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EPAC1 AS A THERAPEUTIC TARGET FOR PANCREATIC DUCTAL ADENOCARCINOMA

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EPAC1 AS A THERAPEUTIC TARGET FOR PANCREATIC DUCTAL ADENOCARCINOMA

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DISSERTATION

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY

THE UNIVERSITY OF TEXAS MEDICAL BRANCH

SEPTEMBER, 2015

Acknowledgements

First and foremost, I would like thank my advisor Dr. Xiaodong Cheng, who has been an incredible mentor and provided me with great guidance and advice during my PhD studies. Dr. Cheng strikes an excellent balance between letting his students explore their independent ideas while giving general direction to ensure proper progress and development of his students' career, and for that I am very grateful. I would also like to thank my committee members, Drs. Celia Chao, Marxa Figueiredo, Carmen Dessauer, and Yingzi Cong, for their tremendous advice and insight. I would also like to give special thanks to Dr. Fang Mei, who was instrumental in training me and has always done a great job of organizing the lab and ensuring we all have what we needed for our experiments. I also want to thank Dr. Hui Wang, who joined our lab in my last year, but has given me great advice and helped me tremendously in the immunology part of my work. I also want to thank our collaborators Drs. Mark Hellmich, who was an excellent resource for advice during the development of the *in vivo* PDA model, and Jia Zhou, whose lab carried out the chemical syntheses of some of the compounds utilized in my studies. Last, but not least, I would like to Yaohua Hu and Drs. Jingbo Yan, Yingmin Zhu, and Tamara Tsalkova who were/are wonderful colleagues and have made the lab a great and fun place to be!

EPAC1 AS A THERAPEUTIC TARGET FOR PANCREATIC DUCTAL ADENOCARCINOMA

Publication No. 5

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The University of Texas Medical Branch, 2015

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Abstract

The recent discovery of a new family of cAMP sensor proteins, exchange protein activated by cAMP (EPAC), ushered in a new era in cAMP signaling. EPAC is composed of two main isoforms, EPAC1 and EPAC2, whose roles in mediating cAMP effects in various cellular contexts remains relatively unclear, and must be elucidated to examine the therapeutic potential of these proteins. This work investigated the role of EPAC1 in promoting invasion and metastasis of pancreatic ductal adenocarcinoma (PDA) and immune regulation, through modulation of the activity of regulatory T-cells (Treg), a subset of T-cells that suppress the immune system and maintain immune tolerance.

In three common PDA cell lines, AsPC-1, PANC-1, and MIA PaCa-2, suppression/inhibition of EPAC1 reduced invasion/migration in transwell invasion/migration and wound healing assays. In an *in vivo* orthotopic metastatic PDA mouse model, suppression/inhibition of EPAC1 significantly reduced local invasion and distant metastasis of MIA PaCa-2 as determined by *in vivo* bioluminescence imaging and histological evaluation of the number of metastatic foci in the liver. Mechanistically, FACS-based and protein fractionation assays showed that EPAC1 facilitates activation and trafficking of integrin β1, both of which play a critical role in mediating cancer cell motility.

Inhibition/knockout of EPAC1 in either Treg or effector T-cells (Teff) decreased Treg-mediated suppression of Teff, while the impact of EPAC1 inhibition/knockout was additive, as determined by an *in vitro* suppression assay. Mechanistically, inhibition/knockout of EPAC1 up-regulated STAT3 activation and desensitized T-cells to

TGF-β1 signaling. These results suggest that EPAC1 enhances the potency of Treg cells and simultaneously sensitizes Teff cells to suppression.

PDA has a dismal prognosis with ~ 95% mortality rate, in large part because of its high metastatic potential and dense desmoplastic microenvironment, which efficiently recruits and induces Treg cells to quell the body's antitumor immune response. The findings presented in this work suggest that EPAC1 inhibitors might have excellent therapeutic potential as anti-PDA agents that can concurrently reduce PDA metastasis and enhance antitumor immunity.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

EPAC	Exchange protein directly activated by cAMP
CBD	cAMP binding domain
DEP	dishevelled/Egl-10/pleckstrin domain
REM	RAS exchange motif
RA	Ras association domain
CDC25HD	CDC25-homology domain
ERM	Ezrin-radixin-moesin
Rap1	Ras-proximate-1
PDA	Pancreatic ductal adenocarcinoma
Rim	Rab3 interacting molecule
RIAM	Rap1-GTP-interacting adaptor molecule
EGFR	Epidermal growth factor recepror
ESI-09	3-(5-*tert*-butyl-isoxazol-3-yl)-2-[(3-chloro-phenyl)-hydrazono]- 3-oxo-propionitrile
007-AM	8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′, 5′-cyclic monophosphate, acetoxymethyl ester 007-AM
BIM	Bisindolylmaleimide I
NPC	2,6-Diamino-N-([1-(1-oxotridecyl)-2-piperidinyl] methyl) hexanamide
Treg	Regulator T-cells
Teff	Effector T-cells
OVA	Ovalbumine
GJ	gap junctions

Cx43	connexin 43
Stattic	6-Nitrobenzo[b]thiophene-1,1-dioxide

Chapter 1: Introduction

The discovery of EPAC and rethinking cAMP signaling

Cyclic adenosine monophosphate (cAMP), discovered more than 6 decades ago, is one of the most common second messengers that is involved in the regulation of virtually all biological functions [1]. Nearly all of cAMP's effects are transduced by the classic protein kinase A (PKA) family, or the recently identified exchange protein activated by cAMP (EPAC) family, composed of two main isoforms, EPAC1 and EPAC2 [2, 3]. The discovery of EPAC has necessitated the reexamination of cAMP signaling to discern the roles of PKA and EPAC and possible interplay between these two cAMP sensors.

More than 15 years of research have now firmly established that many of cAMP's functions that had been exclusively ascribed to PKA are in fact also mediated by EPAC. For instance, EPAC proteins regulate leptin signaling [4], cardiac function [5], exocytosis and secretion [6, 7], endothelial barrier tight junctions [8, 9], immune responses [10], gap junction (GJ) permeability [11, 12], and cell adhesion [13, 14]. Moreover, EPAC proteins have been implicated in the pathophysiology of several diseases, including cancer, cardiac hypertrophy, cognitive impairment, renal disease, diabetes, and metabolic dysfunction [15-17].

Despite the fact that the same second messenger activates both PKA and EPAC, it is now clear that these two families can act synergistically or antagonistically, depending on their expression, distribution, and cellular context [18, 19]. For example, EPAC increases, while PKA decreases, phosphorylation of Akt/PKB [20, 21]. On the other hand, PKA and

EPAC work in concert to stimulate secretion of the gastrointestinal hormone neurotensin from enteroendocrine cells [22].

EPAC structure, activation, and substrates

EPAC1 and EPAC2 are products of different, but highly homologous genes [2, 3]. EPAC2 is predominantly expressed in the adrenal glands, brain, pancreas, and liver, while EPAC1 is more ubiquitously expressed [2, 3]. EPAC proteins act as guanine nucleotide exchange factors and facilitate the activation of the small GTPase Ras-related-proteins Rap1 and Rap2, but each isoform of EPAC performs distinct functions because of different tissue localization and interactions with various intracellular proteins [16]. The vast majority of EPAC's effects are mediated by Rap1/2, but recent studies have revealed some Rap-independent functions, including regulation of insulin secretion through interaction with Rim (Rab3 interacting molecule) and the calcium sensor Piccolo [6, 23, 24], and possibly nuclear transport through interaction with the small GTPase Ran [25].

EPAC1 and EPAC2 have very similar multi-domain structures with a regulatory region at the N-terminal, and a catalytic region at the C-terminal. EPAC are normally inactive as the regulatory region is usually positioned in a manner that blocks the catalytic region and prevents the binding of Rap1/2 (*Fig. 1A*)[i]. Binding of cAMP is thought to induce a conformational change that exposes the catalytic site of the protein and leads to its activation [16].

The architecture of the two EPAC differs only in the regulatory region, which contains the cAMP binding domain (CBD) (*Fig. 1B*). EPAC1 has one cAMP binding domain (CBD), cAMP-B, while EPAC2 has two CBDs, cAMP-B and cAMP-A, which

[i] Reproduced with permission from Pharm Rev (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;65:670-709), copyright (2013)

has a lower affinity for cAMP [16]. The regulatory region also has a dishevelled/Egl-10/pleckstrin (DEP) domain, which determines protein localization through interactions with the membrane lipid phosphatidic aid (PA) and ezrin-radixin-moesin (ERM) proteins [16]. The catalytic region is composed of a RAS exchange motif (REM), a RAS association (RA) domain, and a CDC25-homology domain (CDC25HD). Recent studies show that in addition to the DEP domain, the RA and CBD domains also participate in the spatio-temporal regulation of EPAC [16, 26].

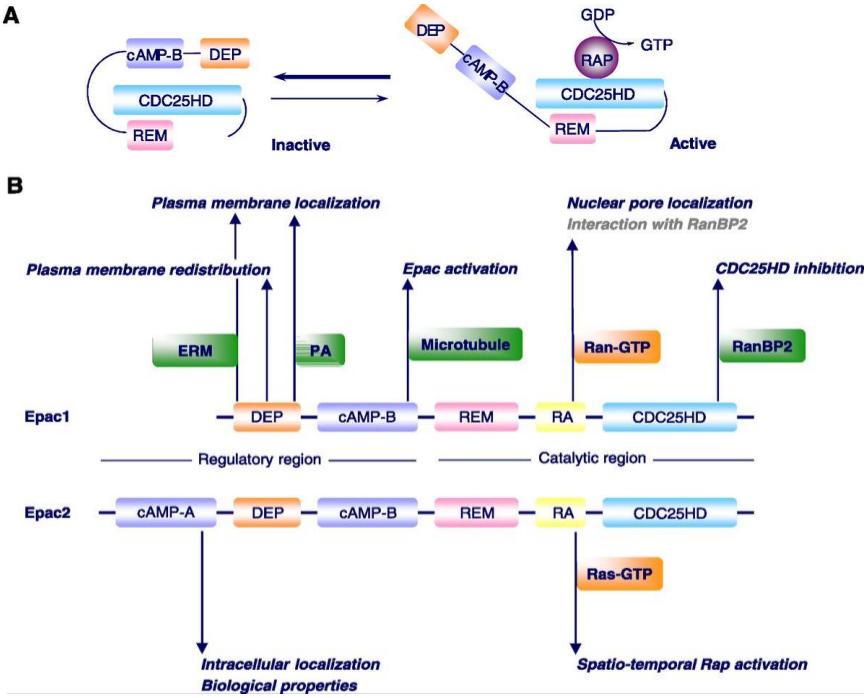

Figure 1. Activation and structure of EPAC. A) The N-terminal normally blocks the catalytic C-terminal from binding to the protein substrate. Binding of cAMP to its binding domain relieves auto-inhibition. B) The regulatory and catalytic regions are composed of distinct domains, which serve particular roles in facilitating the enzymatic activity of the protein, or spatial regulation via interaction with other proteins as shown. cAMP-A, low-affinity cAMP-binding domain; cAMP-B, a higher affinity cAMP-binding domain; RANBP2, RAN-binding protein-2; PA, phosphatidic acid.

Current state of pancreatic ductal adenocarcinoma

We have achieved tremendous advances in the treatment of most cancers, but unfortunately the outlook for pancreatic ductal adenocarcinoma (PDA) has remained stagnant over the past several decades. PDA has a bleak prognosis with ~ 95% mortality rate and it is one of the leading causes of cancer-related deaths [27, 28]. Depending on the sex of the patient, PDA is estimated to be the eighth or ninth cause of cancer deaths worldwide, and the fourth or fifth in developed countries [27, 28]. In the United States alone, ~46,000 diagnoses of PDA are expected in 2014, and ~ 40,000 of these patients are expected to die [29].

In the United States, the current standard of care for PDA generally involves surgical resection when possible, preceded (neoadjuvant) or followed (adjuvant) by chemotherapy alone, or in combination with radiotherapy (chemoradiation) [30]. Although there is some disagreement about the efficacy of post-operative radiotherapy [30]. The main chemotherapy agents utilized are gemcitabine and fluoropyrimidines, like 5-fluoruracil, and they are often combined with other agents like platinum-based drugs, such as Oxaliplatin [31]. However, the clinical outcome of these various treatments is not favorable in most cases, and improvements are usually very modest with success often measured by mere months of prolonged survival [30, 31].

All but one of the current chemotherapy agents utilized in the treatment of PDA are non-specific drugs that target different parts of the cell replication machinery, and as such, they often have intolerable side-effects. More recently, the targeted drug erlotinib, an inhibitor of epidermal growth factor recepror (EGFR) signaling, was approved by the FDA for treatment of PDA [32, 33]. EGFR is over-expressed in as much as 55% of PDA [34],

and erolitinib was able to improve patient survival when combined with gemcitabine [33]. However, the median survival was only increased by ~ half a month, and some studies indicate that KRAS mutations, which are present in ~90% of PDA [35], are associated with resistance to erlotinib [36].

Clearly there is a dire need for novel and more effective targeted treatment strategies for PDA. In particular, therapies that address some of the factors that have made this neoplasm difficult to treat are needed. A multitude of factors contribute to PDA's resistant to various form of treatments; chief among them are its aggressive and highly metastatic nature, and the strong desmoplastic response that creates an immunosuppressive tumor microenvironment that limits the body's immune response to the cancer cells [37].

PDA's metastatic potential

One of the biggest factors behind PDA's poor prognosis is its aggressive local spread and strong potential for distant metastasis. The early signs of the disease are non-specific and there is a lack of reliable diagnostic markers, and therefore, the vast majority of patients is diagnosed after metastasis had occurred [38]. In addition, PDA is a unique cancer as small metastatic foci develop quite early in the progression of the disease [37]. In fact, in a genetic mouse model in which PDA develops spontaneously due to pancreas-specific *Kras* and *p53* mutations, PDA cells were detected in the blood and liver tissue before distinct carcinoma cells formed in the parent pancreatic tissue [39]. Such a pattern of early and aggressive dissemination makes curative surgical resection and local radiotherapy ineffective, and distant spread is the cause of death in ~90% of pancreatic

cancer patients [37, 38]. Even when curative surgical resection is possible, relapse with distant metastasis happens in the majority of patients [40, 41].

The invasion and metastasis process of PDA is a complex one that is governed by numerous biochemical, genetic, and epigenetic alterations. A detailed summary of these changes is beyond the scope of this work, but briefly, several key events are required for tumor cells to become metastatic. These changes include the loss of cell-cell adhesion molecules, such as E-cadherins, which normally inhibit invasion, as well as alterations in the expression levels of other adhesion molecules, including ICAM-1, VCAM-1, and ELAM-1 [38]. Additionally, the expression, distribution, and function of integrins are deregulated in a manner conducive to invasion and metastasis [42]. Finally, to enable cells to break through extracellular matrix and enter into the body's circulation, matrix metalloproteases (MMP), which degrade stromal proteins, are frequently over-expressed in PDA [38].

While the basic steps leading to the formation of an invasive cell have been well elucidated, there are numerous other changes that are not well understood. Moreover, our understanding of the various deregulator steps leading to aforementioned basic changes remains rudimentary [37]. In particular, the genetic mutations that occur in PDA are some of the most heterogeneous observed in cancer [37]. It is critical that we identify these various mutations and alterations in gene expression to develop tailored therapies that are able to target cancer-specific pathways, or circumvent ones that lead to drug resistance.

PDA's immunosuppressive microenvironment

Although inflammation can be pro-tumorigenic, solid carcinomas like PDA usually elicit anti-tumorigenic adaptive and innate immune responses. Which are

positively correlated with patient survival [43, 44]. Even though cancerous cells express mostly self-antigens, the genetic changes that accompany the development of PDA modify the expression patterns and structure of certain surface proteins enough to invoke an inflammatory response designed to eliminate the growing tumor. For instance, antibodies (humoral response) and cytotoxic and helper T-cells (cellular response) specific to the glycoproteins mesothelin and mucin-1 (MUC-1) have been detected in PDA patients [45, 46]. Even intracellular proteins like certain Ras mutants induce T-cell responses in some patients [47].

Since the 1970s researchers have sought to exploit features of the body's spontaneous immune response to cancer to develop effective immunotherapies. Not only can immunotherapy eradicate existing tumor cells, but it also has the potential to provide the immune surveillance necessary to prevent cancer recurrence. A wide array of treatment modalities have undergone clinical trials, including the administration of cytokines that enhance the activity of tumor-specific immune cells, adoptive transfer of engineered autologous T-cells, and administration of monclonoal antibodies against cancer-specific antigens or whole-cell or synthetic peptide vaccines [48]. Unfortunately the field of cancer immunotherapy has not produced many breakthroughs. But with our improved understanding of the intricacies of the immune system and technical advancements in immunological engineering, the field has been revitalized and some promising achievements have been made. In fact, there are currently two available immunotherapies: in 2010 the FDA approved sipuleucel-T, an activated autologous T-cell treatment, for advanced prostate cancer [49], and in ipilimumab, a monoclonal antibody approved in 2011 for non-resectable advanced melanoma [50].

Despite these recent advances, there remain significant hurdles facing cancer immunotherapy. Chief among these hurdles is the immunosuppressive microenvironments of solid tumors [51]. Cross-talk between tumor infiltrating immune cells, tumor cells, and stromal cells leads to the reprogramming of the anti-tumor immune response and development of an immunosuppressive milieu that is suitable for tumor growth [43, 52]. Indeed, the ability to escape immune surveillance is now considered a hallmark of cancer [52]. Given the strong desmoplastic response normally seen with PDA, this tumor is especially adept at evading immune rejection, and its immunosuppressive microenvironment is hugely problematic for the development of efficacious PDA-specific immunotherapies [37].

Perhaps the biggest driver of the immunosuppressive tumor microenvironment is regulatory T-cells (Treg), a subset of T-cells that suppress other immune cells, in both the adaptive and innate immune systems [53, 54]. These cells are crucial to maintaining peripheral tolerance and preventing detrimental immune reactions to innocuous antigens and exaggerated responses to infections [55]. There are two varieties of Treg cells, natural Treg (nTreg), which develop in the thymus, and induced Treg (iTreg), which develop from naïve T cells under the stimulation of cytokines, such as TGF-β1, in peripheral tissues in specific contexts [55]. Tumors recruit nTreg cells with chemokines and induce the generation of Treg cells from non-suppressor T-cells under the stimulation of cytokines (*Fig. 2*)[ii], produced by tumor cells and tumor-associated myeloid-derived suppressor cells (MDSC) [56]. Tumor cells and MDSC also express 2,3-dioxygenase (IDO), which activates Treg cells and aids in their expansion [57, 58]. Treg cells in turn suppress natural killer cells

[ii] Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Cancer (Colombo MP, Piconese S. Regulatory-T-cell inhibition versus depletion: the right choice in cancer immunotherapy. Nat Rev Cancer 2007;7(11):880-887), copyright (2007)

and T-cells that are primed to attack the tumor. In fact, Treg cells accompany cancer development from the early pre-invasive stages and progressively increase in numbers as the tumor cells de-differentiate and increase their metastatic potential [52].

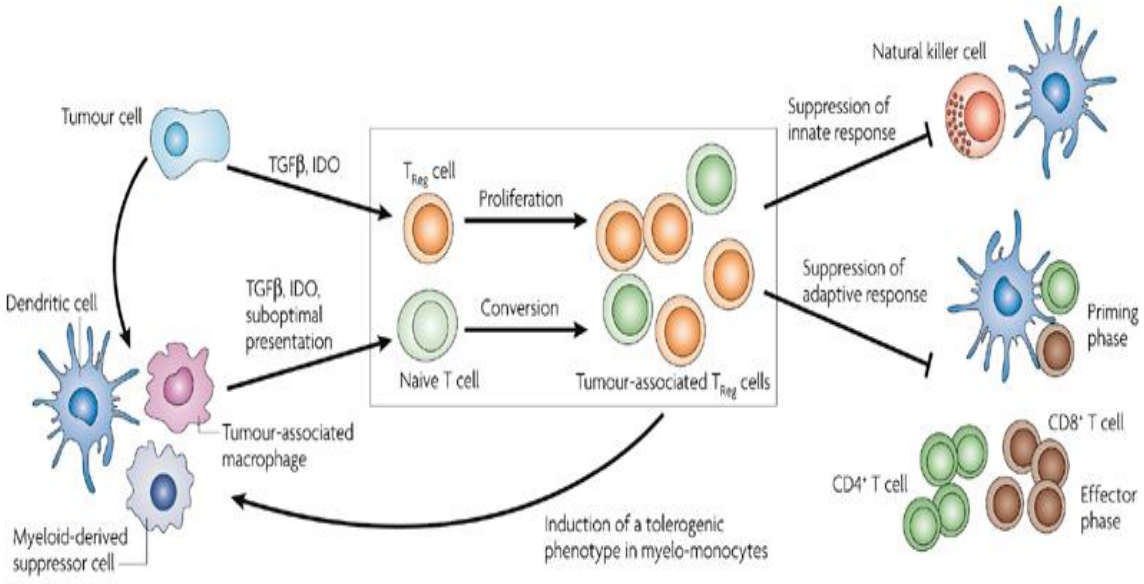

Figure 2. Treg cells in tumor microenvironment. nTreg cells are recruited to the tumor microenvironment or induced by cytokines from tumor or tumor-associated myeloid suppressor cells. Treg cells in turn down-regulate the anti-tumor immune response by suppressing other tumor infiltrating immune cells like natural killer cells and tumor-specific $CD4^+$ and $CD8^+$ T-cells.

Numerous clinical observations support the notion that eliminating tumor infiltrating Treg cells would enhance the efficacy of cancer immunotherapy. Patients with PDA have a higher number of circulating Treg cells and this increased prevalence is generally associated with worse prognosis [59, 60]. Therapies aimed at eliminating Treg cells have shown some promising results in clinical trials [48]. Furthermore, several studies have shown that after administering tumor vaccines, the number of vaccine-specific Treg cells increases significantly; diminishing the effectiveness of the treatment [61-63]. Therefore,

treatments that down-regulate Treg activity have great potential as cancer immunotherapies, especially if administered in conjunction with cancer vaccines.

Dissertation goals

Recent findings, which will be summarized in subsequent chapters, have suggested that EPAC1 mediates cAMP's pro-migratory and immunosuppressive effects. In this dissertation, I will examine the role of EPAC1 in promoting invasion and metastasis of pancreatic ductal adenocarcinoma (PDA), and facilitating immunosuppression mediated by regulatory T-cells (Treg), a subset of T-cells with general suppressor function and a key player in the development of cancer [53, 64]. I will argue that inhibition of EPAC1 is potentially a viable therapeutic strategy that can reduce PDA metastasis and simultaneously enhance the anti-tumor immune response.

Chapter 2: Inhibition of EPAC1 Reduces Invasion and Metastasis of PDA

Introduction

A recent report showed that in tumor samples from patients with pancreatic ducal adenocarcinoma (PDA), EPAC1 was over-expressed in cancerous tissue, compared to normal adjacent pancreatic tissue; both at the mRNA and protein levels [65]. However, the consequences of this over-expression have not been investigated and it is unclear whether enhanced expression of EPAC1 in PDA is detrimental and promotes the malignant phenotype of this tumor, or a protective mechanism that develops as an attempt to control PDA.

cAMP plays a complex and context dependent role in regulating migration [66, 67]. Several studies have shown that EPAC1 mediates cAMP's pro-migratory roles in a variety of cell types. For example, EPAC1 promotes adhesion and migration of white blood cells [68], cells of epithelial origin [14], and vascular smooth muscle cells [69]. In addition, EPAC1 regulates invasion and metastasis of several cancers, including melanoma, prostate cancer, and ovarian cancer [13, 70-75], but there is some controversy about EPAC1's role in each cancer and if it's pro- or anti-migratory.

In melanoma, there is a consensus that EPAC1 enhances invasion and metastasis, as has been shown in several *in vitro* and *in vivo* studies [73-75]. In prostate cancer, some results suggest that EPAC1 promotes metastasis and proliferation [76-78], while other studies contradict these results [72]. Although it has been suggested the inhibitory effect observed for EPAC1 in prostate cancer is actually the result of PKA activation by 8-(4-

Chlorophenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate (8CPT) , the EPAC agonist employed in the study [79]. In ovarian cancer, EPAC1 seems to have pro-migratory effects in some cell lines (Ovcar3) [13], and anti-migratory effects in others (ES-2) [71]. In order improve the development of anti-metastasis therapeutic strategies, it is of paramount importance that we determine the role of EPAC1 in the context of each cancer and elucidate the mechanism by which it boosts or attenuates migration in each case.

Based on the studies summarized here, we speculated that EPAC1 plays an essential role in regulating invasion and metastasis of PDA. In this chapter I will show that EPAC1 facilitates invasion and metastasis of PDA, and EPAC1 inhibitors have therapeutic potential as anti-metastasis for PDA.

Materials and Methods

Small molecule agonists/antagonists

8-(4-Chlorophenylthio)-2′-O-methyladenosine-3′, 5′-cyclic monophosphate, acetoxymethyl ester (007-AM), is a selective EPAC agonist [80, 81] (BioLog Life Science Institute , Bremen, Germany). Bisindolylmaleimide I (BIM I) and 2,6-Diamino-N-([1-(1-oxotridecyl)-2-piperidinyl] methyl) hexanamide (NPC 15437) are selective PKC antagonists [82, 83] (Santa Cruz, Dallas, TX, USA). ESI-09 is a highly selective EPAC inhibitor recently identified in our lab [84]. 8-(4- Chlorophenylthio)-2'-O- methyladenosine-3', 5'- cyclic monophosphate, acetoxymethyl ester (007-AM) was initially provided by and subsequently purchased from BioLog Life Science Institute (Bremen, Germany).

Antibodies

The following antibodies were used for Western blotting: EPAC1 (#4155), EPAC2 (#4156), Akt (#9272), phosphor-Akt (T308) (#4056), phosphor-Akt (S473) (#9271), Integrin β1 (#9699), Na^+/K^+ ATPase (#3010), and ACTIN (#4968) (Cell Signaling Technology, Danvers, MA, USA). Rap1 antibody (SC-65) (Santa Cruz, Dallas, TX, USA). For FACS analysis the following antibodies were used: FITC-conjugated Integrin β1 clone 12G10 (abcam, Cambridge, MA, USA) and FITC-conjugated Integrin β1 clone K-20 (Santa Cruz, Dallas, TX, USA).

Cell lines

The pancreatic ductal adenocarcinoma cell lines AsPC-1, PANC-1, and MIA PaCa-2 were purchased from ATCC and maintained in glutamine containing RPMI 1640 medium (Thermo Scientific HyClone, Logan, UT) supplemented with 10 mM HEPES buffer and

10% fetal bovine serum (complete medium) (Invitrogen, Grand Island, NY) at 37°C under 5% CO_2. Stable cell lines with suppressed EPAC1 (*Epac1*-KD) were generated using MISSION^{TM} TRC lentiviral based shRNA according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). Cells with stable firefly luciferase expression were generated using a lentiviral based pGL4 luciferase reporter according to the manufacturer's protocol (Promega, Madison, WI, USA).

Animals

Animal experiments were conducted in 6- to 8-week-old female athymic BALB/c nu/nu mice (Charles River Laboratories, Wilmington, MA). Use of all animals was in accordance with the guidelines of the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Western blotting

Cellular Proteins were collected in sodium dodecyl sulfate (SDS) lysis buffer (2% SDS, 10% glycerol, 60 mM Tris, pH 6.8), sonicated for 5 s, and denatured at 100°C for 3 min. Protein samples, 5-30 μg depending on level of protein expression, were then resolved on resolved on 10% SDS-PAGE gel, and transferred to a PVDF membrane ((Invitrogen, Grand Island, NY). The membranes were then incubated at room temperature in Tris-buffered saline (TBS)/5% non-fat milk and 0.1% Tween-20 for 1 hr to block non-specific interactions. Following the blocking step, membranes were incubated with the appropriate primary antibody, diluted in TBS/0.1% Tween-20 (dilution buffer), overnight at 4°C, then washed four times (10 min) in dilution buffer. After the washing step the appropriate secondary antibody (peroxidase-conjugated) was added in dilution buffer and incubated at room temperature for 1 hour. The washing step was repeated and the signal

was visualized with a West Pico Chemiluminescent Kit (GenDEPOT, Barker, TX). Densitometry was performed using software ImageJ. Primary and secondary antibodies were diluted as recommended by the manufacturer.

Cell viability assay

Cells were incubated for the specified period of time in a 96-well cell culture plate (~2000/well) (Corning, Corning, NY) in complete culture medium at 37°C under 5% CO_2. Cell viability was then measured by an alamarBlue cell viability assay (lifetechnologies, Carlsbad, CA) according to the manufacturer's instructions. Where indicated, the specified concentrations of ESI-09, BIM I, or NPC 15437 were added to the medium.

Luciferase activity

Luciferase-transduced WT or *Epac1*-KD cells were lysed and luciferase activity was determined by a Luciferase Assay System assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. Bioluminescence was measured with an LmaxII 384 microplate reader (Molecular Devices, Sunnyvale, CA).

Rap1 activation assay

Cells were treated with 10 μM 007-am for 10 min with or without pre-treatment with 10 μM ESI-09 for 5 min. The activation of Rap1 was examined using an active Rap1 (GTP-bound) pull down assay as described previously [20].

Phosphorylation of Akt

Cells were starved of serum for 24 hours before treatment with ESI-09 for 5 min, followed by treatment with 10 μM 007-AM for 15 min. Vehicle-treated cells, and cells treated with 10 μM 007-AM only, were used as negative and positive controls

respectively. Approximately 10 µg of protein were resolved by SDS-PAGE and Western blotting was performed as described earlier. Akt phosphorylation was probed with anti-phospho-AKT T308 and S473 antibodies (1:1000). Phosphorylation levels were determined with densitometry and expressed as a percentage of basal Akt phosphorylation (negative control).

Transwell migration/invasion assay

The top chambers of 8 micron inserts (Costar Inc) were coated with BD Matrigel™ Basement Membrane Matrix (50 µg/mL) (BD biosciences, San Jose, CA, USA). Cells were starved of serum for 24 hr, detached with 0.25% trypsin-EDTA, and added to the top chamber of the inserts in serum free RPMI medium containing 0.25% BSA (2 × 105 cells in 100 µL). The bottom chamber was filled with 600 µL of RPMI containing 10% FBS and 5 µM ESI-09 where indicated. Then the cells were incubated at 37 °C in 5% CO2 for 20 hours. Cells were removed from the top chamber and migrated cells were fixed in methanol (15 min) and stained with crystal violet (15 min). The numbers of migrated cells were counted in four different fields. For experiments with the EPAC agonist 007-AM, the cells were treated with 007-AM (10 µM) alone or in combination with the EPAC inhibitor ESI-09 (5 µM) or PKC inhibitors BIM I (1 µM) or NPC 15437 (1 µM) for 10 minutes in serum free RPMI/0.25% BSA before adding them to the top chamber. The bottom chamber was filled with RPMI/4% FBS containing the same combination of drugs used for pretreatment.

Wound healing assay

Cells were grown to 95-100% confluency before a scratch wound was made. The medium was changed to RPMI 10% FBS containing 5 µM ESI-09 or DMSO vehicle. The

cells were then incubated at 37 °C in 5% CO_2. The wound was imaged at 0 and 22 hrs after changing the medium. Healing rate was determined by calculating the percentage of wound closure according to the following equation: %wound closure = (initial wound width – wound width at 22 hrs)/initial wound width×100. To make the results comparable across all assays, the widths of the initial wounds were all normalized to a 1 mm distance. For experiments with 007-AM, the cells were treated with 007-AM (10 μM) alone or with ESI-09 (5 μM) or BIM I (1 μM) in serum free RPMI for 30 minutes before adding 10% FBS.

Surface protein isolation

MIA PaCa-2 cells were seeded on a fibronectin matrix (Sigma-Aldrich, St. Louis, MO, USA) and starved of serum for 24 hr and treated with with 007-AM (10 μM) alone or with ESI-09 (10 μM) or BIM I (1 μM) in serum free RPMI for 45 min before surface proteins were biotinylated with EZ-link sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA) and isolated according to the manufacturer's instructions. Briefly, 0.25 mg/mL of EZ-link sulfo-NHS-SS-biotin was added to the cells and incubated at 4 °C for 30 min. Biotinylation was quenched and the cells were harvested and centrifuged at 500 g for 3 min. Cells were solubilized with the kit's lysis buffer containing the protease inhibitor phenylmethanesulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA) and incubated on ice for 30 min. The samples were centrifuged at 10,000 g for 2 min at 4 °C and the supernatant containing biotinylated membrane proteins was incubated with NeutrAvidin gel slurry for 60 min at room temperature. Then surface proteins were eluted from the column with elution buffer containing 50 mM dithiothreitol (DTT). Approximately 15 μg of biotinylated protein were separated by SDS-PAGE surface int. β1 was probed by

Western blotting as described earlier. The plasma membrane protein Na^+/K^+ ATPase was used as a loading control. To obtain total int. β1 cells were treated as described for surface int. β1 isolation and lysed with SDS lysis buffer (2% SDS, 10% glycerol, 60 mM Tris, pH 6.8).

Flow cytometry analysis of activation and cell-surface expression of integrin β1

MIA PaCa-2 cells were starved of serum for 24 hr and detached with 0.25% trypsin-EDTA for 5 minutes. Cells were then treated with 007-AM (10 μM) alone or in combination with ESI-09 (10 μM) or NPC 15437 (1 μM) in serum free RPMI/0.25% BSA and incubated for 15 min at 37 °C in 5% CO2. Cells were fixed in 4% paraformaldehyde for 12 min, then active int. β1 was stained with 12G10 (1:100), which only recognizes the active conformation of int. β1 [85], and total int. β1 with K20 (1:5) for 30 min at 4 °C in PBS containing 3% BSA. The samples were analyzed using FACS (FACS Calibur, Becton Dickinson, USA). The level of active int. β1 was determined by normalizing the mean fluorescence intensity (MFI) of active int. β1 staining to total int. β1 MFI.

Orthotopic mouse model and in vivo imaging

WT or Epac1-KD MIA PaCa-2 cells, transduced with firefly luciferase, were grown to 80-90% confluency and detached with 0.25% trypsin-EDTA, washed with RPMI/10%FBS, and suspended in PBS/Matrigel (1:1) and saved on ice. Mice were anesthetized with isoflurane inhalation to effect. A small nose cone was used to maintain anesthesia during the procedure. A small incision (~10 mm) was made through the skin overlying the spleen/pancreas. The spleen/pancreas were exteriorized and MIA PaCa-2 injected into the parenchyma of the pancreas (50 μL of the PBS/Matrigel suspension

containing 1.5 × 106 cells). The spleen/pancreas were then returned into the abdominal cavity and the incision (both muscle and skin layers) were re-approximated with surgical sutures. Sutures were removed 1 week post procedure. Treatment with ESI-09 (I.P injection of10 mg/kg) was initiated two days after injection of cells. For in vivo imaging, mice were injected with D-Luciferin (150 mg/kg in PBS) (PerkinElmer, Waltham, MA, USA), anesthetized with isoflurane, and then imaged with the IVIS 200 Pre-clinical In Vivo Imaging System (PerkinElmer, Waltham, MA, USA).

Statistical Analysis

Student t test was used for data analysis in this study and results were considered as statistically significant if P values were <0.05.

Results [3,4]

Characterization of EPAC expression and activity in PDA

To confirm the expression of EPAC1 in PDA, we probed its protein levels in the PDA cell lines AsPC-1, PANC-1, and MIA PaCa-2 by Western blotting. All three cell lines had strong expression of EPAC1, which was successfully suppressed with shRNA (*Fig. 3A*). None of the cell lines had a detectable level of EPAC2 (*Fig.* 3B).

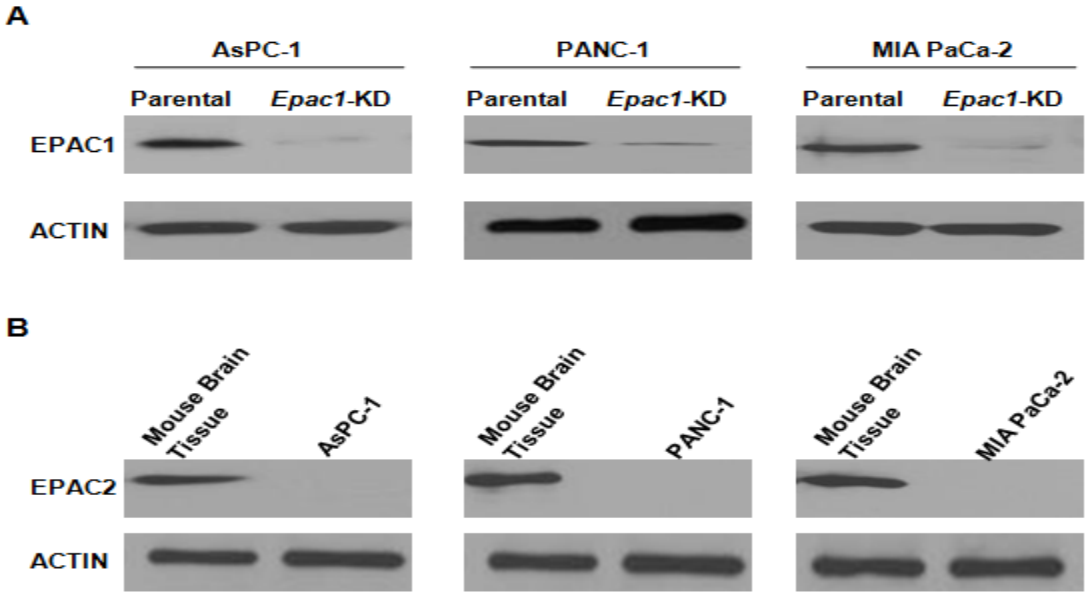

Figure 3. EPAC expression in PDA. A) Expression of EPAC1 in the PDA cells lines AsPC-1, PANC-1, and MIA PaCa-1 was probed by Western blotting, and knockdown of EPAC1 by shRNA was confirmed. B) EPAC2 expression was also probed in all three cell lines. Mouse Brain tissue was used as a positive control for EPAC2 expression.

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[3] All figures and data based on the AsPC-1 and PANC1 cell lines were reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved. (Almahariq M, Tsalkova T, Mei FC, Chen H, Zhou J, Sastry SK, Schwede F, Cheng X. A novel epac-specific inhibitor suppresses pancreatic cancer cell migration and invasion. *Mol Pharmacol*. 2013;83:122-128)

[4] All figures and data based on the MIA PaCa-2 cell line were reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved. (Almahariq M, Chao C, Mei FC, Hellmich MR, Patrikeev I, Motamedi M, Cheng X: Pharmacological Inhibition and Genetic Knockdown of Exchange Protein Directly Activated by cAMP 1 Reduce Pancreatic Cancer Metastasis In Vivo. *Molecular Pharmacol*ogy 2015;87 (2):142-149)

Recently, our group identified the first EPAC-specific inhibitor, ESI-09, (*Fig. 4A*) [84], which was utilized in combination with the EPAC agonist 007-AM to test the functional activity of EPAC1. EPPAC1 is known to up-regulate phosphorylation of AKT (phospho-AKT) at the T307 and S473 residues [20]**.** Treatment with 007-AM increased phospho-AKT level at these residues, but this trend was inhibited by ESI-09 in a dose dependent manner (*Fig* 4B). To test ESI-09's ability to specifically target EPAC1 in context of PDA cells, we treated the cells with ESI-09 prior to stimulation with epidermal growth factor (EGF). ESI-09 was unable to inhibit EGF-stimulated phosphoryaltion of AKT.

As mentioned previously, EPAC proteins primarily act as guanine exchange factors (GEF) for the small GTPase proteins Rap1/2 [86, 87], so we examined EPAC1's GEF activity to further confirm its functionality in PDA. 007-AM was able to stimulate Rap1 activation as determined by increased levels of GTP-bound Rap1, while ESI-09 decreased those levels (*Fig. 4C*). Together, these results confirm EPAC1's expression and activity in PDA cells and show that ESI-09 is able to specifically target EPAC1 in this context.

EPAC1 facilitates invasion and migration of PDA cells

As discussed earlier in this chapter, studies have shown that EPAC1 plays a role in the migration of melanoma and ovarian cancer, and migration and proliferation of prostate cancer [13, 70-75]. To investigate whether EPAC1 affects proliferation and/or migration of PDA, we employed genetic and pharmacologic approaches to suppress and inhibit EPAC1, respectively. Suppression of EPAC1 did not alter the proliferation rate of AsPC-1, PANC1, or MIA PaCa-2 cells (*Fig. 5*). On the other hand, genetic suppression

of EPAC1, or pharmacologic inhibition by ESI-09 significantly inhibited invasion/migration of PDA cells as determined by transwell invasion/migration and wound healing assays (*Fig. 6*).

Additionally, treatment with the EPAC-specific activator 007-AM significantly increased invasion/migration in those assays, but ESI-09 completely negated the impact of 007-AM. (*Fig. 7*). To rule out the possibility that ESI-09 inhibited invasion/migration by lowering cell viability, we monitored cell proliferation in the presence of this compound. ESI-09 did not affect cell viability at the concentrations utilized in the employed assays (*Fig. 8*). These results show that in PDA cells, inhibition of EPAC1 decreases invasion/migration without affecting cell proliferations.

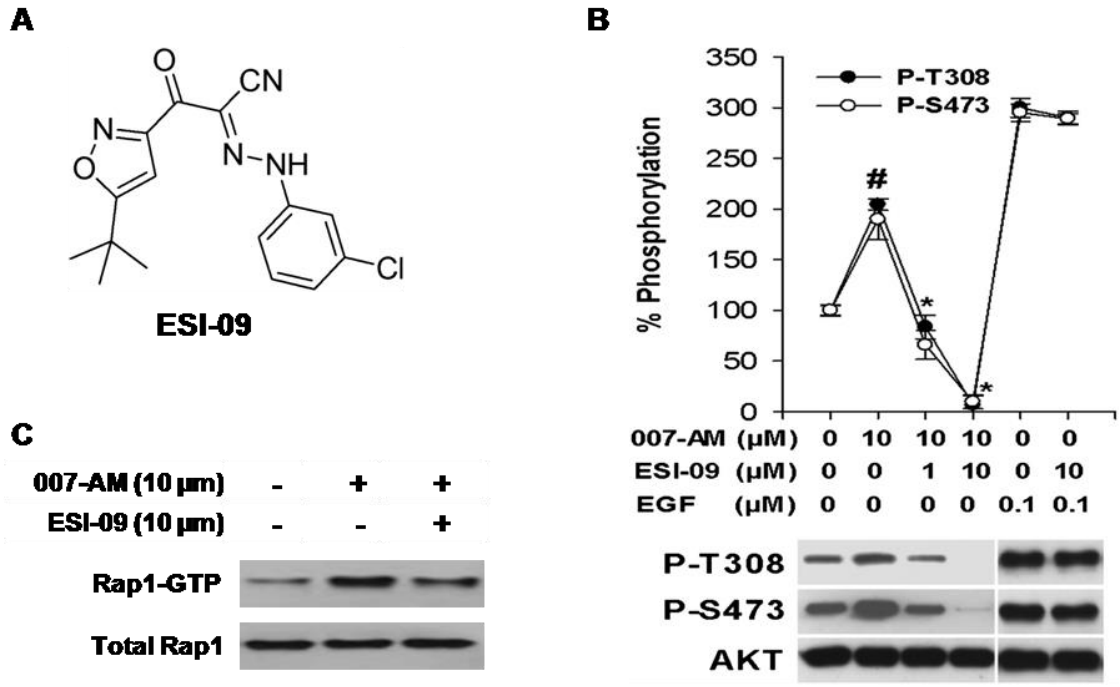

Figure 4. EPAC1 activity in PDA. A) Chemical structure of the EPAC inhibitor ESI-09. B) Serum-starved AsPC-1 cells were treated (15 min) with 007-AM or EGF after pretreatment (5 min) with the indicated concentrations of ESI-09. phospho-AKT T308, phosphor-AKT S473, and total AKT levels were probed by Western blotting (representative blot shown). Posphorylation levels were determined by densitometry and expressed as a percentage of total AKT. C) Serum-starved MIA PaCa-2 cells were stimulated (10 min) with 007-AM after pretreatment (5 min) with ESI-09. Data points represent mean ± S.D. (n=3). $^{\#}$Significantly higher than vehicle group ($P < 0.02$). *Significantly lower that 007-am stimulated group ($P < 0.02$).

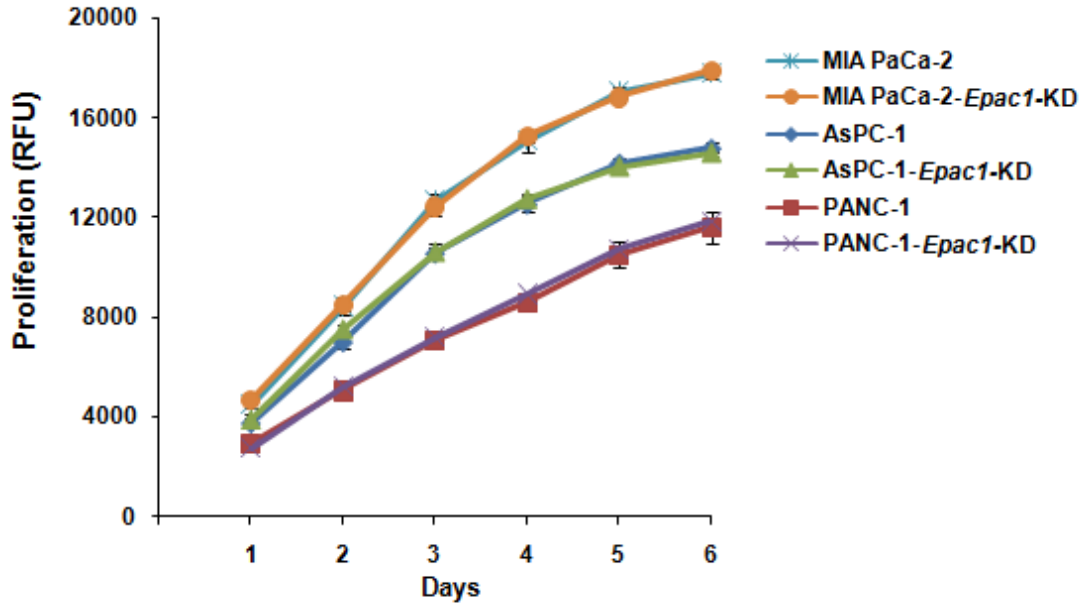

Figure 5. Suppression of EPAC1 has no impact on PDA proliferation. The proliferation of parental or *Epac1*-KD AsPC-1, PANC-1, and MIA PaCa-2 cells was monitored for 6 days by an alamarBlue metabolic activity assay. RFU, relative fluorescence unit.

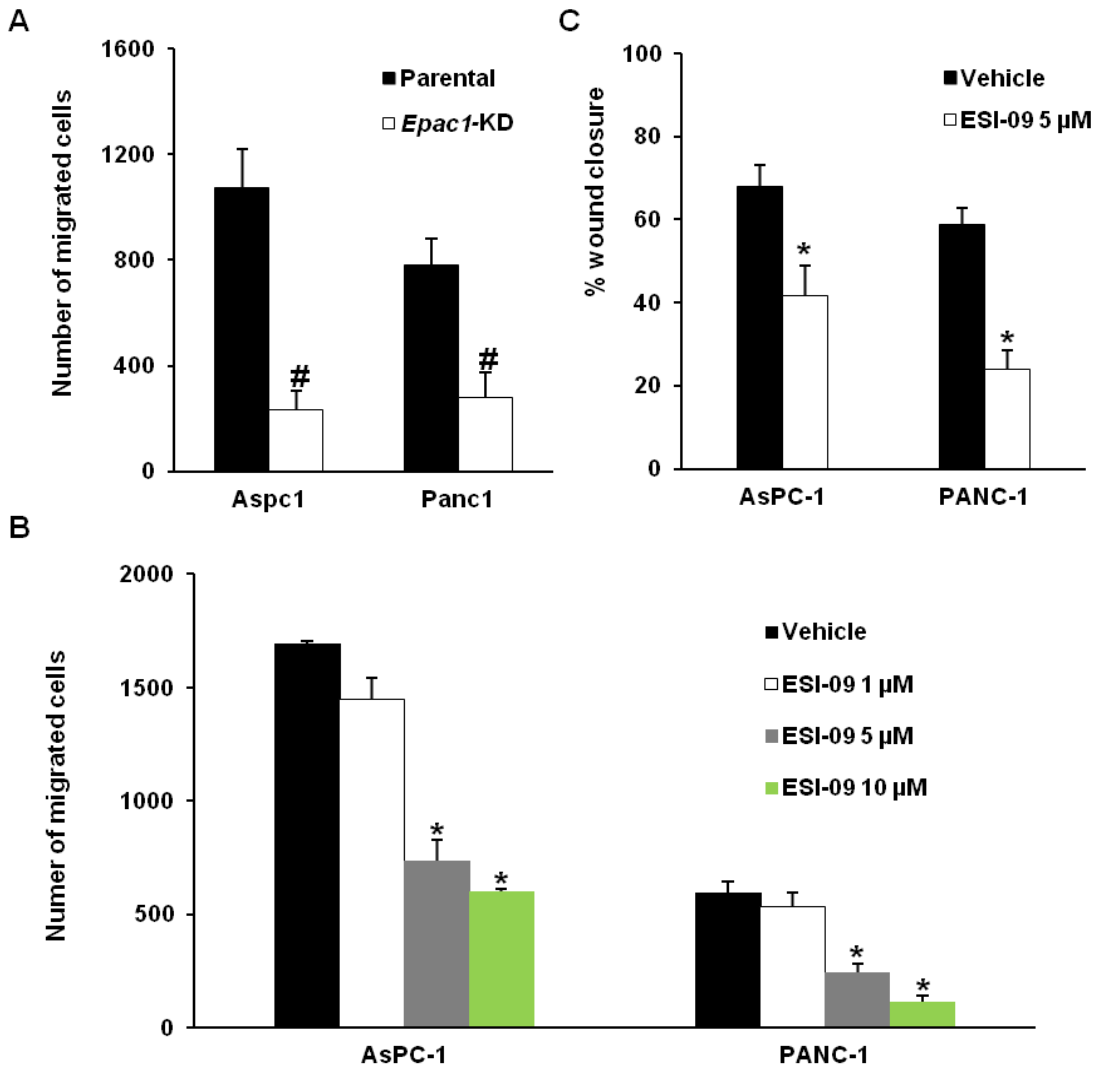

Figure 6. EPAC1 suppression or inhibition decrease PDA cell migration and invasion. A) Cells were serum starved overnight, detached, and their invasion/migration ability was measured by a transwell invasion/migration assay. B) Invasion/migration was measured as described in (A) in the presence or absence of the indicated concentrations of ESI-09. C) Cells were serum starved overnight, a scratch wound was made, and wound healing rate was followed in the presence or absence of ESI-09. Bars represent mean ± S.D. (n = 3). *Significantly lower than vehicle group ($P < 0.05$). #Significantly lower than parental cells group ($P < 0.02$).

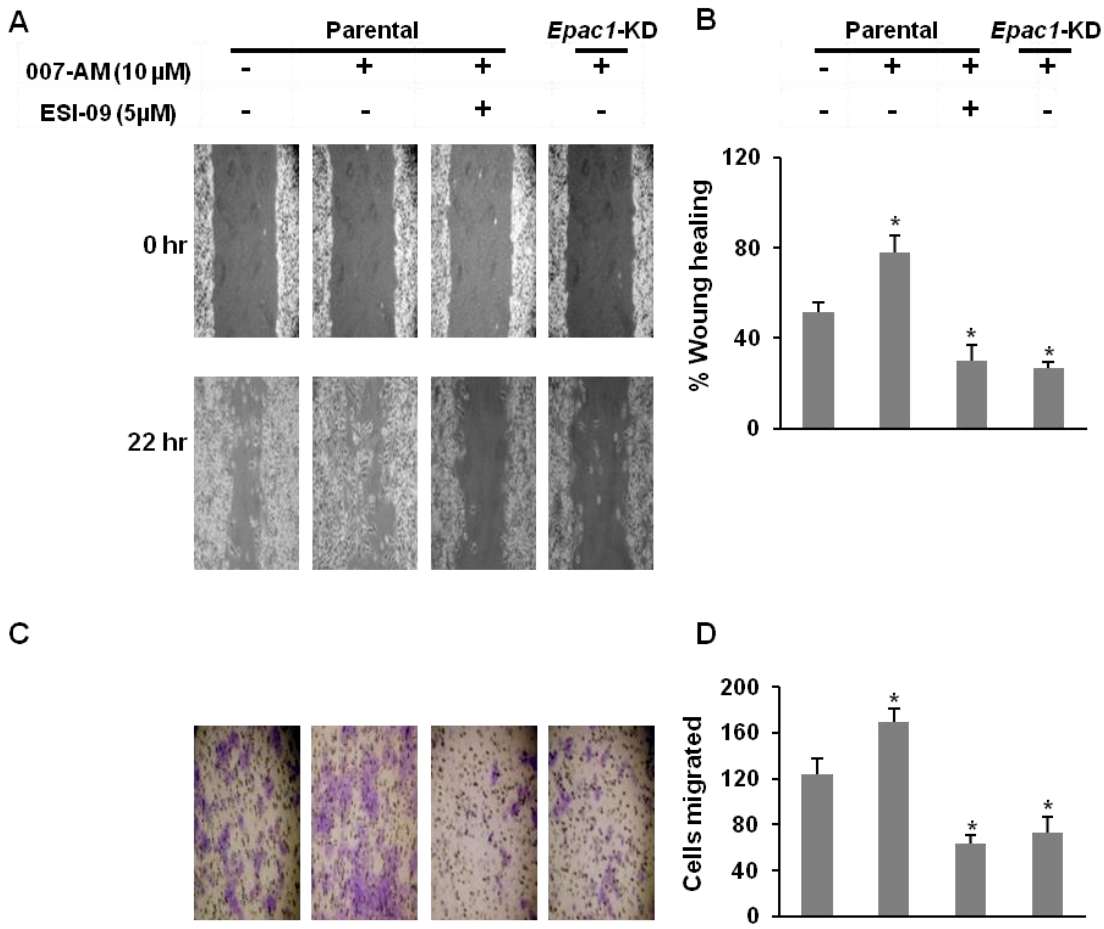

Figure 7. EPAC1 suppression or inhibition decrease PDA cell migration and invasion. MIA PaCa-2 cells were serum starved overnight, detached, and their invasion/migration ability was measured by transwell invasion/migration and wound healing assays. A) Representative images showing an increase in wound healing rate with 007-AM treatment, and a decrease by *Epac1*-KD or ESI-09 treatment. B, quantification of wound-closure presented as the distance traveled by the edge of the wound relative to the wound's initial size. C) Representative images showing an increase in invasion/migration with 007-AM treatment and a decrease by *Epac1*-KD or ESI-09 treatment. D) Quantification of the number of migrating cells. * Significantly higher or lower than vehicle-treated parental cell ($P < 0.03$). Data is presented as mean ± S.D. (n=3).

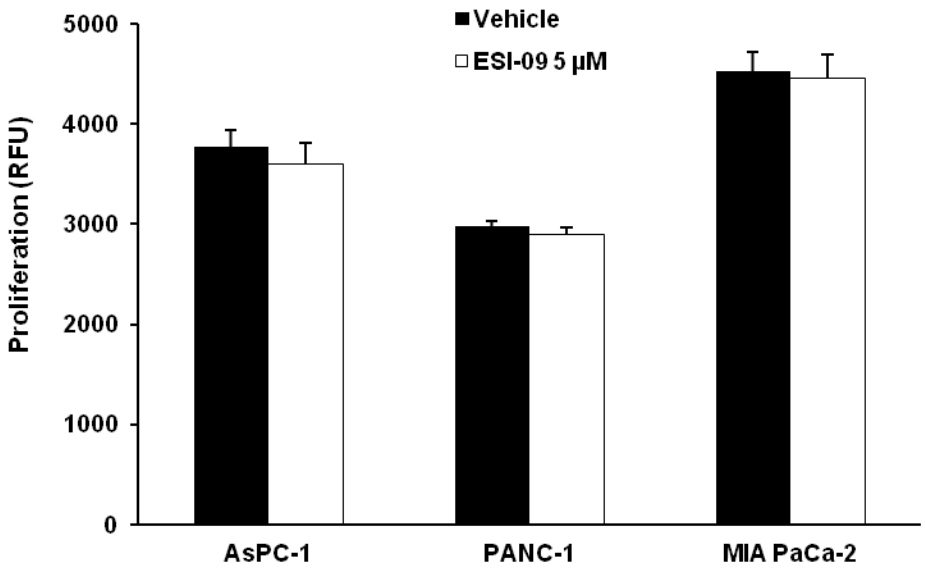

Figure 8. ESI-09 does not affect PDA cell viability. The viability of AsPC-1, PANC-1, and MIA PaCa-2 cells was monitored for 24 hours in the presence or absence of ESI-09 (5 μM) by an alamarBlue metabolic activity assay. RFU, relative fluorescence unit.

EPAC1 promotes trafficking of integrin β1

The expression, distribution, trafficking, and function of integrins are frequently altered in tumor cells in a manner that promotes cancer migration [42]. Integrin β1 is particularly important for invasion of PDA and plays an essential role in facilitating its metastasis [88, 89]. Several reports have implicated EPAC1 and its effector Rap1 in integrin β1-mediated adhesion and migration of endothelial progenitor and immune cells [68, 90]. Hence we hypothesized that EPAC1 facilitates invasion/migration of PDA through an integrin β1-related pathway. Neither activation, nor pharmacologic inhibition or knockdown of EPAC1 altered expression levels of integrin β1 (*Fig. 9A & B*). However, when cells were treated with the EPAC activator 007-AM (45 mins), followed by biotinylation and isolation of surface proteins, the plasma membrane fraction of integrin

β1 was significantly increased, and this observed rise was completely negated by ESI-09 treatment or knockdown of EPAC1 (*Fig. 9C & D*) [91]. In fact, inhibition or suppression of EPAC1 reduced the membrane fraction of integrin β1 below the basal level determined for vehicle-treated parental MIA PaCa-2 cells (*Fig. 9C & D*). Additionally, after cells were trypsinized, recovery of cell surface integrin β1, even after only 15 min, was significantly enhanced by EPAC activation and attenuated by its inhibition or suppression (*Fig. 9E*). These results suggest that EPAC1 facilitates trafficking of integrin β1 to the plasma membrane during invasion/migration.

EPAC1 promotes trafficking of integrin β1 through PKC

PKC regulates integrin trafficking and has been shown to promote migration of cancer cells [92, 93]. Of note, during the integrin trafficking cycle, PKCε mediates the movement of integrin β1 from the perinuclear recycling compartment (PNRC) to the plasma membrane [42]. Numerous studies have shown than EPAC activates PKC in various cell contexts, including PKCε [94, 95]. Therefore, we reasoned that EPAC1 enhances trafficking of integrin β1 to the plasma membrane in part through PKC activation. Similarly to the impact of inhibition and suppression of EPAC1, inhibition of PKC with the PKC-specific inhibitor BIM I had no impact on total integrin β1 expression (*Fig. 10A & B*), but completely abrogated the increase in surface integrin β1 seen with EPAC1 activation by 007-AM (*Fig. 10C & D*). Furthermore, inhibition of PKC negated the rise in invasion/migration observed with EPAC1 activation in transwell invasion/migration and wound-healing assays (*Fig. 10E & F*). To confirm the specificity of the observed response to BIM I treatment, we utilized another PKC-specific inhibitor (NPC 15437). This inhibitor also blocked 007-AM's stimulatory effect on invasion/migration of MIA

PaCa-2 and integrin β1 trafficking (*Fig. 11*). BIM I and NPC 15437 had had no impact on the viability of MIA PaCa-2 cells during the time frame of the essay (*Fig. 12*). Together, these findings suggest that EPAC1 promotes integrin β1 trafficking through the PKC pathway. Noticeably though, PKC inhibition (*Fig 10E & F*) reduced invasion/migration to a lower extent than inhibition of EPAC1 did (*Fig. 7 C & D*). This suggested the presence of a possible additional mechanism by which EPAC1 facilitates invasion/migration of PDA cells.

EPAC1 promotes activation of integrin β1

Integrins are usually present in an inactive conformation that has a low affinity for their ligand. A series of signaling events that involve the recruitment of various adaptor proteins to the cytosolic domain of the integrin are required for activation (inside-outside signaling) [96]. Several reports have shown that Rap1 plays a role in the integrin activation cascade [97, 98]. To determine if EPAC1 facilitates activation of integrin β1 in PDA, we probed the activation status of integrin β1 after EPAC1 activation or inhibition/suppression using an antibody that only recognizes the active conformation of integrin β1 (12G10) [85]. To account for the change in total surface integrin β1 in response altering EPAC1 activity, the fluorescnece intensity of the integrin's active conformation was normalized to the intensity of total surface integrin β1 staining. Activation of EPAC1 by 007-AM significantly increased the fraction of active integrin β1, and inhibition/suppression of EPAC1 lowered it below basal levels (*Fig. 13*). Moreover, inhibition of PKC with NPC 15437 did not affect integrin β1 activation (*Fig. 13*). These results suggest that in addition to regulating integrin trafficking, EPAC1 mediates activation of integrin β1, and this effect is most likely PKC-independent.

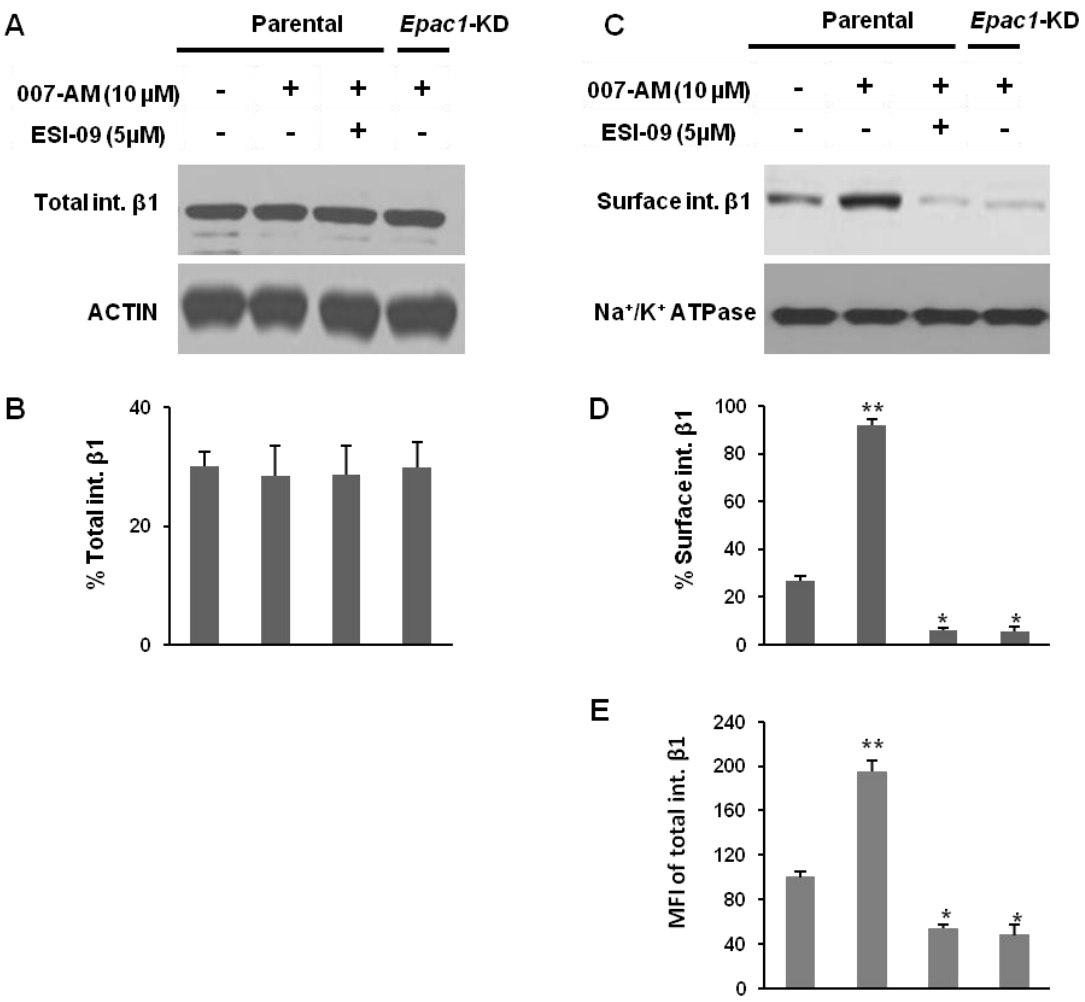

Figure 9. EPAC1 increases trafficking of integrin β1 to the plasma membrane. A-D) MIA PaCa-2 cells were treated with 007-AM in the presence or absence of ESI-09 and total or plasma-membrane proteins were isolated. A and C) Representative plots of total and membrane-bound integrin β1, respectively. Na^+/K^+ ATPase was used as a loading control for the membrane protein fraction. B) and D) Quantification of total and membrane-bound integrin β1, respectively. Integrin β1 levels were determined by densitometry and presented as a percentage of loading control. E) Cells were trypsinized and recovery of surface integrin β1was probed by FACS. Data is presented as mean fluorescence intensity (MFI) and normalized to vehicle treated WT MIA PaCa-2 cells. Bars represent mean ± S.D. (n=3). ** Significantly higher than vehicle-treated WT cells ($P < 0.01$). * Significantly lower than vehicle-treated WT cells ($P < 0.02$).

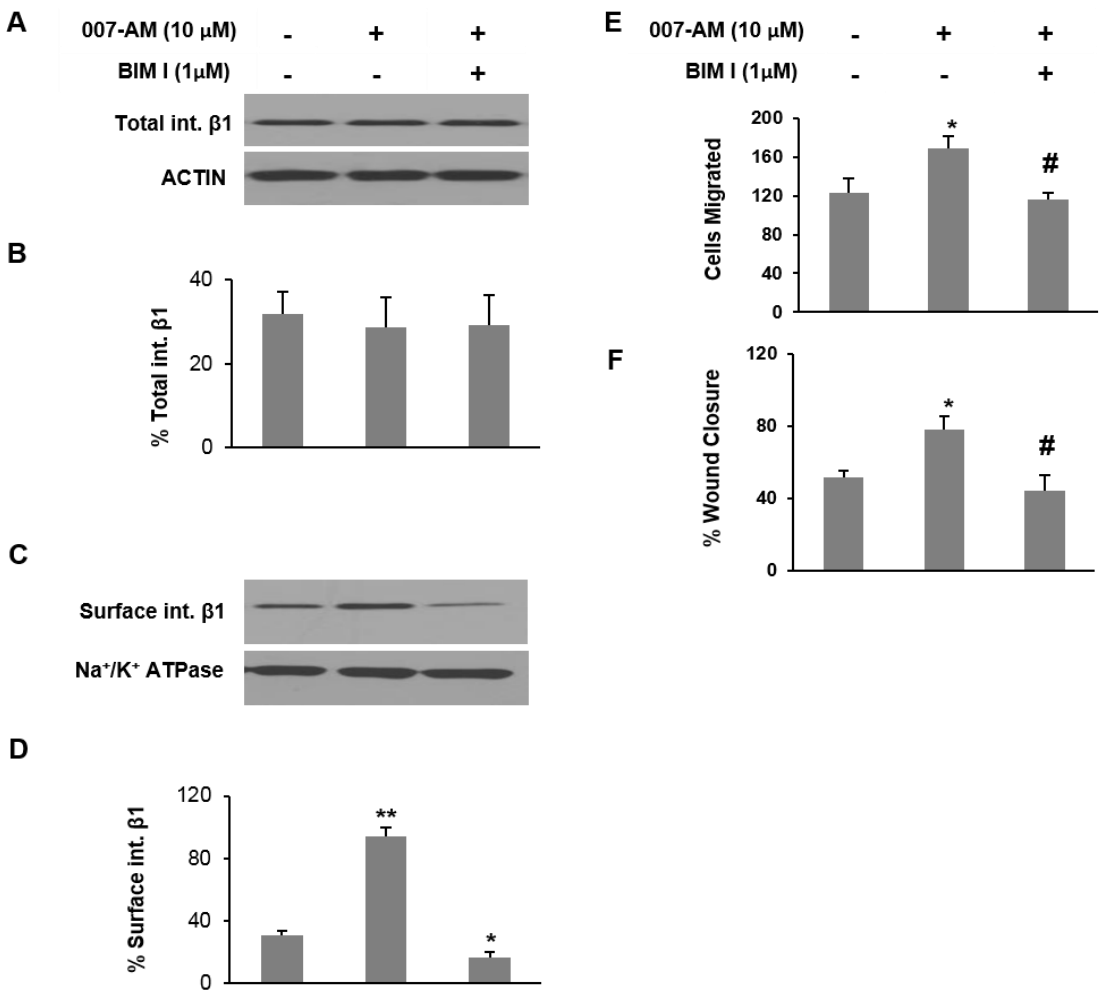

Figure 10. EPAC1 increases trafficking of integrin β1 to the plasma membrane through PKC. MIA PaCa-2 cells were treated with 007-AM in the presence or absence of BIM I. A and C) Representative plots of total and plasma membrane-bound integrin β1, respectively. B and D) Quantification of total and plasma membrane-bound integrin β1, respectively. Integrin β1 levels were determined by densitometry and presented as a percentage of loading control. E) Invasion/migration was examined by a trans-well invasion/migration assay. F) Wound healing rate was examined in a wound-healing assay. Wound-closure rate is presented as the distance traveled by the edge of the wound relative to the wound's initial size. Bars represent mean ± S.D. (n=3). ** Significantly higher than vehicle-treated cells ($P < 0.01$). *Significantly higher or lower than vehicle-treated cells ($P < 0.03$). # Significantly lower than 007-AM treated cells.

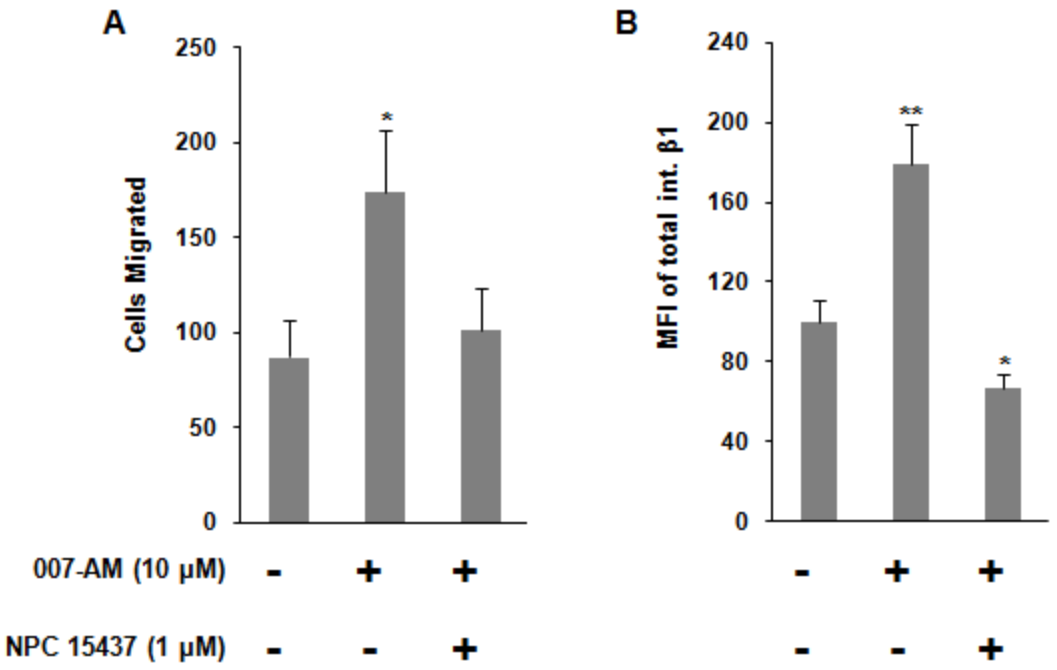

Figure 11. NPC 15437 negates 007-AM's impact on MIA PaCa-2's migration and trafficking of integrin β1. A) Cells were treated with 007-AM in the presence or absence of NPC 15437 and invasion/migration was examined by a transwell migration. B) Cells were trypsinized, treated with 007-AM in the presence or absence of NPC 15437, and recovery of surface integrin β1 was probed by FACS using the integrin β1 antibody K20 by FACS. Mean fluorescence intensity (MFI) was normalized to vehicle-treated cells. Bars represent mean ± S.D. (n=3). ** Significantly higher than vehicle-treated WT cells ($P < 0.02$). * Significantly higher or lower than vehicle-treated WT cells ($P < 0.05$).

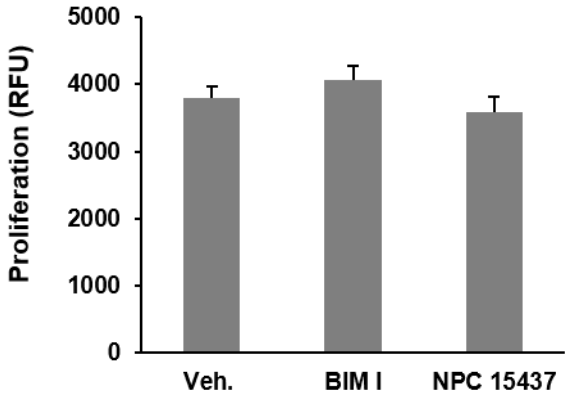

Figure 12. Effects of utilized PKC inhibitors on viability of MIA PaCa-2. Cells were treated with vehicle DMSO, 1 BIM I, or 1 μM NPC 15437 for 24 hrs, and cell viability was determined by an almarBlue metabolic activity assay. RFU, relative fluorescence unit. Bars represent mean ± S.D. (n=3).

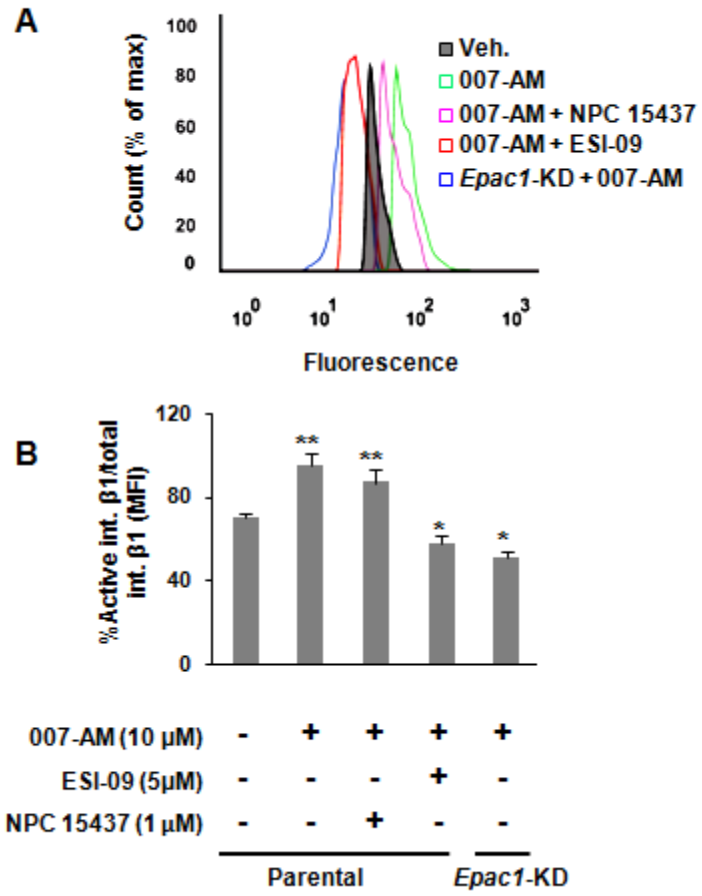

Figure 13. EPAC1 facilitates activation of integrin β1. Cells were treated with 007-AM in the presence or absence of ESI-09 or NPC 15437 and activation of integrin β1 was probed by FACS using the antibody 12G10, which only binds to the active form of integrin β1. Total integrin β1 was probed with the antibody K-20. A) A representative histogram showing the binding of 12G10. B) Quantification of active int. β1 relative to total int. β1 (MFI_{active}/MFI_{total}). Bars represent mean ± S.D. (n=3). ** Significantly higher than vehicle-treated WT cells ($P < 0.03$). * Significantly lower than vehicle-treated WT cells ($P < 0.04$).

Genetic suppression of EPAC1 reduces PDA metastasis

To determine if EPAC1 facilitates PDA invasion and metastasis *in vivo* we utilized an orthotopic PDA metastasis mouse model. WT and *Epac1*-KD MIA PaCa-2 cells were transduced with luciferase and cells with comparable luciferase activity (*Fig. 14A*) were injected into the pancreas of athymic nude mice. Local invasion and metastasis were monitored *in vivo* using bioluminescence imaging, and at the end of the experiment, metastasis was further quantified by the number of metastatic foci in the liver, which is one of the most common sites of PDA metastasis [99]. Suppression of EPAC1 reduced local and distant spread of MIA PaCa-2 cells (*Fig. 14B*) and significantly decreased metastasis to the liver (*Fig. 14C & D*).

Pharmacological inhibition of EPAC reduces PDA metastasis

To determine if inhibition of EPAC1 is a potentially viable therapeutic strategy for reducing metastasis of PDA, we employed the EPAC inhibitor ESI-09. Our group has previously shown that ESI-09 has excellent pharmacological activity *in vivo* and is capable of protecting mice from a lethal dose of rickettsial infection and recapitulating EPAC1 knockout phenotype [100]. Treatment with ESI-09 (10 mg/kg daily starting 2 days post tumor injection) appeared to reduce local and distant spread of MIA PaCa-2 cells and significantly decreased metastasis to the liver (*Fig. 15A & B*). These results suggest that EPAC1 is a potential target for developing anti-metastasis agents for treatment of PDA.

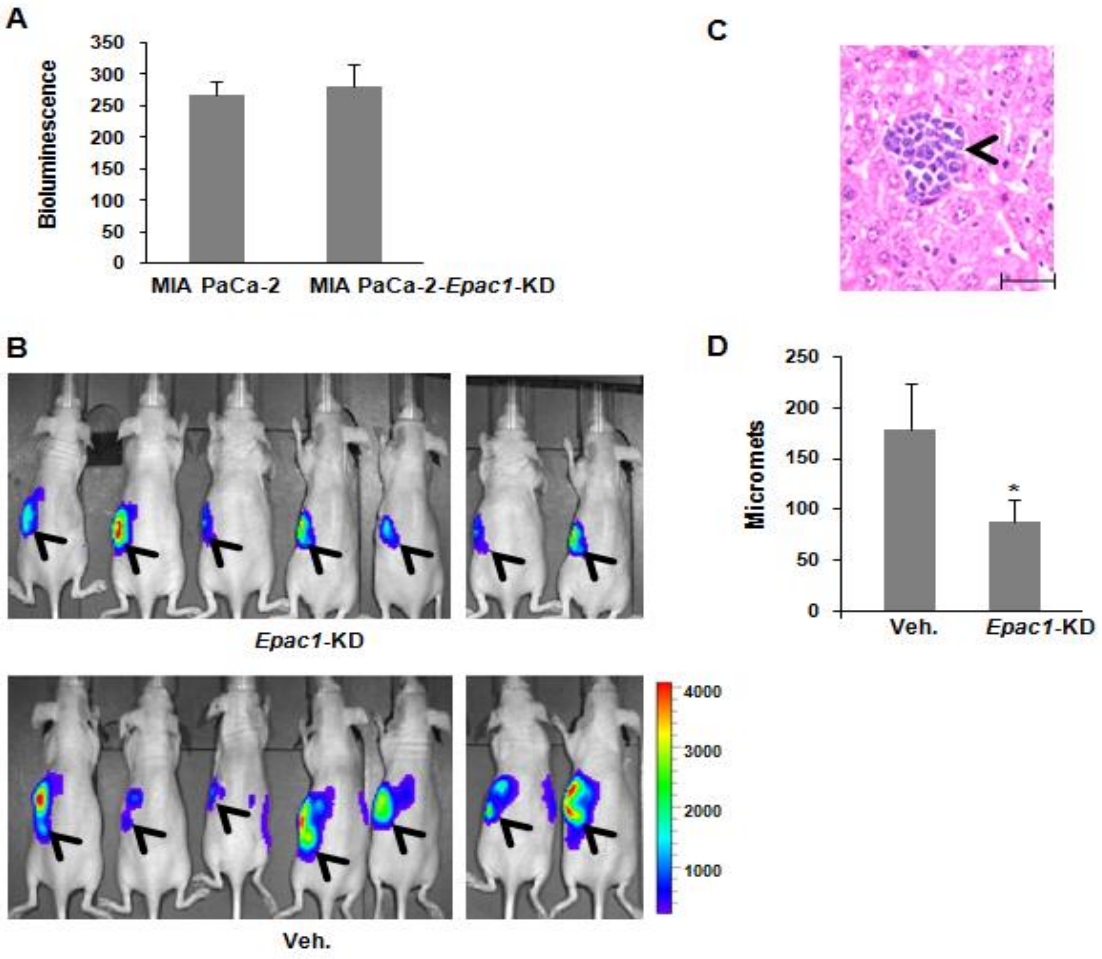

Figure 14. Knockdown of EPAC1reduces PDA invasion and metastasis. A) Luciferase activity in luciferase-transduced WT and *Epac1*-KD MIA PaCa-2 cells was determined by an *in vitro* luciferase assay system. B) Cells were injected into the pancreas of athymic nude mice and *in vivo* bioluminescence imaging was performed 3 weeks post injection. Arrowheads show signal from the primary tumor and local invasion. C) A representative image of H&E staining of the liver showing a metastatic focus of cells (micromets) of MIA PaCa-2 cells (arrowhead), scale bar 10 µm. D) Quantification of liver micromets (number of micromets/H&E slide). For each mouse, the number of micromets is the average of two slides taken ~ 20 µm apart. Bars represent mean ± S.D. (n=7). * Significantly lower than vehicle-treated group ($P < 0.02$).

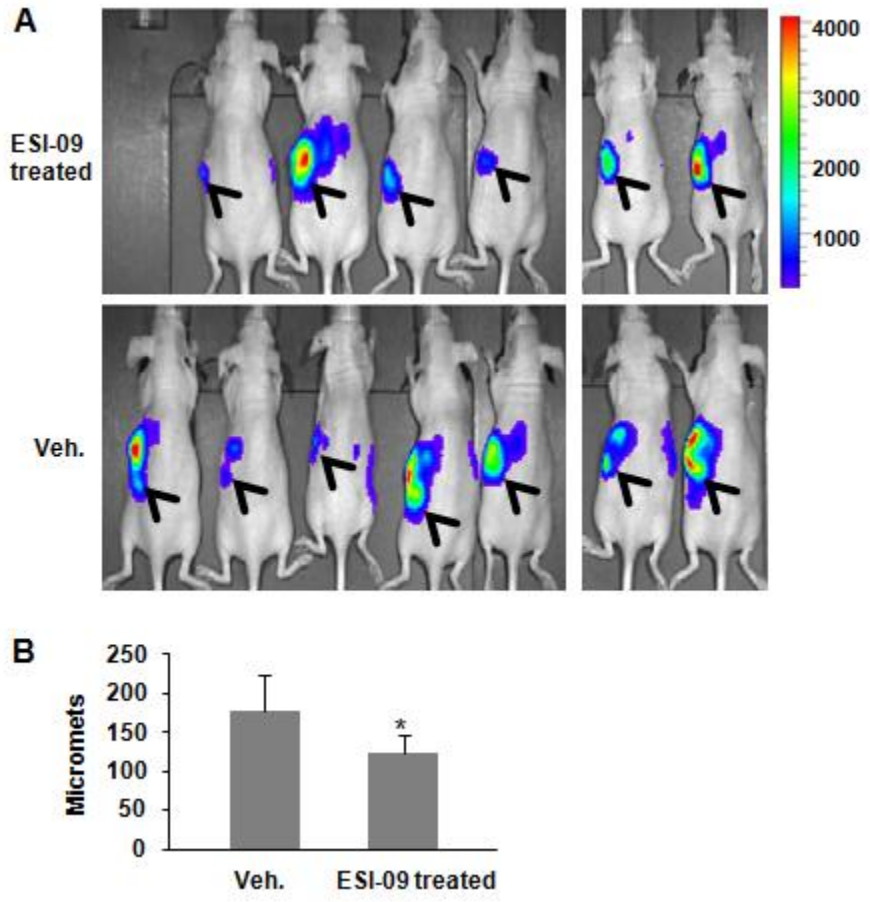

Figure 15. Pharmacologic inhibition of EPAC1 reduces metastasis of MIA PaCa-2. Luciferase-transduced MIA PaCa-2 cells were injected into the pancreas of athymic nude mice and animals were treated with ESI-09 (daily I.P injection of 10 mg/kg) or vehicle. A) *In vivo* bioluminescence image taken 3 weeks post injection of cells. Arrowheads show signal from the primary tumor and local invasion. One mouse in the treatment group died because of an erroneous injection procedure of ESI-09. B) Quantification of liver micromets (number of micromets/H&E slide). For each mouse, the number of micromets is the average of two slides taken ~ 20 µm apart. Bars represent mean ± S.D. * significantly lower than vehicle-treated group ($P < 0.04$).

Discussion

The results presented in this chapter show that EPAC1 facilitates invasion and metastasis of PDA. Genetic suppression of EPAC1 in three different PDA cell lines, AsPC-1, PANC-1, and MIA PaCa-2, inhibited their invasion/migration *in vitro* and the latter's metastasis in an *in vivo* orthotopic metastatic mouse model. This conclusion suggests that EPAC1's pro-migratory effect is a general one in the context of PDA. Although, further studies on other available PDA cell lines and/or cells extracted from patient tumor specimen are needed to definitively determine if EPAC1's role in PDA migration can be generalized. In addition, EPAC1 does not appear to affect proliferation of PDA cells.

Mechanistically, the results of this study suggest that EPAC1 promotes invasion/migration of PDA by enhancing integrin β1 trafficking, which plays a crucial role in controlling cell mobility. During cell migration, integrins are internalized from the cell surface into endosomes and accumulate in the perinuclear recycling compartment (PNRC) before being shuttled to the leading edge of the migrating cell [42, 101]. This process is dynamic and occurs rapidly with each step being tightly regulated by various interactions of a complex network of proteins [101]. The PKC isoform PKCε is particularly important for mediating movement of integrin β1 vesicles from the PNRC pool to the plasma membrane [42]. The PKC-specific inhibitors BIM I and NPC 15437 negated the stimulatory impact of the EPAC agonist 007-AM on trafficking of integrin β1and invasion/migration of PDA. The inhibitor NPC 15437 preferentially targets novel isoenzymes of PKC like PKCε [83]. Therefore, our results suggest that it is through the PKCε pathway that EPAC1 promotes integrin β1 trafficking (*Fig. 16*). This is in

agreement with numerous reports showing EPAC activates PKC, including PKCε, through the PLC/PIP-2 pathway [17, 94, 95].

Interestingly, while the impact of EPAC1 inhibition/suppression on integrin β1 trafficking was mimicked by PKC inhibition, the latter did not suppress invasion/migration of MIA PaCa-2 to the same extent as the former did; prompting us to investigate additional mechanisms underlying EPAC1's role in promoting PDA migration. In addition to integrin trafficking, cells control their adhesion and migration by regulating the activation status of integrins [96]. Integrins usually exist in an inactive confirmation that possesses a low affinity for their ligands, and a series of coordinated intracellular signaling events that involve the recruitment of various adaptor proteins to the cytosolic domain of the integrin are required for activation (inside-outside signaling) [96].

Our results show that EPAC1 facilitates the activation process for integrin β. This is in concordance with several reports showing the EPAC effector Rap1 activates integrins, including integrin β1, through Rap1-GTP-interacting adaptor molecule (RIAM), without affecting the overall surface expression levels of these proteins [102, 103]. RIAM promotes the binding of the cytosolic domain of an integrin to the adaptor protein talin, which is a critical last step during the activation sequence [96]. On the other hand, inhibition of PKC had no impact on the activation status of integrin β1; suggesting EPAC1's role in this mechanism is PCK independent (*Fig. 16*).

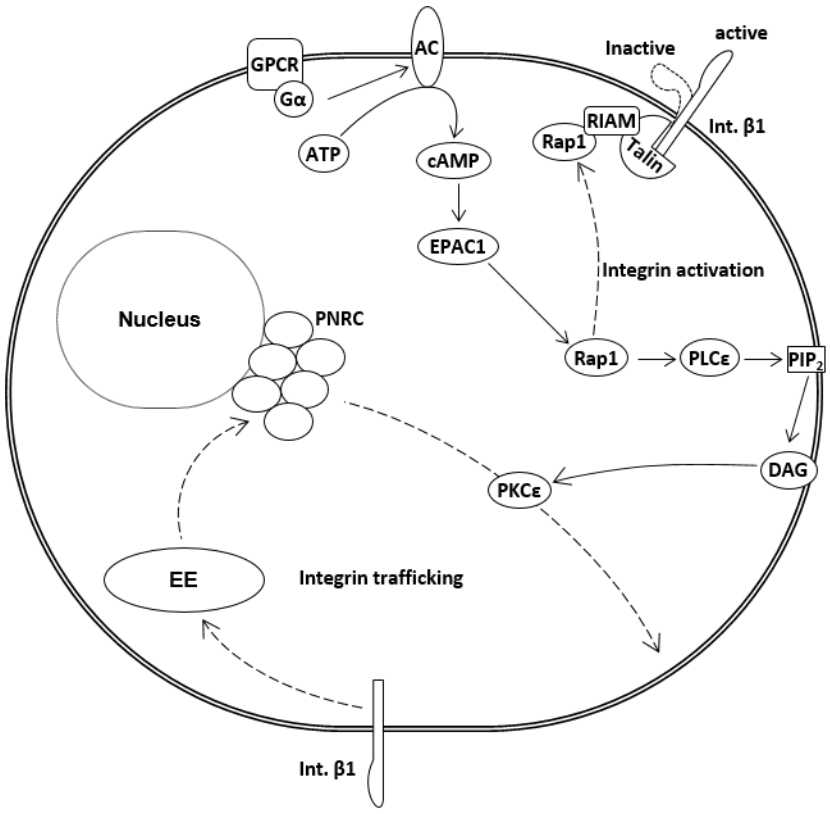

Figure 16. EPAC1 mediates integrin β1 trafficking and activation. cAMP production by adenylyl cyclase (AC) leads to EPAC1 activation, which in turn activates Rap1. Rap1 in conjunction with Rap1-GTP-interacting adaptor molecule (RIAM) and talin leads to activation of integrinβ1. Rap1, through the PLC/DAG pathway, also activates PKCε, which facilitates trafficking of integrin vesicles from the perinuclear recycling compartment (PNRC) to the plasma membrane. EE, early endosomes.

Many proteins show promising potential as therapeutic targets *in vitro*, but prove to be undruggable *in vivo.* To determine the druggability of EPAC1, we utilized the small ESI-09, an EPAC-specific inhibitor recently discovered in our lab [84]. This drug was able to reduce invasion and metastasis of MIA PaCa-2, suggesting that EPAC1 is a potentially viable target for anti-metastasis agents. This finding has significant clinical implications as there is a dire need for mechanism-based therapeutic strategies for pancreatic cancer,

especially ones that target the integrin β1 activation and/or trafficking pathways. Numerous studies have shown that integrin β1 mediates the malignant phenotype of PDA and facilitates the loss of epithelial integrity and oncogenic transformation in epithelial tumors [104-106]. In fact, constitutive activation of integrin β1 is correlated with higher grade carcinomas [105]. There are currently no available small molecules that target integrin β1, but monocolonal antibodies and synthetic peptides against this integrin have shown significant clinical efficacy [107]. Hence, EPAC-inhibitors might provide a new approach to target integrin β1 in cancer treatment.

Studies have shown that functional up-regulation of integrin β1 is one of the main mechanisms that lead to cancer resistance to anti-angiogenic therapies. The functionality of this integrin was significantly enhanced in tumor specimen from patients that exhibited resistance to the angiogenic inhibitor bevacizumab [108]. However, neutralizing integrin β1 monoclonal antibodies were able to ameliorate this resistance in xenograft models [108]. Therefore, it is feasible the combination of EPAC1 inhibitors with anti-angiogenic therapies might be particularly effective in the treatment of PDA.

Chapter 3: EPAC1 Modulates Regulatory T-Cell-mediated Suppression

Introduction

Cyclic adenosine monophosphate (cAMP) is a potent regulator of the immune system, including innate and adaptive immunity [10, 109, 110]. While many of these regulatory functions are transduced by the classic cAMP receptor PKA, recent findings have revealed a crucial role for EPAC1. EPAC1 is expressed in T and B-lymphocytes, monocytes, and macrophages and mediates cAMP's roles in these cells under various contexts in the immune system [10, 111-113]. For example, through Rap1, EPAC1 positively regulates integrin-mediated adhesion of monocytes and T-lymphocytes [90, 114], while in B-lymphocytes it is involved in induction of growth arrest [115], and in macrophages it inhibits Fc receptor-mediated phagocytosis [116, 117].

Several lines of indirect evidence have raised the possibility that EPAC1 exerts broad control over the immune system through mediating the activity of regulatory T-cells (Treg), a subset of T-lymphocytes that helps to maintain peripheral tolerance and regulate the immune response [118]. Treg cells produce high levels of intracellular cAMP and suppress effector T-lymphocytes (Teff) by direct transfer of cAMP through gap junctions (GJ) [64, 119, 120]. It has been suggested that upon transfer to Teff, cAMP, through PKA, activates the inducible cAMP early repressor (ICER), an isoform of cAMP response element modulator (CREM) that is known to suppress Teff cytokines such as IL-2 and IFNγ[121, 122]. A recent study however, showed that ICER deficiency in Teff and/or Treg cells had no significant impact on suppression of the former by the latter, inferring a potential role for EPAC1 in the suppression process [123]. Other findings support

this notion, such as the fact that stimulation of human $CD4^+CD25^+$Treg cells leads to significant elevation of the main EPAC1 effector Rap1 [124], and transgenic mice expressing a constitutively active Rap1 had lower levels of pro-inflammatory cytokines [125, 126]. However, Rap1 has many GEFs [127], and it remains unclear whether EPAC1 is the GEF at play in Treg cells.

Moreover, studies have shown that EPAC1 plays an essential role in the formation of GJ in cardiac cells as it facilitates the accumulation of connexin 43 (Cx43), a subunit of GJ, at the site of formation of these direct intercellular connections [11, 12, 128]. Given that Cx43 is the main GJ subunit in the immune system [129, 130], it is feasible that in addition to mediating cAMP signaling in Treg-mediated suppression, EPAC1 facilitates GJ formation between Treg and Teff. However, these possibilities have not been explored. Shedding light on EPAC1's role in Treg-mediated suppression is critical and has significant therapeutic implications.

In this chapter I will investigate the role of EPAC1 in Treg-mediated suppression using genetic and pharmacologic approaches. I will show that EPAC1 plays a dual role in enhancing Treg suppression of Teff: it boosts Treg suppressive potency while sensitizing Teff to suppression.

Materials and Methods

Antibodies

The following antibodies were used: EPAC1 (# 4155), EPAC2 (# 4156), phospho-STAT3 (Tyr705) (# 9131), STAT3 (# 4904), SHP-1 (# 3759), SHP-2 (# 3397), SOCS3 (# 2923), LCK (# 2752), p-LCK (Try505) (# 2751), JAK2 (# 3230), SMAD2 (# 5339), p-SMAD2 (Ser465/467) (# 3108), and SMAD4 (# 9515) (Cell Signaling Technology, Inc., Danvers, MA). Actin–Cy3 (C 5838) (Sigma-Aldrich, St. Louis, MO). SMAD7 (sc-11392) (Santa Cruz Biotechnology, Dallas, TX).

Mice

The generation of EPAC1 global knockout mice was described previously [131]. Mice used in this study were backcrossed to the C57BL/6 background for more than 10 generations and derived from wild-type or homozygous littermates. The mice were housed with a 12-h–12-h light-dark cycle, with free access to water and food. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch or the University of Texas Health Science Center at Houston.

In vitro suppression assay

Single-cell suspensions were prepared from spleens of 8-10 week old female mice and Teff ($CD4^+CD25^-$) and Treg ($CD4^+CD25^+$) cells were isolated using an EasySep™ Mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada). The purity of Treg was determined by FACS by staining isolated cells with PerCP-Cy 5.5-conjugated anti-CD4 and PE-conjugated anti-CD25 antibodies (BD Biosciences, San Jose, California). Treg cells used were ≥90% pure. 5 × 104Teff cells

were cultured in complete RPMI (RPMI 1640, 10% FCS, 2 mmol/L L-glutamine, penicillin/streptomycin) alone (positive control), or with Treg (2.5× 104). Wells with Treg cells alone were used as a negative control. Plates were pre-coated with 5 µg/mL anti-CD3 antibody and 2 µg/mL soluble anti-CD28 antibody (Life Technologies, Grand Island, NY) was added to the medium. Plates were incubated at 37°C in 5% CO2 for 96 hrs. Where indicated, ESI-09 (5 µM) and/or stattic (50 ng/mL, Sigma-Aldrich, St. Louis, MO) in DMSO were added to the medium. Cell proliferation was determined based on DNA content using a CyQUANT Cell Proliferation Assay (Life Technologies, Grand Island, NY). Teff proliferation was calculated by subtracting the determined cell number from the negative control wells from that determined for the co-culture wells.

Detection of gap junction formation

Regulatory and effector T-cells were incubated with Calcein, AM (2 µM) and Calcein Red-Orange (2 µM) (Invitrogen, Grand Island, New York), respectively, in serum free RPMI-1640 culture medium at 37°C in 5% CO2 for 30 min. The cells were then washed three times and co-cultured as described for the suppression assay at a 1:1 ratio for 24 hrs. GJ formation was determined by the percentage of cells positive for both dyes.

p-STAT3 studies

Single-cell suspensions were prepared from spleens of 10-12 week old female mice and Teff and Treg cells were isolated as described earlier. The isolated cells (3 × 106/well) were then cultured with plate-bound anti-CD3 (5µg/mL) and soluble anti-CD28 (2µg/mL) in complete RPMI 1640 medium at 37°C and 5% CO2. Where indicated, 5 µM ESI-09 was added to the medium. Total cellular proteins were extracted and Western

blotting was performed as described in Chapter 2, and protein expression level was estimated by densitometry.

TGF-β1 studies

Single-cell suspensions were prepared from spleens of 10-12 week old female mice and $CD4^+$ T-cells were isolated using an EasySep™ Mouse $CD4^+$ T-Cell Isolation Kit (Stemcell Technologies, Vancouver, Canada). Isolated cells were stimulated with plate-bound anti-CD3 (5 μg/mL) and soluble anti-CD28 (2 μg/mL) in the presence or absence of TGF-β1 (2 ng/mL) and/or ESI-09 (5 μM) for 17 hr at 37°C and 5% CO2. Total cellular proteins were extracted and the levels of p-SMAD2, SMAD4, and SMAD7 were determined by Western blotting as described in Chapter 2 using the appropriate antibodies. The supernatants were collected and IL-2 levels were determined as described below.

IL-2 ELISA

At the end of their indicated incubation periods, supernatants from the Treg suppression and TGF-β1 sensitivity assays were collected and assayed for IL-2 using a Mouse IL-2 DuoSet Kit (R&D Systems, Minneapolis, MN).

Quantitative real-time PCR

Single-cell suspensions were prepared from spleens of 10-12 week old female mice and $CD4^+$ T-cells were isolated as described earlier. Isolated cells were stimulated with plate-bound anti-CD3 (5 μg/mL) and soluble anti-CD28 (2 μg/mL) for 17 hr at 37°C and 5% CO2. Then total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA synthesis was performed using 1 μg of total RNA with M-MLV MultiScribe™ Reverse Transcriptase (Life Technologies, Grand Island, NY) and

quantitative real-time PCR (qRT-PCR) was performed using iTaq™ Universal SYBR®GreenSupermix (Bio-Rad, Hercules, CA) in a LightCycler thermal cycler (Roche,Nutley, NJ). The amplification program included the initial denaturation step at 95 °C for 2 min, and 40 cycles of denaturation at 95 °C for 15 s, annealing at 62 °C for 15 s, and extension at 68 °C for 30 s. Gapdh was used as an internal control to which the target gene signal was normalized. The sequences of the primers used for qRT-PCR are listed in Table 1.

Table 1. qRT-PCR primer sequences.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
Smad7	CCTCCTCCTTACTCCAGATACC	TCTTGGACACAGTAGAGCCTC
Smad4	TGGATGGACGACTTCAGGTG	AGGTGAGACAACCCGCTCAT
Gapdh	TGCACCACCAACTGCTTAG	GCAGGGATGATGTTCTGG

Oral immunization with ovalbumine

Prior to immunization, mice were orally administered 0.2 mL of an isotonic bicarbonate solution (8 parts Hanks' balanced salt solution and 2 parts of 7.5% sodium bicarbonate) to neutralize stomach acidity. Mice were then orally primed with ovalbumine (OVA) (100 μg in 0.2 mL of PBS) on day 1 and boosted with the same dose on day 21. For ESI-09 treatment, mice were orally gavaged with the drug (50 mg/kg in vegetable oil). Drug treatment was initiated 3 days prior to priming and continued daily until 2 days after boosting. Mice were bled on day 28 (one week after boosting). All animals used were 8-12 week old female mice.

Determining total and OVA-specific IgG

For direct ELISA assays, 96-well plates (Corning Life Sciences, Lowell, MA) were coated with ovalbumine (Grade V, Sigma-Aldrich, St. Louis, MO, 3 μg/mL, 100 μL/well) overnight at 4 ˚C. Plates were then incubated with blocking buffer (100 μL 1% BSA in PBS) for 2 hrs at room temperature. After blocking, sera from OVA-immunized mice, diluted in dilution buffer (PBS/0.05% Tween-20), were added (100 μL/well) and incubated for 2 hrs at room temperature. HRP-conjugated secondary anti-mouse IgG antibody (100 μL/well 1:2500 diluted, Cell Signaling Technology, Inc., Danvers, MA) was added and incubated at room temperature for 1 hr. Plates were then incubated with 100 μL per well of o-phenylenediamine (OPD) (Sigma-Aldrich, St. Louis, MO). Optical density was determined at 450 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA). Total serum-IgG levels from naïve 10-12 week old female mice were determined using the same described assay, but the plates were initially coated with anti-mouse IgG (BioLegend, San Diego, CA).

Immunophenotyping of spleen cells

Spleen cells were isolated from 10-12 week old female WT and *Epac1*$^{-/-}$ mice and stained with the indicated surface markers (BD Biosciences, San Jose, California) using standard flow cytometry staining protocols. Cells were sorted using a BD FACSCalibur Flow Cytometr. Six mice were used for each group.

Statistical Analysis

Student t test was used for data analysis in this study and results were considered as statistically significant if P values were <0.05.

Results [5]

EPAC1 regulates Treg-mediated suppression

To address whether EPAC1 modulates Treg cell function, we generated *Epac1*$^{-/-}$ mice [131]. We confirmed the deletion of EPAC1 in CD4^{+} T-cells (*Fig. 17A*) and verified that EPAC2 level was low and not up-regulated in T-cells as a result of EPAC1 deletion (*Fig. 17B*). The suppressive potency of WT and *Epac1*$^{-/-}$ Treg was examined using a standard in *vitro assay*, in which proliferation of CD4^{+}CD25^{-} T cells (Teff) was monitored in the presence or absence of CD4^{+}CD25^{+}Treg cells (*Fig. 17C*). *Epac1*$^{-/-}$and WT Teff proliferated at the same rate when stimulated with anti-CD3/CD28 alone. As expected, the addition of WT Treg suppressed the proliferation of WT Teff significantly (~75% suppression). However, the addition of *Epac1*$^{-/-}$ Treg only suppressed WT Teff by ~45%, indicating an attenuated suppressive potency for *Epac1*-deficient cells. On the other hand, the ability of WT Treg cells to suppress *Epac1*$^{-/-}$ Teff was also compromised (~50% suppression), and combining *Epac1*$^{-/-}$ Treg and *Epac1*$^{-/-}$ Teff resulted in the least amount of Teff suppression (~20%). To confirm our observations of EPAC1's impact on Treg-mediated suppression based on genetic manipulation, we repeated the suppression assay using the EPAC-specific inhibitor ESI-09 [84]. Pharmacologic inhibition recapitulated the effect of EPAC1 deletion and reduced Treg-mediated suppression significantly (~25% suppression) [132].

[5] All figures and data in this chapter were originally published in Almahariq M, Mei FC, Wang H, Cao AT, Yao S, Soong L, Sun J, Cong Y, Chen J, Cheng X: Exchange Protein Directly Activated by cAMP Modulates Regulatory T-cell-Mediated Immunosuppression. *Biochemical Journal* 2015;465 (2):295-303

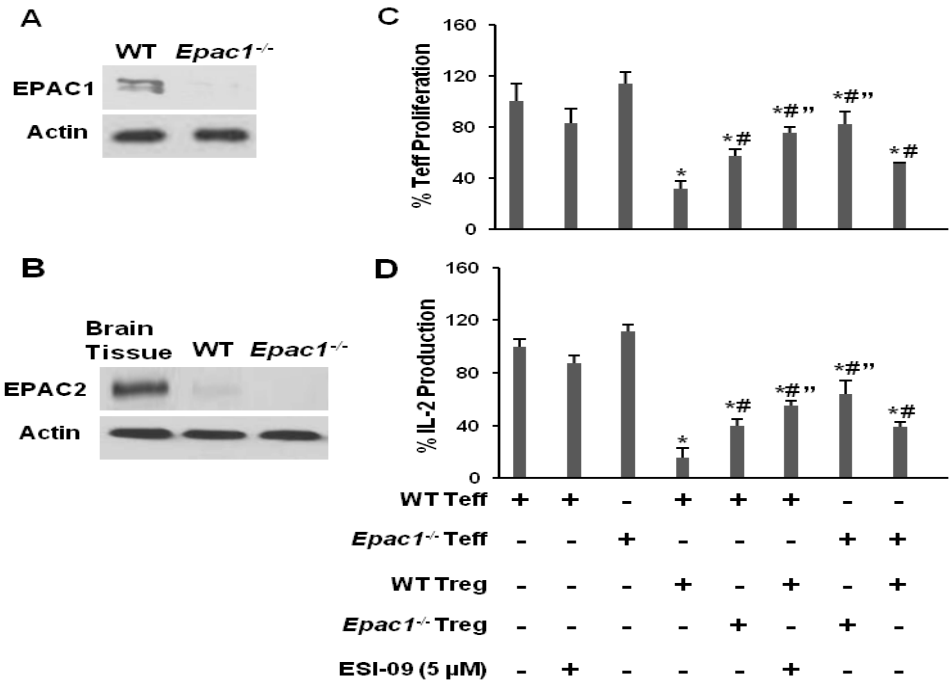

Figure 17. Deletion or inhibition of EPAC1 diminishes Treg-mediated suppression. A and B) $CD4^+$ T-cells were isolated from 10-12 week old female WT and *Epac1*$^{-/-}$ mice and probed for EPAC1 and EPAC2 expression. Brain tissue was used as a positive control for detection of EPAC2. C) Teff cells (5 × 104) were stimulated with plate-bound anti-CD3 (5 µg/mL) and soluble anti-CD28 (2 µg/mL) in the presence or absence of Treg cells (2.5 × 104) and/or ESI-09 (5 µM). Proliferation was measured by determining DNA content 96 hrs later. Samples containing only Treg were used as negative controls. D) At the end of the proliferation assay, the concentration of IL-2 in the supernatants was measured by ELISA. Data from the Teff only samples (positive controls) was normalized to the WT Teff positive control. Data from the co-culture assays was normalized to the positive control matching the genotype of the Teff cells in the assay. Bars represent mean ± S.D. of at least 3 experiments. * Significantly lower than the corresponding positive control samples ($P < 0.05$). # Significantly higher than WT Teff/WT Treg co-culture samples ($P < 0.05$). " Significantly higher than mixed WT/*Epac1*$^{-/-}$co-culture samples ($P < 0.05$).

Since Treg are also known to inhibit Teff's production of IL-2 [55], an important cytokine for T-cell proliferation, we examined the concentration of this cytokine in the co-culture assays. Treg suppression of IL-2 production by Teff followed a pattern similar to that described for suppression of Teff proliferation (*Fig. 17D*). Together, these results indicate that EPAC1 plays a role in regulating both Treg suppressive potency and Teff susceptibility to Treg-mediated suppression.

EPAC1 has no impact on GJ mediated cAMP transfer between Treg and Teff

Treg cells are known to suppress Teff cells by direct transfer of cAMP through gap junctions (GJ) [64, 119, 120], and EPAC1 has been shown to play an essential role in the formation of GJ by facilitating the trafficking of their subunits [11]. We hypothesized that EPAC1 might regulate the formation of GJ between Treg and Teff cells. However, GJ-mediated communication between Treg and Teff cells was not affected by inhibition or deletion of EPAC1, as determined by transfer of the membrane-impermeable dye calcein (*Fig. 18*). These results suggest it is unlikely that cAMP transfer between Treg and Teff is affected by EPAC1.

EPAC1 modulates T cell sensitivity by attenuating STAT3 activation

Our group and others have previously shown that EPAC1 weakens activation (phosphorylation) of signal transducer and activator of transcription 3 (STAT3) in non-immune cells [131, 133]. STAT3 is a known downstream signaling target of the T-cell receptor (TCR)[134, 135], and its activation in Treg and Teff has been shown to decrease suppression of the latter by the former [136]. Hence, we reasoned that EPAC1 could affect Treg-mediated suppression by attenuating STAT3 signaling. We examined STAT3 activation levels by measuring phospho-STAT3 (p-STAT3-Tyr705) in *Epac1*$^{-/-}$ Treg and

Teff to determine if EPAC1 alters STAT3 signaling in these cells. Upon activation of the T-cell receptor, p-STAT3 levels increased to a significantly higher level in both *Epac1*$^{-/-}$ Treg and Teff cells, and were sustained longer when compared to their WT counterparts (*Fig. 19A*). Furthermore, addition of stattic, an inhibitor of STAT3 activation, enhanced Treg suppressive potency and abolished *Epac1*$^{-/-}$ Teff's resistance to Treg suppression (*Fig. 19B*). Additionally, stattic treatment negated the effect of the EPAC-specific inhibitor ESI-09 on Treg activity (*Fig. 19B*). Taken together, these findings suggest that EPAC1 increases Treg suppressive potency and enhances Teff sensitivity to suppression by reducing the level of STAT3 phosphorylation in these cells.

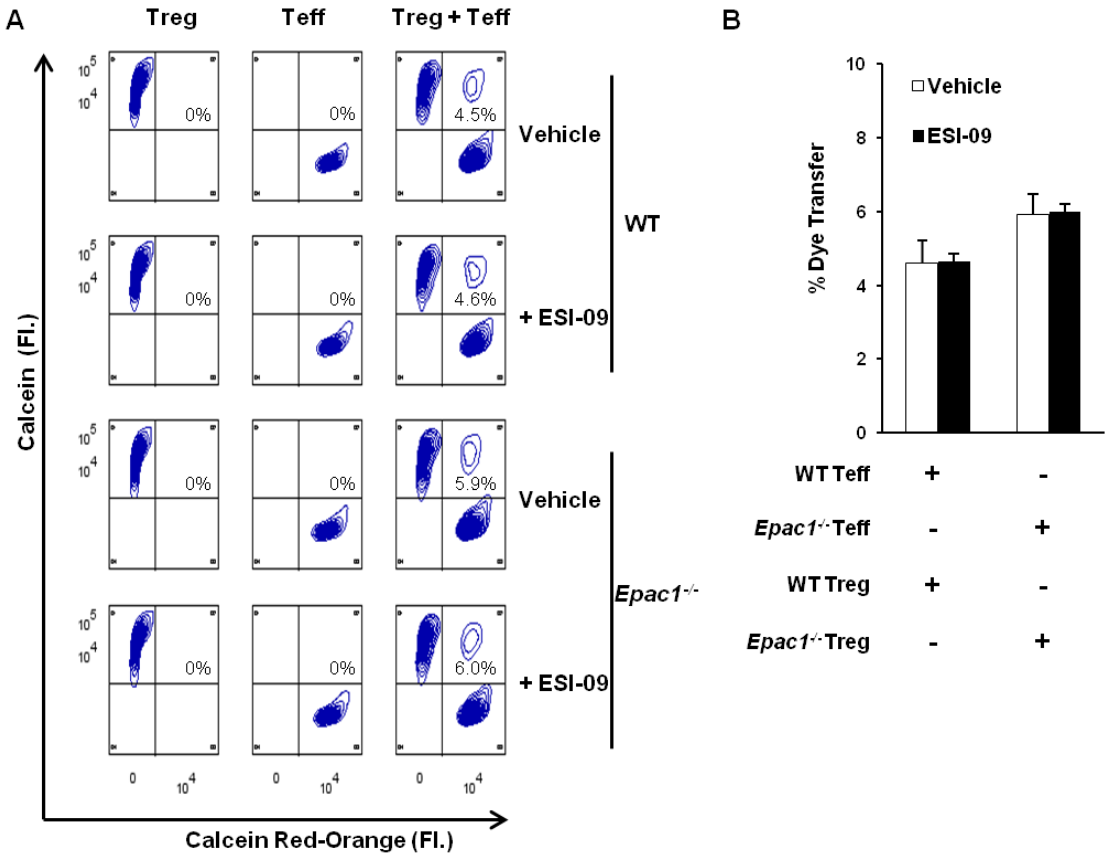

Figure 18. Deletion or inhibition of EPAC1 has no impact on gap junction (GJ) mediated intercellular communication between Teff and Treg cells. Treg and Teff cells were loaded with Calcein-AM and Calcein Red-Orange-AM, respectively, and co-cultured for 24 hrs at a 1:1 ratio under plate-bound anti-CD3 (5 µg/mL) and soluble anti-CD28 (2 µg/mL) stimulation. Cellular transfer between Treg and Teff cells was determined by the percentage of cells positive for both dyes. A) Representative plots showing dye transfer between Treg and Teff. B) Quantification of dye transfer from three experiments. Bars represent mean ± S.D.

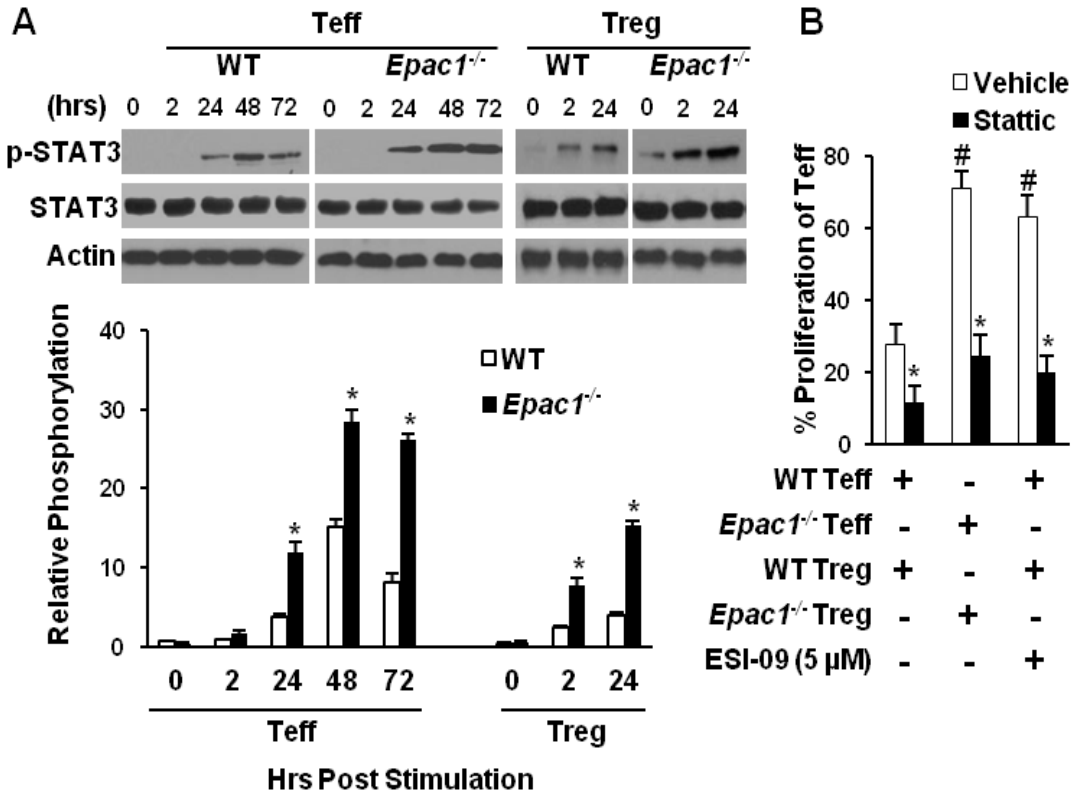

Figure 19. EPAC1 modulates Treg-mediated suppression through the STAT3 pathway. A) Teff or Treg cells were stimulated with plate bound anti-CD3 (5 µg/mL) and soluble anti-CD28 (2 µg/mL) and p-STAT3 (Tyr705) was probed by Western blotting at the indicated time points (one representative blot is shown). p-STAT3 levels were determined by densitometry and expressed as a percentage of total STAT3. * Significantly higher than WT counterpart ($P < 0.01$). B) The suppression assay was carried out as described in Fig. 17 in the presence or absence of the STAT3 inhibitor stattic (50 ng/mL) and/or ESI-09 (5 µM). # Significantly higher than vehicle treated WT cells ($P < 0.01$). * Significantly lower than vehicle treated counterpart ($P < 0.05$). Bars represent mean ± SD of at least three experiments.

EPAC1 regulates STAT3 activation by an atypical mechanism

Phosphorylation of STAT3 in response to cytokine and TCR stimulation is typically attenuated by suppressor of cytokine signaling 3 (SOCS3) [137, 138] and the protein tyrosine phosphatases (PTP) SHP-1 and SHP-2 [139-141]. We hypothesized that the lack of EPAC1 might potentially alter the induction of these proteins. However, the levels of SOCS3 induction were similar in WT and *Epac1*$^{-/-}$ CD4^{+} T-cells, while SHP-1 and SHP-2 were unexpectedly higher in *Epac1*$^{-/-}$ cells (*Fig. 20A*).

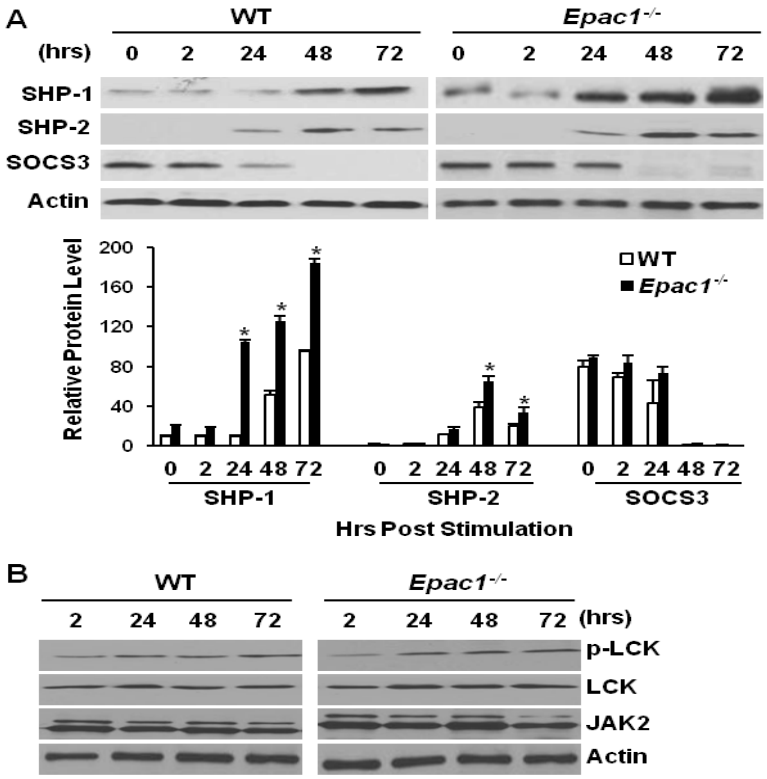

Figure 20. EPAC1 alters STAT3 activation independently of canonical regulatory pathways. A and B) CD4^{+} T-cells were stimulated with plate bound anti-CD3 (5 µg/mL) and soluble anti-CD28 (2 µg/mL) and the indicated proteins were probed by Western blotting at the indicated time points (one representative blot is shown). p-LCK (Tyr505). * Significantly higher than WT counterpart ($P < 0.01$). Data presented as a percentage of the loading control actin and bars represent mean ± S.D. (n=3).

We further examined the levels of JAK2, the main activator of STAT3 in response to cytokine stimulation, and activation of LCK (p-LCK-Try505), the main kinase triggering STAT3 phosphorylation downstream of TCR stimulation [141, 142]. However, there was no difference between WT and *Epac1*$^{-/-}$ T cells (*Fig. 20B*). These results suggest that EPAC1 most likely exerts its impact on STAT3 independently of the canonical regulatory pathways of STAT3 activation.

EPAC1 sensitizes CD4^{+} T-cells to TGF-β1 signaling

Several recent studies have shown that TGF-β1 plays a significant role in Treg-mediated suppression [143-145]. Not only does TGF-β1 serve to suppress Teff, but it also maintains the suppressive capacity of Treg through autocrine signaling [146]. Given the well documented cross-talk between STAT3 and TGF-β1 signaling [147-149] and that *Epac1*$^{-/-}$ have elevated levels of p-STAT3 upon T-cell activation, we hypothesized that EPAC1 might modulate TGF-β1 sensitivity in T-cells.

TGF-β1 is known to suppress IL-2 production by T-cells [150], so we measured WT and *Epac1*$^{-/-}$ Teff's sensitivity to TGF-β1 by determining their IL-2 production in the presence or absence of TGF-β1. While TGF-β1 suppressed IL-2 production by ~90% in WT Teff, it only suppressed production by ~50% and ~60% in *Epac1*$^{-/-}$ Teff and ESI-09 treated WT Teff, respectively (*Fig. 21A*). TGF-β1 induced phosphorylation of SMAD2 was also significantly inhibited in WT CD4^{+} T-cells after the addition of ESI-09 and in *Epac1*$^{-/-}$ CD4^{+} T-cells (*Fig. 21B*), indicating a reduced TGF-β1 response. Furthermore, upon TCR stimulation, SMAD7, which inhibits TGF-β1 signaling, rose to significantly higher levels at the mRNA and protein levels in *Epac1*$^{-/-}$ CD4^{+} T-cells and ESI-09-treated WT CD4^{+} T-cells, compared to their vehicle-treated WT counterparts (*Fig. 21C & D*).

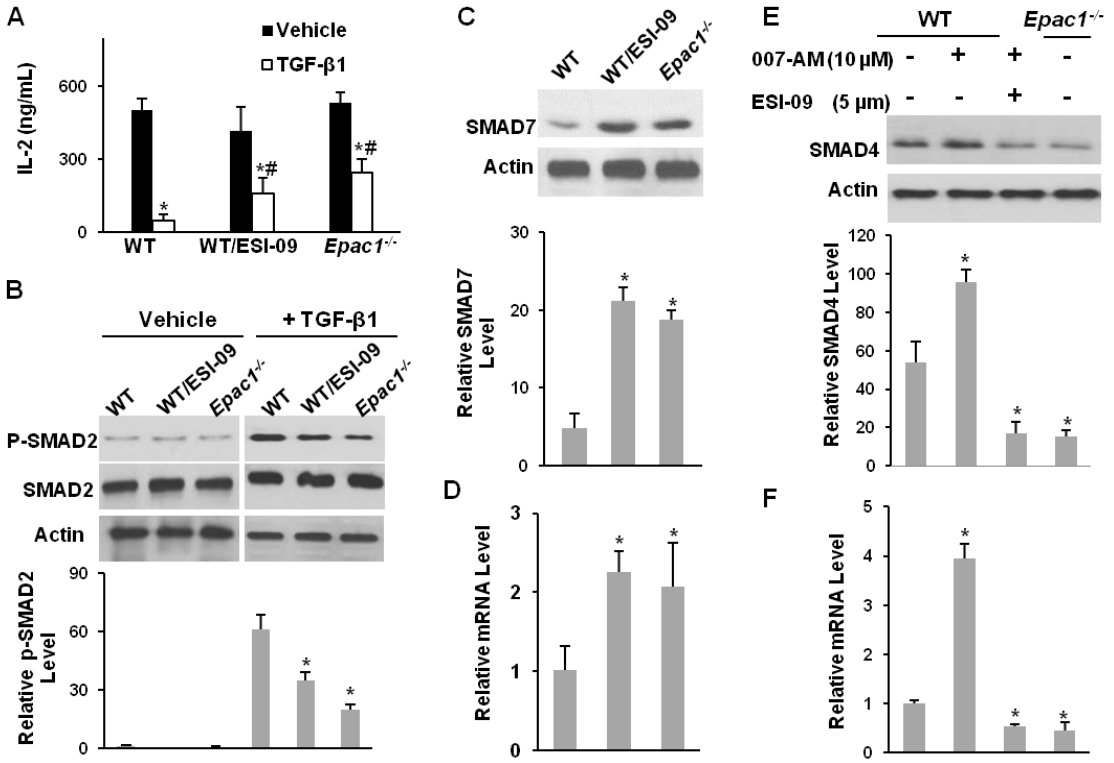

Figure 21. Inhibition of EPAC1 leads to resistance to TGF-β1 signaling. $CD4^+$ T-cells were stimulated with plate bound anti-CD3 (5 μg/mL) and soluble anti-CD28 (2 μg/mL) for 17 hrs in the presence or absence of TGF-β1 (2 ng/mL), ESI-09 (5 μM), and 007-AM (10 μM) as indicated. A) Supernatants were assayed for IL-2 concentration by ELISA. * Significantly lower than vehicle treated counterpart ($P < 0.03$). # Significantly higher than TGF-β1-treated WT cells ($P < 0.05$). B) The level of p-SMAD2 was determined by Western blotting (one representative blot is shown) and expressed as a percentage of total SMAD2. * Significantly lower than TGF-β1-treated WT cells ($P < 0.02$). C-F) The protein and mRNA levels of SMAD7 or SMAD4 were determined by Western blotting (one representative blot is shown) and qRT-PCR, respectively. Protein levels were expressed as percentage of the loading control actin. mRNA levels were expressed relative to *Gapdh*RNA levels. * Significantly higher or lower than vehicle treated WT cells ($P < 0.03$). Bars represent mean ± S.D. (n=3).

We also examined the level of SMAD4, which is essential for nuclear translocation of activated SMAD2 in response to TGF-β1 stimulation. We found that EPAC1 regulates the mRNA and protein levels of SMAD4. Stimulation of $CD4^+$ T-cells in the presence of the EPAC agonist 007-AM significantly increased the mRNA and protein levels of SMAD4, while pharmacologic inhibition or genetic deletion of EPAC1 significantly reduced SMAD4 levels (*Fig. 21E & F*). Together, the results presented here suggest that EPAC1 potentially regulates TGF-β1 signaling in $CD4^+$ T-cells by multiple mechanisms including regulation of p-STAT3 and SMAD4 levels.

Epac1$^{-/-}$ mice are resistant to developing oral tolerance

To test whether EPAC1 might regulate Treg-mediated suppression *in vivo*, we utilized a mouse model in which mice are administered a low dose of ovalbumine (OVA) orally. This model has been shown to induce activation and expansion of antigen-specific Treg, which actively suppress the immune response to OVA and lead to lower levels of IgG production [144, 151-153]. As expected, intragastric administration of low dose OVA (100 μg) led to a weak response in WT mice as determined by serum OVA-IgG levels (*Fig. 22A*). Conversely, a significantly higher level of OVA-IgG was detected in *Epac1*$^{-/-}$mice (*Fig. 22A*). To confirm that the altered immune response was a direct consequence of EPAC1 suppression, rather than a compensatory mechanism as a result of global knockdown of EPAC1, we inhibited EPAC pharmacologically by feeding WT mice with an EPAC-specific inhibitor, ESI-09 [84, 154]. Similarly to genetic deletion of EPAC1, pharmacologic inhibition also resulted in a higher anti-OVA IgG titer (*Fig. 22A*). Furthermore, the basal level of total serum-IgG was higher in naïve unchallenged *Epac1*$^{-/-}$ mice than in their WT counterparts (*Fig. 22B*), indicating a generally heightened immune

response in the absence of EPAC1. Taken together, these findings support a potential role for EPAC1 in modulation of Treg activity *in vivo*. While we cannot rule out other possible roles for EPAC1 in controlling the immune response, we found EPAC1 to be dispensable for immune cell development as similar populations of various immune cells were detected in WT and *Epac1*$^{-/-}$ mice (Table 2).

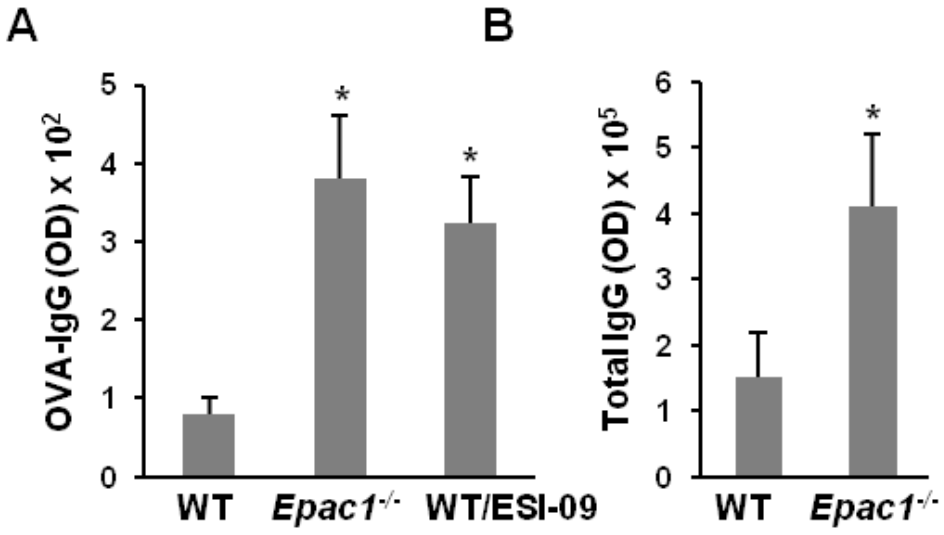

Figure 22. Deletion or inhibition of EPAC1 heightens the immune response. A) 10-12 week old female mice were administered 100 µg OVA orally on days 1 and 21. ESI-09 treated mice were administered the drug daily (50 mg/kg orally). Serum was collected on day 28 and anti-OVA IgG levels were determined by ELISA (n=5). B) Serum was collected from naive 10-12 week old female mice and total IgG levels were determined by ELISA (n=8). Data is shown as means ± SD. * Significantly higher than WT group ($P < 0.05$).

Table 2. Immunophenotyping of spleen cells. Spleen cells were isolated from 10-12 week old female WT and *Epac1*$^{-/-}$ mice and stained with the indicated surface markers.

Cell Population	Surface Markers	% of Spleen Cells	
		WT	***Epac1*$^{-/-}$**
CD4$^+$ T-cells	CD3$^+$CD4$^+$	17.6 ± 2.7	16.8 ± 3.5
Regulatory T-cells	CD4$^+$CD25$^+$	0.9 ± 0.1	1.1 ± 0.2
CD8$^+$ T-cells	CD3$^+$CD8$^+$	5.9 ± 0.9	6.2 ± 1.7
B-cells	CD3$^-$CD19$^+$	50.8 ± 6.2	53.5 ± 3.1
Natural Killer T-cells	CD3$^+$NK1.1$^+$	0.9 ± 0.2	0.8 ± 0.1
Natural Killer Cells	CD3$^-$NK1.1$^+$	2.3 ± 0.3	2.2 ± 0.4
Dendritic Cells	CD11c$^+$	4.5 ± 0.7	4.4 ± 1.0
Neutrophils	CD11b$^+$Ly6G$^+$F4/80$^-$	1.1 ± 0.1	1.1 ± 0.3
Monocytes/Macrophages	CD11b$^+$Ly6C$^+$Ly6G$^-$	1.5 ± 0.2	1.5 ± 0.2

Discussion

Recent studies have suggested that some of cAMP effects in Treg cells, a subset of T-cells that exert a broad regulatory suppressive function in the immune system, are regulated independently of PKA [122, 123, 125]. However, the potential role of the other major cAMP sensor, EPAC, had not been directly investigated. Our current study revealed that EPAC1, the EPAC isoform expressed in Treg and Teff cells [123], plays an essential role in Treg-mediated suppression.

We examined Treg-mediated suppression by an *in vitro* suppression assay using genetic and pharmacologic approaches to suppress or inhibit EPAC1. The suppressive capacity of Treg cells was reduced in the absence of EPAC1. Furthermore, the lack of EPAC1 in Teff rendered them resistant to suppression by Treg, and inhibition/suppression of this protein in both cell populations had an additive effect on compromising Treg-mediated suppression. Our findings highlight a critical role for EPAC1 in Treg-mediated suppression; in agreement with a recent report showing that significant inhibition of the PKA pathway has no impact on Treg suppression [123].

One of the main contact-dependent suppression mechanisms employed by Treg cells is the direct transfer of cAMP into Teff cells through gap junctions [64, 119, 120], EPAC1 facilitates the accumulation of the ubiquitous subunit Cx43 at the site of GJ formation in cardiac cells [11]. Cx43 is the main connexin in the immune system [129, 130], and thus we deemed it possible that EPAC1 mediates cAMP transfer between Treg and Teff cells by maintaining GJ permeability. Since formation of GJ requires trafficking of connexins in both cell types, we hypothesized that a lack of EPAC1 in Treg or Teff would partially hinder their communication, while a lack of EPAC1 in both Treg and Teff would have an

additive negative impact. However, this possibility is unlikely since neither genetic deletion, nor pharmacologic inhibition of EPAC1 reduced GJ communication between these cells as determined by membrane impermeable dye transfer.

On the other hand, our results show that EPAC1 regulates Treg-mediated suppression through the STAT3 and TGF-β1 pathways. In the absence of EPAC1, both Treg and Teff cells had higher levels of p-STAT3, and inhibition of STAT3 activation restored the potency of *Epac1*$^{-/-}$ Treg-mediated suppression. These results are consistent with previous reports showing that activation of STAT3 in Treg and Teff synergistically leads to desensitization to suppression of the latter by the former [136, 155, 156]. The levels of SMAD7, which inhibits TGF-β1 signaling, were elevated after suppression/inhibition of EPAC1, in concordance with previous studies showing hyper-activation of STAT3 leads to desensitization to TGF-β1 signaling by inducing SMAD7 [147, 149]. Consequently, in the absence of EPAC1, CD4^{+} T-cells were resistant to TGF-β1 as demonstrated by the lower activation levels of SMAD2 and reduced IL-2 production in response to stimulation by TGF-β1.

Interestingly, we found that even in the absence of TGF-β1 stimulation, activation of EPAC1 induced expression of SMAD4, while inhibition/deletion of EPAC1 blunted its expression. These results suggest the presence of a previously unidentified connection between EPAC1 and SMAD4. The lower levels of SMAD4 in EPAC1 deficient CD4^{+} T-cells most likely contributed to their resistance to TGF-β1 stimulation.

Treg cells utilize membrane-bound TGF- β1 as one of their main mechanisms of suppressing Teff cells -β1 [143, 157, 158]. Concurrently, this cytokine also serves to maintain the suppressive potency of Treg-cells through autocrine and paracrine signaling [146].

Therefore, our data are consistent with a model in which EPAC1, through attenuation of p-STAT3 and promotion of SMAD4 expression, plays an essential role in sensitizing Treg and Teff cells to TGF-β1 signaling, and as such, its inhibition in both cells has an additive impact on compromising Treg-mediated suppression.

Our results show that EPAC1 regulates STAT3 activation independently of the canonical regulatory loops involving SOCS3 and SHP-1/2. A recent study showed that SMAD4 inhibits STAT3 phosphorylation in non-immune cells [159]. Hence, it is feasible that by inducing SMAD4, EPAC1 indirectly blunts STAT3 phosphorylation. Additionally, studies have shown that TGF-β1 signaling, through SMAD2, inhibits STAT3 activation and nuclear translocation [148]. Therefore, it is possible that EPAC1 mediates a regulatory loop in which it promotes expression of SMAD4, which maintains low levels of p-STAT3 and consequently reduces SMAD7 levels; leading to additional up-regulation of TGF-β1 signaling and-SMAD2 activation. The latter in turn further suppresses p-STAT3. However, more studies are needed to confirm and fully elucidate the details of this potential pathway.

In order to provide insight as to whether EPAC1 facilitates Treg-mediated suppression *in vivo*, we employed an active oral tolerance model. Intragasric administration of low dose ovalbumine induces the production of antigen-specific Treg cells, which in turn suppress the immune response to the administered protein in an antigen nonspecific manner [144, 151-153]. However, after oral administration of OVA, *Epac1*$^{-/-}$ and ESI-09-treated WT mice still produced significant levels of OVA-specific IgG unlike their WT counterparts. Furthermore, while the composition of immune cells was similar between *Epac1*$^{-/-}$ and WT mice, the former had significantly higher basal IgG

levels even in the absence of an antigen challenge. These findings are consistent with our *in vitro* data and suggest that Treg-mediated suppression is attenuated in the absence of EPAC1. Nonetheless, our results cannot rule out other possible roles for EPAC1 in the function of other immune cells that might affect oral tolerance and antibody production, including antigen presenting cells and B-cells.

In conclusion, our study shows that EPAC1 facilitates cAMP signaling during Treg-mediated suppression. Inhibition of EPAC1 leads to resistance of Teff to Treg suppression and concurrently diminishes the suppressive potency of the latter. The impact of EPAC1 on these cells seems to be mediated by regulation of STAT3 activation and TGF-β1 signaling. These findings have significant potential clinical implications as they validate EPAC1 as a potential target for fine tuning Treg cell activity and TGF-β1-mediated immune suppression. Strategies that rely on depletion of Treg cells, even transiently, or broadly target TGF-β1 signaling usually lead to serious systemic side effects and signs of severe autoimmune disease [146, 160]. In fact, Ipilimumab**,** one of two cancer immunotherapies currently approved by FDA, is an anti-CTLA-4 antibody that eliminates Treg, but despite its efficacy, it has potentially fetal autoimmune side effects and is only used in advanced non-resectable cases of melanoma [50]**.** Neither genetic deletion, nor long term pharmacologic inhibition of EPAC1 had observable side effects in mice as we have shown previously [131]. Hence, pharmacologic modulators of this protein might have excellent therapeutic potential in vaccine adjuvant development, infection control, autoimmune disease, and cancer immunity, among other fields.

Chapter 4: Summary, Conclusions, and Future Directions

Background and significance

The cAMP signaling pathway plays a role in mediating nearly all biological functions. The pervasiveness of this signaling network lends itself to therapeutic exploitation and the design of drugs than can target multiple cell functions in different systems within the body. In fact, drugs targeting proteins involved in the regulation of cAMP comprise a very large portion of the medications currently available for the treatment of a wide range of diseases, from simple headaches to schizophrenia and congestive heart failure [161].

However, the ubiquitous nature of cAMP signaling also means greater potential for detrimental systemic drug side effects, and possible off-target effects that actually work antagonistically to the intended impact of a given treatment. Hence, it is imperative that we investigate the regulation of cAMP signaling in various contexts to unravel the complexity of this network and enable the design of targeted drugs with a wide therapeutic index, and whose potential impact on different systems within the body is synergistic.

The discovery of exchange protein activated by cAMP (EPAC) has ushered in a new era of cAMP signaling and revealed an additional player in the cAMP network that can potentially serve as a target for therapeutic intervention. In this dissertation, I investigated the role of EPAC1 in promoting invasion and metastasis of pancreatic ductal adenocarcinoma (PDA) and immune regulation through modulation of the activity of regulatory T-cells (Treg). The findings of my studies suggest that EPAC1 can potentially

be exploited as a multi-mechanistic drug target for the treatment of PDA as explained below.

EPAC1 promotes PDA metastasis

In three common PDA cell lines, AsPC-1, PANC-1, and MIA PaCa-2, genetic suppression or pharmacologic inhibition reduced invasion/migration *in vitro*. And in an *in vivo* orthotopic metastatic PDA mouse model, genetic suppression or pharmacologic inhibition of EPAC1 significantly reduced metastasis of MIA PaCa-2 to the liver. These observations suggest that EPAC1 plays a general role in promoting invasion and metastasis of PDA and that EPAC1 inhibitors have potential therapeutic efficacy and should be explored as anti-metastasis agents for PDA and other carcinomas in which EPAC1 has been shown to facilitate local and distant spread, including melanoma, ovarian cancer, and possibly prostate cancer [13, 70-75].

Mechanistically, our results suggest that EPAC1 facilitates invasion/migration of PDA by promoting integrin β1 activation and trafficking to the plasma membrane, two processes that play a critical role in cancer motility, and particularly PDA [104, 105]. Furthermore, it seems that EPAC1 enhances integrin β1 trafficking through the PKC pathway, as inhibition of PKC abrogated the impact of EPAC activation on this process. On the other hand, inhibition of PKC had no impact on EPAC's ability to upregulate the activation of integrin β1, suggesting a PKC-independent mechanism.

EPAC1 modulates Treg-mediated suppression

The results presented in this work reveal an essential role in Treg-mediated suppression of effector T-cells (Teff). Knockout or pharmacologic inhibition of EPAC1 in Treg cells reduced their suppressive potency. In addition, a lack of EPAC1 in Teff

cells rendered them resistant to suppression by Treg cells. Suppression or inhibition of EPAC1 in both cell populations additively reduced Treg-mediated suppression. These results suggest that EPAC1 plays a dual role in the suppression process: it enhances the potency of Treg cells and simultaneously sensitizes Teff cells to suppression.

At the molecular level, our results show that EPAC1 regulates Treg-mediated suppression through the STAT3 and TGF-β1 pathways. In the absence of EPAC1, both Treg and Teff cells had higher levels of p-STAT3, and inhibition of STAT3 activation restored the potency of *Epac1*$^{-/-}$ Treg-mediated suppression. Moreover, the levels of SMAD7, which inhibits TGF-β1 signaling, were elevated after suppression/inhibition of EPAC1, in concordance with previous studies showing hyper-activation of STAT3 leads to desensitization to TGF-β1 signaling by inducing SMAD7 [147, 149]. Consequently, in the absence of EPAC1 $CD4^+$ T-cells were resistant to TGF-β1 as demonstrated by the lower activation levels of SMAD2 and reduced IL-2 production in response to stimulation by TGF-β1.

Treg cells utilize membrane-bound TGF- β1 as one of their main mechanisms of suppressing Teff cells. Concurrently, this cytokine also serves to maintain the suppressive potency of Treg-cells through autocrine and paracrine signaling [146]. Therefore, our data are consistent with a model in which EPAC1 plays an essential role in sensitizing Treg and Teff cells to TGF-β1 signaling, and as such, its inhibition in both cells has an additive impact on compromising Treg-mediated suppression.

In an *in vivo* active oral tolerance model, in which Treg cells are usually induced and suppress production of antibodies to the administered antigen [144, 151-153], genetic knockout or pharmacologic inhibition of EPAC1 reduced oral tolerance and significantly

elevated the levels of antibodies produced against the administered protein, ovalbumine (OVA). Furthermore, the composition of immune cells was similar between *Epac1*$^{-/-}$ and WT mice, indicating the difference in antibody production between these two groups was not the result of altered differentiation or stability of various immune cells. These findings provide support for a role of EPAC1 in Treg-mediated suppression *in vivo.*

Future directions

PDA has a dismal prognosis with ~ 95% mortality rate [27, 28]. The bleak outlook of this neoplasm is largely due to its high metastatic potential and exceptionally dense desmoplastic microenvironment, which maintains an immunosuppressive milieu that quells the body's antitumor immune response [37]. There is a dire need for targeted therapies that address one or both of these issues, and the results presented here suggest that EPAC1 inhibitors should be explored as anti-PDA therapeutic agents. On the one hand, inhibition of EPAC1in PDA cells has the potential to reduce metastasis, and on the other, its inhibition in the host's immune cells can reduce Treg-mediated suppression and augment anti-tumor immune responses.

One of the key questions to be answered in the future is whether pharmacologic inhibition or genetic knockdown of EPAC1 enhances anti-tumor immunity *in vivo*. This question can be addressed using a syngenic mouse PDA cell line, such as PANC-02 [162]. These cells can be injected into mice subcutaneously, a site that normally does not lead to metastasis and allows for easy and direct assessment of tumor growth and the anti-tumor immune response [163]. If inhibition of EPAC1 reduces Treg-mediated suppression in the tumor microenvironment, then tumor growth should be reduced significantly. At the end of the experiment, tumors can be resected to evaluate the composition of tumor-

infiltrating immune cells and examine if a higher number of tumor-specific lymphocytes is present.

Additionally, the aforementioned heterotopic *in vivo* PANC-02 model can be used to determine the efficacy of combining EPAC1 inhibitors with other therapies aimed at enhancing anti-tumor immunity. For instance, one can administer an EPAC1 inhibitor in conjunction with anti-PD-L1 monoclonal antibodies, which attack the PD-L1 antigen used by many cancers, including PDA, to suppress the body's anti-tumor response [164, 165]. This antibody is currently in clinical trials, but early indications suggest a reduced efficacy in PDA patients [164]. It would be interesting to see if inhibition of EPAC1 could boost the efficacy of anti-PD-L1 in the treatment of PDA.

Finally, if the experiments proposed here confirm EPAC1 inhibition as a potential strategy to combat PDA's immunosuppressive microenvironment, one should then examine this strategy's ability to lower both growth and metastasis of PDA in an orthotopic immune-competent mouse model. One could use the syngenic mouse cell line PACN-02, or the transgenic mouse strain expressing pancreas-specific $Trp53^{R172H}$ and $Kras^{G12D}$ mutations, which lead to spontaneous development of metastatic PDA [166]. This model can test they potential synergistic therapeutic effect of inhibiting EPAC1 in the host and tumor cells simultaneously.

References

1. Berthet J, Rall TW, Sutherland EW. The relationship of epinephrine and glucagon to liver phosphorylase. Iv. Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates. *J Biol Chem*. 1957;224:463-475
2. de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a rap1 guanine-nucleotide-exchange factor directly activated by cyclic amp. *Nature*. 1998;396:474-477
3. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. A family of camp-binding proteins that directly activate rap1. *Science*. 1998;282:2275-2279
4. Fukuda M, Williams KW, Gautron L, Elmquist JK. Induction of leptin resistance by activation of camp-epac signaling. *Cell Metab*. 2011;13:331-339
5. Métrich M, Lucas A, Gastineau M, Samuel JL, Heymes C, Morel E, Lezoualc'h F. Epac mediates beta-adrenergic receptor-induced cardiomyocyte hypertrophy. *Circ Res*. 2008;102:959-965
6. Ozaki N, Shibasaki T, Kashima Y, Miki T, Takahashi K, Ueno H, Sunaga Y, Yano H, Matsuura Y, Iwanaga T, Takai Y, Seino S. Camp-gefii is a direct target of camp in regulated exocytosis. *Nat Cell Biol*. 2000;2:805-811
7. Seino S, Shibasaki T, Minami K. Dynamics of insulin secretion and the clinical implications for obesity and diabetes. *J Clin Invest*. 2011;121:2118-2125
8. Cullere X, Shaw SK, Andersson L, Hirahashi J, Luscinskas FW, Mayadas TN. Regulation of vascular endothelial barrier function by epac, a camp-activated exchange factor for rap gtpase. *Blood*. 2005;105:1950-1955
9. Kooistra MR, Corada M, Dejana E, Bos JL. Epac1 regulates integrity of endothelial cell junctions through ve-cadherin. *FEBS Lett*. 2005;579:4966-4972
10. Shirshev SV. Role of epac proteins in mechanisms of camp-dependent immunoregulation. *Biochemistry (Mosc)*. 2011;76:981-998
11. 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;97:655-662
12. Duquesnes N, Derangeon M, Métrich M, Lucas A, Mateo P, Li L, Morel E, Lezoualc'h F, Crozatier B. Epac stimulation induces rapid increases in connexin43 phosphorylation and function without preconditioning effect. *Pflugers Arch*. 2010;460:731-741
13. Rangarajan S, Enserink JM, Kuiperij HB, de Rooij J, Price LS, Schwede F, Bos JL. Cyclic amp induces integrin-mediated cell adhesion through epac and rap1 upon stimulation of the beta 2-adrenergic receptor. *J Cell Biol*. 2003;160:487-493
14. Enserink JM, Price LS, Methi T, Mahic M, Sonnenberg A, Bos JL, Taskén K. The camp-epac-rap1 pathway regulates cell spreading and cell adhesion to laminin-5 through the alpha3beta1 integrin but not the alpha6beta4 integrin. *J Biol Chem*. 2004;279:44889-44896
15. Gloerich M, Bos JL. Epac: Defining a new mechanism for camp action. *Annu Rev Pharmacol Toxicol*. 2010;50:355-375

16. 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;65:670-709
17. Almahariq M, Mei FC, Cheng X. Cyclic amp sensor epac proteins and energy homeostasis. *Trends Endocrinol Metab*. 2014;25:60-71
18. Mei FC, Qiao J, Tsygankova OM, Meinkoth JL, Quilliam LA, Cheng X. Differential signaling of cyclic amp: Opposing effects of exchange protein directly activated by cyclic amp and camp-dependent protein kinase on protein kinase b activation. *J Biol Chem*. 2002;277:11497-11504
19. Cheng X, Ji Z, Tsalkova T, Mei F. Epac and pka: A tale of two intracellular camp receptors. *Acta Biochim Biophys Sin (Shanghai)*. 2008;40:651-662
20. Mei FC, Qiao J, Tsygankova OM, Meinkoth JL, Quilliam LA, Cheng X. Differential signaling of cyclic amp: Opposing effects of exchange protein directly activated by cyclic amp and camp-dependent protein kinase on protein kinase b activation. *J Biol Chem*. 2002;277:11497-11504
21. Brennesvik EO, Ktori C, Ruzzin J, Jebens E, Shepherd PR, Jensen J. Adrenaline potentiates insulin-stimulated pkb activation via camp and epac: Implications for cross talk between insulin and adrenaline. *Cell Signal*. 2005;17:1551-1559
22. Li J, O'Connor KL, Cheng X, Mei FC, Uchida T, Townsend CM, Evers BM. Cyclic adenosine 5'-monophosphate-stimulated neurotensin secretion is mediated through rap1 downstream of both epac and protein kinase a signaling pathways. *Mol Endocrinol*. 2007;21:159-171
23. Kashima Y, Miki T, Shibasaki T, Ozaki N, Miyazaki M, Yano H, Seino S. Critical role of camp-gefii--rim2 complex in incretin-potentiated insulin secretion. *J Biol Chem*. 2001;276:46046-46053
24. Fujimoto K, Shibasaki T, Yokoi N, Kashima Y, Matsumoto M, Sasaki T, Tajima N, Iwanaga T, Seino S. Piccolo, a ca2+ sensor in pancreatic beta-cells. Involvement of camp-gefii.Rim2. Piccolo complex in camp-dependent exocytosis. *J Biol Chem*. 2002;277:50497-50502
25. Liu C, Takahashi M, Li Y, Dillon TJ, Kaech S, Stork PJ. The interaction of epac1 and ran promotes rap1 activation at the nuclear envelope. *Mol Cell Biol*. 2010;30:3956-3969
26. 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;219:652-658
27. Kamangar F, Dores GM, Anderson WF. Patterns of cancer incidence, mortality, and prevalence across five continents: Defining priorities to reduce cancer disparities in different geographic regions of the world. *J Clin Oncol*. 2006;24:2137-2150
28. Raimondi S, Maisonneuve P, Lowenfels AB. Epidemiology of pancreatic cancer: An overview. *Nat Rev Gastroenterol Hepatol*. 2009;6:699-708
29. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin*. 2014;64:9-29
30. Tempero MA, Berlin J, Ducreux M, Haller D, Harper P, Khayat D, Schmoll HJ, Sobrero A, Van Cutsem E. Pancreatic cancer treatment and research: An international expert panel discussion. *Ann Oncol*. 2011;22:1500-1506

31. Andrén-Sandberg A. Pancreatic cancer: Chemotherapy and radiotherapy. *N Am J Med Sci*. 2011;3:1-12
32. Dowell J, Minna JD, Kirkpatrick P. Erlotinib hydrochloride. *Nat Rev Drug Discov*. 2005;4:13-14
33. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W, Group NCIoCCT. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: A phase iii trial of the national cancer institute of canada clinical trials group. *J Clin Oncol*. 2007;25:1960-1966
34. Fjällskog ML, Lejonklou MH, Oberg KE, Eriksson BK, Janson ET. Expression of molecular targets for tyrosine kinase receptor antagonists in malignant endocrine pancreatic tumors. *Clin Cancer Res*. 2003;9:1469-1473
35. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008;321:1801-1806
36. Pao W, Wang TY, Riely GJ, Miller VA, Pan Q, Ladanyi M, Zakowski MF, Heelan RT, Kris MG, Varmus HE. Kras mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Med*. 2005;2:e17
37. Oberstein PE, Olive KP. Pancreatic cancer: Why is it so hard to treat? *Therap Adv Gastroenterol*. 2013;6:321-337
38. Keleg S, Büchler P, Ludwig R, Büchler MW, Friess H. Invasion and metastasis in pancreatic cancer. *Mol Cancer*. 2003;2:14
39. Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, Reichert M, Beatty GL, Rustgi AK, Vonderheide RH, Leach SD, Stanger BZ. Emt and dissemination precede pancreatic tumor formation. *Cell*. 2012;148:349-361
40. Cress RD, Yin D, Clarke L, Bold R, Holly EA. Survival among patients with adenocarcinoma of the pancreas: A population-based study (united states). *Cancer Causes Control*. 2006;17:403-409
41. Winter JM, Cameron JL, Campbell KA, Arnold MA, Chang DC, Coleman J, Hodgin MB, Sauter PK, Hruban RH, Riall TS, Schulick RD, Choti MA, Lillemoe KD, Yeo CJ. 1423 pancreaticoduodenectomies for pancreatic cancer: A single-institution experience. *J Gastrointest Surg*. 2006;10:1199-1210; discussion 1210-1191
42. Caswell PT, Norman JC. Integrin trafficking and the control of cell migration. *Traffic*. 2006;7:14-21
43. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell*. 2010;140:883-899
44. Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. *Nat Immunol*. 2013;14:1014-1022

45. Kotera Y, Fontenot JD, Pecher G, Metzgar RS, Finn OJ. Humoral immunity against a tandem repeat epitope of human mucin muc-1 in sera from breast, pancreatic, and colon cancer patients. *Cancer Res*. 1994;54:2856-2860
46. Johnston FM, Tan MC, Tan BR, Porembka MR, Brunt EM, Linehan DC, Simon PO, Plambeck-Suess S, Eberlein TJ, Hellstrom KE, Hellstrom I, Hawkins WG, Goedegebuure P. Circulating mesothelin protein and cellular antimesothelin immunity in patients with pancreatic cancer. *Clin Cancer Res*. 2009;15:6511-6518
47. Kubuschok B, Neumann F, Breit R, Sester M, Schormann C, Wagner C, Sester U, Hartmann F, Wagner M, Remberger K, Schilling M, Pfreundschuh M. Naturally occurring t-cell response against mutated p21 ras oncoprotein in pancreatic cancer. *Clin Cancer Res*. 2006;12:1365-1372
48. Kirkwood JM, Butterfield LH, Tarhini AA, Zarour H, Kalinski P, Ferrone S. Immunotherapy of cancer in 2012. *CA Cancer J Clin*. 2012;62:309-335
49. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, Redfern CH, Ferrari AC, Dreicer R, Sims RB, Xu Y, Frohlich MW, Schellhammer PF, Investigators IS. Sipuleucel-t immunotherapy for castration-resistant prostate cancer. *N Engl J Med*. 2010;363:411-422
50. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-723
51. Vasievich EA, Huang L. The suppressive tumor microenvironment: A challenge in cancer immunotherapy. *Mol Pharm*. 2011;8:635-641
52. Wörmann SM, Diakopoulos KN, Lesina M, Algül H. The immune network in pancreatic cancer development and progression. *Oncogene*. 2013
53. Beyer M, Schultze JL. Regulatory t cells in cancer. *Blood*. 2006;108:804-811
54. Dodson LF, Hawkins WG, Goedegebuure P. Potential targets for pancreatic cancer immunotherapeutics. *Immunotherapy*. 2011;3:517-537
55. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. Regulatory t cells: How do they suppress immune responses? *Int Immunol*. 2009;21:1105-1111
56. Colombo MP, Piconese S. Regulatory-t-cell inhibition versus depletion: The right choice in cancer immunotherapy. *Nat Rev Cancer*. 2007;7:880-887
57. Curti A, Trabanelli S, Salvestrini V, Baccarani M, Lemoli RM. The role of indoleamine 2,3-dioxygenase in the induction of immune tolerance: Focus on hematology. *Blood*. 2009;113:2394-2401
58. Munn DH. Indoleamine 2,3-dioxygenase, tregs and cancer. *Curr Med Chem*. 2011;18:2240-2246
59. Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS, Linehan DC. Prevalence of regulatory t cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol*. 2002;169:2756-2761

60. Liyanage UK, Goedegebuure PS, Moore TT, Viehl CT, Moo-Young TA, Larson JW, Frey DM, Ehlers JP, Eberlein TJ, Linehan DC. Increased prevalence of regulatory t cells (treg) is induced by pancreas adenocarcinoma. *J Immunother*. 2006;29:416-424
61. Ebert LM, MacRaild SE, Zanker D, Davis ID, Cebon J, Chen W. A cancer vaccine induces expansion of ny-eso-1-specific regulatory t cells in patients with advanced melanoma. *PLoS One*. 2012;7:e48424
62. Chakraborty NG, Chattopadhyay S, Mehrotra S, Chhabra A, Mukherji B. Regulatory t-cell response and tumor vaccine-induced cytotoxic t lymphocytes in human melanoma. *Hum Immunol*. 2004;65:794-802
63. Zhou G, Drake CG, Levitsky HI. Amplification of tumor-specific regulatory t cells following therapeutic cancer vaccines. *Blood*. 2006;107:628-636
64. Vignali DA, Collison LW, Workman CJ. How regulatory t cells work. *Nat Rev Immunol*. 2008;8:523-532
65. Lorenz R, Aleksic T, Wagner M, Adler G, Weber CK. The camp/epac1/rap1 pathway in pancreatic carcinoma. *Pancreas*. 2008;37:102-103
66. Howe AK. Cross-talk between calcium and protein kinase a in the regulation of cell migration. *Curr Opin Cell Biol*. 2011;23:554-561
67. Burdyga A, Conant A, Haynes L, Zhang J, Jalink K, Sutton R, Neoptolemos J, Costello E, Tepikin A. Camp inhibits migration, ruffling and paxillin accumulation in focal adhesions of pancreatic ductal adenocarcinoma cells: Effects of pka and epac. *Biochim Biophys Acta*. 2013;1833:2664-2672
68. Carmona G, Chavakis E, Koehl U, Zeiher AM, Dimmeler S. Activation of epac stimulates integrin-dependent homing of progenitor cells. *Blood*. 2008;111:2640-2646
69. Yokoyama U, Minamisawa S, Quan H, Akaike T, Jin M, Otsu K, Ulucan C, Wang X, Baljinnyam E, Takaoka M, Sata M, Ishikawa Y. Epac1 is upregulated during neointima formation and promotes vascular smooth muscle cell migration. *Am J Physiol Heart Circ Physiol*. 2008;295:H1547-1555
70. Gao L, Feng Y, Bowers R, Becker-Hapak M, Gardner J, Council L, Linette G, Zhao H, Cornelius LA. Ras-associated protein-1 regulates extracellular signal-regulated kinase activation and migration in melanoma cells: Two processes important to melanoma tumorigenesis and metastasis. *Cancer Res*. 2006;66:7880-7888
71. Bastian P, Balcarek A, Altanis C, Strell C, Niggemann B, Zaenker KS, Entschladen F. The inhibitory effect of norepinephrine on the migration of es-2 ovarian carcinoma cells involves a rap1-dependent pathway. *Cancer Lett*. 2009;274:218-224
72. Grandoch M, Rose A, ter Braak M, Jendrossek V, Rübben H, Fischer JW, Schmidt M, Weber AA. Epac inhibits migration and proliferation of human prostate carcinoma cells. *Br J Cancer*. 2009;101:2038-2042
73. Baljinnyam E, De Lorenzo MS, Xie LH, Iwatsubo M, Chen S, Goydos JS, Nowycky MC, Iwatsubo K. Exchange protein directly activated by cyclic amp increases melanoma cell migration by a ca2+-dependent mechanism. *Cancer Res*. 2010;70:5607-5617

74. Baljinnyam E, Umemura M, De Lorenzo MS, Iwatsubo M, Chen S, Goydos JS, Iwatsubo K. Epac1 promotes melanoma metastasis via modification of heparan sulfate. *Pigment Cell Melanoma Res*. 2011;24:680-687
75. Baljinnyam E, Umemura M, Chuang C, De Lorenzo MS, Iwatsubo M, Chen S, Goydos JS, Ishikawa Y, Whitelock JM, Iwatsubo K. Epac1 increases migration of endothelial cells and melanoma cells via fgf2-mediated paracrine signaling. *Pigment Cell Melanoma Res*. 2014
76. Bailey CL, Kelly P, Casey PJ. Activation of rap1 promotes prostate cancer metastasis. *Cancer Res*. 2009;69:4962-4968
77. 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;108:998-1011
78. 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;113:1488-1500
79. Menon J, Doebele RC, Gomes S, Bevilacqua E, Reindl KM, Rosner MR. A novel interplay between rap1 and pka regulates induction of angiogenesis in prostate cancer. *PLoS One*. 2012;7:e49893
80. Vliem MJ, Ponsioen B, Schwede F, Pannekoek WJ, Riedl J, Kooistra MR, Jalink K, Genieser HG, Bos JL, Rehmann H. 8-pcpt-2'-o-me-camp-am: An improved epac-selective camp analogue. *Chembiochem*. 2008;9:2052-2054
81. Chepurny OG, Leech CA, Kelley GG, Dzhura I, Dzhura E, Li X, Rindler MJ, Schwede F, Genieser HG, Holz GG. Enhanced rap1 activation and insulin secretagogue properties of an acetoxymethyl ester of an epac-selective cyclic amp analog in rat ins-1 cells: Studies with 8-pcpt-2'-o-me-camp-am. *J Biol Chem*. 2009;284:10728-10736
82. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F. The bisindolylmaleimide gf 109203x is a potent and selective inhibitor of protein kinase c. *J Biol Chem*. 1991;266:15771-15781
83. Sullivan JP, Connor JR, Shearer BG, Burch RM. 2,6-diamino-n-([1-(1-oxotridecyl)-2-piperidinyl] methyl)hexanamide (npc 15437): A novel inhibitor of protein kinase c interacting at the regulatory domain. *Mol Pharmacol*. 1992;41:38-44
84. Almahariq M, Tsalkova T, Mei FC, Chen H, Zhou J, Sastry SK, Schwede F, Cheng X. A novel epac-specific inhibitor suppresses pancreatic cancer cell migration and invasion. *Mol Pharmacol*. 2013;83:122-128
85. Byron A, Humphries JD, Askari JA, Craig SE, Mould AP, Humphries MJ. Anti-integrin monoclonal antibodies. *J Cell Sci*. 2009;122:4009-4011
86. de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a rap1 guanine-nucleotide-exchange factor directly activated by cyclic amp. *Nature*. 1998;396:474-477
87. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. A family of camp-binding proteins that directly activate rap1. *Science*. 1998;282:2275-2279

88. Vogelmann R, Kreuser ED, Adler G, Lutz MP. Integrin alpha6beta1 role in metastatic behavior of human pancreatic carcinoma cells. *Int J Cancer*. 1999;80:791-795
89. Grzesiak JJ, Tran Cao HS, Burton DW, Kaushal S, Vargas F, Clopton P, Snyder CS, Deftos LJ, Hoffman RM, Bouvet M. Knockdown of the β(1) integrin subunit reduces primary tumor growth and inhibits pancreatic cancer metastasis. *Int J Cancer*. 2011;129:2905-2915
90. Lorenowicz MJ, van Gils J, de Boer M, Hordijk PL, Fernandez-Borja M. Epac1-rap1 signaling regulates monocyte adhesion and chemotaxis. *J Leukoc Biol*. 2006;80:1542-1552
91. Almahariq M, Chao C, Mei FC, Hellmich MR, Patrikeev I, Motamedi M, Cheng X. Pharmacological inhibition and genetic knockdown of exchange protein directly activated by camp 1 reduce pancreatic cancer metastasis in vivo. *Mol Pharmacol*. 2015;87:142-149
92. Wang J, Wu J, Hong J, Chen R, Xu K, Niu W, Peng C, Liu E, Liu S, Agrez M, Niu J. Pkc promotes the migration of colon cancer cells by regulating the internalization and recycling of integrin αvβ6. *Cancer Lett*. 2011;311:38-47
93. Al-Alem LF, McCord LA, Southard RC, Kilgore MW, Curry TE. Activation of the pkc pathway stimulates ovarian cancer cell proliferation, migration, and expression of mmp7 and mmp10. *Biol Reprod*. 2013;89:73
94. Borland G, Bird RJ, Palmer TM, Yarwood SJ. Activation of protein kinase calpha by epac1 is required for the erk- and ccaat/enhancer-binding protein beta-dependent induction of the socs-3 gene by cyclic amp in cos1 cells. *J Biol Chem*. 2009;284:17391-17403
95. Hucho TB, Dina OA, Levine JD. Epac mediates a camp-to-pkc signaling in inflammatory pain: An isolectin b4(+) neuron-specific mechanism. *J Neurosci*. 2005;25:6119-6126
96. Banno A, Ginsberg MH. Integrin activation. *Biochem Soc Trans*. 2008;36:229-234
97. Bos JL. Linking rap to cell adhesion. *Curr Opin Cell Biol*. 2005;17:123-128
98. Han J, Lim CJ, Watanabe N, Soriani A, Ratnikov B, Calderwood DA, Puzon-McLaughlin W, Lafuente EM, Boussiotis VA, Shattil SJ, Ginsberg MH. Reconstructing and deconstructing agonist-induced activation of integrin alphaiibbeta3. *Curr Biol*. 2006;16:1796-1806
99. Paik KY, Choi SH, Heo JS, Choi DW. Analysis of liver metastasis after resection for pancreatic ductal adenocarcinoma. *World J Gastrointest Oncol*. 2012;4:109-114
100. Gong B, Shelite T, Mei FC, Ha T, Hu Y, Xu G, Chang Q, Wakamiya M, Ksiazek TG, Boor PJ, Bouyer DH, Popov VL, Chen J, Walker DH, Cheng X. Exchange protein directly activated by camp plays a critical role in bacterial invasion during fatal rickettsioses. *Proc Natl Acad Sci U S A*. 2013;110:19615-19620
101. Shin S, Wolgamott L, Yoon SO. Integrin trafficking and tumor progression. *Int J Cell Biol*. 2012;2012:516789
102. Lafuente EM, van Puijenbroek AA, Krause M, Carman CV, Freeman GJ, Berezovskaya A, Constantine E, Springer TA, Gertler FB, Boussiotis VA. Riam,

an ena/vasp and profilin ligand, interacts with rap1-gtp and mediates rap1-induced adhesion. *Dev Cell*. 2004;7:585-595
103. Reedquist KA, Ross E, Koop EA, Wolthuis RM, Zwartkruis FJ, van Kooyk Y, Salmon M, Buckley CD, Bos JL. The small gtpase, rap1, mediates cd31-induced integrin adhesion. *J Cell Biol*. 2000;148:1151-1158
104. Grzesiak JJ, Bouvet M. The alpha2beta1 integrin mediates the malignant phenotype on type i collagen in pancreatic cancer cell lines. *Br J Cancer*. 2006;94:1311-1319
105. Lee YC, Jin JK, Cheng CJ, Huang CF, Song JH, Huang M, Brown WS, Zhang S, Yu-Lee LY, Yeh ET, McIntyre BW, Logothetis CJ, Gallick GE, Lin SH. Targeting constitutively activated β1 integrins inhibits prostate cancer metastasis. *Mol Cancer Res*. 2013;11:405-417
106. Onodera Y, Nam JM, Bissell MJ. Increased sugar uptake promotes oncogenesis via epac/rap1 and o-glcnac pathways. *J Clin Invest*. 2014;124:367-384
107. Barkan D, Chambers AF. B1-integrin: A potential therapeutic target in the battle against cancer recurrence. *Clin Cancer Res*. 2011;17:7219-7223
108. Jahangiri A, Aghi MK, Carbonell WS. B1 integrin: Critical path to antiangiogenic therapy resistance and beyond. *Cancer Res*. 2014;74:3-7
109. Serezani CH, Ballinger MN, Aronoff DM, Peters-Golden M. Cyclic amp: Master regulator of innate immune cell function. *Am J Respir Cell Mol Biol*. 2008;39:127-132
110. Mosenden R, Taskén K. Cyclic amp-mediated immune regulation--overview of mechanisms of action in t cells. *Cell Signal*. 2011;23:1009-1016
111. Bryce PJ, Dascombe MJ, Hutchinson IV. Immunomodulatory effects of pharmacological elevation of cyclic amp in t lymphocytes proceed via a protein kinase a independent mechanism. *Immunopharmacology*. 1999;41:139-146
112. Grandoch M, Roscioni SS, Schmidt M. The role of epac proteins, novel camp mediators, in the regulation of immune, lung and neuronal function. *Br J Pharmacol*. 2010;159:265-284
113. Mosenden R, Taskén K. Cyclic amp-mediated immune regulation--overview of mechanisms of action in t cells. *Cell Signal*. 2011;23:1009-1016
114. de Bruyn KM, Rangarajan S, Reedquist KA, Figdor CG, Bos JL. The small gtpase rap1 is required for mn(2+)- and antibody-induced lfa-1- and vla-4-mediated cell adhesion. *J Biol Chem*. 2002;277:29468-29476
115. Grandoch M, López de Jesús M, Oude Weernink PA, Weber AA, Jakobs KH, Schmidt M. B cell receptor-induced growth arrest and apoptosis in wehi-231 immature b lymphoma cells involve cyclic amp and epac proteins. *Cell Signal*. 2009;21:609-621
116. Aronoff DM, Canetti C, Serezani CH, Luo M, Peters-Golden M. Cutting edge: Macrophage inhibition by cyclic amp (camp): Differential roles of protein kinase a and exchange protein directly activated by camp-1. *J Immunol*. 2005;174:595-599
117. Bryn T, Mahic M, Enserink JM, Schwede F, Aandahl EM, Taskén K. The cyclic amp-epac1-rap1 pathway is dissociated from regulation of effector functions in monocytes but acquires immunoregulatory function in mature macrophages. *J Immunol*. 2006;176:7361-7370

118. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. Foxp3+ regulatory t cells in the human immune system. *Nat Rev Immunol*. 2010;10:490-500
119. Bopp T, Becker C, Klein M, Klein-Hessling S, Palmetshofer A, Serfling E, Heib V, Becker M, Kubach J, Schmitt S, Stoll S, Schild H, Staege MS, Stassen M, Jonuleit H, Schmitt E. Cyclic adenosine monophosphate is a key component of regulatory t cell-mediated suppression. *J Exp Med*. 2007;204:1303-1310
120. Fassbender M, Gerlitzki B, Ullrich N, Lupp C, Klein M, Radsak MP, Schmitt E, Bopp T, Schild H. Cyclic adenosine monophosphate and il-10 coordinately contribute to ntreg cell-mediated suppression of dendritic cell activation. *Cell Immunol*. 2010;265:91-96
121. Bodor J, Feigenbaum L, Bodorova J, Bare C, Reitz MS, Gress RE. Suppression of t-cell responsiveness by inducible camp early repressor (icer). *J Leukoc Biol*. 2001;69:1053-1059
122. Schmidt A, Oberle N, Krammer PH. Molecular mechanisms of treg-mediated t cell suppression. *Front Immunol*. 2012;3:51
123. Vang AG, Housley W, Dong H, Basole C, Ben-Sasson SZ, Kream BE, Epstein PM, Clark RB, Brocke S. Regulatory t cells and camp suppress effector t cells independently of pka-crem/icer: A potential role for epac. *Biochem J*. 2013
124. Li L, Godfrey WR, Porter SB, Ge Y, June CH, Blazar BR, Boussiotis VA. Cd4+cd25+ regulatory t-cell lines from human cord blood have functional and molecular properties of t-cell anergy. *Blood*. 2005;106:3068-3073
125. Li L, Greenwald RJ, Lafuente EM, Tzachanis D, Berezovskaya A, Freeman GJ, Sharpe AH, Boussiotis VA. Rap1-gtp is a negative regulator of th cell function and promotes the generation of cd4+cd103+ regulatory t cells in vivo. *J Immunol*. 2005;175:3133-3139
126. Abreu JR, Krausz S, Dontje W, Grabiec AM, de Launay D, Nolte MA, Tak PP, Reedquist KA. Sustained t cell rap1 signaling is protective in the collagen-induced arthritis model of rheumatoid arthritis. *Arthritis Rheum*. 2010;62:3289-3299
127. 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;275:34901-34908
128. Lee TM, Lin SZ, Chang NC. Both pka and epac pathways mediate n-acetylcysteine-induced connexin43 preservation in rats with myocardial infarction. *PLoS One*. 2013;8:e71878
129. Oviedo-Orta E, Hoy T, Evans WH. Intercellular communication in the immune system: Differential expression of connexin40 and 43, and perturbation of gap junction channel functions in peripheral blood and tonsil human lymphocyte subpopulations. *Immunology*. 2000;99:578-590
130. Mendoza-Naranjo A, Bouma G, Pereda C, Ramírez M, Webb KF, Tittarelli A, López MN, Kalergis AM, Thrasher AJ, Becker DL, Salazar-Onfray F. Functional gap junctions accumulate at the immunological synapse and contribute to t cell activation. *J Immunol*. 2011;187:3121-3132
131. Yan J, Mei FC, Cheng H, Lao DH, Hu Y, Wei J, Patrikeev I, Hao D, Stutz SJ, Dineley KT, Motamedi M, Hommel JD, Cunningham KA, Chen J, Cheng X. Enhanced leptin sensitivity, reduced adiposity, and improved glucose homeostasis

in mice lacking exchange protein directly activated by cyclic amp isoform 1. *Mol Cell Biol*. 2013;33:918-926
132. Almahariq M, Mei FC, Wang H, Cao AT, Yao S, Soong L, Sun J, Cong Y, Chen J, Cheng X. Exchange protein directly activated by camp modulates regulatory t-cell-mediated immunosuppression. *Biochem J*. 2015;465:295-303
133. Sands WA, Woolson HD, Milne GR, Rutherford C, Palmer TM. Exchange protein activated by cyclic amp (epac)-mediated induction of suppressor of cytokine signaling 3 (socs-3) in vascular endothelial cells. *Mol Cell Biol*. 2006;26:6333-6346
134. Anderson P, Sundstedt A, Li L, O'Neill EJ, Li S, Wraith DC, Wang P. Differential activation of signal transducer and activator of transcription (stat)3 and stat5 and induction of suppressors of cytokine signalling in t(h)1 and t(h)2 cells. *Int Immunol*. 2003;15:1309-1317
135. Murray PJ. The jak-stat signaling pathway: Input and output integration. *J Immunol*. 2007;178:2623-2629
136. Goodman WA, Young AB, McCormick TS, Cooper KD, Levine AD. Stat3 phosphorylation mediates resistance of primary human t cells to regulatory t cell suppression. *J Immunol*. 2011;186:3336-3345
137. Babon JJ, Kershaw NJ, Murphy JM, Varghese LN, Laktyushin A, Young SN, Lucet IS, Norton RS, Nicola NA. Suppression of cytokine signaling by socs3: Characterization of the mode of inhibition and the basis of its specificity. *Immunity*. 2012;36:239-250
138. Chueh FY, Yu CL. Engagement of t-cell antigen receptor and cd4/cd8 co-receptors induces prolonged stat activation through autocrine/paracrine stimulation in human primary t cells. *Biochem Biophys Res Commun*. 2012;426:242-246
139. Xu D, Qu CK. Protein tyrosine phosphatases in the jak/stat pathway. *Front Biosci*. 2008;13:4925-4932
140. Lorenz U. Shp-1 and shp-2 in t cells: Two phosphatases functioning at many levels. *Immunol Rev*. 2009;228:342-359
141. Yu M, Lee WW, Tomar D, Pryshchep S, Czesnikiewicz-Guzik M, Lamar DL, Li G, Singh K, Tian L, Weyand CM, Goronzy JJ. Regulation of t cell receptor signaling by activation-induced zinc influx. *J Exp Med*. 2011;208:775-785
142. Lund TC, Coleman C, Horvath E, Sefton BM, Jove R, Medveczky MM, Medveczky PG. The src-family kinase lck can induce stat3 phosphorylation and dna binding activity. *Cell Signal*. 1999;11:789-796
143. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by cd4(+)cd25(+) regulatory t cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med*. 2001;194:629-644
144. Zhang X, Izikson L, Liu L, Weiner HL. Activation of cd25(+)cd4(+) regulatory t cells by oral antigen administration. *J Immunol*. 2001;167:4245-4253
145. Marie JC, Letterio JJ, Gavin M, Rudensky AY. Tgf-beta1 maintains suppressor function and foxp3 expression in cd4+cd25+ regulatory t cells. *J Exp Med*. 2005;201:1061-1067
146. Tran DQ. Tgf-β: The sword, the wand, and the shield of foxp3(+) regulatory t cells. *J Mol Cell Biol*. 2012;4:29-37

147. Jenkins BJ, Grail D, Nheu T, Najdovska M, Wang B, Waring P, Inglese M, McLoughlin RM, Jones SA, Topley N, Baumann H, Judd LM, Giraud AS, Boussioutas A, Zhu HJ, Ernst M. Hyperactivation of stat3 in gp130 mutant mice promotes gastric hyperproliferation and desensitizes tgf-beta signaling. *Nat Med*. 2005;11:845-852
148. Guo X, Wang XF. Signaling cross-talk between tgf-beta/bmp and other pathways. *Cell Res*. 2009;19:71-88
149. Luwor RB, Baradaran B, Taylor LE, Iaria J, Nheu TV, Amiry N, Hovens CM, Wang B, Kaye AH, Zhu HJ. Targeting stat3 and smad7 to restore tgf-β cytostatic regulation of tumor cells in vitro and in vivo. *Oncogene*. 2013;32:2433-2441
150. Das L, Levine AD. Tgf-beta inhibits il-2 production and promotes cell cycle arrest in tcr-activated effector/memory t cells in the presence of sustained tcr signal transduction. *J Immunol*. 2008;180:1490-1498
151. Piccirillo CA, Letterio JJ, Thornton AM, McHugh RS, Mamura M, Mizuhara H, Shevach EM. Cd4(+)cd25(+) regulatory t cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med*. 2002;196:237-246
152. Chung Y, Lee SH, Kim DH, Kang CY. Complementary role of cd4+cd25+ regulatory t cells and tgf-beta in oral tolerance. *J Leukoc Biol*. 2005;77:906-913
153. Hadis U, Wahl B, Schulz O, Hardtke-Wolenski M, Schippers A, Wagner N, Müller W, Sparwasser T, Förster R, Pabst O. Intestinal tolerance requires gut homing and expansion of foxp3+ regulatory t cells in the lamina propria. *Immunity*. 2011;34:237-246
154. Tsalkova T, Mei FC, Li S, Chepurny OG, Leech CA, Liu T, Holz GG, Woods VL, Cheng X. Isoform-specific antagonists of exchange proteins directly activated by camp. *Proc Natl Acad Sci U S A*. 2012;109:18613-18618
155. Pasare C, Medzhitov R. Toll pathway-dependent blockade of cd4+cd25+ t cell-mediated suppression by dendritic cells. *Science*. 2003;299:1033-1036
156. Goodman WA, Levine AD, Massari JV, Sugiyama H, McCormick TS, Cooper KD. Il-6 signaling in psoriasis prevents immune suppression by regulatory t cells. *J Immunol*. 2009;183:3170-3176
157. Annunziato F, Cosmi L, Liotta F, Lazzeri E, Manetti R, Vanini V, Romagnani P, Maggi E, Romagnani S. Phenotype, localization, and mechanism of suppression of cd4(+)cd25(+) human thymocytes. *J Exp Med*. 2002;196:379-387
158. Ostroukhova M, Qi Z, Oriss TB, Dixon-McCarthy B, Ray P, Ray A. Treg-mediated immunosuppression involves activation of the notch-hes1 axis by membrane-bound tgf-beta. *J Clin Invest*. 2006;116:996-1004
159. Zhao S, Venkatasubbarao K, Lazor JW, Sperry J, Jin C, Cao L, Freeman JW. Inhibition of stat3 tyr705 phosphorylation by smad4 suppresses transforming growth factor beta-mediated invasion and metastasis in pancreatic cancer cells. *Cancer Res*. 2008;68:4221-4228
160. Ellis JS, Wan X, Braley-Mullen H. Transient depletion of cd4+ cd25+ regulatory t cells results in multiple autoimmune diseases in wild-type and b-cell-deficient nod mice. *Immunology*. 2013;139:179-186
161. Pierre S, Eschenhagen T, Geisslinger G, Scholich K. Capturing adenylyl cyclases as potential drug targets. *Nat Rev Drug Discov*. 2009;8:321-335

162. Corbett TH, Roberts BJ, Leopold WR, Peckham JC, Wilkoff LJ, Griswold DP, Schabel FM. Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in c57bl/6 mice. *Cancer Res*. 1984;44:717-726
163. Little EC, Wang C, Watson PM, Watson DK, Cole DJ, Camp ER. Novel immunocompetent murine models representing advanced local and metastatic pancreatic cancer. *J Surg Res*. 2012;176:359-366
164. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM. Safety and activity of anti-pd-l1 antibody in patients with advanced cancer. *N Engl J Med*. 2012;366:2455-2465
165. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM, Sznol M. Safety, activity, and immune correlates of anti-pd-1 antibody in cancer. *N Engl J Med*. 2012;366:2443-2454
166. Hingorani SR, Wang L, Multani AS, Combs C, Deramaudt TB, Hruban RH, Rustgi AK, Chang S, Tuveson DA. Trp53r172h and krasg12d cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell*. 2005;7:469-483

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- **Almahariq, M,** Mei, FC Cheng, X: The Pleiotropic Role of Exchange Protein Directly Activated by cAMP 1 (EPAC1) in Cancer: Implications for Therapeutic Intervention. *Acta Biochimica et biophysica Sinica* 2015, In Press
- **Almahariq M**, Chao C, Mei FC, Hellmich MR, Patrikeev I, Motamedi M, Cheng X: Pharmacological Inhibition and Genetic Knockdown of Exchange Protein Directly Activated by cAMP 1 Reduce Pancreatic Cancer Metastasis In Vivo. *Molecular Pharmacol*ogy 2015;87 (2):142-149
- **Almahariq M**, Mei FC, Wang H, Cao AT, Yao S, Soong L, Sun J, Cong Y, Chen J, Cheng X: Exchange Protein Directly Activated by cAMP Modulates Regulatory T-cell-Mediated Immunosuppression. *Biochemical Journal* 2015;465 (2):295-303
- **Almahariq, M,** Mei, FC, Cheng, X: Cyclic AMP Censor EPAC Proteins and Energy Homeostasis. *Trends in Endocrinology and Metabolism* 2014 25(2): 60-71.
- **Almahariq M**, Tsalkova T, Mei FC, Chen H, Zhou J, Sastry SK, Schwede F, Cheng X: A Novel EPAC-Specific Inhibitor Suppresses Pancreatic Cancer Cell Migration and Invasion. *Moleclar Pharmacolgy* 2013, 83(1):122-128

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