 CAMYEL activation is induced by Epac agonists in a concentration-dependent manner. cAMP and cAMP analogs were added to a 96-well white plate with 100µL CAMYEL in HEK293 lysate and incubated for 5 min before measuring activation. **(A)** cAMP analogs activate the CAMYEL construct in a concentration-dependent manner. **(B)** Structures of cAMP analogs used in the assay of CAMYEL activation. *n=3.*

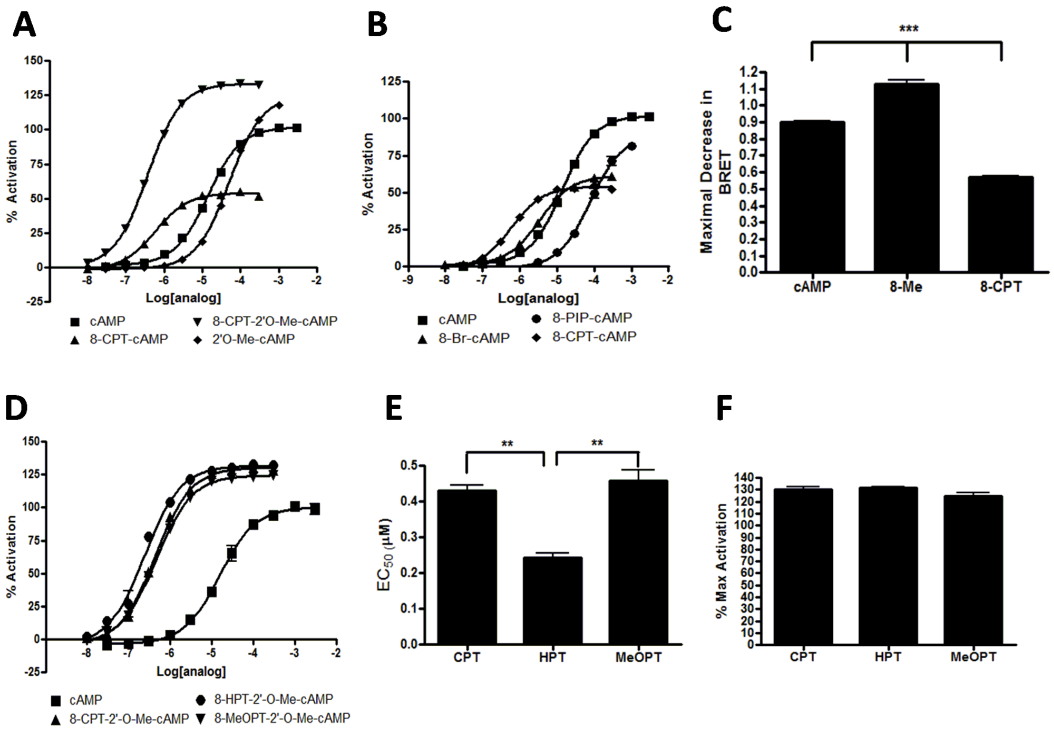


Figure 3.3 CAMYEL can assess if cAMP analogs are agonists, super agonists, or partial agonists. cAMP and cAMP analogs were added and assayed as in Figure 3.2. **(A)** Activation of CAMYEL by cAMP analogs. **(B)** Activation of CAMYEL is decreased by cAMP analogs modified with 8-R substitutions. **(C)** The maximum decrease in BRET (measured by 465/535nm) in response to 300μM cAMP, 8-Me, or CPT. **(D)** Activation assays of the cAMP analogs and their **(E)** EC_{50} and **(F)** maximum activation compared to that by cAMP. *n=3, ** p<0.01, ***p<0.001.*

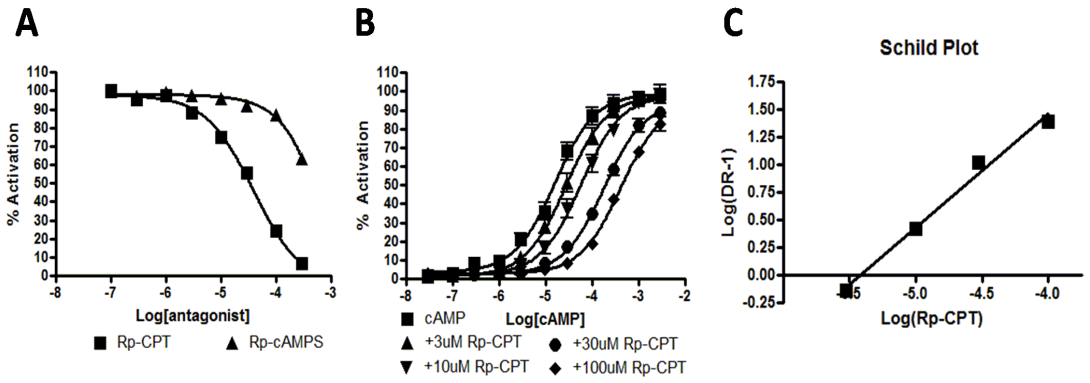


Figure 3.4 Rp-substituted cAMP analogs are competitive inhibitors and addition of 8-CPT enhances binding affinity. cAMP and cAMP analogs were added and assayed as in Figure 3.2. (**A)** Rp-cAMPS and Rp-CPT inhibit CAMYEL pre-activated by 100µM cAMP in a concentration-dependent manner. **(B)** Rp-CPT was pre-incubated with CAMYEL for 5 min before adding cAMP. The concentration-dependent activation of CAMYEL by cAMP was determined 5 min after activation by cAMP. **(C)** Schild Plot obtained from Figure 4B. *n=3-6.*

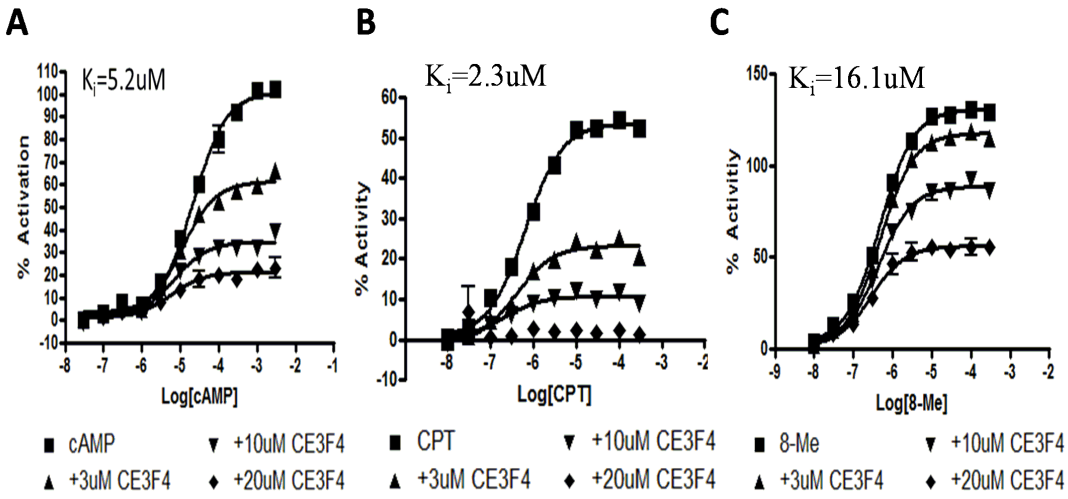


Figure 3.5 CE3F4, an Epac-specific inhibitor, inhibits CAMYEL activation. CAMYEL was pre-incubated with CE3F4 (3µM, 10µM, 20µM) for 5 min before assessing the ability of **(A)** cAMP, **(B)** 8-Me, or **(C)** CPT to activate CAMYEL. CE3F4 inhibited the ability of the Epac agonists to activate CAMYEL in a concentration-dependent manner. *n=3.*

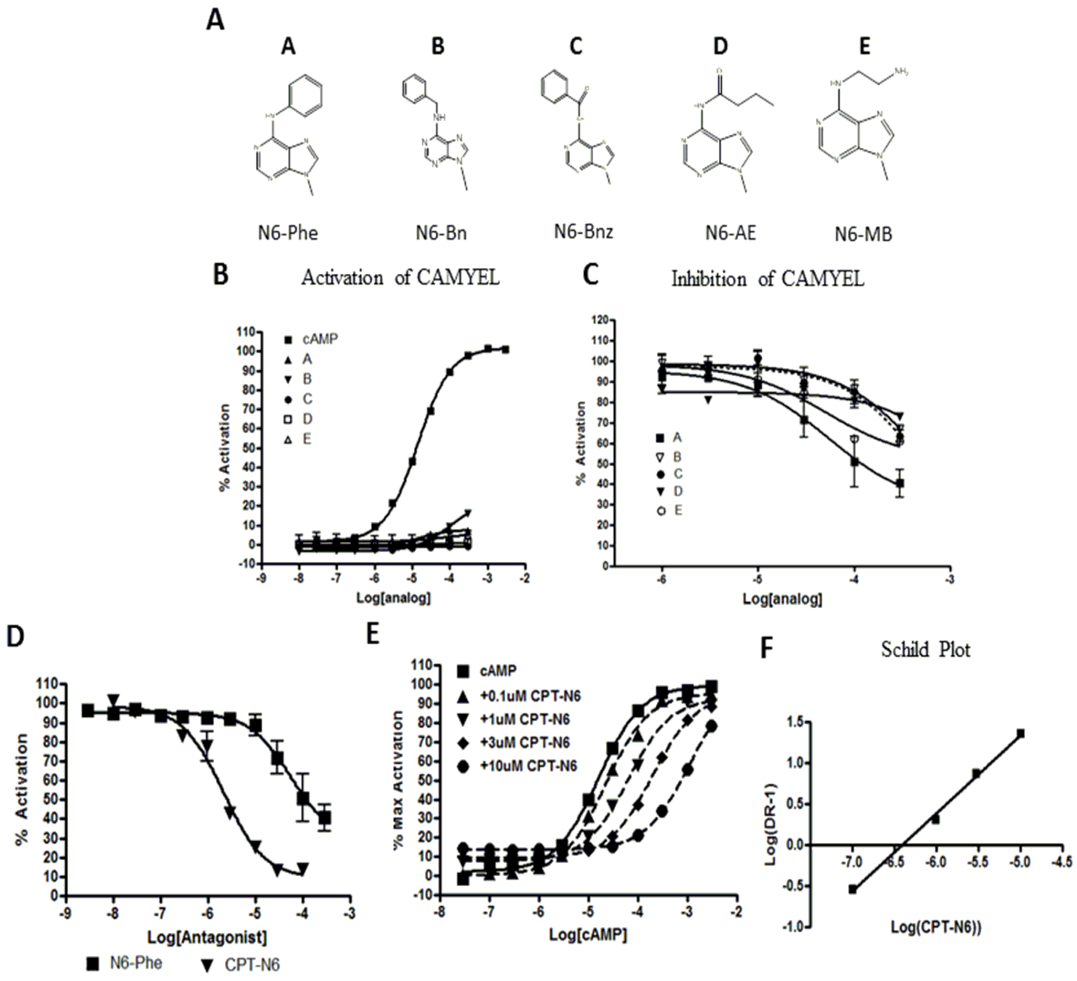


Figure 3.6 N6-modified cAMP analogs are competitive inhibitors of Epac1, as assessed in the CAMYEL assay. (A) Structures of the adenine ring of cAMP showing various N6-substitutions. N6-modified cAMP analogs were tested for their ability to: **(B)** activate CAMYEL or **(C)** inhibit 100µM cAMP-activated CAMYEL. **(D)** CAMYEL activated by 100µM cAMP is inhibited by N6-Phe or CPT-N6 in a concentration-dependent manner. **(E)** CPT-N6 inhibits CAMYEL activation by cAMP in a concentration-dependent manner. **(F)** Schild plot obtained from data shown in 6E. *n=3-6*.

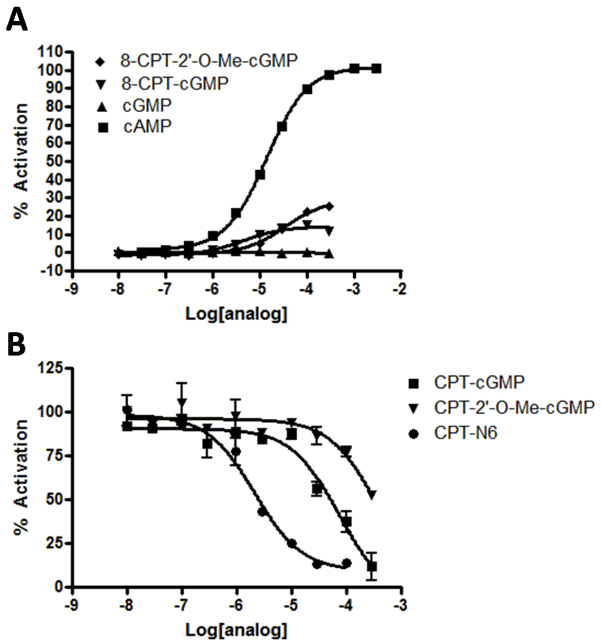


Figure 3.7 cGMP analogs as inhibitors of Epac1, as assessed in the CAMYEL assay. (A) Concentration-dependent activation of CAMYEL by cGMP and cGMP analogs. **(B)** Concentration-dependent inhibition by cGMP and cGMP analogs of CAMYEL pre-activated with 100µM cAMP. *n=3.*

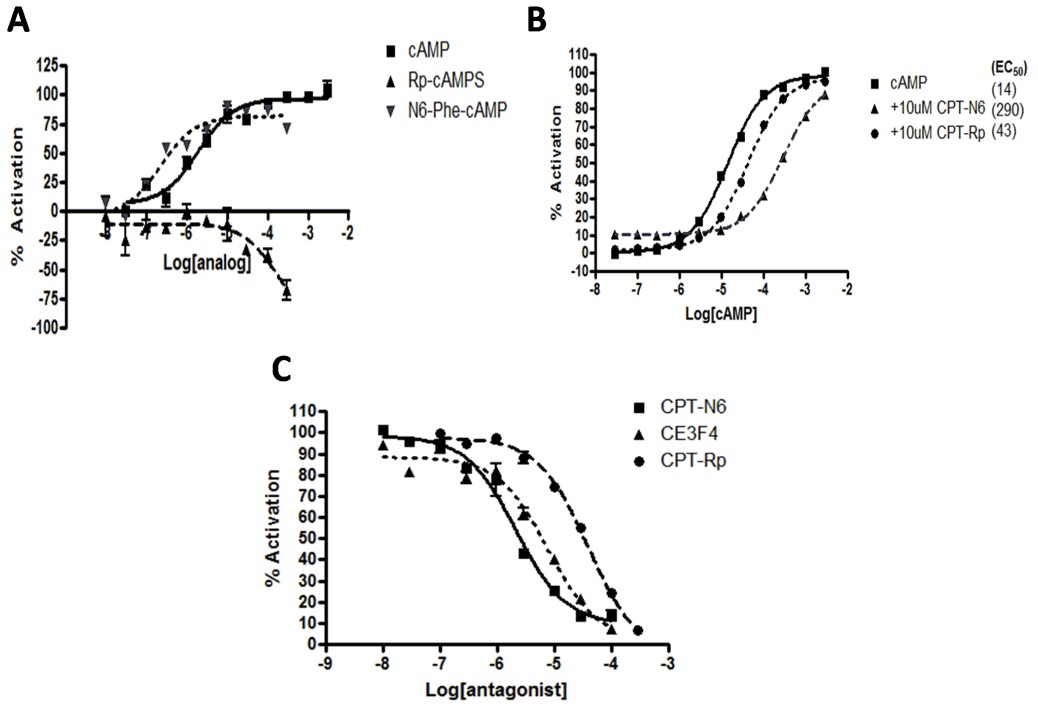


Figure 3.8 Comparison of inhibitors of Epac, as assessed using Epac2-cAMPS or the CAMYEL assay. (A) cAMP, Rp-cAMPS, or N6-cAMP-Phe were added to 100µL Epac2-cAMPS lysate in a black 96-well plate. After 5 min, their ability to activate Epac2-CNBD was measured (results normalized to cAMP). **(B)** Activation of CAMYEL by cAMP pre-incubated for 5 min with 10µM CPT-N6 or 10µM Rp-CPT. **(C)** CAMYEL was activated with 100µM cAMP before assessment of the ability of CE3F4, CPT-N6, and Rp-CPT to inhibit CAMYEL. *n=3.*

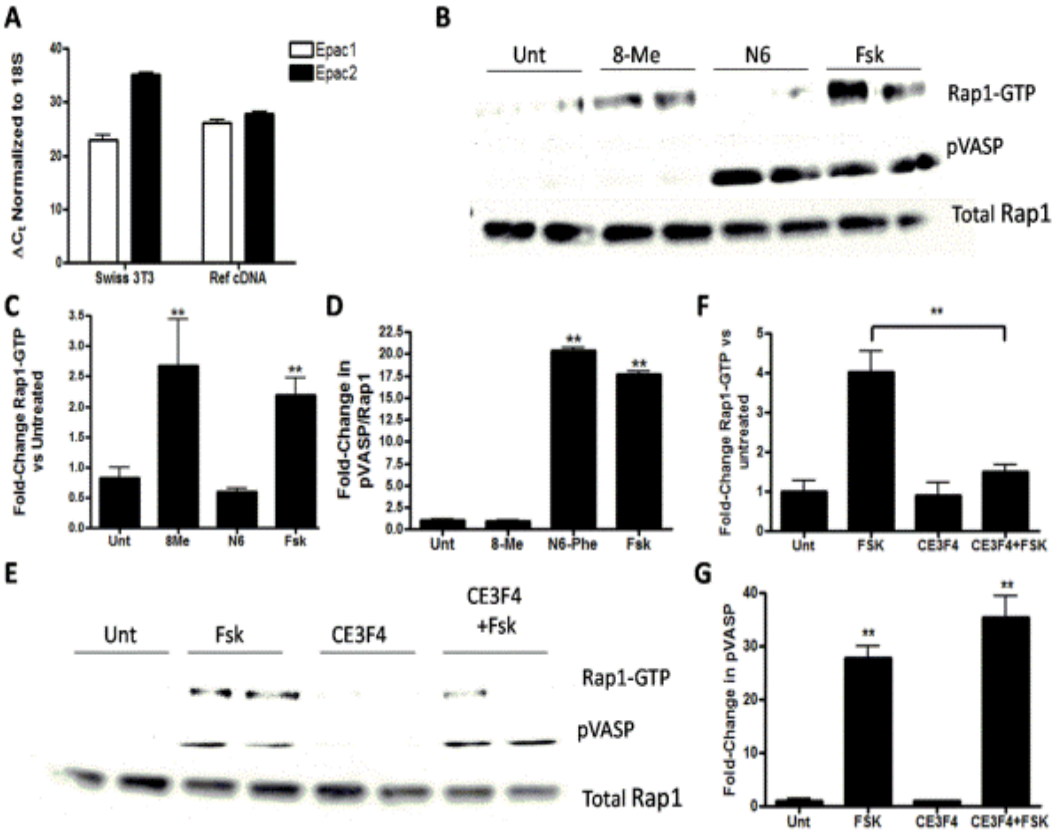


Figure 3.9 Epac1 activates Rap1 while PKA activation mediates VASP phosphorylation in Swiss 3T3 cells. **(A)** QT-PCR analysis of Epac1 and Epac2 mRNA expression in Swiss 3T3 cells and mouse reference cDNA **(B)** Rap1 activation and VASP phosphorylation were assessed by Western blotting of Swiss 3T3 cells that were untreated (Unt) or incubated with 8-ME, N6 or Forskolin (Fsk) for 5 min. **(C)** Pooled results (n=3) for Rap1 activation and **(D)** VASP phosphorylation by 8-ME, N6 or forskolin. **(E)** Inhibition of forkolin-promoted Rap1 activation but not VASP phosphorylation by 20µM CE3F4. **(F)** and **(G)** Pooled results for effect of 20µM CE3F4 on forskolin-promoted Rap1 activation and VASP phosphorylation, respectively *n=4, **p<0.01*.

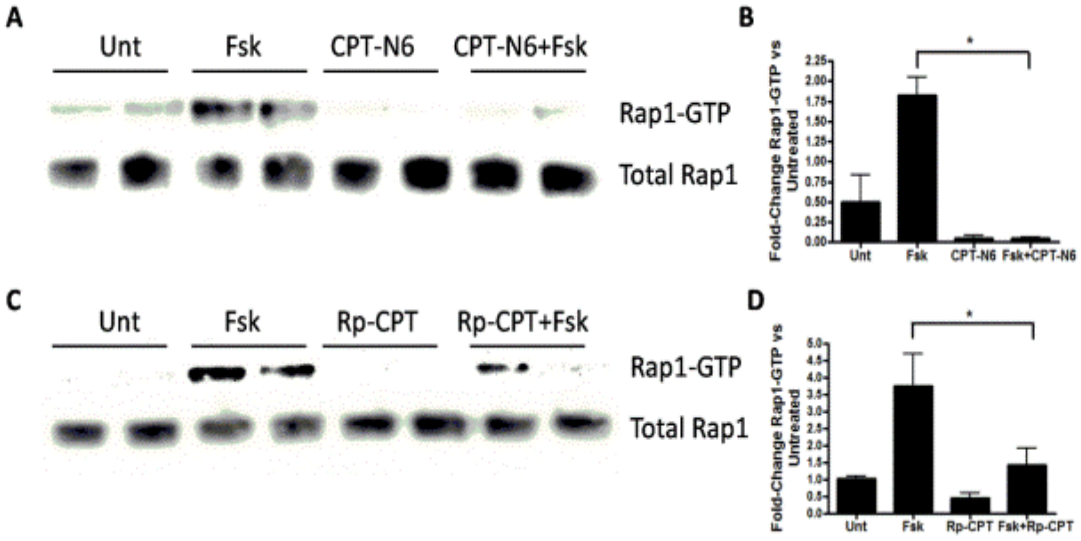


Figure 3.10 CPT-N6 and Rp-CPT inhibit forskolin-promoted Epac activation in Swiss 3T3 cells. Swiss 3T3 cells were incubated with 50µM CPT-N6 **(A, B)** or 100 µM Rp-CPT **(C, D)** for 30min and were then untreated (Unt) or incubated with forskolin (10µM). Cells were harvested 5 min later and the levels of activated Rap1 were quantified. **(B)** and **(D)** show pooled results for *n=5, *p<0.01*.

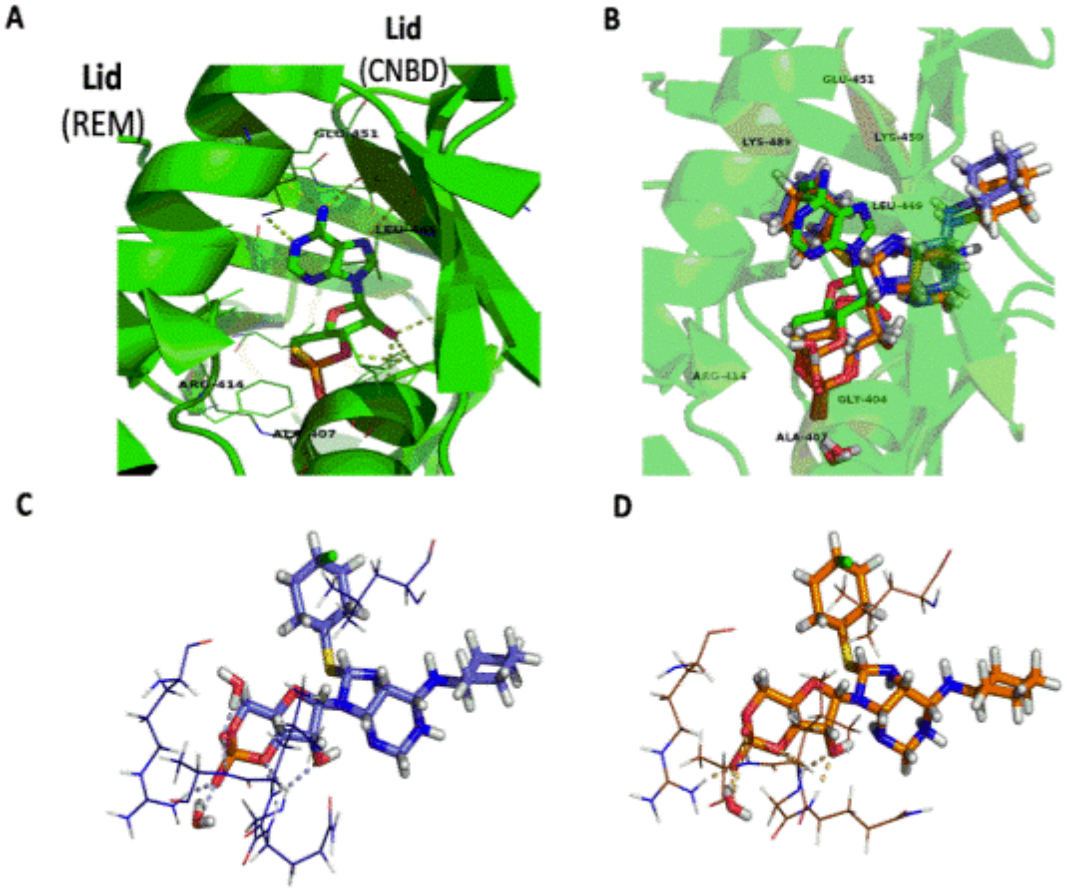


Figure 3.11 Molecular Model of CPT-N6 bound to Epac1. (A) The solved structure of Epac2 CNBD (green) with Sp-cAMPS (PDB ID: 3CF6) and **(B)** representative structures from the holo MD of CPT-N6 docked to the Epac1 model (trajectory 1 in purple, trajectory 2 in orange) are shown for comparison. **(C)** A closer look at the CPT-N6-bound Epac1 models from trajectory 1 and **(D)** trajectory 2, showing potential interactions. Ligands and water molecules are shown as sticks, protein residues as lines, and bonds as dotted lines. Protein secondary structure is shown as a cartoon in **(A)** and **(B)**. Residues are labeled for Epac1 and Epac 2 (in parentheses) for comparison in **(C)** and **(D)**. Figures were created using PyMOL.

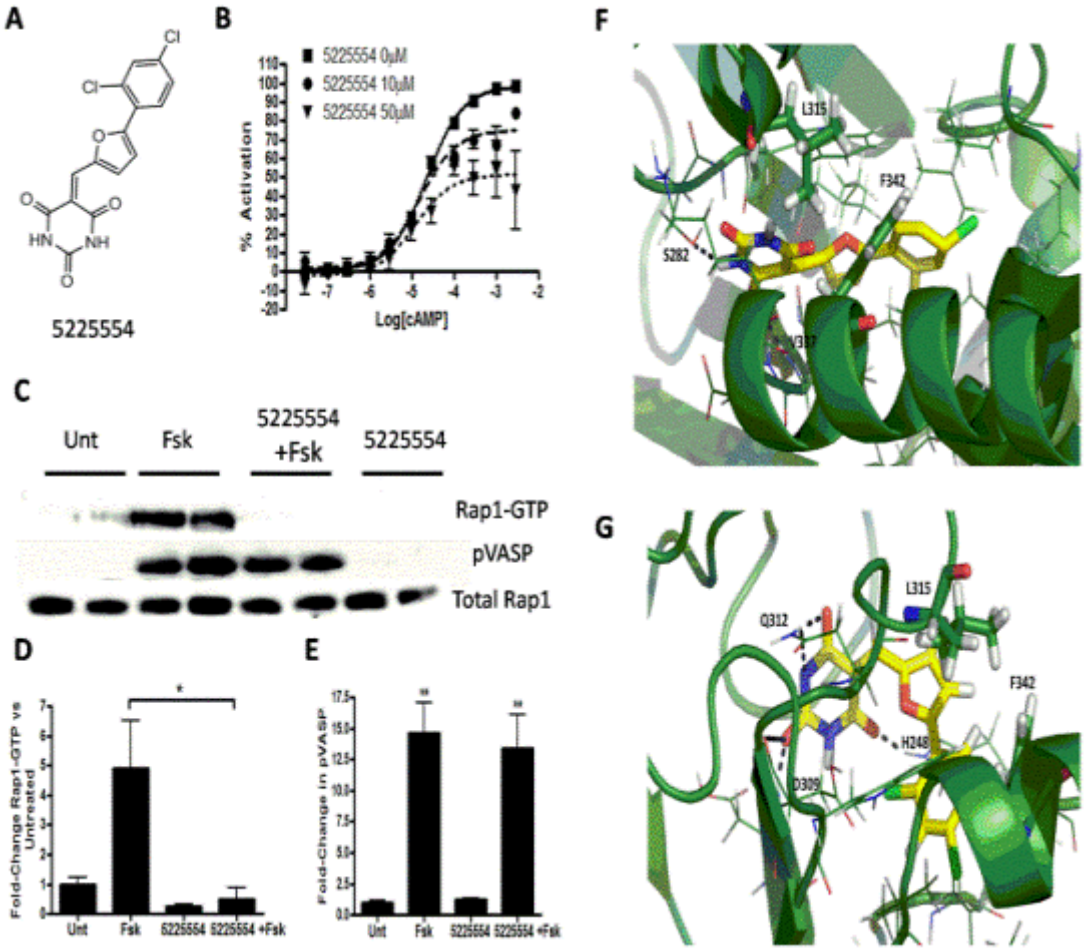


Figure 3.12 Chembridge compound 5225554 is an allosteric inhibitor of Epac. **(A)** The structure of 5225554**. (B)** Pre-incubation with increasing concentrations of 5225554 decreases cAMP-promoted activation of CAMYEL. **(C)** Swiss 3T3 cells were treated with 100μM compound 5225554 for 30 min prior to incubation with forskolin (10μM) for 5 min. Activated Rap1 and VASP phosphorylation were then assessed by Western blotting. **(D)** and **(E)** Pooled results (n=3) for effect of 5225554 on forskolin-promoted Rap1 activation and VASP phosphorylation, respectively. **(F)** and **(G)** Virtual docking of Compound 5225554 near the hinge region in the inactive Epac1 model. Protein secondary structure is shown as a cartoon. 5225554 is yellow, surrounding residues are shown as lines. L315 and F342 are shown as sticks to highlight proximity; black dotted lines depict potential hydrogen bonding. PyMOL was used to generate **F** and **G**. * $p<0.05$, ** $p<0.01$.

3.8 References

1. Insel, P. A., Zhang, L., Murray, F., Yokouchi, H., and Zambon, A. C. (2012) Cyclic AMP is both a pro-apoptotic and anti-apoptotic second messenger. *Acta Physiol. Oxf. Engl.* 204, 277–287

2. Billington, C. K., Ojo, O. O., Penn, R. B., and Ito, S. (2013) cAMP regulation of airway smooth muscle function. *Pulm. Pharmacol. Ther.* 26, 112–120

3. Beavo, J. A., and Brunton, L. L. (2002) Cyclic nucleotide research -- still expanding after half a century. *Nat. Rev. Mol. Cell Biol.* 3, 710–718

4. Ozaki, N., Shibasaki, T., Kashima, Y., Miki, T., Takahashi, K., Ueno, H., Sunaga, Y., Yano, H., Matsuura, Y., Iwanaga, T., Takai, Y., and Seino, S. (2000) cAMP-GEFII is a direct target of cAMP in regulated exocytosis. *Nat. Cell Biol.* 2, 805–811

5. Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) A family of cAMP-binding proteins that directly activate Rap1. *Science* 282, 2275–2279

6. De Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A., and Bos, J. L. (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 396, 474–477

7. Schwede, F., Maronde, E., Genieser, H., and Jastorff, B. (2000) Cyclic nucleotide analogs as biochemical tools and prospective drugs. *Pharmacol. Ther.* 87, 199–226

8. Qiao, J., Mei, F. C., Popov, V. L., Vergara, L. A., and Cheng, X. (2002) Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP. *J. Biol. Chem.* 277, 26581–26586

9. Consonni, S. V., Gloerich, M., Spanjaard, E., and Bos, J. L. (2012) cAMP regulates DEP domain-mediated binding of the guanine nucleotide exchange factor Epac1 to phosphatidic acid at the plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* 109, 3814–3819

10. Liu, C., Takahashi, M., Li, Y., Song, S., Dillon, T. J., Shinde, U., and Stork, P. J. S. (2008) Ras is required for the cyclic AMP-dependent activation of Rap1 via Epac2. *Mol. Cell. Biol.* 28, 7109–7125

11. De Rooij, J., Rehmann, H., van Triest, M., Cool, R. H., Wittinghofer, A., and Bos, J. L. (2000) Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. *J. Biol. Chem.* 275, 20829–20836

12. Boute, N., Jockers, R., and Issad, T. (2002) The use of resonance energy transfer in high-throughput screening: BRET versus FRET. *Trends Pharmacol. Sci.* 23, 351–354

13. Jiang, L. I., Collins, J., Davis, R., Lin, K.-M., DeCamp, D., Roach, T., Hsueh, R., Rebres, R. A., Ross, E. M., Taussig, R., Fraser, I., and Sternweis, P. C. (2007) Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. *J. Biol. Chem.* 282, 10576–10584

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

15. Matthiesen, K., and Nielsen, J. (2011) Cyclic AMP control measured in two compartments in HEK293 cells: phosphodiesterase K(M) is more important than phosphodiesterase localization. *PloS One* 6, e24392

16. Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45

17. Franke, B., Akkerman, J. W., and Bos, J. L. (1997) Rapid Ca2+-mediated activation of Rap1 in human platelets. *EMBO J.* 16, 252–259

18. *LigPrep* (2009) Schrodinger, LLC, New York, NY

19. Roy, A., Kucukural, A., and Zhang, Y. (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nat. Protoc.* 5, 725–738

20. Rehmann, H., Arias-Palomo, E., Hadders, M. A., Schwede, F., Llorca, O., and Bos, J. L. (2008) Structure of Epac2 in complex with a cyclic AMP analogue and RAP1B. *Nature* 455, 124–127

21. Henzler, A. M., and Rarey, M. (2010) In Pursuit of Fully Flexible Protein-Ligand Docking: Modeling the Bilateral Mechanism of Binding. *Mol. Inform.* 29, 164–173

22. *Protein Preparation Wizard SS* (2009) SChrodinger, LLC, New York, NY

23. Case, D. A., Darden, T. A., Cheatham, T. E., Simmerling, C. L., Wang, J., Duke, R. E., Luo, R., Walker, R. C., Zhang, W., Merz, K. M., Roberts, B., Hayik, S., and Roitberg, A. (2012) *AMBER 12*, University of California, San Francisco

24. Wang, J., Wang, W., Kollman, P. A., and Case, D. A. (2006) Automatic atom type and bond type perception in molecular mechanical calculations. *J. Mol. Graph. Model.* 25, 247–260

25. Pastor, R. W., Brooks, B. R., and Szabo, A. (1988) An analysis of the accuracy of Langevin and molecular dynamics algorithms. *Mol. Phys.* 65, 1409–1419

26. Christen, M., Hünenberger, P. H., Bakowies, D., Baron, R., Bürgi, R., Geerke, D. P., Heinz, T. N., Kastenholz, M. A., Kräutler, V., Oostenbrink, C., Peter, C., Trzesniak, D., and van Gunsteren, W. F. (2005) The GROMOS software for biomolecular simulation: GROMOS05. *J. Comput. Chem.* 26, 1719–1751

27. Daura, X., Gademann, K., Jaun, B., Seebach, D., van Gunsteren, W. F., and Mark, A. E. (1999) Peptide Folding: When Simulation Meets Experiment. *Angew. Chem. Int. Ed.* 38, 236–240

28. Enserink, J. M., Christensen, A. E., de Rooij, J., van Triest, M., Schwede, F., Genieser, H. G., Døskeland, S. O., Blank, J. L., and Bos, J. L. (2002) A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. *Nat. Cell Biol.* 4, 901–906

29. Courilleau, D., Bouyssou, P., Fischmeister, R., Lezoualc'h, F., and Blondeau, J.-P. (2013) The (R)-enantiomer of CE3F4 is a preferential inhibitor of human exchange protein directly activated by cyclic AMP isoform 1 (Epac1). *Biochem. Biophys. Res. Commun.*

30. Courilleau, D., Bisserier, M., Jullian, J.-C., Lucas, A., Bouyssou, P., Fischmeister, R., Blondeau, J.-P., and Lezoualc'h, F. (2012) Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac. *J. Biol. Chem.* 287, 44192–44202

31. Altschuler, D. L., and Ribeiro-Neto, F. (1998) Mitogenic and oncogenic properties of the small G protein Rap1b. *Proc. Natl. Acad. Sci. U. S. A.* 95, 7475–7479

32. Sechi, A. S., and Wehland, J. (2004) ENA/VASP proteins: multifunctional regulators of actin cytoskeleton dynamics. *Front. Biosci. J. Virtual Libr.* 9, 1294–1310

33. Zhang, Y., and Skolnick, J. (2004) Scoring function for automated assessment of protein structure template quality. *Proteins* 57, 702–710

34. Sherman, W., Day, T., Jacobson, M. P., Friesner, R. A., and Farid, R. (2006) Novel procedure for modeling ligand/receptor induced fit effects. *J. Med. Chem.* 49, 534–553

35. *Small-Molecule Drug Discover Suite 2013-3: Glide version 6.1* (2013) Schrodinger, LLC, New York, NY

36. Rehmann, H., Schwede, F., Døskeland, S. O., Wittinghofer, A., and Bos, J. L. (2003) Ligand-mediated activation of the cAMP-responsive guanine nucleotide exchange factor Epac. *J. Biol. Chem.* 278, 38548–38556

37. Christensen, A. E., Selheim, F., de Rooij, J., Dremier, S., Schwede, F., Dao, K. K., Martinez, A., Maenhaut, C., Bos, J. L., Genieser, H.-G., and Døskeland, S. O. (2003) cAMP analog mapping of Epac1 and cAMP kinase. Discriminating analogs demonstrate that Epac and cAMP kinase act synergistically to promote PC-12 cell neurite extension. *J. Biol. Chem.* 278, 35394–35402

38. Dao, K. K., Teigen, K., Kopperud, R., Hodneland, E., Schwede, F., Christensen, A. E., Martinez, A., and Døskeland, S. O. (2006) Epac1 and cAMP-dependent protein kinase holoenzyme have similar cAMP affinity, but their cAMP domains have distinct structural features and cyclic nucleotide recognition. *J. Biol. Chem.* 281, 21500–21511

39. Tsalkova, T., Mei, F. C., and Cheng, X. (2012) A fluorescence-based high-throughput assay for the discovery of exchange protein directly activated by cyclic AMP (EPAC) antagonists. *PloS One* 7, e30441

40. McPhee, I., Gibson, L. C. D., Kewney, J., Darroch, C., Stevens, P. A., Spinks, D., Cooreman, A., and MacKenzie, S. J. (2005) Cyclic nucleotide signalling: a molecular approach to drug discovery for Alzheimer's disease. *Biochem. Soc. Trans.* 33, 1330–1332

41. Wu, J., Jones, J. M., Nguyen-Huu, X., Ten Eyck, L. F., and Taylor, S. S. (2004) Crystal structures of RIalpha subunit of cyclic adenosine 5'-monophosphate (cAMP)-dependent protein kinase complexed with (Rp)-adenosine 3',5'-cyclic monophosphothioate and (Sp)-adenosine 3',5'-cyclic monophosphothioate, the phosphothioate analogues of cAMP. *Biochemistry (Mosc.)* 43, 6620–6629

42. Gjertsen, B. T., Mellgren, G., Otten, A., Maronde, E., Genieser, H. G., Jastorff, B., Vintermyr, O. K., McKnight, G. S., and Døskeland, S. O. (1995) Novel (Rp)-cAMPS analogs as tools for inhibition of cAMP-kinase in cell culture. Basal cAMP-kinase activity modulates interleukin-1 beta action. *J. Biol. Chem.* 270, 20599–20607

43. Harper, S. M., Wienk, H., Wechselberger, R. W., Bos, J. L., Boelens, R., and Rehmann, H. (2008) Structural dynamics in the activation of Epac. *J. Biol. Chem.* 283, 6501–6508

44. Tsalkova, T., Blumenthal, D. K., Mei, F. C., White, M. A., and Cheng, X. (2009) Mechanism of Epac activation: structural and functional analyses of Epac2 hinge mutants with constitutive and reduced activities. *J. Biol. Chem.* 284, 23644–23651

45. Vigil, D., Cherfils, J., Rossman, K. L., and Der, C. J. (2010) Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? *Nat. Rev. Cancer* 10, 842–857

Chapter Four: The Hinge Region of the Cyclic Nucleotide Binding Domain of Epac as a Target for Allosteric Inhibitors

4.1 Abstract

Epac, a guanine nucleotide exchange factor for the low molecular weight G protein Rap, is an effector of cAMP signaling and has been implicated to have roles in numerous diseases, including diabetes mellitus, heart failure, and cancer. We used a computational molecular modeling approach to predict potential binding sites for allosteric modulators of Epac and to identify molecules that might bind to these regions. We found that the conserved hinge region of the cyclic nucleotide binding domain (CNBD) of Epac is a potentially druggable region of the protein. Using a BRET-based assay (CAMYEL), we assessed the predicted compounds for their ability to bind Epac and modulate its activity. We identified a thiobarbituric acid derivative, 5376753, that allosterically inhibits Epac activity and used Swiss 3T3 and HEK293 cells to test the compound's ability to modulate the activity of Epac or PKA, determined by Rap1 activity or VASP phosphorylation, respectively. Compound 5376753 selectively inhibited Epac in biochemical and cell migration studies. These results document the utility of a computational approach to identify a domain for allosteric regulation of Epac and a novel compound that binds to the hinge region of the CNBD of Epac1 and Epac2 to prevent their activation by cAMP.

4.2 Introduction

Increases in the second messenger cAMP occur following activation of adenylyl cyclases, which catalyze the conversion of ATP to cAMP, by G_s-linked GPCRs. The effects of cAMP signaling in mammalian cells are mediated by two main intracellular effectors: protein kinase A (PKA[1]) and exchange protein activated by cAMP (Epac). Epac is a guanine nucleotide exchange factor (GEF) that activates the low-molecular weight G-proteins Rap1 and Rap2 and has been implicated in the regulation of cAMP-mediated events that include proliferation, apoptosis, differentiation, secretion, migration, and adhesion(1)(2)(3). It has been proposed that dysregulation of Epac signaling occurs in a variety of diseases including autism, heart failure, diabetes mellitus, and cancer(4)(5)(6)(7)(8). Thus, there is considerable interest in developing compounds that modulate its activity.

The two isoforms of Epac, Epac1 and Epac2, have similar domain structures and modes of activation (Fig 1A). Epac has a regulatory region that sterically blocks Rap1 from interacting with the catalytic region. The regulatory region is composed of a Dishevelled, Egl-10, Pleckstrin (DEP) domain, which mediates the subcellular localization of Epac through its binding to phosphatidic acid and a cyclic nucleotide binding domain (CNBD) responsible for its activation(9)(10). Epac2 possesses a second lower affinity CNBD that is involved in subcellular localization but not the activation of Epac2. The catalytic region contains a CDC25 homology domain (CDC25HD), which interacts with Rap1 and facilitates the exchange of GTP for GDP, a Ras exchange motif (REM), and a Ras association motif (RA)(11).

In its auto-inhibited state the CNBD interacts with the CDC25HD by an ionic latch that sterically prevents interaction with Rap. Initiation of Epac activation by cAMP occurs when it interacts with the phosphate binding cassette (PBC) of the CNBD and induces conformational changes that relieve the steric hindrance between the PBC and the hinge of the CNBD, allowing the "hinge" to rotate(12)(13). This results in the release of the ionic latch due to an increase in the entropic penalty associated with preserving the auto-inhibited state and allowing the CNBD to move away from the CDC25HD(14). Thus, binding of cAMP induces a conformational change in Epac in which the regulatory region moves away from the catalytic region, facilitating an interaction of the CDC25HD with Rap and in turn, exchange of GDP for GTP (Fig 1B)(15).

In a previous study we reported on the use of CAMYEL to identify competitive or allosteric inhibitors of Epac *in vitro*(16). CAMYEL is a BRET-based cAMP sensor based on Epac1 that is sandwiched between citrine and *Renilla* luciferase(17). The inactive state yields a strong BRET signal that results from the transfer of energy from luciferase to citrine. Activation by cAMP induces conformational changes that result in a decrease in the BRET signal as the luciferase moves away from citrine. We verified the results from the CAMYEL assay by conducting studies with Swiss 3T3 cells and monitoring activation of Epac's downstream target, Rap1b. In the current studies we identified the hinge of the CNBD of Epac1 as a novel region to target with allosteric inhibitors and demonstrate that a thiobarbituric acid derivative prevents Epac activation *in vitro* and in cells.

4.3 Experimental Procedures

Cell lines and transfections: Swiss 3T3 and HEK293 cells were grown in 10-cm culture dishes at 37°C, 5% CO_2 in DMEM supplemented with 5% FBS, 1% penicillin, and 1% streptomycin. Transient transfection of pcDNA3 CAMYEL into HEK293 cells was carried out using TransIT LT-1 (Mirus Bio) according to the manufacturer's instructions. Lysis and BRET measurements were performed 48-72 h after transfection. For Rap1 pull-down assays in HEK293 cells, the cells were split into 10-cm culture dishes, allowed to adhere overnight, transfected the next day with Epac2 plasmid, serum-starved in DMEM for 24 h before subsequent assays.

Isolation and culture of adult rat cardiac fibroblasts (CFs): Ethical approval for the care and use of animals was granted by the UCSD Institutional Animal Care and Use Committee. CFs were isolated from adult (8–10 wk), male Sprague-Dawley rats, as described (18). Briefly, the rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection. The heart was excised and digested with collagenase II (Worthington Biochemical, Lakewood, NJ) via a modified reverse-Langendorff apparatus. CFs were separated from cardiac myocytes by gravity separation and grown to confluency in 10-cm culture dishes at 37°C, 10% CO_2 in DMEM supplemented with 10% FBS, 1% penicillin, and 1% streptomycin. Studies with the CFs were undertaken with cells that were passaged no more than 2 times.

BRET/FRET assay: HEK293 cells expressing CAMYEL were harvested and lysed in CAMYEL Assay Buffer (40mM Hepes, pH7.2, 140mM KCl, 10mM NaCl, 1.5mM $MgCl_2$) with 0.5% Triton X100 and 1% Complete protease inhibitor cocktail (Roche) as described(19). After centrifugation at 20,000× *g* for 10 min, the supernatant was removed and diluted to the desired volume. 100µL was added to 96-well white plates and incubated for 5 min at room temperature with the indicated treatments. Inhibitors were added 5 min before those treatments. Coelenterazine was added to a final concentration of 2 µM immediately before assessing BRET. Emission from RLuc and Citrine was measured simultaneously at 465 nm and 535 nm in a DTX-800 Plate Reader (Beckman Coulter). Apparent activation curves were determined by fitting the data to a sigmoidal dose-response curve.

Immunoblot analysis: Whole-cell lysates were prepared in 150 mM Na_2CO_3 buffer (pH 11) and homogenized by sonication. Equal amounts of protein (assayed using a dye-binding reagent; Bio-Rad) were separated by SDS/PAGE using 10% polyacrylamide precast gels (Invitrogen) and transferred to a polyvinylidene difluoride membrane with the X-Cell II Blot Module (Invitrogen). Membranes were blocked in PBS Tween (1%) containing 5% bovine serum albumin and incubated with primary antibody 18 h at 4°C. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and ECL reagent (Amersham Pharmacia, Pittsburgh, PA). Bands were compared with molecular weight standards to confirm migration of proteins at the appropriate size. Quantification of protein expression densitometry was performed using ImageJ

software (NIH, Bethesda, MD). Epac2 (5B1), pVASP S157, and Rap1A/Rap1B antibodies were purchased from Cell Signaling (Danvers, MA). Epac1 (A-5) antibody was purchased from Santa Cruz. β-tubulin antibody was purchased from Abcam.

Rap1 activation assay: The Rap1-GTP–binding domain (RBD) of mammalian RalGDS was expressed in *E. coli* as a GST fusion protein, as described (20). The purified GST-RalGDS-RBD was used to detect activated Rap1. 10-cm plates of Swiss 3T3 cells were washed twice with ice-cold PBS and then lysed with 1mL Tris-Lysis Buffer (100 mM Tris, pH 7.5, 300 mM NaCl, 50 mM $MgCl_2$, 20% glycerol, 1% NP-40, 2 mM DTT, 2 mM vanadate, aprotinin, and 5 μg/ml leupeptin) at the indicated times. The lysates were centrifuged for 10 min, and the supernatants were incubated with 10 μg GST-RBD on glutathione–Sepharose beads at 4°C for 60 min. The beads were washed three times and subjected to SDS-PAGE and Western blot analysis with either an anti-pVASP mAb or anti-Rap1 pAb. For control of the input amount of Rap1 protein, we analyzed 20 μl of the cell lysates.

CF migration: Migration of CFs was assayed by using the Boyden chamber method. The cells were maintained in serum-free conditions for 24 h and then suspended in serum-free DMEM at a density of 1×10^5 CFs/100 μl in uncoated chamber inserts. Basal migration was assessed by adding serum-free DMEM (600 μl) to the lower chambers. Cells were allowed to migrate for 16 h, then fixed in 10% formalin and stained with Hema 3 (Fisher Scientific). Cells on the upper surface of the membrane

were mechanically removed with a cotton swab. Cells that migrated were counted from 4 different fields (0.1 mm^2/field).

Cell viability assay: Swiss 3T3 cells were seeded at 5 x 10^4 cells/mL in 24-well plates in DMEM with 10% FBS. 24 h later the cells were treated with the indicated compounds or 1% DMSO. Viability was assessed 48 h later using the CellTiter-Glo assay according to the manufacturer's instructions (Promega).

Mapping of druggable hot spots in the inactive Epac structure: When mutated, certain amino acid residues near a hinge region of Epac can substantially alter its activity(21). To investigate potential druggability there, we used FTMap and small organic molecules to sample the surface of apo-Epac2 in order to find favorable locations to dock(22)(23). These small molecular probes were then clustered to find "hot spots" and ranked based on their average energy. Cluster locations were visualized in PyMOL(24).

Computational modeling of apo-Epac1 and virtual screening for allosteric modulators: I-TASSER was used to generate a 3D model of the apo-Epac1 structure in its inactive state(25)(26). This model and a library of compounds from Chembridge were prepared with the Schrödinger Protein Preparation Wizard and the LigPrep Program, respectively (27)(28). A preliminary screen centered on the Epac hinge was performed; docking poses were generated using Schrödinger Induced Fit Docking (IFD). A library of 450,000 compounds from Chembridge was filtered to yield

101,691 by removing promiscuous binders and small molecules with undesired, toxic, or reactive functional groups, more than 4 chiral centers, more than 10 rotatable bonds, salts or undesired atoms, or properties outside the following ranges: 250<MW<460, 50<PSA<150, and -2<cLogP<5.0. Schrödinger IFD was used with the prepared protein and ligands, the latter of which were represented as 169,381 different optimized compound "models" generated with LigPrep to incorporate ligand flexibility (including tautomeric, stereochemical, and ionization variations)(14)(15).

Apo-Epac1 Molecular Dynamics (MD): To explore protein flexibility further, MD was performed with the apo-Epac1 model. The system was first prepared (parameterized and solvated with Xleap) for AMBER MD as described (16). Second, while the protein was kept fixed, the water positions were allowed to minimize. Third, the energy of the whole system was minimized. Next, the water was heated from 0K to 300K during 20 ps of MD at constant volume using the Langevin thermostat. The system was then equilibrated during 100 ps of subsequent MD at constant pressure. After minimization and equilibration, four independent production MD runs were performed on the Gordon Compute Cluster at the San Diego Supercomputer Center, totaling 200 ns. All MD simulations were performed under constant pressure periodic boundary conditions with isotropic position scaling, a reference pressure of 1 atm, and a pressure relaxation time of 2 ps. The system was maintained at a reference temperature of 300K using the Langevin thermostat with a collision frequency of 2 ps^{-1} (29). A simple Leapfrog integrator was used to solve Newton's equations of motion and propagate the trajectory with a time step of 0.002 ps. Long-range electrostatic

interactions were handled with the particle-mesh Ewald procedure using the sander Amber module; long-range van der Waals interactions were estimated by a continuum model. Reference (30) provides further computational details.

Incorporating Epac flexibility into virtual screening: The MD trajectories were concatenated and clustered using GROMACS (RMSD cutoff 0.125nm) around the Epac1 hinge (L315 and F342)(31). The central member from each of the 30 largest clusters was considered representative of the set of structures and combined to create an "ensemble" to be used in virtual screening. PDB2PQR was used to generate pdbqt files for the ensemble of structures and the library of Chembridge compounds(32)(33). These files were used as inputs for the AutoDock Vina molecular docking program on UCSD's NSF Center for Theoretical Biological Physics (CTPB) Linux cluster. The docking was centered around the apo-Epac1 hinge (residues Leu-315 and Phe-342) for both the IFD described above and these Vina screens. The results were used to determine compound priority for purchase and experimental validation.

Epac1-5225554 and Epac1-5376753 poses near the hinge: For each of the two most experimentally promising ligands, IFD docking was performed near the hinge and the top poses for each were compared. Ligand Interaction Diagram screenshots and pdb structures were saved in Maestro(34). PyMOL was used to generate docking pose images with these pdb files.

Reagents: Reagents were purchased as follows: cAMP and cAMP analogs from Biolog (Bremen, Germany), hinge compounds from Chembridge (San Diego, CA), Coelenterazine from Nanolight (Pinetop, AZ), Forskolin (Fsk) from Abcam Biochemicals (Cambridge, MA), complete protease inhibitors from Roche (Indianapolis, IN), phosphatase inhibitor Cocktail 3 and PDGF-BB from Sigma-Aldrich (St. Louis, MO) and pLX304-Epac2 from the DNASU plasmid repository. pcDNA3 CAMYEL was a kind gift from Dr. Paul Sternweis (UT Southwestern). CE3F4 was kindly provided by Frank Lezoualc'h (INSERM UMR-1048, Toulouse, France).

Statistical analysis: Z' factor was calculated as described in (35) Z' = 1-((3SDmax + 3SDmin)/ΔM), where ΔM = (mean max value – mean min value), and SDmax and SDmin are the respective standard deviations. Calculations and statistics were performed using GraphPad Prism 6.0 software (GraphPad Software, La Jolla, CA). Numerical values are presented as means ± se. All experiments were performed in triplicate or quadruplicate. Analysis of data from experiments with multiple comparisons was done using ANOVA with Bonferroni's correction. Values of $p < 0.05$ were considered significant.

4.4 Results

Identification of the hinge region of Epac as a druggable region

We sought to identify novel regions of Epac that might be targeted with small molecules. Prior experimental work investigating the mechanism of Epac activation had shown that upon cAMP binding at the CNBD, subsequent bending at a hinge region is an important component of protein activity (21). This bend is maintained via hydrophobic interactions among three residues: Leu-408, Phe-435, and Leu-439 (which are conserved in Epac1 and Epac2)(21). This provided an intriguing possibility for structure-based drug design. Our interest in pursuing small molecule inhibitors with an affinity for the Epac surface near the hinge was further confirmed by the results of FTMap sampling of potential druggable sites, showing that the 5^{th} largest cluster of probes was located near Leu-408 and Phe-435 on Epac2 (Fig. 2A).

Screening of compounds that bind to the hinge region of Epac

An I-TASSER generated model of Epac1 was used for (molecular dynamics) MD and virtual screening. First, the model was used for an Induced Fit Docking (IFD) screen of the Chembridge library. Ligprep-prepared compound "models" introduced ligand variety in the IFD protocol, which also allows for small accommodations in the protein upon binding. To incorporate additional flexibility of the protein in virtual screening efforts, MD was performed with an Epac1 model. The clustered trajectory provided a collection or "ensemble" of structures representative of the most stable conformations that could be used in a virtual screening approach with the Chembridge

compound library(36). We used the faster Autodock Vina software to screen the same library against this ensemble of structures (made of the largest 30 clusters from the MD trajectory). There were unique top hits from the two different approaches so both sets were considered for experimental validation.

Based on results of this computational screen, we used the CAMYEL assay to test the 133 compounds that had the highest score (16). The CAMYEL assay has a Z'-factor of 0.725, indicating that it satisfies the requirement of >0.5 to be used in HTS assays. We treated HEK293 cell lysates with CAMYEL in 96-well white plates with either 10µM or 100µM of each compound for 5 min followed by activation of CAMYEL with 100µM cAMP. Due to the high fluorescent background of the compounds at 100µM, we analyzed the inhibition of Epac by the compounds at 10µM (Fig 3.1A)

The screen of the 133 compounds revealed 4 compounds that decreased Epac activation in the CAMYEL assay; we further characterized these compounds for their ability to inhibit Epac1 *in vitro* and in cells. We have shown that incubation of Swiss 3T3 cells with the adenylyl cyclase activator, forskolin (to increase cellular cAMP) activates PKA and Epac1, as assessed by VASP phosphorylation at S157 and activation of Rap1, respectively (16). Studies with Swiss 3T3 cells indicated that incubation of 100µM 5225554 for 30 min inhibited forskolin-stimulated Rap1 activation (Epac-mediated signaling) without altering VASP phosphorylation (PKA-mediated signaling) (Fig 3B, 3C). The other three compounds did not inhibit Epac1 in cells (data not shown). Interestingly, the IFD screen alone ranked the top experimental compound overall among the highest in the library, furthering demonstrating that

every system is different and that a one-size-fits-all approach to virtual screening likely does not exist (37).

A thiobarbituric acid derivative is a more effective inhibitor of Epac in cells than is the barbituric acid derivative

Having confirmed the inhibitory effect of compound 5225554, we sought to determine the importance of the barbituric acidic head group for binding to Epac1. We thus tested similar molecules, which we obtained from Chembridge (Fig 4A). One of these, 5376753, a thiobarbituric acid derivative, was even more effective than 5225554 but was not in the top 10 compounds in the IFD screen that identified 5225554. 5376753 was able to inhibit Epac1 in Swiss 3T3 cells at lower concentrations (10μM) than the barbituric acid derivative, 5225554 (100μM) (Figs 4B, 4C). Substitution of methyl groups for hydrogen on the nitrogen of the acidic head group decreased the ability of this compound to inhibit Epac, indicating the importance of the N-H group for binding to Epac. To verify that 5376753 inhibits Epac, we tested the effect of 5376753 on Epac activation by cAMP analogs. Swiss 3T3 cells were treated with the partial agonist 8-CPT-cAMP (CPT) or the Epac-specific activator 8-CPT-2'-O-Me-cAMP (8-Me) alone or with 10μM 5376753(38). In addition to inhibiting Epac activation by the endogenous ligand cAMP, 5376753 also inhibited activation of Epac by CPT and 8-Me (Figs 4D, 4E).

Proposed Ligand Binding of 5225554 and 5376753

The IFD scores for 5225554 and 5376753 in the Epac1 model were -11.3 and -12.2 kcal/mol, respectively. The Ligand Interaction Diagram (Figure 5A) of the top pose from IFD of 5225554 shows potential interactions with Trp-283 (Pi-Pi), Val-337, and Asp-338 (H-bond). Selecting "polar interactions" within PyMol suggests that there could be another bond between the ligand and Thr-281 (Figure 5B). For the IFD top pose of 5376753, the Ligand Interaction Diagram (Figure 5C) is similar but shows one more potential Pi-Pi interaction with Trp-283 and the H-bond to Thr-281. Using PyMol for visualization (Figure 5D), it appears that Phe-342 is flipped outward about 100 degrees to accommodate the 5376753 docked slightly closer to hinge. Showing the polar interactions also highlighted another potential bond with Arg-336.

All residues involved in the potential interactions between Epac1 and these two ligands are conserved between Epac1 and Epac2 but only D338 is also conserved in PKA. This is consistent with our experimental results. Although the top IFD poses of 5225554 and 5376753 were very similar, there were small potential differences in structure. Phe-342 appears to open up away from 5376753 to allow a closer docking of the dichlorophenyl group than in 5225554. Also 5376753 may have an additional pi-pi bond to this same group, as well as a hydrogen bond to Arg-336 not seen in the predicted pose of 5225554. There may be additional reasons why 5376753 is more active as an inhibitor in cells, but it is encouraging that the computational modeling is in agreement with the experimental results in terms of the rank of the compounds.

5225554 and 5376753 noncompetitively inhibit Epac

We next sought to assess the mechanism by which the compounds inhibit Epac activation. We previously showed that the CAMYEL assay can determine if an inhibitor is acting competitively or noncompetitively (16). We compared the inhibition of CAMYEL by 5225554 and 5376753 to the inhibition by the competitive inhibitor 8-CPT-N6-Phenyl-cAMP (CPT-N6) and by the uncompetitive inhibitor CE3F4(39). Treatment of CAMYEL with 10µM of 5225554 or 5376753 reduced the maximal activation induced by cAMP without changing the apparent affinity towards cAMP (Fig 6A). This is similar to the inhibition induced by treatment with 10µM of the allosteric inhibitor CE3F4 (Fig 6B). Incubation of CAMYEL with 3µM of the competitive inhibitor CPT-N6 followed by activation with cAMP resulted in a right-shift of the activation curve without decreasing the maximal activation (Fig. 6C). Thus, 5225554 and 5376753 allosterically inhibit Epac.

The inhibition of Epac by 5376753 is not due to loss in cell viability

We conducted additional studies to rule out the possibility that the inhibition of Epac might be due to an inhibition of cell viability. We assessed the ability of varying concentrations of 5225554 or 5376753 to induce death of Swiss 3T3 cells and found that < 50µM of both compounds did not significantly decrease viability (Fig 7).. 5376753 inhibits Epac at lower concentrations than does 5225554 while both have the same ability to induce cell death at higher concentrations, indicating that the loss in viability is an off-target effect not related to ability to bind and inhibit Epac.

5376753 is a dual Epac1 and Epac2 inhibitor

The hinge region of the CNBD of Epac is highly conserved between Epac1 and Epac2(40). We sought to determine if 5376753 inhibits both Epac1 and Epac2. To test this, we used HEK293 cells, which express low amounts of endogenous Epac1 protein and no detectable Epac2 protein (Fig 8E). Stimulation of non-transfected HEK293 cells with forskolin induced Rap1 activation and phosphorylation of VASP. Treatment with either 5376753 or the Epac1-specific uncompetitive inhibitor, CE3F4, inhibited the activation of Rap1 (Fig 8A, 8B). We used HEK293 cells transfected with Epac2 cDNA to test for inhibition of Epac2. Treatment of Epac2-overexpressing HEK293 cells with 5376753 or CE3F4 and then forskolin revealed that 5376753, but not CE3F4, inhibited Rap1 activation (Fig. 8C, 8D). Thus, CE3F4 is an Epac1-specific inhibitor but 5376753 inhibits both Epac1 and Epac2 in cells.

5376753 inhibits primary cardiac fibroblast migration

We tested if 5376753 will inhibit biological functions of Epac, in particular, a cAMP-promoted increase in cell migration of cardiac fibroblasts (CFs). Small increases in cAMP induced by low levels of forskolin (1µM) stimulate migration of CFs in an Epac-dependent manner(41). We assessed the ability of 5376753 to inhibit migration of primary rat CFs induced by forskolin (1µM) or 8-Me (50µM) and found that migration induced by both treatments was blocked by 10µM 5376753 (Fig 9A). To verify that this effect resulted from inhibition of Epac and not global inhibition of migration, we assessed if 5376753 altered the migration of CFs induced by platelet-derived growth factor (PDGF) and found that 5376753 did not block PDGF-BB

(10ng/mL)-promoted migration (Fig 9B)(42). Thus, 5376753 selectively inhibits Epac-mediated migration in fibroblasts.

4.5 Discussion

Previous work has shown that movement of the hinge region of the CNBD is a key determinant in the activation of Epac. Studies of a F435G Epac2 mutant (Phe-435 is a conserved residue in the hinge) showed that it had GEF activity similar to that of the cAMP-activated wild-type protein but was also capable of partially activating Rap1 in the absence of cAMP(21). Conversely, an Epac F435W mutation with a bulkier residue in that position inhibits the bending of the hinge and catalytic activity of Epac. Together, those results suggest that Phe-435 allows for GEF activity after binding of cAMP and conformational changes that transition Epac into an active state; a bulkier residue does not permit this essential movement of the hinge region. This mechanism implies that binding of small molecules to this region have the potential to prevent Epac activation.

Having identified the hinge of the CNBD as a possible druggable region, we computationally and experimentally screened compounds for their ability to bind to this region. Using an *in vitro* BRET-based assay that we have validated for the identification of competitive or allosteric modulators of Epac activity (16), in the current study we assessed the activity of allosteric modulators of Epac activity identified by the computational homology model of Epac1. We found that compound 5225554, a barbituric acid derivative, identified by the computational screen is a

novel, allosteric inhibitor of Epac. We verified that 5225554 inhibits Epac1 in Swiss3T3 cells by assessing its inhibition of Rap1 activation induced by forskolin.

The current results showed that compound 5376753, the thiobarbituric acid modification of 5225554, has a greater ability to decrease the conformational changes of Epac necessary for its activation and to inhibit Epac signaling in cells. This greater inhibition may be attributable to increased pi stacking between Trp-283 and the dichlorophenyl group that is lacking in 5225554. Substitution of the hydrogens on the nitrogen atoms of the thiobarbituric acid head group with methyls reduced the ability of the compound to inhibit Epac. This result is in agreement with the proposed binding of this compound, in which a hydrogen bond forms between the N-H group of the thiobarbituric acid group on 5376753 and Thr-281 of Epac1. Thiobarbituric acid derivatives are more lipophilic than barbituric acids and thus, 5376753 may also be more cell-permeable(43).

Although 5225554 inhibits Epac signaling in fibroblasts at high concentrations, its more limited solubility and toxic effects at the concentrations needed for Epac inhibition make it unsuitable as an Epac inhibitor in cells. By contrast, inhibition of cell viability by 5376753 does not occur at lower concentrations that inhibit Epac. Moreover, CFs treated with 10μM 5376753, which blocks Epac-promoted migration, migrate in response to PDGF-BB. PDGF-BB induces migration in fibroblasts by activating another Rap1GEF, C3G, a response that occurs in the presence of 5376753(44). Further investigation into SAR studies of 5376753 may facilitate the development of compounds that have increased binding affinity and enhanced solubility.

In summary, this study identified 5376753 as a novel, first generation noncompetitive inhibitor of Epac1 and Epac2. The hinge region of the CNBD is highly conserved between both Epac isoforms but differs from that of the CNBD of the PKA regulatory subunit. We verified that 5376753 is an Epac-selective inhibitor that does not decrease PKA-mediated VASP phosphorylation. It is intriguing to hypothesize that since targeting the hinge of Epac with small molecules leads to its inhibition, perhaps targeting the hinge region of PKA might also be a site for inhibiting PKA signaling as well.

GEFs are not considered "classical" drug targets due to their lack of binding pockets for small molecules but as shown here, we discovered that the hinge region of the CNBD has potential as a possible druggable region of Epac(45). It will be of interest to determine if other compounds that interact with the hinge region of Epac can be allosteric activators as well as allosteric inhibitors.

4.6 Acknowledgements

We thank Dr. Paul Sternweis for the pcDNA3 CAMYEL plasmid and Dr. Frank Lezoualc'h for the CE3F4 compound. This work was supported in part by the National Institutes of Health Molecular Biological Approaches to Endocrinology Training Grant (5T32DK007541-27). Work in the JAM group was supported in part by National Science Foundation Grant MCB1020765, National Institutes of Health Grant GM31749, the Howard Hughes Medical Institute, the National Biomedical

Computation Resource, the Center for Theoretical Biological Physics, and NSF Supercomputer Centers. All authors declare that there are no conflicts of interest.

The contents of Chapter 4, in full, is in preparation: **Brown, L.M.,** Rogers K.E., Aroonsakool, N, McCammon J.A., and Insel, P.A. (2014) "The hinge region of the cyclic nucleotide binding domain of Epac as a target for allosteric inhibitors." *Journal TBD.* The dissertation author was the primary investigator and author of this paper. All co-authors have given written permission for its use and the reproduction of all associated data in this dissertation.

4.7 Figures

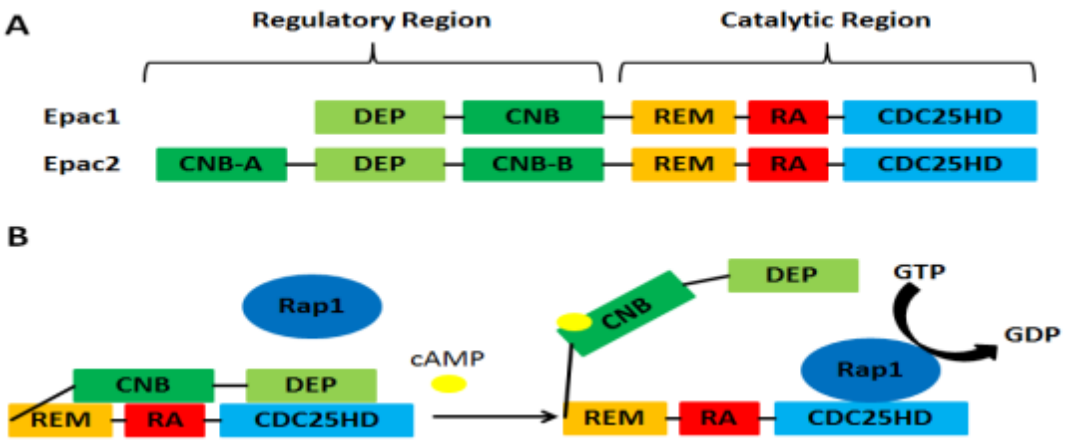


Figure 4.1 Domain structure and activation of Epac. (A) A schematic demonstrating the domain structure of Epac1 and Epac2. Both proteins have N-terminal regulatory and C-terminal catalytic regions. The regulatory region contains a cyclic nucleotide binding domain (CNB), Dishevelled Egl-10, Pleckstrin domain (DEP). The catalytic region is composed of a CDC25 Homology Domain (CDC25HD), ras exchange motif (REM), and ras association domain (RA). **(B)** cAMP binding to the CNB domain induces intramolecular movement that allows Rap1 to interact with the CDC25HD and facilitate the exchange of GTP for GDP.

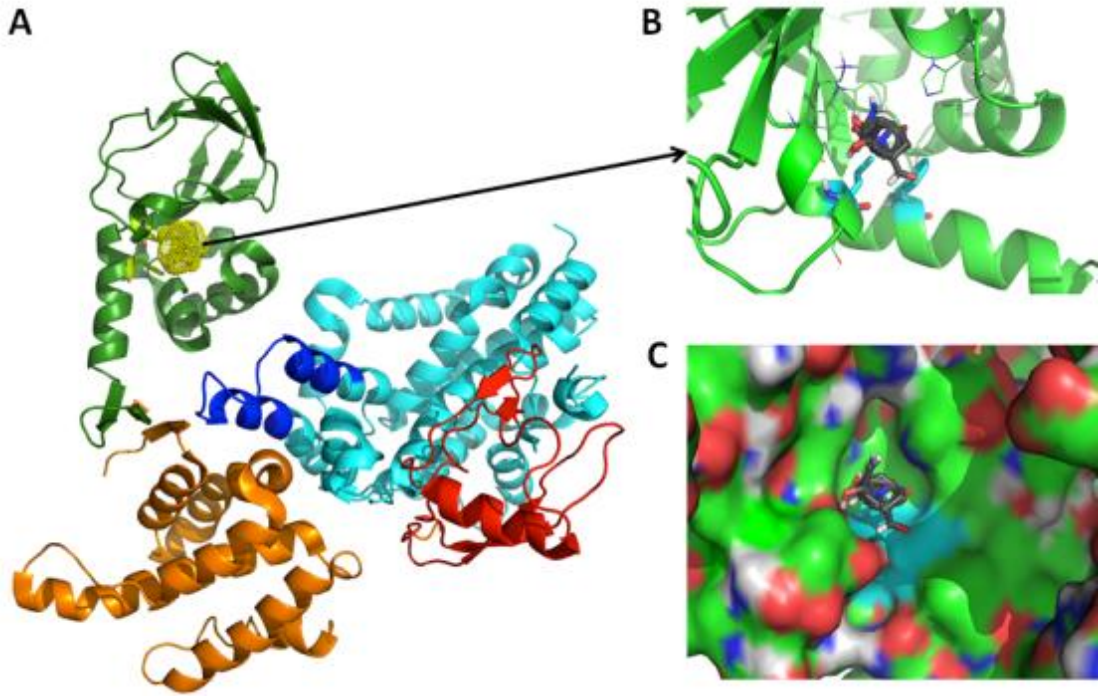


Figure 4.2 Hot Spot near Epac Hinge. FTMap survey of the Epac structure revealed potentially druggable sites near residues L315 and F342, important residues for hinge mobility. **(A)** Epac shown as cartoon with domains colored corresponding to Fig.1. Molecular probes are shown as yellow dots (A) or as gray sticks (B-C). **(B)** Residues shown as lines except for L315 and F342, which are shown as cyan sticks, and the protein as a cartoon. **(C)** Protein shown as surface with L315 and F342 highlighted in cyan to show proximity to molecular probes.

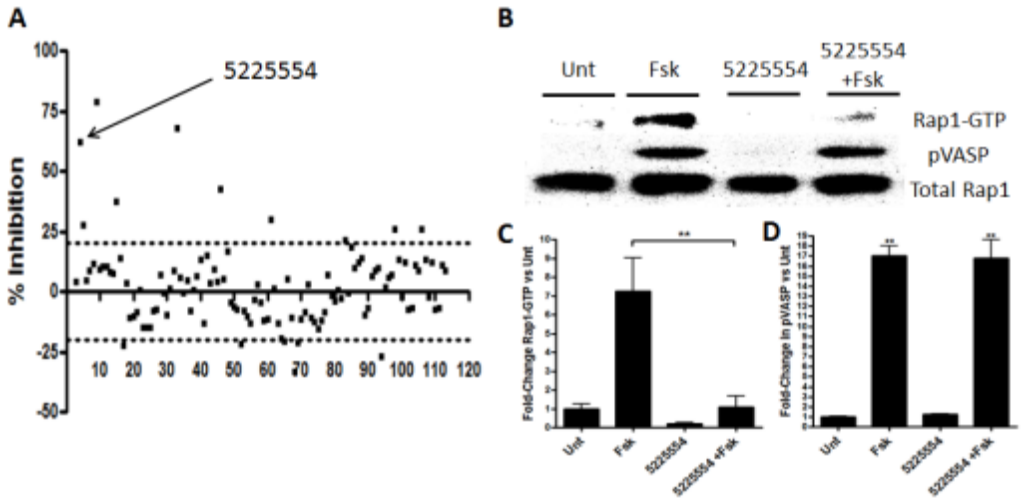


Figure 4.3 Screening of 133 compounds identified 5225554 as an Epac1 inhibitor. **(A)** 10µM of each compound was added to 100µL of CAMYEL reagents with HEK293 cell lysate in a 96-well plate and allowed to bind for 5 min. 100µM cAMP was added to the wells and the activation of CAMYEL was assessed after 5 min. The dotted line represents +/- 3 standard deviations. **(B)** Rap1 activation and VASP phosphorylation were assessed by Western blotting of Swiss 3T3 cells that were untreated (Unt), incubated with 5225554 (100µM) for 30 min, or stimulated with Forskolin (Fsk, 10µM) for 5 min after incubation with 52255554. Rap1 activation was quantified in **(C)** and phosphorylation was quantified in **(D)**. *n=4*. ***p* <0.01.

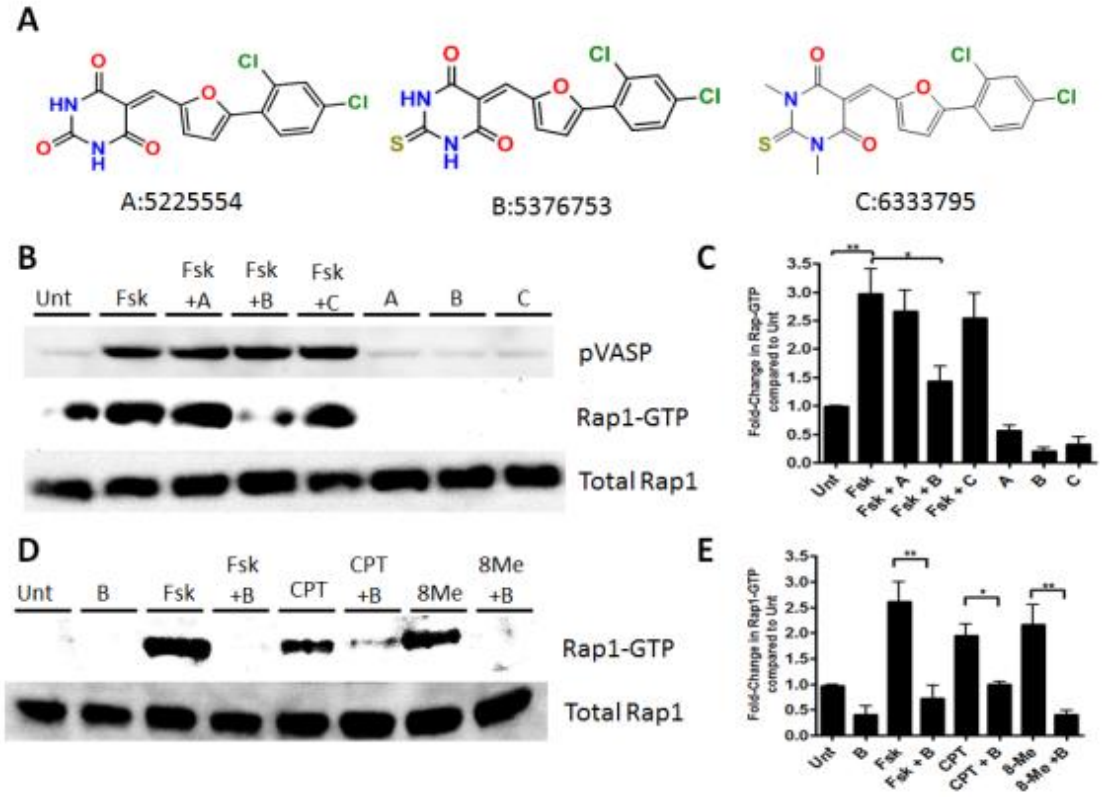


Figure 4.4 A thiobarbituric acidic head group enhances the ability of the compound to inhibit Epac in Swiss 3T3 cells. (A) The structures of the three different inhibitors are shown. **(B)** Rap1 activation and VASP phosphorylation were assessed by Western blotting of Swiss 3T3 cells that were untreated (Unt), incubated with compounds A (5225554), B (5376753), or C (6333795) (all at 10µM) for 30 min, and/or stimulated with Forskolin (Fsk, 10µM) for 5 min after incubation with the compounds. **(C)** Quantification of activated Rap1 from (B). **(D)** Western blot assessment of Rap1 activation of Swiss 3T3 cells treated with Fsk (10µM), CPT (50µM), or 8-Me (50µM) alone or with compound B (10µM). **(E)** Quantification of activated Rap1 from (D). n=4. *p<0.05. **p<0.01.

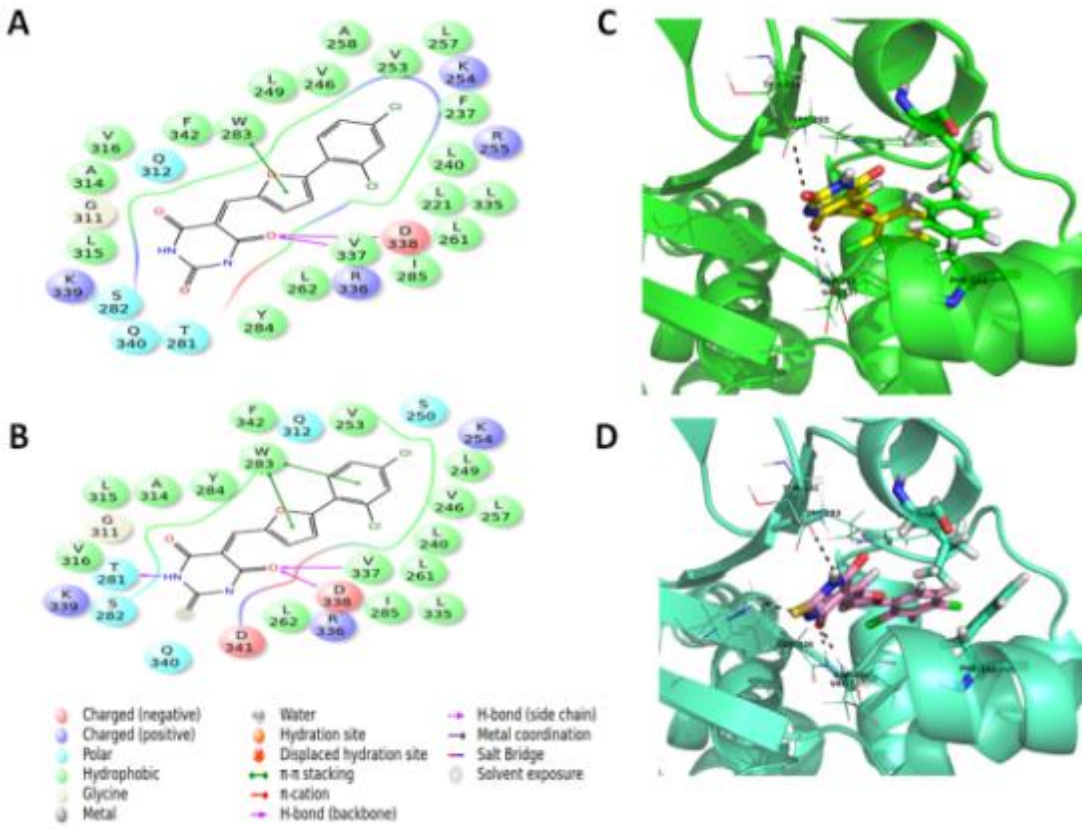


Figure 4.5 Ligand Interactions. Potential interactions between Epac and ligands 5225554 **(A, C)** and 5376753 **(B, D)** identified in Maestro **(A, B)** and PyMol **(C, D)**. The legend is shown for the interaction diagrams. Poses show residues L315 and F342 near the hinge and ligands in sticks (yellow, B and pink, D). Residues with potential interactions are shown as sticks with protein in cartoon representation and bonds as dotted dark gray lines **(B, D)**.

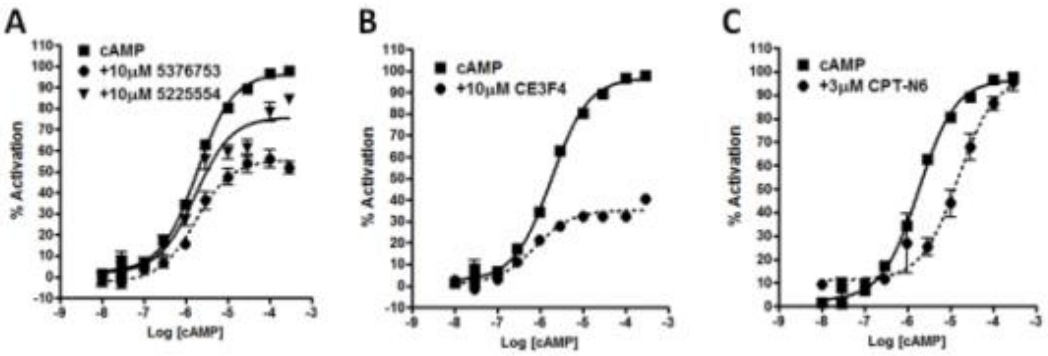


Figure 4.6 5376753 and 5225554 are allosteric inhibitors of Epac. 5376753 or 5225554 **(A)**, the uncompetitive Epac inhibitor CE3F4 **(B)**, or the competitive Epac inhibitor CPT-N6 **(C)** were added to a 96-well plate with 100µL CAMYEL reagents with HEK293 lysate and incubated for 5 min before addition of cAMP. Activation of CAMYEL was measured 5 min after the addition of cAMP. *n=3.*

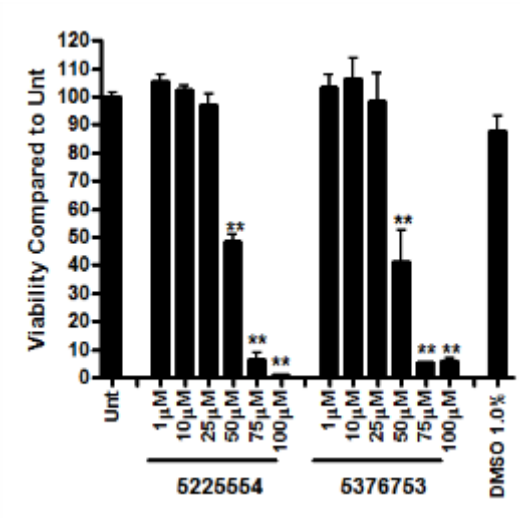


Figure 4.7 Effect of 5225554 and 5376753 on viability of Swiss 3T3 cells. Swiss 3T3 cells were seeded into 24-well plates at 50,000 cells/well in DMEM supplemented with 10%FBS. The next day they were treated with the indicated concentrations of either compound. Viability was assessed using the CellTiter-Glo assay 48hr later. *n=6.* $^{**}p < 0.01$.

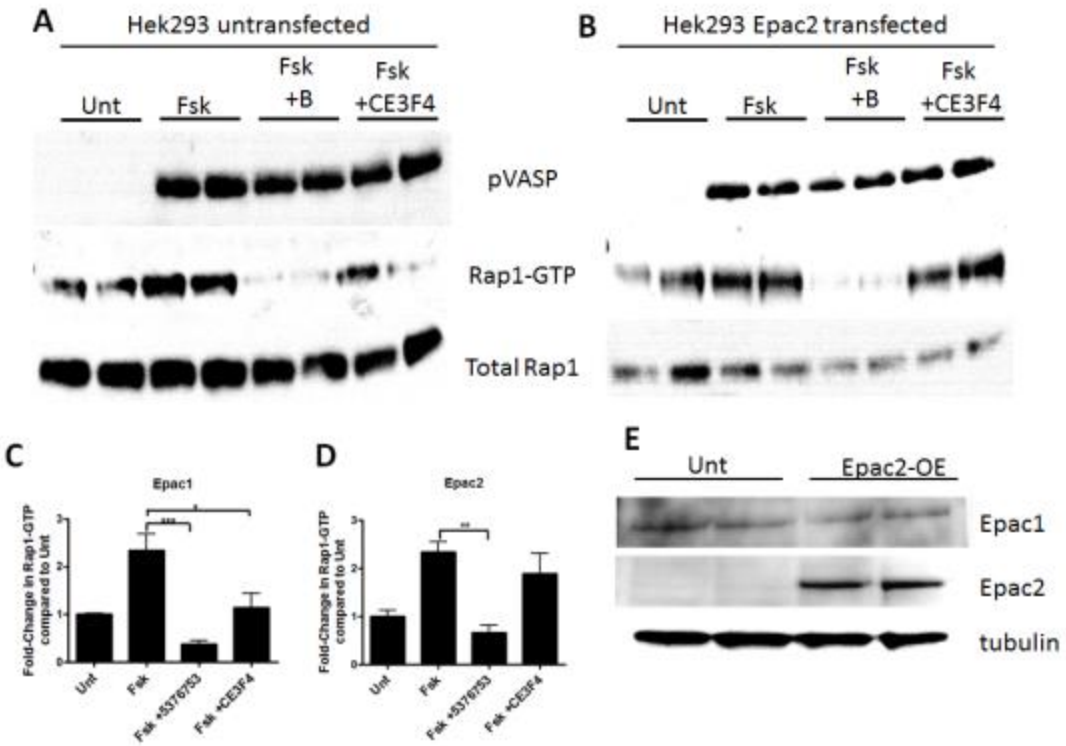


Figure 4.8 5376753 can inhibit Epac1 and Epac2 in HEK293 cells. Rap1 activation and VASP phosphorylation were assessed by Western blotting of HEK293 cells that were non-transfected **(A)** or transfected with Epac2 **(B)** and that were then either left untreated (Unt), or incubated with forskolin (Fsk 10µM) for 5 min alone or in the presence of inhibitors. 5376753 (10µM) or CE3F4 (20µM) were added 30 min prior to addition of forskolin. The Rap1 activation was quantified in **(C)** and **(D)** respectively. **E)** The expression of Epac1 and Epac2 protein was assessed by Western blotting of non-transfected (Unt) or Epac-2 transfected (Epac2-OE) cells. *n=5.* *p<0.05, **p<0.01, ***p<0.001.

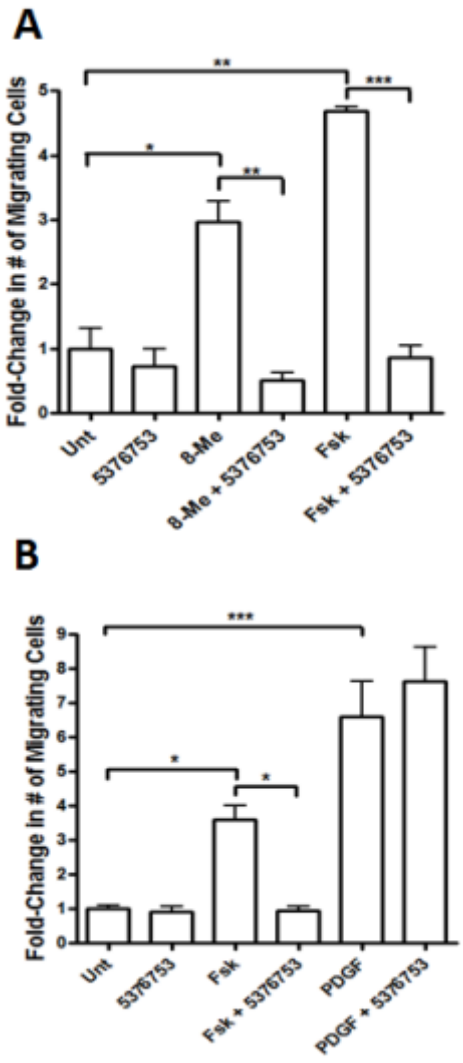


Figure 4.9 5376753 inhibits Epac-mediated migration of rat cardiac fibroblasts. Adult rat cardiac fibroblasts were seeded onto transwells and serum-starved overnight. 5376753 (10µM) was added to the wells 30 min before addition of Fsk (1µM), 8-Me (50µM) or PDGF-BB (10ng/mL). The cells were counted 16 hr later to determine the number of migrating cells. n=4. *p<0.05, **p<0.01, ***p<0.001.

4.8 References

1. Bos JL. Epac: a new cAMP target and new avenues in cAMP research. Nat Rev Mol Cell Biol. 2003 Sep;4(9):733–8.

2. Schmidt M, Dekker FJ, Maarsingh H. Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. Pharmacol Rev. 2013 Apr;65(2):670–709.

3. Laurent A-C, Breckler M, Berthouze M, Lezoualc'h F. Role of Epac in brain and heart. Biochem Soc Trans. 2012 Feb;40(1):51–7.

4. Srivastava DP, Woolfrey KM, Jones KA, Anderson CT, Smith KR, Russell TA. An autism-associated variant of Epac2 reveals a role for Ras/Epac2 signaling in controlling basal dendrite maintenance in mice. PLoS Biol. 2012;10(6):e1001350.

5. Ulucan C, Wang X, Baljinnyam E, Bai Y, Okumura S, Sato M. Developmental changes in gene expression of Epac and its upregulation in myocardial hypertrophy. Am J Physiol Heart Circ Physiol. 2007 Sep;293(3):H1662–1672.

6. Baljinnyam E, Iwatsubo K, Kurotani R, Wang X, Ulucan C, Iwatsubo M. Epac increases melanoma cell migration by a heparan sulfate-related mechanism. Am J Physiol Cell Physiol. 2009 Oct;297(4):C802–813.

7. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med. 2001 Dec 3;194(11):1625–38.

8. Lorenz R, Aleksic T, Wagner M, Adler G, Weber CK. The cAMP/Epac1/Rap1 pathway in pancreatic carcinoma. Pancreas. 2008 Jul;37(1):102–3.

9. Qiao J, Mei FC, Popov VL, Vergara LA, Cheng X. Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP. J Biol Chem. 2002 Jul 19;277(29):26581–6.

10. Consonni SV, Gloerich M, Spanjaard E, Bos JL. cAMP regulates DEP domain-mediated binding of the guanine nucleotide exchange factor Epac1 to phosphatidic acid at the plasma membrane. Proc Natl Acad Sci U S A. 2012 Mar 6;109(10):3814–9.

11. Liu C, Takahashi M, Li Y, Song S, Dillon TJ, Shinde U. Ras is required for the cyclic AMP-dependent activation of Rap1 via Epac2. Mol Cell Biol. 2008 Dec;28(23):7109–25.

12. Harper SM, Wienk H, Wechselberger RW, Bos JL, Boelens R, Rehmann H. Structural dynamics in the activation of Epac. J Biol Chem. 2008 Mar 7;283(10):6501–8.

13. Rehmann H, Arias-Palomo E, Hadders MA, Schwede F, Llorca O, Bos JL. Structure of Epac2 in complex with a cyclic AMP analogue and RAP1B. Nature. 2008 Sep 4;455(7209):124–7.

14. Das R, Mazhab-Jafari MT, Chowdhury S, SilDas S, Selvaratnam R, Melacini G. Entropy-driven cAMP-dependent allosteric control of inhibitory interactions in exchange proteins directly activated by cAMP. J Biol Chem. 2008 Jul 11;283(28):19691–703.

15. De Rooij J, Rehmann H, van Triest M, Cool RH, Wittinghofer A, Bos JL. Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. J Biol Chem. 2000 Jul 7;275(27):20829–36.

16. Brown LM, Rogers KE, McCammon JA, Insel PA. Identification and Validation of Modulators of Epac Activity: Structure-function Implications for Epac Activation and Inhibition. J Biol Chem. 2014 Feb 4;

17. Jiang LI, Collins J, Davis R, Lin K-M, DeCamp D, Roach T. Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. J Biol Chem. 2007 Apr 6;282(14):10576–84.

18. Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA. Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. Proc Natl Acad Sci U S A. 2005 Jan 11;102(2):437–42.

19. Matthiesen K, Nielsen J. Cyclic AMP control measured in two compartments in HEK293 cells: phosphodiesterase K(M) is more important than phosphodiesterase localization. PloS One. 2011;6(9):e24392.

20. Franke B, Akkerman JW, Bos JL. Rapid Ca2+-mediated activation of Rap1 in human platelets. EMBO J. 1997 Jan 15;16(2):252–9.

21. Tsalkova T, Blumenthal DK, Mei FC, White MA, Cheng X. Mechanism of Epac activation: structural and functional analyses of Epac2 hinge mutants with constitutive and reduced activities. J Biol Chem. 2009 Aug 28;284(35):23644–51.

22. Brenke R, Kozakov D, Chuang G-Y, Beglov D, Hall D, Landon MR. Fragment-based identification of druggable “hot spots” of proteins using Fourier domain correlation techniques. Bioinforma Oxf Engl. 2009 Mar 1;25(5):621–7.

23. Kozakov D, Hall DR, Chuang G-Y, Cencic R, Brenke R, Grove LE. Structural conservation of druggable hot spots in protein-protein interfaces. Proc Natl Acad Sci U S A. 2011 Aug 16;108(33):13528–33.

24. The PyMOL Molecular Graphics System. Schrödinger, LLC.

25. Roy A, Kucukural A, Zhang Y. I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc. 2010 Apr;5(4):725–38.

26. Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics. 2008;9:40.

27. Schrödinger Suite 2013 Protein Preparation Wizard. New York, NY: Schrödinger, LLC; 2013.

28. Schrödinger Release 2013-3: LigPrep. New York, NY: Schrödinger, LLC; 2013.

29. Pastor RW, Brooks BR, Szabo A. An analysis of the accuracy of Langevin and molecular dynamics algorithms. Mol Phys. 1988 Dec 20;65(6):1409–19.

30. Case DA, Darden TA, Cheatham TE, Simmerling CL, Wang J, Duke RE. AMBER 12. University of California, San Francisco; 2012.

31. Christen M, Hünenberger PH, Bakowies D, Baron R, Bürgi R, Geerke DP. The GROMOS software for biomolecular simulation: GROMOS05. J Comput Chem. 2005 Dec;26(16):1719–51.

32. Dolinsky TJ, Nielsen JE, McCammon JA, Baker NA. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. Nucleic Acids Res. 2004 Jul 1;32(Web Server issue):W665–667.

33. Dolinsky TJ, Czodrowski P, Li H, Nielsen JE, Jensen JH, Klebe G. PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. Nucleic Acids Res. 2007 Jul;35(Web Server issue):W522–525.

34. Schrödinger Release 2014-1: Maestro. New York, NY: Schrödinger, LLC; 2014.

35. Zhang, Chung, Oldenburg. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen. 1999;4(2):67–73.

36. Lin J-H, Perryman AL, Schames JR, McCammon JA. Computational drug design accommodating receptor flexibility: the relaxed complex scheme. J Am Chem Soc. 2002 May 22;124(20):5632–3.

37. Nichols SE, Baron R, McCammon JA. On the use of molecular dynamics receptor conformations for virtual screening. Methods Mol Biol Clifton NJ. 2012;819:93–103.

38. Rehmann H, Schwede F, Døskeland SO, Wittinghofer A, Bos JL. Ligand-mediated activation of the cAMP-responsive guanine nucleotide exchange factor Epac. J Biol Chem. 2003 Oct 3;278(40):38548–56.

39. Courilleau D, Bisserier M, Jullian J-C, Lucas A, Bouyssou P, Fischmeister R. Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac. J Biol Chem. 2012 Dec 28;287(53):44192–202.

40. Selvaratnam R, Mazhab-Jafari MT, Das R, Melacini G. The auto-inhibitory role of the EPAC hinge helix as mapped by NMR. PloS One. 2012;7(11):e48707.

41. Yokoyama U, Patel HH, Lai NC, Aroonsakool N, Roth DM, Insel PA. The cyclic AMP effector Epac integrates pro- and anti-fibrotic signals. Proc Natl Acad Sci U S A. 2008 Apr 29;105(17):6386–91.

42. Siegbahn A, Hammacher A, Westermark B, Heldin CH. Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. J Clin Invest. 1990 Mar;85(3):916–20.

43. Mark LC, Burns JJ, Brand L, Campomanes CI, Trousof N, Papper EM. The passage of thiobarbiturates and their oxygen analogs into brain. J Pharmacol Exp Ther. 1958 May;123(1):70–3.

44. Takahashi M, Rikitake Y, Nagamatsu Y, Hara T, Ikeda W, Hirata K. Sequential activation of Rap1 and Rac1 small G proteins by PDGF locally at leading edges of NIH3T3 cells. Genes Cells Devoted Mol Cell Mech. 2008 Jun;13(6):549–69.

45. Vigil D, Cherfils J, Rossman KL, Der CJ. Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? Nat Rev Cancer. 2010 Dec;10(12):842–57.

Chapter Five: Conclusions and Discussion

The Epac proteins were discovered 16 years in a search for proteins that mediate the effects of cAMP independently of PKA(1)(2) . Since their discovery many cAMP-mediated effects have been attributed to Epac-signaling. Epac is a guanine nucleotide exchange factor (GEF) for the low molecular weight G-protein Rap. While Rap1 was originally thought to oppose Ras-mediated transformation, it is now known to have biological effects independent of Ras. The main downstream signaling from Rap1 is activation of integrins, which are involved in adhesion and migration of cells. This activity provided the first indication as to the possible consequences of activation of Epac and indeed, Epac was found to increase adhesion of cells to fibronectin(3). Since that time the downstream signaling effects of Rap and Epac have greatly expanded and now include regulation of secretion, proliferation, migration, apoptosis, and differentiation(4)(5). Besides its role in normal physiology Epac has also been implicated to have important roles in different diseases that include autism, diabetes mellitus, heart failure, and cancer(6)(7)(8)(9)(10).

While the downstream signaling has been an intensive focus of Epac research, less attention has been paid to understanding the mechanism by which Epac is regulated. Epac1 was reported to be uniquely up-regulated in CLL but no mechanism for this increased expression was known. I sought to determine the mechanism by which Epac1 expression is regulated by analyzing proteins that bind to the promoter

region of Epac1 in CLL cell lines. I found that Lef-1, a transcription actor up-regulated in CLL, is involved in the increased expression of Epac1 by a non-canonical β-catenin-independent mechanism. To assist in understanding how the increased up-regulation of Epac affects CLL biology I sought to inhibit its function. Knockdown of Epac by siRNA in CLL cells proved difficult and no pharmacological inhibitors of Epac had as-yet been developed. The development of inhibitors of Epac would assist in understanding the consequences of its dysregulation in cancer and other diseases.

Epac activation is measured in cells by assessing the activation of Rap1 by a pull-down method where the activated Rap1 is precipitated out by binding to RalGDS and the amount of activated Rap1 is assessed by Western blot(11). This assay works but it is time-consuming and expensive. To assist in identifying modulators of Epac activity I designed a BRET-based assay to screen for modulators of Epac activity. Using this assay I then screened cAMP analogs and compounds predicted to be allosteric inhibitors of Epac for their ability to modulate the activity of Epac. The following sections detail the significance of these findings and propose future directions to further explore underlying mechanisms.

5.1 Regulation of Epac expression by Lef-1

CLL is the most common leukemia in the Western World. Due to its high prevalence and the ease of obtaining samples from patients (isolating the peripheral blood mononuclear cells from a blood sample is all that is needed to obtain patient CLL cells) there has been much research focused on understanding the genetic

changes that take place in the disease in order to try to develop new diagnostic tools and therapeutics. Analysis of the genes that are specifically up-regulated in CLL patients compared to normal B-cells and other B-cell lymphomas revealed that Epac1 is uniquely up-regulated in CLL(12). This was an interesting discovery as it was the first strong implication that Epac may have a role in cancer and because cAMP was known to be pro-apoptotic in CLL(13)(14). Further research on the determining the downstream effects of activating either PKA or Epac revealed that they have opposing roles with PKA promoting apoptosis while Epac inhibits apoptosis(15).

Although Epac was reported to be up-regulated in CLL, it had only been analyzed at the mRNA level and no mechanism for its increased expression had been determined. I confirmed that Epac1 was up-regulated at the mRNA level and showed this increase at the protein level in CLL cells. I further showed that Lef-1 appears to mediate this increased expression in CLL cells. Lef-1 expression is increased in CLL and high Lef-1 expression correlates with a poor prognosis(16). Similarly to the higher expression of Lef-1, higher expression of Epac1 is found in the aggressive form of CLL. PDE7B, a PDE that is uniquely up-regulated in CLL, also has a putative Lef-1 site and its increased expression is associated with a worse prognosis(17)(18)(19). This indicates that the increased Lef-1 expression may be responsible for the changes in the cAMP pathway that occur in CLL and that these changes may influence the aggressiveness of the disease.

Increased Lef-1 expression precedes the initiation of the disease, indicating that increased Lef-1 is not sufficient to induce the development of CLL(20)(21). It would be interesting to determine at which point in the development and progression

of CLL Epac is up-regulated. Since increased Rap1 activity in B-cells and overexpression of the Epac effector TCL-1 can lead to leukemia, it would be interesting to determine if the increased expression of Epac1 is necessary for the initiation of CLL or if the transformation of CLL cells is responsible for the increase in Epac expression(22)(23)(24). The development of Epac1 inhibitors would help to illuminate the role of Epac in CLL biology.

5.2 Development of a High Throughput Screen (HTS) to Identify Epac Modulators

Understanding how cAMP mediates its effects is important to understanding normal physiology and pathophysiology. While many tools have been developed to study this important signaling pathway, most are targeted to GPCRs, ACs, PKA, and PDEs. The work on Epac is more limited(25). When this project was started, the only Epac-specific modulator was the cAMP analog 8-CPT-2'O-Me-cAMP which preferentially activates Epac (26). This analog is a "super agonist" of Epac1 but only a partial agonist of Epac2(27). Initial screens for additional Epac modulators used competitive assays where the displacement of either a radioligand or a fluorescent ligand from Epac was measured(28)(29). These screens identified compounds that appeared to compete with cAMP or cAMP analogs for binding, but they were unsuitable for use with cells due to poor solubility, high toxicity, or were false positives due to fluorescent quenching. Further chemical screens eventually identified

ESI-09 (Epac1-specific), ESI-05 (Epac2-specific), and HJC197 (Epac1 and Epac2) as potential inhibitors(30)(31)(32). However, the specificity of ESI-05 and HJC197 have been questioned(33). Another screen for inhibitors for Epac used a very robust assay of measuring the displacement of a fluorescent GDP analog from Rap1 when activated by Epac. This succeeded in identifying the uncompetitive inhibitor CE3F4(34).

While the Epac-Rap1 assay is very robust it requires the production of high quality protein from bacteria. I wanted to use an assay that while still being robust was more cost-effective and quick. Previous work had designed a BRET-cAMP sensor with Epac that took advantage of the intramolecular movements that occur upon activation to detect cAMP levels in cells(35). I adapted this construct (CAMYEL) to an *in vitro* assay that could be quickly and reliably used to detect modulators of Epac activity. I demonstrated that CAMYEL can accurately predict binding of cAMP and analogs that agrees with the biochemical data obtained from studies with radio-labeled cAMP. I also further showed that based on the maximal activation CAMYEL can predict if a ligand is a partial agonist, full agonist, or "super agonist" and that this agrees with the data obtained from measuring the abilities of the ligands to activate purified Epac *in vitro*. After analyzing the abilities of cAMP analogs to bind and modulate CAMYEL's activity, I showed that analogs with substitutions at the N6 position of cAMP act as competitive inhibitors and that through computational modeling this appears to be due to their inability to stabilize the lid of the CNBD of activated Epac. I verified that these compound work in cells as well to inhibit Rap1 activation by Epac. While these compounds inhibit Epac, they are not specific, and activate PKA.

To try to develop a Epac specific inhibitor we combined the modifications on the Epac-specific activator with the modifications on the Epac inhibitor to make 8-CPT-2'O-Me-N^6-Phe-cAMP (8-Me-N6) (Fig 5.1A) A comparison of the ability of cAMP analogs to activate PKA in Swiss 3T3 cells indicated that the addition of the 2'O-Me group successfully removed the ability of this compound to activate PKA (Fig 5.1B). While this compound was still able to bind to Epac and inhibit its activation by cAMP it, unexpectedly, had a decreased binding affinity and may act as a partial agonist (Fig 5.1C). The decreased binding affinity was unexpected as 8-Me and 8-CPT-N^6-Phe-cAMP bind Epac1 with similar affinity. More structural studies on Epac1, especially an X-Ray crystal structure would greatly assist in explaining the decreased binding affinity. While this analog does not bind to Epac1 well it is possible that a similar analog with 3 similar modifications on cAMP that are less bulky would be an Epac specific inhibitor that would have increased affinity for Epac while still maintaining low affinity for PKA.,

5.3 Identification of the Hinge of Epac as a Druggable Region

In addition to demonstrating that CAMYEL can identify competitive inhibitors we also showed that it can also identify allosteric inhibitors such as the uncompetitive inhibitor CE3F4. To try to identify additional allosteric modulators we computationally probed an Epac1 model for site that may bind small molecules and that are also important for its activation. Activation of PKA or Epac by cAMP is initiated when the phosphate binding cassette releases its inhibitory interactions on the

hinge region of the CNBD. This allows the hinge to rotate which leads to activation of the protein. While the hinge is highly conserved in cyclic nucleotide binding proteins, the amino acid sequence differs between Epac and PKA (Fig 1.3) (36). Mutation of the hinge region indicated that bulky amino acids prevent the activation of Epac1(37). Computational probing of an Epac1 homology model indicated that the hinge is also likely a druggable region (Fig 4.2). Therefore a small molecule binding to this region may inhibit the movement necessary for Epac activation.

We conducted a screen of compounds predicted to bind to this region and found one novel compound, Chembridge 5225554, that inhibited Epac *in vitro* and in cells (Fig 4.3). We tested a few similar compounds that were commercially available and found that the thiobarbituric acid equivalent, Chembridge 5376753, had a greater ability to bind to Epac and inhibit its activation. This compound inhibited Epac1 and Epac2 equally in cells. It would be interesting to test similar compounds with substitutions at the linker region or on the lipophilic region to determine if binding and inhibition can be enhanced. Preliminary experiments using compounds with different combinations of chlorines on the lipophilic head group did not show any significant difference, but substitution of a NO_2 group for the *para* chlorine removed its ability to inhibit Epac (data not shown). Epac1 and Epac2 are highly conserved in this region and this compound inhibits both proteins. Further screening of compounds may reveal some that interact with the nonconserved residues and would thus be isoform-specific.

5.4 Summary

GEF's are typically considered 'undruggable' due to a lack of binding pockets(38). However, the discovery of Brefeldin A, which inhibits Sec7 activation of Arf, demonstrated that GEFs can be targeted by small molecules(39). Brefeldin A is an "interfacial" inhibitor that binds at the interface of Sec7 and Arf and stabilizes the complex preventing Arf from being released. This type of inhibitor is rare and identifying similar compounds for other GEF-GTPase combinations, while potentially being very specific, would be difficult(40).

The approach taken here to computationally predict novel binding sites and compounds that bind to these regions followed by the experimental testing may work for other GEFs. We identified the hinge of the CNBD as a possible druggable region and while other GEFs do not have a similar region other CNBD containing proteins such as PKA do. It would be interesting to determine if this approach to inhibit Epac would work with PKA as well.

This dissertation helps to provide an understanding of the regulation of Epac in cancer and helps to provide the foundation for competitive cAMP analog inhibitors and allosteric inhibitors of Epac. These modulators of Epac activity will be important for understanding the role of Epac in various cancers and determining if Epac can be a therapeutic target. The inhibitors described in this dissertation will need to be improved upon in terms of affinity, solubility, and toxicity before any therapeutic potential can be reached.

5.5 Figures

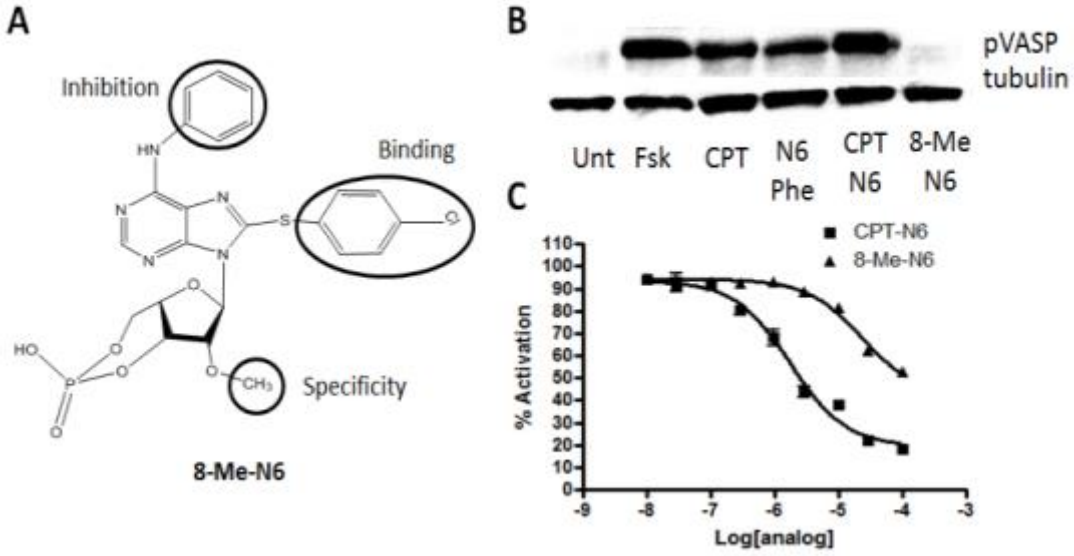


Figure 5.1 8-CPT-2'O-Me-N^6-Phe-cAMP. (A) The structure of 8-CPT-2'O-Me-N^6-Phe-cAMP (8-Me-N6) is shown. **(B)** Swiss 3T3 cells were treated with forskolin (10µM) or the indicated cAMP analogs (50µM) for 5 min and the activation of PKA was assessed by analysis of pVASP by Western Blot. **(C)** CAMYEL was activated with 100uM cAMP and the ability of CPT-N6 or 8-Me-N6 to competitively displace cAMP was analyzed. *n=3.*

5.6 References

1. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M. A family of cAMP-binding proteins that directly activate Rap1. Science. 1998 Dec 18;282(5397):2275–9.

2. De Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature. 1998 Dec 3;396(6710):474–7.

3. Rangarajan S, Enserink JM, Kuiperij HB, de Rooij J, Price LS, Schwede F. Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. J Cell Biol. 2003 Feb 17;160(4):487–93.

4. Schmidt M, Dekker FJ, Maarsingh H. Exchange protein directly activated by cAMP (epac): a multidomain cAMP mediator in the regulation of diverse biological functions. Pharmacol Rev. 2013 Apr;65(2):670–709.

5. Bos JL. Epac: a new cAMP target and new avenues in cAMP research. Nat Rev Mol Cell Biol. 2003 Sep;4(9):733–8.

6. Srivastava DP, Woolfrey KM, Jones KA, Anderson CT, Smith KR, Russell TA. An autism-associated variant of Epac2 reveals a role for Ras/Epac2 signaling in controlling basal dendrite maintenance in mice. PLoS Biol. 2012;10(6):e1001350.

7. Laurent A-C, Breckler M, Berthouze M, Lezoualc'h F. Role of Epac in brain and heart. Biochem Soc Trans. 2012 Feb;40(1):51–7.

8. Kai AKL, Lam AKM, Chen Y, Tai ACP, Zhang X, Lai AKW. Exchange protein activated by cAMP 1 (Epac1)-deficient mice develop β-cell dysfunction and metabolic syndrome. FASEB J Off Publ Fed Am Soc Exp Biol. 2013 Oct;27(10):4122–35.

9. Baljinnyam E, Umemura M, De Lorenzo MS, Iwatsubo M, Chen S, Goydos JS. Epac1 promotes melanoma metastasis via modification of heparan sulfate. Pigment Cell Melanoma Res. 2011 Aug;24(4):680–7.

10. Misra UK, Pizzo SV. Epac1-induced cellular proliferation in prostate cancer cells is mediated by B-Raf/ERK and mTOR signaling cascades. J Cell Biochem. 2009 Nov 1;108(4):998–1011.

11. Franke B, Akkerman JW, Bos JL. Rapid Ca2+-mediated activation of Rap1 in human platelets. EMBO J. 1997 Jan 15;16(2):252–9.

12. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med. 2001 Dec 3;194(11):1625–38.

13. Mentz F, Merle-Beral H, Ouaaz F, Binet JL. Theophylline, a new inducer of apoptosis in B-CLL: role of cyclic nucleotides. Br J Haematol. 1995 Aug;90(4):957–9.

14. Kim DH, Lerner A. Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. Blood. 1998 Oct 1;92(7):2484–94.

15. Tiwari S, Felekkis K, Moon E-Y, Flies A, Sherr DH, Lerner A. Among circulating hematopoietic cells, B-CLL uniquely expresses functional EPAC1, but EPAC1-mediated Rap1 activation does not account for PDE4 inhibitor-induced apoptosis. Blood. 2004 Apr 1;103(7):2661–7.

16. Erdfelder F, Hertweck M, Filipovich A, Uhrmacher S, Kreuzer K-A. High lymphoid enhancer-binding factor-1 expression is associated with disease progression and poor prognosis in chronic lymphocytic leukemia. Hematol Rep. 2010 Jan 26;2(1):e3.

17. Zhang L, Murray F, Zahno A, Kanter JR, Chou D, Suda R. Cyclic nucleotide phosphodiesterase profiling reveals increased expression of phosphodiesterase 7B in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2008 Dec 9;105(49):19532–7.

18. Peiró AM, Tang C-M, Murray F, Zhang L, Brown LM, Chou D. Genetic variation in phosphodiesterase (PDE) 7B in chronic lymphocytic leukemia: overview of genetic variants of cyclic nucleotide PDEs in human disease. J Hum Genet. 2011 Sep;56(9):676–81.

19. Zhang L, Murray F, Rassenti LZ, Pu M, Kelly C, Kanter JR. Cyclic nucleotide phosphodiesterase 7B mRNA: an unfavorable characteristic in chronic lymphocytic leukemia. Int J Cancer J Int Cancer. 2011 Sep 1;129(5):1162–9.

20. Chadeau-Hyam M, Vermeulen RCH, Hebels DGAJ, Castagné R, Campanella G, Portengen L. Pre-diagnostic Transcriptomic Markers of Chronic Lymphocytic Leukemia Reveal Perturbations 10 years before diagnosis. Ann Oncol Off J Eur Soc Med Oncol ESMO. 2014 Feb 22;

21. Gutierrez A Jr, Tschumper RC, Wu X, Shanafelt TD, Eckel-Passow J, Huddleston PM 3rd. LEF-1 is a prosurvival factor in chronic lymphocytic leukemia and is expressed in the preleukemic state of monoclonal B-cell lymphocytosis. Blood. 2010 Oct 21;116(16):2975–83.

22. Minato N. Rap G protein signal in normal and disordered lymphohematopoiesis. Exp Cell Res. 2013 Sep 10;319(15):2323–8.

23. Misra UK, Kaczowka SJ, Pizzo SV. Interaction between TCL1 and Epac1 in the activation of Akt kinases in plasma membranes and nuclei of 8-CPT-2-O-Me-cAMP-stimulated macrophages. Cell Signal. 2008 Jan;20(1):130–8.

24. Bichi R, Shinton SA, Martin ES, Koval A, Calin GA, Cesari R. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. Proc Natl Acad Sci U S A. 2002 May 14;99(10):6955–60.

25. Schwede F, Maronde E, Genieser H, Jastorff B. Cyclic nucleotide analogs as biochemical tools and prospective drugs. Pharmacol Ther. 2000 Sep;87(2-3):199–226.

26. Enserink JM, Christensen AE, de Rooij J, van Triest M, Schwede F, Genieser HG. A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK. Nat Cell Biol. 2002 Nov;4(11):901–6.

27. Courilleau D, Bouyssou P, Fischmeister R, Lezoualc'h F, Blondeau J-P. The (R)-enantiomer of CE3F4 is a preferential inhibitor of human exchange protein directly activated by cyclic AMP isoform 1 (Epac1). Biochem Biophys Res Commun. 2013 Oct 4;

28. McPhee I, Gibson LCD, Kewney J, Darroch C, Stevens PA, Spinks D. Cyclic nucleotide signalling: a molecular approach to drug discovery for Alzheimer's disease. Biochem Soc Trans. 2005 Dec;33(Pt 6):1330–2.

29. Tsalkova T, Mei FC, Cheng X. A fluorescence-based high-throughput assay for the discovery of exchange protein directly activated by cyclic AMP (EPAC) antagonists. PloS One. 2012;7(1):e30441.

30. Almahariq M, Tsalkova T, Mei FC, Chen H, Zhou J, Sastry SK. A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion. Mol Pharmacol. 2013 Jan;83(1):122–8.

31. Tsalkova T, Mei FC, Li S, Chepurny OG, Leech CA, Liu T. Isoform-specific antagonists of exchange proteins directly activated by cAMP. Proc Natl Acad Sci U S A. 2012 Nov 6;109(45):18613–8.

32. Chen H, Tsalkova T, Mei FC, Hu Y, Cheng X, Zhou J. 5-Cyano-6-oxo-1,6-dihydro-pyrimidines as potent antagonists targeting exchange proteins directly activated by cAMP. Bioorg Med Chem Lett. 2012 Jun 15;22(12):4038–43.

33. Rehmann H. Epac-inhibitors: facts and artefacts. Sci Rep. 2013;3:3032.

34. Courilleau D, Bisserier M, Jullian J-C, Lucas A, Bouyssou P, Fischmeister R. Identification of a tetrahydroquinoline analog as a pharmacological inhibitor of the cAMP-binding protein Epac. J Biol Chem. 2012 Dec 28;287(53):44192–202.

35. Jiang LI, Collins J, Davis R, Lin K-M, DeCamp D, Roach T. Use of a cAMP BRET sensor to characterize a novel regulation of cAMP by the sphingosine 1-phosphate/G13 pathway. J Biol Chem. 2007 Apr 6;282(14):10576–84.

36. Selvaratnam R, Mazhab-Jafari MT, Das R, Melacini G. The auto-inhibitory role of the EPAC hinge helix as mapped by NMR. PloS One. 2012;7(11):e48707.

37. Tsalkova T, Blumenthal DK, Mei FC, White MA, Cheng X. Mechanism of Epac activation: structural and functional analyses of Epac2 hinge mutants with constitutive and reduced activities. J Biol Chem. 2009 Aug 28;284(35):23644–51.

38. Vigil D, Cherfils J, Rossman KL, Der CJ. Ras superfamily GEFs and GAPs: validated and tractable targets for cancer therapy? Nat Rev Cancer. 2010 Dec;10(12):842–57.

39. Zeghouf M, Guibert B, Zeeh J-C, Cherfils J. Arf, Sec7 and Brefeldin A: a model towards the therapeutic inhibition of guanine nucleotide-exchange factors. Biochem Soc Trans. 2005 Dec;33(Pt 6):1265–8.

40. Pommier Y, Marchand C. Interfacial inhibitors: targeting macromolecular complexes. Nat Rev Drug Discov. 2012 Jan;11(1):25–36.