

CALCIUM-INDUCED STRUCTURAL REARRANGEMENTS RELEASE AUTOINHIBITION IN  
THE RAP-GEF, CALDAG-GEFI.

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## ABSTRACT

Aaron A. Cook: Calcium-induced structural rearrangements release autoinhibition in the Rap-GEF, CalDAG-GEFI  
(Under the direction of Wolfgang Bergmeier and John Sunde)

When blood vessels are injured, extracellular matrix is exposed leading to the recruitment and activation of platelets where they aggregate to staunch bleeding. To aggregate, platelets require the release of intracellular calcium linked to the activation of Rap1B. Rap1B is a small GTPase that is activated by guanine nucleotide exchange factors (GEFs) related to Cdc25. CalDAG-GEFI is a Cdc25-related GEF likely to coordinate increased calcium levels with Rap1B activation in platelets. However, the molecular details for how CalDAG-GEFI activates Rap1B are unknown. Here we show that calcium directly regulates CalDAG-GEFI to activate Rap1B. In particular, purified CalDAG-GEFI robustly activated Rap1B using a fluorescence-based nucleotide exchange assay and exchange was lost when CalDAG-GEFI was treated to chelate calcium. Conversely, exchange was fully restored by reintroduction of calcium. Furthermore, substitution within the calcium binding loop of either EF hand crippled the exchange capacity of CalDAG-GEFI. Indeed, substitution in both EF hands abrogated calcium-dependent nucleotide exchange on Rap1B. Many EF hands undergo calcium-dependent conformational rearrangements. To better understand potential conformational rearrangements that regulate CalDAG-GEFI, the differential uptake in deuterium between active CalDAG-GEFI and the form with both EF hands disabled was measured. These studies highlighted calcium-induced conformational rearrangements in two regions: i) both EF hands, and ii) the linker connecting the

EF hands and Cdc25 domain. Previous work on CalDAG-GEFII showed that the equivalent linker lies across the surface of the Cdc25 domain needed to engage Ras GTPases and proposed that the linker is displaced from this surface by calcium-dependent rearrangements in the EF hands. To test a similar model for CalDAG-GEFI, the linker was mutated and shown to be required for autoinhibition. In particular, substitution of valine 406 in the linker fully restored exchange capacity of CalDAG-GEFI with both EF hands disabled. Overall, the data support a model whereby the EF hands work in concert, undergoing conformational rearrangements that control the placement of the linker to finely tune the activation of CalDAG-GEFI in platelets. In platelets, CalDAG-GEFI engages Rap1B at the inner leaflet of the plasma membrane. Future studies will define the role of membranes on the calcium-dependent regulation of CalDAG-GEFI.

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## LIST OF ABBREVIATIONS AND FIGURES

Å	Angstrom
aC1	Atypical C1 domain with secondary structure that does not bind DAG
ADP	Adenosine diphosphate
BEH	Ethyl-bridged hybrid
BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
B-Raf	v-Raf murine sarcoma viral oncogene homolog B
C1	PKC DAG-binding domain
C18 HPLC	Octadecyl carbon chain (C18)-bonded silica High performance liquid chromatography
CalDAG-GEF	Calcium and diacylglycerol-regulated nucleotide exchange factor containing residues 1-551
CalDAG-GEFI EF1	N-terminal EF hand mutant CalDAG-GEFI with substitution E450A
CalDAG-GEFI EF1+2	EF hand mutant CalDAG-GEFI with substitutions E450A and E479A
CalDAG-GEFI EF1+2+V406E	EF hand mutant CalDAG-GEFI with substitutions E450A and E479A and glutamate substitution for valine at position 406
CalDAG-GEFI EF2	C-terminal EF hand mutant CalDAG-GEFI with substitution E479A
CalDAG-GEFI+V406E	CalDAG-GEFI with substitution V406E
Cdc25	Catalytic domain in Ras/Rap GEFs
cDNA	Complimentary deoxyribonucleic acid
CNB domain	Cyclic nucleotide binding domain
Da	Dalton
DAG	Diacylglycerol
DEP domain	Dishevelled, Egl-10, and Pleckstrin domain
DH domain	Dbl-homology domain
DMSO	Dimethyl sulfoxide

DTS	Dense tubule system
DTT	Dithiothreitol
ECM	Extracellular matrix
EF hand	Canonical calcium binding domain
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis ( $\beta$ -aminoethyl ether)-N, N, N', N'-tetraacetic acid
Em	Emission wavelength
EPAC	Exchange protein directly activated by cyclic adenosine monophosphate
ERK1/2	Extracellular-signal-regulated kinase
Ex	Excitation wavelength
FBS	Fetal bovine serum
FLAG	Peptide tag with sequence DYKDDDDK used for isolating proteins
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
Glu (E)	Glutamate
GP	Glycoprotein
GPCR	G-protein coupled receptor
GRB2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
GTPase	Enzyme that hydrolyzes guanosine triphosphate
H-Ras	Harvey-Rat sarcoma small GTPase
HCl	Hydrochloric acid
HDX	Hydrogen/deuterium exchange

HEK293T	Human embryonic kidney cells 293 with SV40 large T antigen
His-tag	Six histidine residues added to proteins for purification
HisTrap HP	Nickel Sepharose High Performance and designed for simple, high-resolution purification of histidine-tagged proteins
HLH	Helix-loop-helix as in EF hands
HRP	Horseradish peroxidase
IP3	Inositol 1,4,5- triphosphate
IPTG	Isopropyl $\beta$ -D-1-thiogalactopyranoside
ITAM	Immuno-tyrosine based-activated motif
K	Kelvin
K-Ras	Kirsten-Rat sarcoma small GTPase
Kd	Dissociation constant
kDa	Kilodalton
KO	Knock-out (refers to a gene that is no longer expressed)
L	Leucine
LB	Lysogeny broth
M	Molar
M-Ras	Muscle Rat sarcoma small GTPase
MCF2	MCF2 cell line derived transforming sequence
MEF	Mouse embryonic fibroblasts
MEK	Mitogen activated protein kinase
mg	Milligram
MgCl <sub>2</sub>	Magnesium chloride
ml	Milliliter
mM	Millimolar
MS	Mass spectrometry

NaCl	Sodium chloride
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometer
nM	Nanomolar
NMR	Nuclear magnetic resonance
NP40	Nonyl phenoxy polyethoxy ethanol
ORF	Open reading frame
P loop	Phosphate binding loop in small GTPases
p15LIC2	pET15b ligation-independent cloning vector
PAR	Protease activated receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDZ domain	PSD-95, Dlg, ZO domain
pH	Potential of hydrogen
PH domain	Pleckstrin homology domain
Phe (F)	Phenylalanine
PI(3)K	phosphatidylinositol-3-OH kinase
PIP2	Phosphatidylinositol 4,5-diphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristate acetate

Q-Tof	Quad Time of flight
R-Ras	Ras viral related-Rat sarcoma small GTPase
RA domain	Ras associated domain
Ral	Ras-like small GTPase
RalGDS-RBD	Ral guanine disassociation stimulator Rap binding domain
RanBP2	Ran-binding protein 2
Rap1B	Ras-related protein, isoform 1B
Ras	Rat sarcoma small GTPase
RASA3	Ras p21 protein activator 3
RasGRP	Ras guanyl-releasing proteins
REM	Ras-exchange motif
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SH-2/3	Src-homology domain 2 and 3
Sos1	Son-of-sevenless Ras GEF
Src	Sarcoma (as in Src homology domain)
SWI	Switch one region in small GTPases
SWII	Switch two region in small GTPases
TC21	Small GTPase encoded by Rras2
TCR	T-cell receptor
TEV	Tobacco etch virus
Tris	Tris (hydroxymethyl) aminomethane
Trp (W)	Tryptophan
v/v	Volume/volume
Val (V)	Valine

WT	Wild type. Also, refers to CalDAG-GEFI (1-551)
ZnSO <sub>4</sub>	Zinc sulfate
μl	Microliter
μM	Micromolar

## CHAPTER 1: INTRODUCTION

Blood is comprised of three main types of cells: i) red blood cells that carry oxygen to tissue, ii) white blood cells that fight off infection, and iii) platelets that prevent blood loss at sites of vascular injury. The data gathered from my studies and described in this dissertation pertain only to platelet function and principally address the mechanisms for how calcium regulates the activity of calcium and diacylglycerol-regulated guanine nucleotide exchange factor (CalDAG-GEFI), and the small GTPase Ras-associated protein (Rap) 1B in platelets. These two proteins mediate essential platelet functions necessary for proper hemostatic plug formation at the site of vascular injury (1, 2).

When the endothelial lining of blood vessels is injured, the extracellular matrix (ECM), becomes exposed, which serves as a substrate activating platelets as they flow out of the compromised blood vessel (3). One way platelet activation can be quantified is by measuring their change in morphology, from a discoid shape to a star-like shape with numerous filopodia, and by measuring the amount of activated adhesion receptors, integrin  $\alpha$ IIB $\beta$ 3 on their plasma membrane, activated platelets adhere tightly to each other albeit, indirectly. Fibrinogen, a protein secreted in the plasma in blood binds to the activated integrins on adjacent platelets to form platelet aggregates that continue to accumulate, eventually recruiting enough platelets to form a stable hemostatic plug that prevents further bleeding (4).

Hemostatic plugs do not form unless there is integrin  $\alpha$ IIB $\beta$ 3 in the active state on platelet membranes. These integrin's become activated by an integrin activation complex (IAC), formed by proteins that are recruited to the integrins cytoplasmic tail, primarily Kindlin3, vinculin, talin,

and Rap isozymes in response to an increase in calcium concentration in the cytosol (5). Subsequently, the IAC binds to the cytoplasmic tail of the integrins causing them to undergo conformational rearrangements that are transmitted back through the membrane necessary for integrin activation. Increases in the intracellular calcium concentration coincide with high levels of activated integrin on the platelet surface. Improper regulation of calcium cripples the platelets' ability to function normally because they cannot activate  $\alpha\text{IIb}\beta\text{3}$  on their surface (6, 7). For example, integrin activation is dramatically reduced in platelets that have defective calcium channels (8, 9). These platelets are dysfunctional because calcium cannot be released from the dense tubular system (DTS). Clearly, calcium is important for platelet function, but the mechanism linking calcium to integrin  $\alpha\text{IIb}\beta\text{3}$  activation is unknown (10).

The IAC is critical for inside-out activation of integrins. Rap1B is a molecular switch, cycling between active and inactive state. Rap1B is 'on' or active when bound to GTP and 'off' when bound to GDP. Platelets express all Rap isozymes. However, Rap1B is the most abundant isozyme, and comprises nearly 0.1% of total platelet proteins (11). The abundance of Rap1B emphasizes its importance in platelet function. Platelets from genetically modified mice lacking Rap1B, have many defects including a marked reduction in integrin activation in response to agonist stimulation. These findings are important because they provide a link between integrin activation and Rap1B and delayed aggregation (12).

Enzymes that activate small GTPases are guanine nucleotide exchange factors or GEFs. They do so by increasing the rate of nucleotide exchange and are divided into different GEF families that have unique regulatory mechanisms and target different GTPases. One such family of GEFs has specific activity towards the Ras-superfamily of small GTPases. These GEFs are unique because they contain a catalytic, cell-division-cycle (Cdc) 25 domain which is the binding

surface that interacts specifically with Ras-family proteins (13-16). Interestingly, the most abundant GEF in platelets is a Rap-specific GEF, CalDAG-GEFI (17, 18). Platelets from mice lacking CalDAG-GEFI have normal calcium regulation but showed a dramatic reduction in Rap signaling and integrin activation. These findings suggest a link between CalDAG-GEFI, Rap1B, and integrin  $\alpha$ IIb $\beta$ 3 signaling pathways. However, to determine the molecular mechanisms regulating CalDAG-GEFI, my work focused to determining how CalDAG-GEFI is regulated and define whether calcium is directly activating CalDAG-GEFI, required for proper platelet function.

Platelets lacking CalDAG-GEFI have normal calcium mobilization but a marked delay in Rap1B activation and  $\alpha$ IIb $\beta$ 3 integrin activation. However, these platelets eventually do form a hemostatic plug. These data suggest two facts about Rap1B activation: 1) There is more than one phase of Rap1B activation, and 2) the early phase of Rap1B activation requires CalDAG-GEFI activity. Because there are multiple phases to Rap1B activation, we set out to determine the biochemical function of calcium in the first phase of Rap1B activity whether calcium interacts directly with CalDAG-GEFI (2). To answer this question, we developed a functional assay to measure nucleotide exchange on Rap1B; we use purified CalDAG-GEFI and Rap1B to determine whether calcium directly regulates CalDAG-GEFI activity. Thus, my thesis work addressed the following aims:

- 1) Determine whether CalDAG-GEFI activity is calcium-dependent.
- 2) Determine the conformational rearrangements in CalDAG-GEFI caused by calcium binding.
- 3) Define regulatory domains that control CalDAG-GEFI activity.

The following chapters describe key concepts of platelet biology pertinent to my studies: an extensive summary of platelet-receptor activation, calcium mobilization, the biochemical and functional role of calcium-binding EF hand domains, the regulation of Rap isozymes, and how the family of Cdc25 domain GEFs are regulated. These chapters will provide the context necessary to understand the importance of my findings, as discussed in my published work and work that is under review, describing how CalDAG-GEFI is regulated by: a) negative-feedback loops sustained through extracellular-signal-regulated kinase (ERK) 1/2 signaling pathways and b) Calcium-induced structural rearrangements release autoinhibition in the Rap-GEF, CalDAG-GEFI.

## **CHAPTER 2: PLATELET-RECEPTOR SIGNALING, CALCIUM REGULATION, AND EF HAND BIOLOGY**

Platelets are shed into the vascular system from megakaryocytes that reside in bone marrow, spleen and lungs after they mature and undergo apoptosis, releasing platelets. Each megakaryocyte is capable of producing 2000-5000 platelets that are vital for the development and maintenance of our vasculature (19). In the developing fetus, platelets are required for the proper segregation of the lymphatic and blood vascular systems, while in adults, the major functions of platelets are to prevent blood loss and promote wound healing.

Platelets in circulation have a discoid shape and are the smallest cells circulating in the blood stream. Platelets detect an injured vessel when the ECM, lining the outside of the vessel, becomes exposed (20). The exposed ECM then serves as substrates for receptors on the platelet surface (21). When these receptors are activated they transmit signals across the membrane, initiating a cascade of signaling events that result in dramatic changes in platelet morphology and their adhesive properties (22, 23). In this section, I will discuss platelets in the context of three phases of hemostatic plug formation: i) platelet activation and firm adhesion to the ECM, ii) platelet-platelet cohesion, and iii) clot retraction.

### *Platelet receptors signaling*

To begin hemostatic plug formation at a site of vascular injury, platelets first become tethered to the exposed ECM by von Willebrand factor (vWF), a multimeric plasma protein that binds to collagen, a major component of the ECM (23, 24). Platelets can bind collagen through vWF with constitutively active glycoprotein (GP) Ib/IX/V receptors on their surface. The fast

on/off rate of GPIb/V/IX binding to vWF allows platelets to be tethered and detached over a lawn of vWF, required to recruit platelets to sites of injury where the shear forces inhibit the interaction of other platelet receptors that engage the ECM but have slower binding rates. For example, platelets have receptors that directly bind collagen however, without tethering to collagen through vWF, platelets would have a lower incidence of binding to the ECM directly because of the shear forces (20). As a result, platelets would accumulate more slowly at the site of injury, hindering hemostatic plug formation. However, once GP Ib/V/IX engages vWF, signals are transmitted across the membrane to activate proteins at the inner leaflet to produce new signals being transmitted back across the membrane, activating two platelet receptors that directly bind collagen (25).

GP VI and  $\alpha 2\beta 1$  integrins are receptors on the plasma membrane, bind collagen and enhance the calcium signals propagated by GP Ib/IX/V necessary for firm platelet adhesion (26). GPVI is an immunoreceptor tyrosine-based activation motif (ITAM) receptor activated when its cytoplasmic tail is phosphorylated by Fyn/Lyn kinases (27). These kinases are recruited to the Fc gamma chain (ITAM)-containing intracellular tail of GPVI. The interaction, between the cytoplasmic tails, enhances Fyn/Lyn kinase activity and enhances clustering of GPVI within the membrane. Collagen binding also leads to GPVI clustering, and transmits a signal back across the membrane, amplifying the intracellular Fyn/Lyn kinase activity (27). These inside-out signals propagated by GVI clustering, activates another collagen receptor on the surface of platelets,  $\alpha 2\beta 1$  integrin.

The  $\alpha 2\beta 1$  integrin is a cell surface receptor, formed by an alpha-two and beta- three heterodimer that spans the plasma membrane. The inside-out activation of  $\alpha 2\beta 1$  is propagated by phosphorylation of its cytoplasmic tail, at the inner leaflet of the plasma membrane, and the

formation of the integrin activation complex (IAC) (5, 28-30). The IAC includes proteins such as talin, kindlin3 and the small GTPase, Ras-associated protein (Rap) 1. Assembly of the IAC at the cytoplasmic tail of the  $\beta$  subunit leads to a conformational change in  $\alpha 2\beta 1$ , shifting it from a low affinity state to a high affinity state. Clustering of  $\alpha 2\beta 1$  receptors enhances the avidity for collagen (4, 31, 32). Platelets tethered and adhered to collagen form a ring that encircles the injury site and recruit additional platelets (24). These newly recruited platelets adhere to the platelets in the ring, and to one another, forming a monolayer, adhering in concentric circles, eventually closing the wound. An important intracellular event downstream of activated receptors and platelet adhesion is the release of stored calcium into the cytosol (7, 33). Previous work demonstrated a close correlation between calcium mobilization and integrin activation on the platelet surface. Importantly, the kinetics of the activation of Rap1, a crucial member of the IAC, correlates with calcium mobilization and integrin activation; both calcium and Rap1 are the major focus of my studies and in chapter 5, I will discuss the precise mechanism for how Rap isozymes become activated downstream of calcium release.

Protease activated receptor (PAR) isozymes 1 and 4 are two G-protein coupled receptors (GPCR) on the surface of platelets that transmit signals across the membrane to sustain robust calcium signals that began when platelets tethered to collagen (34). PAR receptors are the conduit for transmitting signals from ligands produced by the coagulation pathways. The coagulation pathways activate proteins in the blood plasma at sites of vascular injury and are important throughout the process of hemostatic plug formation. Despite the importance of these pathways, the mechanistic details of how all of the proteins activated by these pathways, will not be discussed. However, thrombin is a protease activated by the coagulation pathway, and is mentioned here, because it activates PAR receptors (35). Thrombin cleaves a portion of the N-

terminus of the PAR receptor, between arginine 41 and serine 42, revealing a new N-terminus that becomes the activating ligand for the receptor itself. All PAR receptors are activated by thrombin that is sustained by high concentrations of intracellular calcium.

Sustained signals through PARs cause platelets to secrete the contents of their granules into the extracellular space. Adenosine diphosphate (ADP), is one of the molecules released from granules and is an important substrate for autocrine and paracrine signaling.

Autocrine and paracrine signals positively regulate platelet activation insuring that platelet signaling, in and around the hemostatic plug, is robust and sustained. (2, 21). Cohesion between platelets, when platelets adhere to one another, builds layers of platelets, stabilizing hemostatic plugs over holes in vessels. Platelet cohesion provides a means for relaying both, outside-in and inside-out signals from surface receptors, unifying the activation state of all the platelets in these layers by promoting granule secretion (22). Interestingly, platelets within the hemostatic plug secrete two types of granules; dense granules, containing ligands such small molecules, and alpha granules containing peptides and proteins (36, 37). The granule releasates activate receptors both on platelets from which they are released and receptors on platelets in close proximity, mechanisms known as autocrine or paracrine signaling, respectively. Although, many receptors are important for autocrine and paracrine signaling, I will only discuss two important receptors on the platelet surface: the P2Y receptors for Adenosine diphosphate (ADP), and  $\alpha$ II $\beta$ 3 integrin receptors for fibrinogen and fibrin.

P2Y1 and P2Y12 receptors on platelets surface is activated by ADP. P2Y1 receptors transmit a weak signal in response to ADP, producing a small burst of calcium in cytosol with no significant impact on the formation of a hemostatic plug (38). In contrast, P2Y12 signals do not

signal through calcium, but provide robust signals through Phosphatidylinositol-3-kinase (PI3K) which sustains the activity of Rap1 isozymes and subsequently,  $\alpha$ IIB $\beta$ 3 integrin (22, 39, 40).

$\alpha$ IIB $\beta$ 3 integrins are platelet-specific adhesion receptors and are the most abundant receptors on their surface (11). These integrins are activated through inside-out signaling pathways and form a high-affinity conformation, by the identical mechanism as  $\alpha$ 2 $\beta$ 1 integrins, as previously discussed. Activated  $\alpha$ IIB $\beta$ 3 in-turn, transmits signals back across the membrane, providing outside-in signals. The  $\alpha$ IIB $\beta$ 3 integrins are important for all phases of platelet adhesion and are thus crucial for hemostatic plug formation.

Fibrinogen, a protein produced in the liver, circulates within the bloodstream. Within the hemostatic plug, fibrinogen is the ‘glue’ that holds platelets together. Like vWF, fibrinogen acts like a tether between platelets, linking them together through binding to activated  $\alpha$ IIB $\beta$ 3 integrins.

The final phase of platelet plug formation requires both, the proteolytic cleavage of fibrinogen to form fibrin, and the contraction of the actin cytoskeleton within each platelet, forming a stable and dense hemostatic plug that can withstand the shear forces exerted by blood flow and has the flexibility to withstand contractions and dilations by the blood vessel (41). To form a more stable hemostatic plug, fibrinogen is cleaved by thrombin to form fibrin. Fibrin self assembles into longer fibrin strands and the platelet- fibrin interaction provides the strength and resilience to the hemostatic plug to withstand the contraction of platelets as part of the final phase of plug formation, clot retraction (37).

Platelet retraction results from rearrangements of their actin cytoskeleton. This in turn, retracts the entire plug, but maintains the platelet-fibrin bridges while trapping immune cells. Platelets adhere tightly to one another and to the ECM and ultimately retract as a unit straining

their adhesions to collagen, and the cohesion between one another. Platelet retraction stabilizes the hemostatic plug, promotes the recruitment of more immune cells, and seals off the wound to prevent further blood loss. Once the platelets are in their final contracted state, they will eventually become necrotic, but will remain in the hemostatic plug throughout the healing process; bound to collagen in the ECM and linked together by fibrin. In the end, the hemostatic plug is broken down by proteases and removed by phagocytic immune cells (22).

As outlined above, a critical event during platelet activation is the release of calcium from the intracellular stores of platelets and the influx of calcium through ion channels in membranes. The concentration of calcium in platelets fluctuates throughout their lifetime in circulation. The release of stored calcium induced by cellular activation, dramatically increases its concentration in the cytoplasm of. High calcium concentrations are sustained throughout all phases of hemostatic plug formation because of autocrine and paracrine signals required for  $\alpha$ II $\beta$ 3 integrins in platelets. Molecular mechanisms of calcium release and calcium regulation downstream of these will be discussed in greater detail in the following section.

### *Mechanisms for regulating calcium in platelets*

In this section I will discuss two main aspects of calcium regulation in platelets, calcium mobilization from inner stores, and calcium transport from the extracellular space into the cytosol. Critical for the generation of the calcium signal is the activation of phospholipase (PLC) isozymes, which are located at the inner leaflet of the plasma membrane (42). Once activated downstream of GPCR or ITAM receptors, PLC beta ( $\beta$ ) and gamma ( $\gamma$ ) isozymes hydrolyze phosphatidylinositol (4,5) phosphate (PIP<sub>2</sub>), on the glycerol side of the phosphodiester bond. PIP<sub>2</sub> hydrolysis produces two important second messengers, diacylglycerol, (DAG), a lipophilic fatty acid chain which remains in the membrane, and inositol triphosphate, (IP<sub>3</sub>) a soluble

compound, that diffuses throughout the cytosol (38). DAG is an important substrate for activating many downstream signaling networks, most notably, protein kinase signaling. DAG also activates some CalDAG-GEF isozymes, but not CalDAG-GEFI. CalDAG-GEFI is the focus of my work thus, activation of other CalDAG-GEF isozymes by DAG is only pertinent because it contrasts the activation mechanism of CalDAG-GEFI. Instead, I will focus on the more pertinent second messenger IP<sub>3</sub>, because it binds to inositol triphosphate receptors (IP<sub>3</sub>R) in the dense tubule system (DTS), causing a dramatic increase in cytosolic calcium concentrations required for CalDAG-GEFI activation (43).

The DTS is an organelle similar to the endoplasmic reticulum of other cell types. It is unique to platelets and is their main storage compartment for calcium. The IP<sub>3</sub>R is the main calcium channel in the DTS, which remains closed in the absence of receptor signaling, keeping calcium sequestered from the cytosol. Once receptors become activated, the activity of PLC increases producing a bolus of IP<sub>3</sub> that binds to and opens IP<sub>3</sub>R channels in the DTS, allowing calcium to flow into the cytoplasmic space. The result of this is a significant increase in the levels of calcium in the cytoplasm, from ~60 nM to nearly 1 μM (7). This dramatic increase in calcium concentration triggers further downstream signals, including the activation of CalDAG-GEFI. Although, calcium released from the DTS produces a rapid increase in calcium concentration in the cytosol, the DTS is only a finite source of free calcium. As previously discussed, there are high concentrations of calcium sustained throughout hemostatic plug formation. Thus, platelets depend on additional proteins that can form calcium channels localized in the plasma membrane (44-47). When calcium stores are depleted from the DTS, calcium sensing proteins in the DTS membrane, the Stromal Interaction Molecules (STIMs), are activated. Activated STIM binds the calcium release-activated calcium channel protein, Orail,

and clusters it in the plasma membrane forming a channel. Calcium is actively transported back into the cytoplasm through the Orai1 channel; a mechanism called store-operated calcium entry (SOCE) (48).

STIM isozymes (1 and 2) are multi-domain, transmembrane proteins, which form dimers in the DTS membrane. STIM proteins sense calcium in the DTS with their N-terminal EF hand domain, which is localized to the inner leaflet of the DTS membrane. In the absence of agonist stimulation, the concentration of calcium in the DTS remains high. Therefore, the EF hand in STIM isozymes are bound to calcium. However, the EF hand has a low affinity for calcium so, once calcium is mobilized from the DTS, it is displaced from the EF hand causing STIM isozymes to cluster (43). Orai isozymes are transmembrane proteins that form dimers when they are in the inactive state and bind to calcium channels in the plasma membrane to prevent calcium transport from the extracellular space into the platelet cytosol. Once STIM isozymes are clustered they recruit Orai dimers away from the calcium channels, allowing them to form tetramers in the plasma membrane which allows for SOCE. SOCE serves two roles: maintain high calcium concentrations in the cytosol and restore calcium to the DTS (46). Once the calcium concentration in the DTS is sufficiently high, calcium binding to the EF hands is restored, the STIM-Orai complex is dissolved.

Platelets use additional ion channels in their plasma membrane to transport calcium into their intracellular space (46). Once released into the cytosol, calcium binds and activates various signaling proteins. Many of these proteins rely on different calcium binding domains, including the well-known EF hand domain. Before moving on to the next chapter, I will briefly discuss the biology of EF hands, namely, how EF hands coordinate calcium binding, and the conformational rearrangements in EF hand proteins induced by calcium.

### *The function and calcium-binding properties of EF hand domains*

Calcium ions serve diverse functions in proteins and bind these proteins to either, enhance protein stability, or regulate enzyme catalysis (49). Proteins that bind calcium ions to enhance their stability, typically, have calcium binding pockets formed by multiple domains within close proximity to each other, and calcium binding is necessary to maintain their secondary structure (50, 51). In contrast, proteins that bind calcium ions to regulate their catalytic activity, have unique calcium binding sites, made up of a single domain, the EF hand.

The EF hand nomenclature comes from the relationship between their two helices connected by a loop, (helix-loop-helix, HLH). Classically, the N- and C-terminal helices are designated by 'E' and 'F' helix, respectively (52). The tertiary structure of the HLH when superimposed on the forefinger and thumb of a person's right hand, is modeled with the linker region represented by the curvature of skin, between the thumb (F helix) and forefinger (E helix) (53). While not a rule, EF hands typically come in pairs but, both EF hands within the pair do not always bind calcium; an example of this is CalDAG-GEFII protein. Using isothermal titration calorimetry, (ITC), Iwig et al., showed that in the isolated pair of EF hands, calcium only bound to the N-terminal EF hand (54).

Calcium ions bind within the loop region in EF hands, with variable affinities. Loop regions, typically have 12 residues, however the number and type of residues in the loop do not predict the calcium binding affinity. Interestingly, the residues conserved in the loop of calcium-binding EF hands are well conserved. In particular, positions 1, 3, 5, 7, and 12 which form ionic bonds with the calcium ion, providing electrostatic interactions. These interactions help stabilize the secondary structure of the HLH. In particular, the glutamate at position 12, provides a bidentate, ionic bond with calcium, which is required for calcium binding (55). In addition,

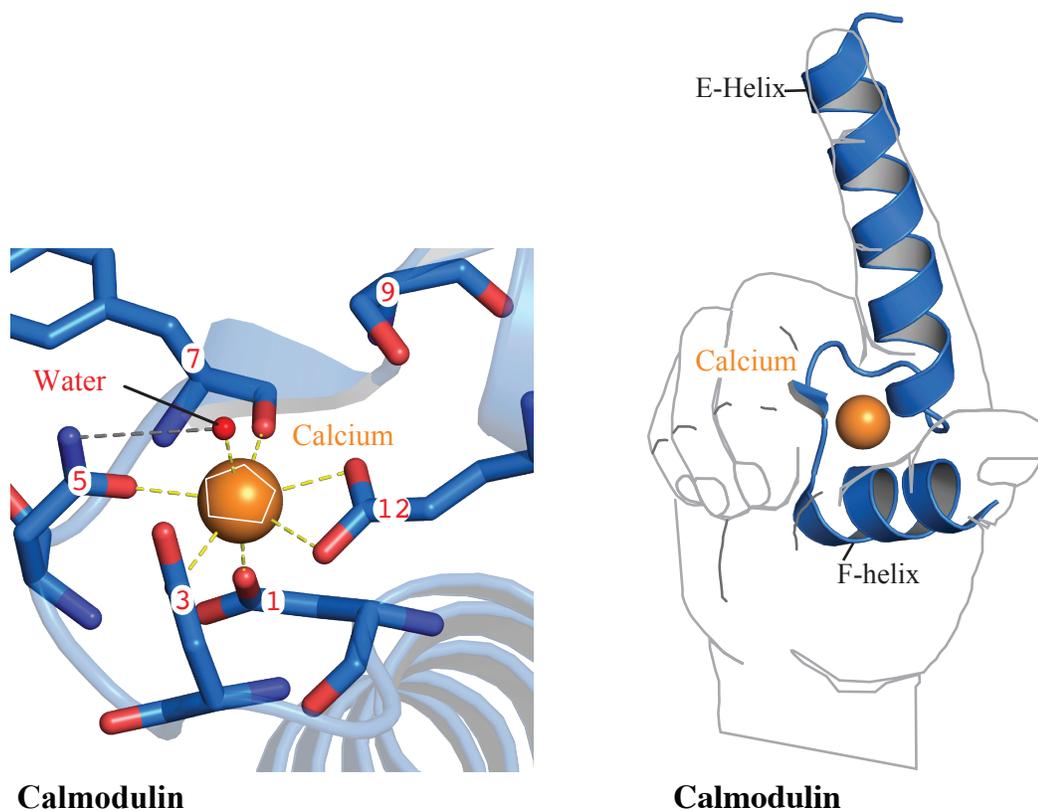
calcium binding within the loop region induces conformational rearrangements between the E and F helices that are transmitted throughout the protein, often regulating enzymatic function allosterically (56).

When calcium binds to the loop region of an EF hand, the E helix undergoes conformational rearrangements relative to the F helix. Figure 1, illustrates the arrangement of a calcium-free HLH, overlaid with the arrangement of an HLH bound to a calcium ion. Calcium binding to EF hands imposes a pentagonal- bi-pyramidal geometric arrangement within the loop that is important for providing the thermodynamic energy required to rearrange the E and F helices, making it possible to unpack previously solvent inaccessible, hydrophobic regions.

EF hands typically pack tightly to the core of the protein through hydrophobic interactions between the EF hands and the protein core. Thus, conformational changes induced by calcium exposes hydrophobic regions in EF hand proteins that are otherwise solvent inaccessible. Exposing these hydrophobic regions promotes protein-protein interactions (57). However, substrate binding is not limited to only the proximity of the hydrophobic regions exposed by the EF hand movement. Often, the movement of EF hands are translated throughout the protein and thus, calcium binding can modulate the tertiary structure of proteins, promoting allosteric binding of substrates or its translocation to the hydrophobic membrane. In addition, the movement of the helices can also displace regions of a protein that are upstream of the E helix or downstream of the F helix. Such is the case for CalDAG-GEFI as proposed by the data presented in chapter 5.

CalDAG-GEFI contains a pair of EF hands, and previous work has shown that in isolation, each EF hand binds one calcium ion. However, the consequences of calcium binding were not described (PDB: 2MA2) (54). As I mentioned, my studies determined that calcium

directly activates CalDAG-GEFI, but the question remained, what was calcium doing to CalDAG-GEFI to enhance its catalytic activity? However, to design experiments to answer this question, it is important to first understand how guanine nucleotide exchange proteins like CalDAG-GEFI, regulate their nucleotide exchange activity. The catalytic domain in CalDAG-GEFI is a cell-cycle-dependent (Cdc) 25 domain. The biochemical and biophysical mechanisms for the regulation of some Cdc25 domain family members have been determined. In the next chapter I will discuss some of the mechanisms regulating other Cdc25 domain proteins, which helped us to design experiments for studies that determine additional ways that CalDAG-GEFI activity may be regulated.



**Figure 1. The pentagonal bipyramidal geometry of calcium coordination of the EF hand domains**

A calcium ion is coordinated by residues 1, 3, 5, 7, 9, 12, and a water molecule. loop region. Once calcium is bound within the loop, the adjoining helices are perpendicular to one another as in the shape of the forefinger and thumb of the right hand.

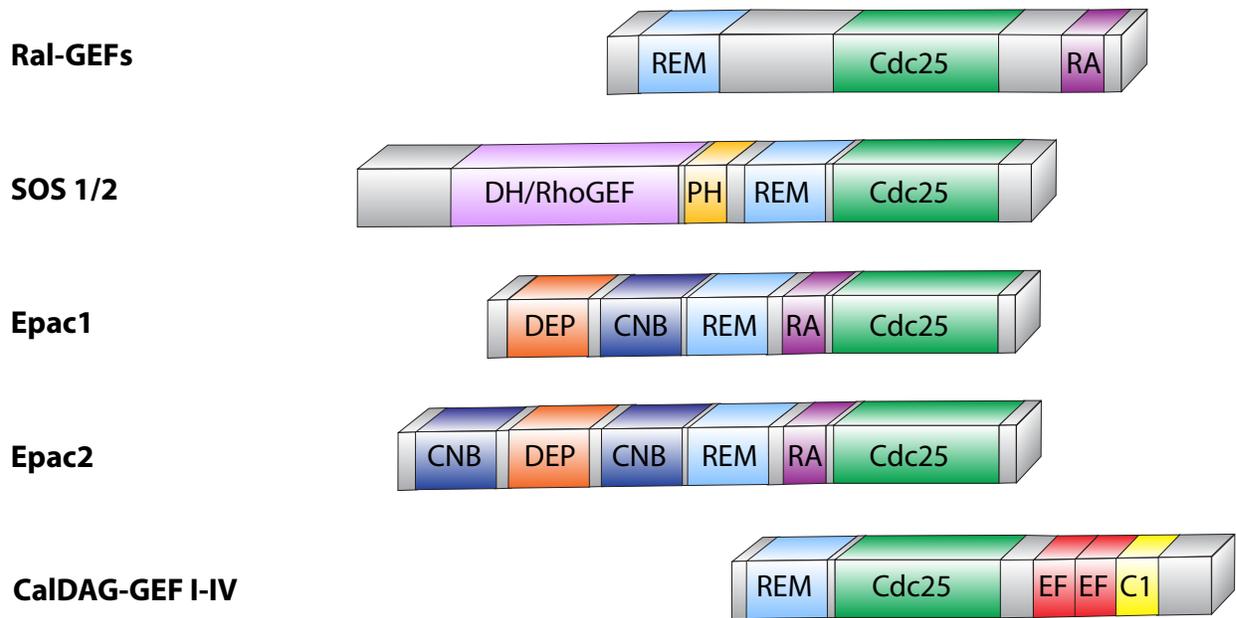
### **CHAPTER 3: MECHANISMS FOR REGULATING SMALL GTPASES BY CDC25 DOMAIN NUCLEOTIDE EXCHANGE FACTORS**

There are approximately 150 small GTPases in eukaryotes. The major isozymes discussed here is Ras associated protein (Rap). Rap is a member of the Rat Sarcoma (Ras) family. However, the most abundant GTPase expressed in platelets are the five Rap isozymes, Rap1A, Rap1B, and Rap2A-C. In particular, Rap1B is expressed 10-fold higher than the other Rap isozymes, accounting for 0.1% of total platelet proteins (58). GTPases function like molecular switches that are switched off when they are bound by guanosine diphosphate (GDP) and switched on when GDP is exchanged for guanosine triphosphate (GTP), a reaction catalyzed by a guanine nucleotide exchange factor (GEF) (59). The rate of nucleotide exchange in small GTPases is intrinsically slow due to the structural flexibility within SWI and SWII, which intermittently displaces the magnesium ion, allowing GTP loading. The concentration of GTP in a cell is nearly 10 times that of GDP thus once GDP is released GTP preferentially binds (60).

Inactive GTPases have a GDP molecule in their nucleotide binding pocket that is stabilized by three conserved secondary structural elements: a) the phosphate binding (P) loop, b) the switch one (SWI) region, and c) the switch two (SWII) region. Furthermore, an obligate magnesium ion stabilizes the GDP (13). The P loop, SWI, and magnesium ion form hydrogen bond networks that interact directly with GDP. Importantly, SWII stabilizes the magnesium ion within those networks. GEF binding releases GDP by inducing conformational changes in the GTPase that push SWII toward the P loop, displacing the magnesium ion, while pulling SWI away from GDP allowing for its release, known as the "push-pull mechanism" (59). Once GDP

is released, GTP is able to bind, switching the GTPase on. Once the GTPase is turned on, the GEF dissociates which allows the magnesium ion to re-coordinate within SWII, stabilizing GTP within the nucleotide-binding pocket. (PDB: 3X1W, 3X1X).

Ras-family small GTPases are activated by multiple GEFs. In turn, some of these GEFs activate multiple small GTPases while others are specific to a particular Ras-family isozyme. The information presented in this chapter provides myriad biochemical and structure/function data to describe the complex and unique regulatory mechanisms for a subset of the GEFs that all share a cell-cycle-dependent (Cdc)25 catalytic domain and activate Ras-family GTPases (Figure 2).



**Figure 2. Cdc25 domain guanine nucleotide exchange factors**

A, Domain architecture which include: Ras exchange motif (REM), cell-division-cycle 25 (Cdc25) domain, EF hand (EF), C1 domain, Ras-associated (RA) domain, Dbl homology (DH) domain, Pleckstrin homology (PH) domain, Rho GEF domain, Dishevelled, Egl-10, and Pleckstrin (DEP) domain, cyclic-nucleotide binding (CNB) domain.

The chapter will conclude by detailing the mechanism for how these small GTPases are switched off.

### *CalDAG-GEF (RasGRP) subfamily*

The calcium and diacylglycerol-regulated guanine nucleotide exchange factor (CalDAG-GEF) family consists of four members (I-IV), also referred to in the literature as Rat sarcoma (Ras) guanyl-nucleotide exchange-releasing protein (RasGRP) (RasGRP1-4). CalDAG-GEF isozymes catalyze nucleotide exchange on both Ras and Rap small GTPases and are composed of an N-terminal Ras exchange motif (REM) domain, a catalytic Cdc25 domain, two EF hands, and a C1 domain.

The CalDAG-GEFII-IV isozymes are primarily regulated by DAG and activate Ras and Rap isozymes. Interestingly, overexpression of these CalDAG-GEF isozymes transforms certain cell types from normal cells to a more cancer-like cell; phenotypes that are associated with aberrant signaling downstream of Ras and/or Rap proteins (61). Indicative of this transformation are changes in cell morphology, an increased rate of cell division, and prolonged cell survival (62). However, the exact regulatory mechanisms for each CalDAG-GEF isozyme attributing to these pathological cellular transformations are not well understood. In contrast, some of the mechanisms regulating each isozyme under physiological conditions are better understood, beginning with CalDAG-GEFII.

The CalDAG-GEFII is a selective nucleotide exchange factor for Ras isozymes, H-, M-, R-Ras1 and R-Ras2, which is highly expressed in distinct portions of the brain and a subset of immune cells (16). Although the role of CalDAG-GEFII in neurons is unclear, CalDAG-GEFII is required for developing and maintaining a healthy immune system, promoting T cell maturation, and proper signaling downstream of B- and T-cell receptors (63).

Recent work published by Iwig *et al.* provided structural and functional data on the molecular mechanisms underlying CalDAG-GEFII function. Data from a crystal structure of the truncated form of CalDAG-GEFII, lacking the C1 domain, suggests that in the absence of calcium CalDAG-GEFII is autoinhibited (PDB: 4L9M). Furthermore, it was shown that calcium binds to the N-terminal EF hand in CalDAG-GEFII, and that calcium binding leads to conformational changes that perturb an autoinhibitory linker region between the Cdc25 domain and EF hand 1.

Data from a second crystal structure of the isolated C1 domain of CalDAG-GEFII revealed another mechanism regulating autoinhibition, suggested by two subunits of the C1 domains that form coiled-coiled homodimers within the unit cell of these crystals. The C1 domain of CalDAG-GEFII shares significant sequence homology with the C1 domains in protein kinase C (PKC) isozymes, for which DAG binding is best understood. The C1 domain of CalDAG-GEFII isozymes binds DAG with nanomolar affinity ( $K_d \approx 1.5$  nM) (64). Functional studies using full-length CalDAG-GEFII with substitutions in residues that prevent dimer formation, revealed an enhanced nucleotide exchange activity, supporting not only homodimer formation in the full-length protein, but suggested that the dimers may be disrupted by DAG binding.

CalDAG-GEFIII is a nucleotide exchange factor for the same repertoire of small GTPases as CalDAG-GEFII: H-, M-, R-Ras, and TC21 (65, 66). Cells overexpressing CalDAG-GEFIII show increased activation of Ras as measured by enhanced levels of phosphorylated extracellular signal-related kinase 1/2 (ERK1/ERK2). This enhanced Ras activity was induced by treating cells with phorbol ester, a DAG mimetic (67). Upon treatment, CalDAG-GEFIII proteins relocalize from the cytosol to both, the perinuclear and plasma membranes (83, 88). This *in vitro*

data provides evidence for how the localization of CalDAG-GEFIII is regulated, but also suggests that DAG binding may release an autoinhibited state, allowing for its translocation to membranes and subsequent activity.

The CalDAG-GEFIV isozymes is a selective nucleotide exchange factor for H-Ras (16, 68). CalDAG-GEFIV has high expression in fetal tissues, specifically the lungs, liver, and spleen, whereas in adult tissue, it is expressed predominantly in leukocytes. CalDAG-GEFIV is the least studied isozyme in the CalDAG-GEF family. However, data has shown that fibroblast-like cells overexpressing CalDAG-GEFIV and treated with phorbol esters had enhanced recruitment of CalDAG-GEFIV to the membrane, had increased activity of H-Ras isozymes, and increased levels of phosphorylated ERK1/ERK2. These data were corroborated by studies using another cell type overexpressing CalDAG-GEFIV, which were also transformed, by a Ras-dependent mechanism, suggesting a possible role for CalDAG-GEFIV as an oncogene (62).

### *Ral GEF subfamily*

All members of the Ras-like (Ral) GEF family activate both RalA and RalB isozymes of Ral GTPases and is comprised of Ral guanine-dissociation stimulator (RalGDS), Ral releasing factor (Rlf), and RalGEF-like (RGL) isozymes (69). RalGEF isozymes are implicated as part of the signaling cascades required for endocytosis, gene transcription, cell proliferation, cell survival, and the production of second messengers. However, it has been difficult to ascertain their true function in these processes because of overlap between their activity and the activity of other Ras and Rap nucleotide exchange factors that are implicated in these same processes (70).

The RalGEF isozymes have three functional domains, an N-terminal REM domain, a Cdc25 domain, and (Ras-associated) RA domain. The REM domain allosterically regulates the activity of RalGEF isozymes, the catalytic Cdc25 domain catalyzes nucleotide exchange on Ral

isozymes, and the RA domain is a binding site for activated Ras isozymes. Activated Ras isozymes bind to RalGEFs inducing conformational rearrangements that release an autoinhibited state necessary for the normal function of Ral isozymes. Not surprising, oncogenic Ras dramatically increases the activation of RalGEFs, leading to enhanced expression of transcription factors associated with tumorigenesis (71). Given the effect of oncogenic Ras has on the activity of Ral isozymes, Ral has become the target of small molecules for cancer therapy (72). Unfortunately, little is known about the mechanism for how RalGEFs are regulated by Ras. Data from a crystal structure of the isolated RA domain of one RalGEF, Rlf reveals very little information about the effect of Ras on the RA domain in the context of the full-length protein (PDB:1RLF) (73).

Data from a crystal structure of truncated Rlf, lacking the RA domain, in complex with Ral, revealed that the activation of Rlf was regulated by residues comprised in a helical hairpin structure located in the REM domain (PDB:5CM8, 5CM9) (74). This hairpin structure contains hydrophobic residues interacting with Ral to stabilize regions in SWI, both enhancing its capacity for nucleotide exchange and promoting the release of Rlf from activated Ral proteins. After GTP binds Ral, active Ral dissociates from the Cdc25 domain in Rlf when the helical hairpin rotates away from the SWI region of Ral. A structural comparison between apo-Rlf protein and Rlf protein in complex with Ral, suggests that once Ral-GTP is released from the catalytic binding pocket, the helical hairpin structure becomes disordered and is more closely associated with the REM domain. Disorder in the helical hairpin in the absence of Ral, suggests that conformational rearrangements in Rlf are necessary to stabilize the secondary structure of the hairpin, a possible allosteric mechanism for activating Rlf isozymes.

Commonly, small GTPases are localized to the membrane and therefore GEFs must be recruited to the membrane to activate them. A proline-rich region at the N-terminus of Rlf, upstream of the REM domain, is thought to serve as a 'flagpole' to recruit Src-homology (SH) 3 domain proteins (PDB:4JGW) (75). This SH3 binding site of Rlf may be a docking site for interactions with a well-known adaptor protein, growth factor receptor-bound protein (Grb) 2, shown to regulate other GEFs by recruiting them to the plasma membrane (76).

### *Sos subfamily*

The Son of Sevenless (Sos) family of GEFs consists of the Sos1 and Sos2 isozymes whose complex regulatory mechanisms are attributed to their elaborate domain architecture. The Sos isozymes contain an N-terminal, histone binding domain, a DH/PH domain, PH/REM helical linker, REM domain, Cdc25 domain, and C-terminal Grb2/E3b binding domain. A wealth of data about the function of some of these regulatory domains has been compiled from biochemical and crystallographic studies defining key aspects for how individual domains contribute to the activity of Sos isozymes such as their cellular localization, their dual specificity for Rho and Ras isozymes, and positive-feedback mechanisms (77).

Inactive Sos isozymes are primarily localized to the cytosol but are recruited to the inner leaflet of the plasma membrane with the help of adaptor proteins that sense changes in the composition of phospholipid composition of the membrane- namely an increase in the concentration of PIP<sub>2</sub>. The DH domain of SOS binds PIP<sub>2</sub>, but sterically occludes by the PH domain, preventing the ability of Sos to activate Rac isozymes until it interacts with PIP<sub>2</sub>. Upon the conversion of PIP<sub>2</sub> in the membrane the PH domain binds and is displaced and the exchange capacity of Sos for Rac isozymes is enhanced, specifically at membranes (78). In addition, the conformational rearrangements in the DH/PH domain enhances the ability for Sos isozymes to

exchange nucleotide on Ras isozymes, suggesting the specificity of Sos for both Rac and Ras isozymes is allosterically regulated.

Data from a crystal structure of a truncated version of Sos1, containing the DH-PH, REM, and Cdc25 domains corroborates the biochemical data for the allosteric regulation of Sos isozymes. These data revealed that in the absence of PIP<sub>2</sub>, the DH-PH domain is in a bent conformation creating contacts between the REM and Cdc25 domain (79). This structural arrangement of the DH-PH domain is thought to be an autoinhibitory mechanism as determined by the reduced capacity for these truncated Sos proteins to exchange nucleotide on Rac and Ras isozymes. Concomitantly, biochemical data showed that Sos1, lacking the DH-PH domain has an enhanced capacity for nucleotide exchange on Rac (76). In addition, data from another crystal structure of Sos1, containing just the REM and Cdc25 in complex with Ras, defines a helical hairpin in the Cdc25 domain that undergoes conformational rearrangement when Ras proteins bind to the Cdc25 domain. These rearrangements break bonding contacts between the REM and Cdc25 domain revealing two hydrophobic patches, one in each domain, creating an allosteric binding site for Ras-GTP. Thus, Sos proteins bind Ras with a 2:1 stoichiometry. Importantly, when Ras-GTP binds at the allosteric site, nucleotide exchange activity by Sos1 at the primary site in the Cdc25 domain is dramatically enhanced. This allosteric mechanism dramatically increases the effective concentrations of active Ras as it clusters around receptors at the cell surface (PDB: 1NVV, 1NVW, 1XD2, 1XD4, 1XDV).

### *Epac subfamily*

There are four isozymes in the subfamily of the Exchange proteins activated by cyclic adenosine monophosphate (cAMP), Epac1 and Epac2A, 2B, and 2C. They all have five functional domains, although Epac1 have slightly different arrangements in their domain

architecture. The N-terminus of Epac1 has a Dishevelled, Egl-10, Pleckstrin (DEP) domain, followed by a cyclic nucleotide-binding domain (CNBD), a REM domain, a Ras-associated domain (RA), and catalytic Cdc25 domain. Epac 2 isoforms contain an N-terminal high affinity-CNBD domain, a DEP domain, a second, low affinity-CNBD, a REM domain, a Ras-associated domain (RA), and catalytic Cdc25 domain (80).

Epac isoforms were first discovered in a database search for unique proteins in cells that could activate Rap isoforms, downstream of increased concentrations of cAMP. Epac1 and Epac2 isoforms are differentially expressed; Epac1 isoforms are most abundant in the heart, liver, blood vessels, and adipose tissue. In the heart, Epac1 signals through  $\beta$ 1-adrenergic receptors and is necessary for cardiac contractility, relaxation, and establishing a consistent heart rate (81). However, sustained, elevated levels of cAMP in cardiac myocytes can lead to cardiac hypertrophy, and/or heart failure. Epac2 isoforms are expressed in the adrenal glands and pancreas (126). Rap1 activation by Epac2 triggers insulin and glucagon secretion in the pancreas without which increases the risk of obesity (82, 83).

Data from crystal structures of Epac2 show that these isoforms remain in an autoinhibited state that is released by conformational rearrangements induced by cAMP binding. The details about the rearrangements have been described using data from crystal structures comparing the conformations of active Epac2 to inactive Epac2 isoforms. Autoinhibition is released when cAMP binds to the CNBD and phenylalanine 435 is displaced exposing the binding site for Rap isoforms in the Cdc25 domain. F435 is a residue within the fifth helix of the N-terminus referred to as the ionic latch and stabilizes Epac2 in a more compact form relative to the active protein (PDB: 2BYV) (84, 85). Furthermore, F435G substitution in Epac2 causes binding of Rap isoforms in the absence of cAMP (PDB: 3CF6) (86).

## **CHAPTER 4: A NEGATIVE-FEEDBACK LOOP REGULATING ERK1/2 ACTIVATION AND MEDIATED BY RASGRP2 PHOSPHORYLATION**

### *Introduction*

The Ras-Raf-MEK-ERK signaling pathway is essential for many cellular processes, including growth, cell-cycle progression, differentiation, and apoptosis. ERK1/2 phosphorylates hundreds of substrates *in vivo*, but these activities are restricted by cell type and subcellular compartment (87). Furthermore, the complexities and dynamics of ERK1/2 signaling are modified by both positive and negative-feedback loops (88). For example, Sos1 is a GEF that activates Ras isoforms. In turn, active Ras allosterically enhances the exchange capacity of Sos1 and this positive feedback loop ultimately impacts ERK1/2 activation (76, 89). Conversely, ERK1/2 phosphorylates both Sos1 and Raf to suppress further activation of ERK1/2 in a negative-feedback loop (90-92).

ERK1/2 is also activated downstream of the small GTPase, Rap1, in platelets. (10, 16, 92, 94). However, in contrast to activation by Ras isoforms, very little is known about potential feedback regulation by ERK1/2 on Rap1 signaling. RasGRP2 (alternative name CalDAG-GEFI) is the predominant GEF that activates Rap1 in platelets. RasGRP2 specifically activates Rap1 but not Ras isoforms both *in vitro* and *in vivo* (17, 18). In this study, using purified proteins and cellular studies, we show that ERK1/2 phosphorylates RasGRP2 to limit its exchange activity. In addition, we show that this connection establishes a negative-feedback loop that ultimately controls the dynamics of active ERK1/2.

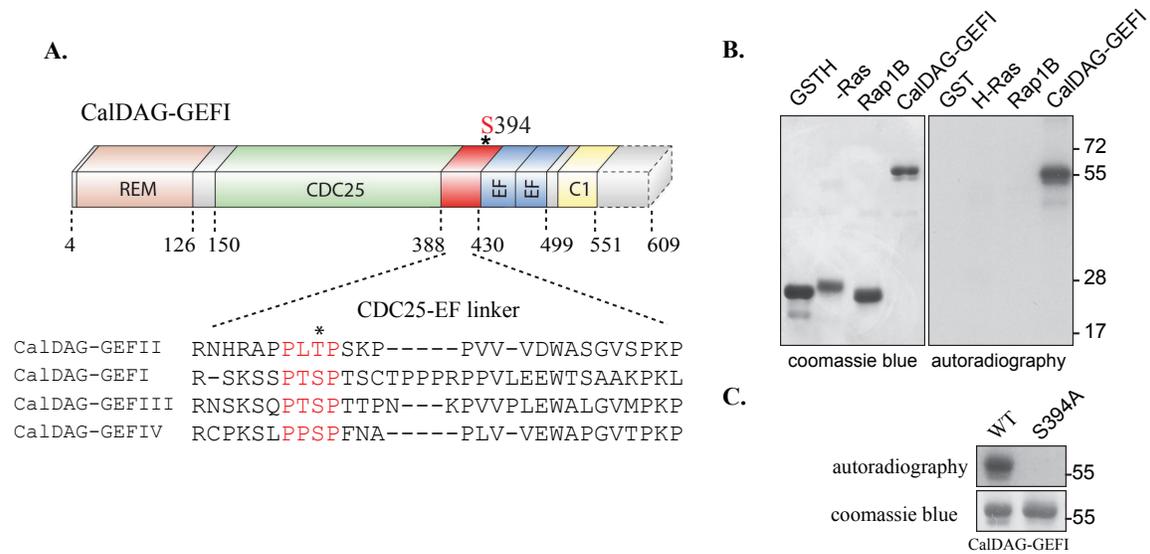
<sup>93</sup> Ren, J., Cook, A. A., Bergmeier, W., and Sondek, J. (2016) A negative-feedback loop regulating ERK1/2 activation and mediated by RasGPR2 phosphorylation. *Biochemical and Biophysical Research Communications*. 474, 193–198

## *Results*

### **ERK2 phosphorylates RasGRP2 at Ser394 in vitro**

RasGRP family members have four conserved functional domains: i) an N-terminal Ras-exchange motif (REM), ii) a catalytic CDC25 domain, and a C-terminal regulatory region comprised of iii) two EF hands and iv) an atypical C1 domain (Fig. 3A). Post-translational modifications to RasGRP2 have previously been identified and are available from several databases that provide mass spectral data (95, 96). From these databases, we noted that RasGRP2 is phosphorylated at Ser394 within the linker between the CDC25 domain and EF hands. In particular, this region occludes the Ras-binding region of RasGRP1 in its autoinhibited state and is conserved in other RasGRP isoforms (54). Furthermore, sequence adjacent to Ser394 matches a consensus motif of many ERK1/2 substrates defined by P-X-S/T-P, where “X” indicates any amino acid and S/T is the site of phosphorylation (Fig. 3A). Thus, we hypothesized that ERK1/2 phosphorylates RasGRP2 at Ser394 to potentially regulate its capacity to activate Rap1.

To test this idea, we initially assessed the capacity of constitutively active ERK2 (ERK2-CA) to phosphorylate purified RasGRP2. Notably, ERK2-CA robustly phosphorylated RasGRP2 but not in other proteins including GST, H-Ras and Rap1b (Fig. 3B). Moreover, ERK2-CA failed to phosphorylate RasGRP2(S394A) indicating that Ser394 is the major site of phosphorylation under these conditions.

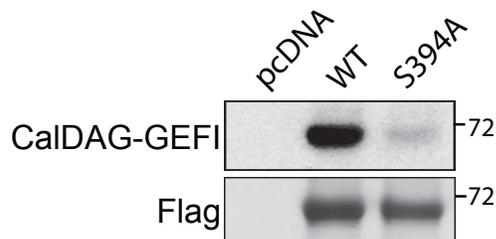
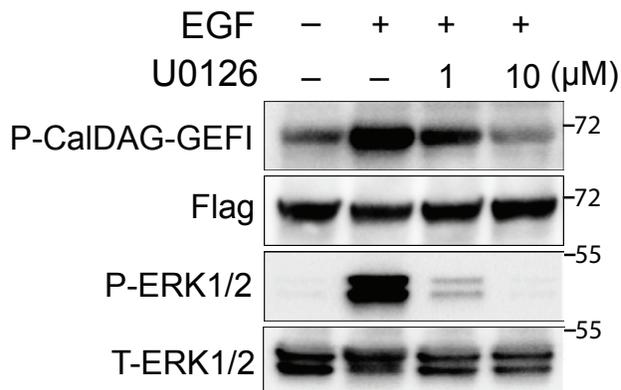


**Figure 3. ERK2 phosphorylates RasGRP2 at Ser394 in vitro**

A. Domain architecture of human RasGRP2. Sequence alignment for the individual RasGRP family members highlights the conserved ERK1/2 phosphorylation motif “PXSP” (red) in the linker between the Cdc25 domain and the EF hands. Asterisk identifies Ser 394 in RasGRP2. B. ERK2 phosphorylates RasGRP2 *in vitro*. C. ERK2 phosphorylates RasGRP2 at Ser394. For panels B and C, bacterially purified proteins (5  $\mu$ g) were incubated with recombinant, constitutively active ERK2 in the presence of ( $^{32}$ P)  $\gamma$ -ATP for 10 min prior to SDS-PAGE and autoradiography

## **The ERK1/2 pathway is required for phosphorylation of RasGRP2 in cells**

HEK239T cells are competent to activate ERK1/2 downstream of EGF activation. Consequently, we transfected these cells with either WT RasGRP2 or RasGRP2(S394A) prior to stimulation with EGF. Without EGF addition, RasGRP2 is basally phosphorylated based on an antibody specific for ERK1/2 substrates (Fig. 4A). Consistent with our *in vitro* data.

**A.****B.**

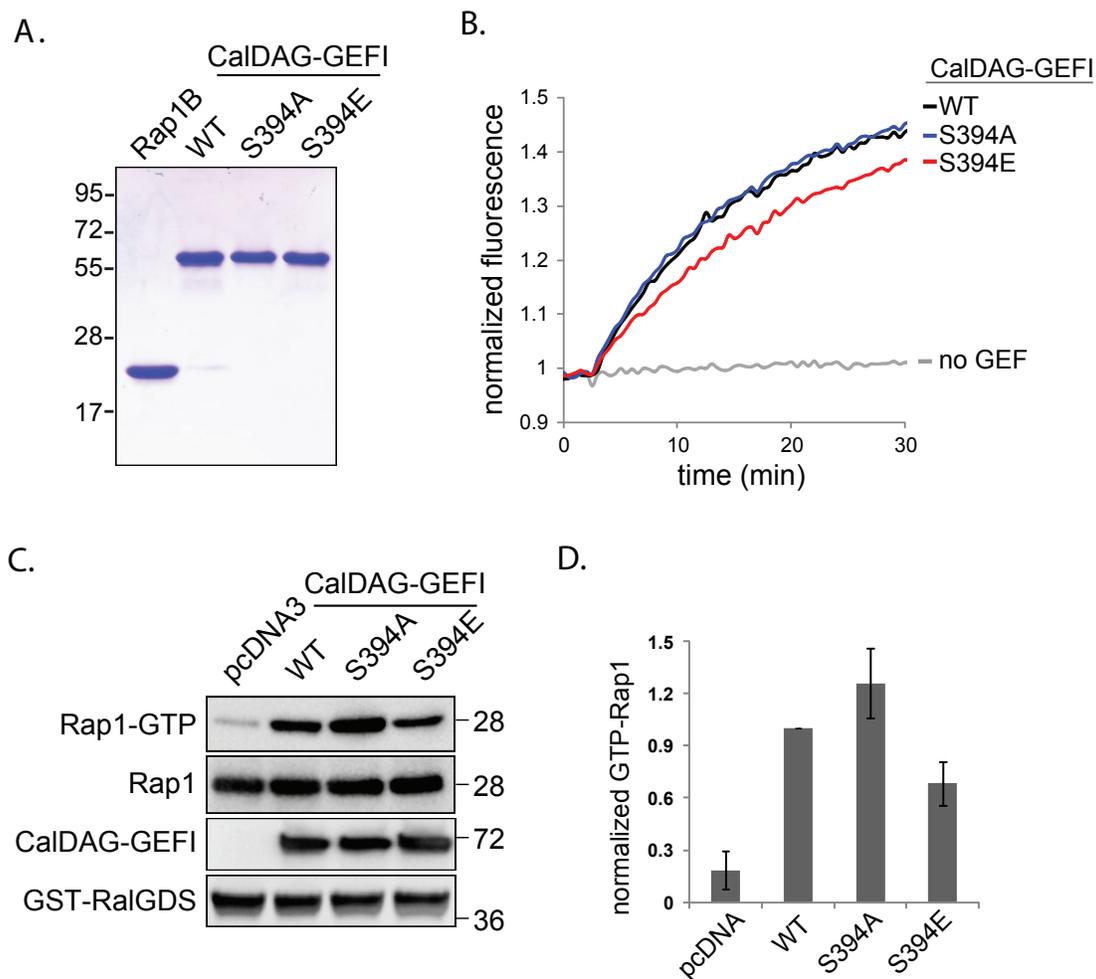
**Figure 4. RasGRP2 is phosphorylated in response to epidermal growth factor (EGF) stimulation in HEK293T cells.**

A. RasGRP2 is basally phosphorylated at Ser394 in HEK293T cells. Plasmids encoding FLAG-tagged RasGRP2 wild-type (WT) or S394A were transfected into HEK293T cells prior to immunoprecipitation of the expressed protein and western blotting as indicated. The parental vector was used as a control and treated similarly. B Phosphorylation of RasGRP2 in response to EGF is eliminated by the MEK inhibitor, U0126. Cells were pretreated with the indicated amount of U0126 or DMSO for 1 hr prior to stimulation with 50 ng/ml EGF for 5 min. FLAG-RasGRP2 was immunoprecipitated from cell lysates and followed by western blotting as indicated. Cell lysates were also blotted for total ERK1/2 (T-ERK1/2) and phos-ERK1/2 (P-ERK1/2)

However, RasGRP2(S394A) is poorly phosphorylated under these conditions. Upon stimulation with EGF, phosphorylation of RasGRP2 is increased and conversely, this phosphorylation is dramatically decreased upon inhibition of MEK1 with the small molecule, U0126 (Fig. 4B).

### **Phospho-mimetic mutation of RasGRP2 impairs nucleotide exchange activity**

A fluorescence-based, guanine nucleotide exchange assay was used to assess the capacities of purified RasGRP2 proteins to activate Rap1b (Fig. 5A-B). The intrinsic rate of nucleotide exchange of Rap1b is low and this rate was increased dramatically by the addition of RasGRP2. A similar increase in exchange activity was seen upon addition of RasGRP2(S394A). However, the phospho-mimetic mutant, RasGRP(S394E), was unable to activate Rap1b to the same degree. Consistent with these in vitro findings, the expression of RasGRP2(S394E) in cells led to reduced levels of active Rap1 when compared to cells expressing RasGRP2 or RasGRP2(S394A) (Fig. 5C-D).



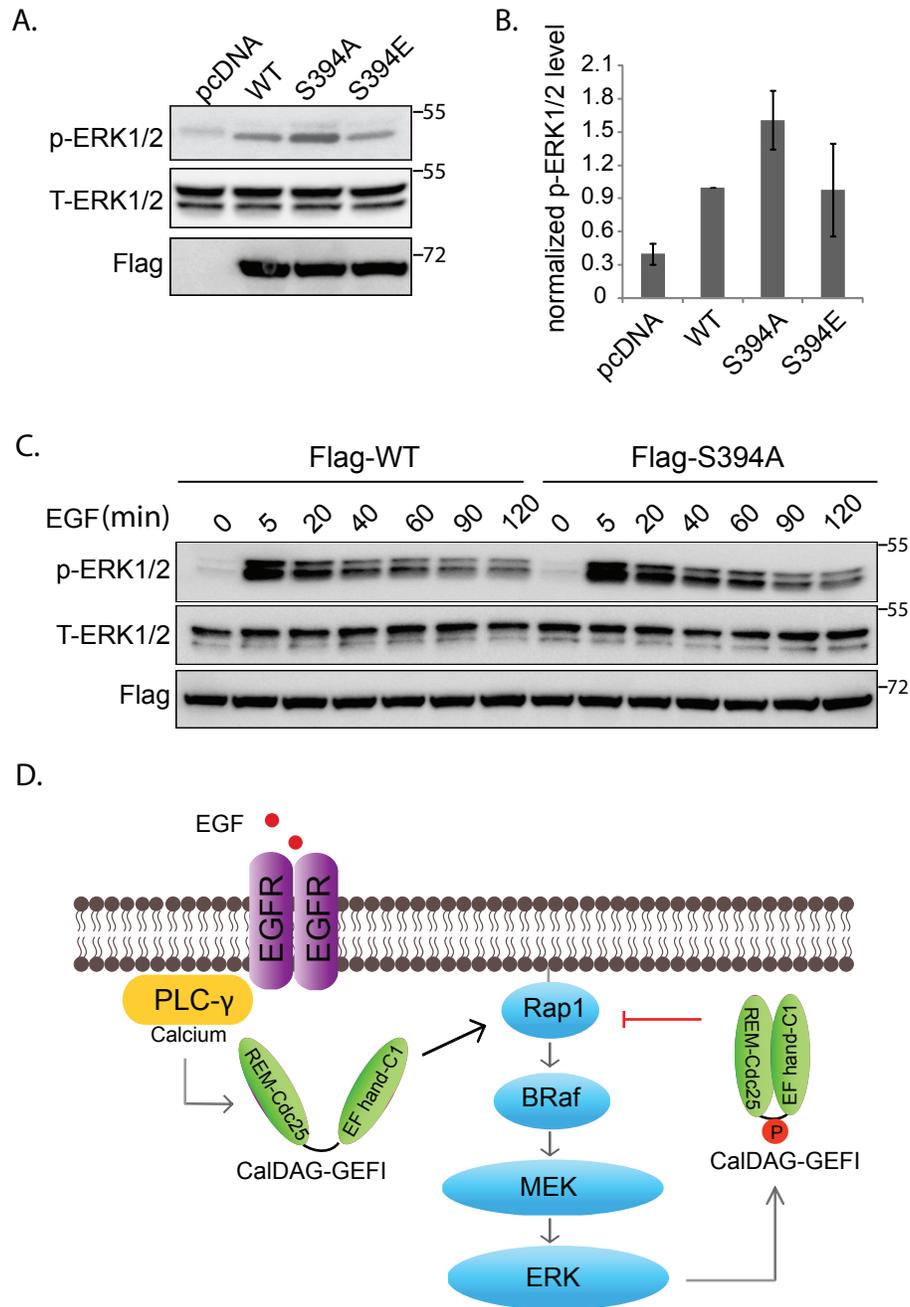
**Figure 5. Phosphorylation of RasGRP2 impairs its nucleotide exchange activity**

A. Purified proteins (3  $\mu$ g) were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. B. *In vitro* nucleotide exchange assay. Equivalent amounts of Rap1b and BODIPY-FL-GDP were preincubated prior to the addition (arrow) of the indicated forms of RasGRP2. C. GST-RalGDS was used to precipitate GTP-Rap1 from HEK293T cells expressing the indicated forms of RasGRP2 prior to western blotting for proteins as indicated. D. GTP-Rap1 levels as shown in C were normalized to that of WT (average  $\pm$  SEM).

## **RasGRP2 phosphorylation negatively regulates ERK1/2 activation**

ERK1/2 activation by Rap1 requires B-Raf (10). The previous experiments indicate that ERK1/2 phosphorylates and inhibits the Rap1 GEF, RasGRP2. Thus, it is possible that these proteins participate in a negative-feedback loop whereby active ERK1/2 leads to decreased active Rap1 through RasGRP2. Since HEK293T cells also express B-Raf in addition to being competent to activate ERK1/2 as shown above, we used this cell line to delineate potential linkages between RasGRP2 and ERK1/2. Consistent with a negative-feedback loop, expression of RasGRP2(S394A), which is not able to be phosphorylated, increased levels of phos-ERK1/2. (Fig. 6A-B). Expression of the phospho-mimetic mutant, RasGPR2(S394E), did not substantially reduce phos-ERK1/2 levels, but this situation might arise due to the already high basal levels of phosphorylated WT RasGRP2 in these cells (Fig.6).

Studies show disruption of other ERK1/2 negative-feedback loops increase the amplitude and duration of ERK1/2 activation upon stimulation (3). If phosphorylation of RasGRP2 at Ser394 is critical for a negative-feedback loop involving active ERK1/2, then we might expect to see an increase in the amplitude and duration of ERK1/2 activation upon expression of RasGRP2(S394A). Indeed, ERK1/2 activation was increased and prolonged in EGF-stimulated cells expressing RasGRP2(S394A) when compared to cells expressing WT RasGRP2 (Fig. 6C).



**Figure 6. Phosphorylation of RasGRP2 negatively regulates ERK1/2**

A. P-ERK1/2 levels in HEK293T cells transfected with the indicated forms of RasGRP2. Cells were lysed 24 hrs after transfection and analyzed by western blotting as indicated. B. Levels of P-ERK1/2 as shown in A for three independent experiments (average  $\pm$  SEM), analyzed with Image J and normalized to that of the cells transfected with WT RasGRP2. C. RasGRP2(S394A) increases the magnitude and duration of ERK1/2 phosphorylation in response

to EGF stimulation. HEK293T cells were transfected with either WT or mutant RasGRP2. Twenty-four hours after transfection, cells were starved for 2 hours and treated with 20 ng/ml EGF for the indicated times prior to lysis. Cell extracts were subjected to SDS-PAGE and immunoblotting as indicated. The result is representative of three independent experiments. (D) Model of the RasGRP2 mediated negative-feedback loop. Active EGFR recruits and activates PLC- $\gamma$  isozymes leading to elevated levels of intracellular calcium and subsequent activation of RasGRP2. Activated RasGRP2 catalyzes the formation of GTP-bound Rap1 to initiate signaling through B-Raf to ERK1/2. ERK1/2 subsequently phosphorylates RasGRP2 at Ser394 to impair the activation of Rap1 and ultimately attenuating the Rap1-ERK pathway.

## *Discussion*

The equivalent of Ser394 and surrounding residues of RasGRP2 are conserved in all members of the RasGRP family suggesting that the negative-feedback loop described here for RasGRP2 is also relevant for signaling by other RasGRP family members. Consistent with this hypothesis, phosphorylation of human RasGRP3 at Ser391, the equivalent of Ser394 in RasGRP2, was identified in two independent phosphoproteomics studies (97, 98).

In addition to ERK1/2, PKA has also been shown to phosphorylate RasGRP2 at multiple sites (99-101). However, these studies have produced conflicting results. In the original studies, phosphorylation by PKA resulted in the inhibition of RasGRP2 in HEK293T cells, while the most recent study showed PKA-mediated activation of RasGRP2 in Cos-7 cells. It is difficult to reconcile these results and perhaps future studies using purified proteins will clarify this issue. It seems likely that the RasGRP proteins are directly targeted by diverse kinases and that the regulation of RasGRP2 by phosphorylation is crucial for a range of cellular processes.

Signaling cascades leading to the activation of ERK1/2 are subject to stringent homeostatic control through both positive and negative-feedback loops. A number of inhibitors that target these cascades are used to treat cancer. However, drug-resistant tumors frequently emerge leading to disease progression. Alteration in the feedback regulation of ERK1/2 pathway is one potential cause of acquired drug resistance. Our study has defined a new negative-feedback loop from ERK1/2 to RasGRP2 and this feedback contributes to the dynamic regulation of ERK1/2 activity that likely has clinical ramifications.

## **CHAPTER 5: CALCIUM-INDUCED STRUCTURAL REARRANGEMENTS RELEASE AUTOINHIBITION IN THE RAP-GEF, CALDAG-GEFI**

### *Introduction*

Human survival depends on our ability to prevent blood loss at sites of vascular injury. Upon damage to the endothelial lining, blood platelets detect exposed extracellular matrix and locally produced thrombin, become activated, and form a hemostatic plug. Critical to plug formation is the engagement of the  $\alpha\text{IIb}\beta\text{3}$  integrins on the cell surface, a process that depends on the inside-out signaling to these receptors (4).

Given the unique high shear environment found in blood vessels, the signal transduction in platelets leading to integrin inside-out activation has been optimized for sensitivity and speed. Upon receptor stimulation, the second messenger calcium, is rapidly released from the dense tubule system (DTS). Calcium also enters through channels in the plasma membrane, effectively increasing intracellular calcium concentrations 50-fold. Calcium plays an important role in the activation of  $\alpha\text{IIb}\beta\text{3}$ . Integrin activation also depends strongly on the focal adhesion proteins, talin-1 and kindlin-3, as well as the small GTPase Ras-related protein (Rap) 1 (2, 4). The Rap1 isozyms, Rap1A and Rap1B, are guanine nucleotide binding proteins and members of the large superfamily of Ras small GTPases. Small GTPases act like molecular switches, cycling between an inactive, GDP-bound, and an active, GTP-bound, state. The biological role of Rap1B in platelets has been well studied. Genetically modified mice lacking the Rap1B isozyms have a

<sup>102</sup> Cook, A. A., Deng, W., Ren, J., Li, R., Sondek, J., and Bergmeier, W. (2018) Calcium-induced structural rearrangements release autoinhibition in the Rap-GEF, CalDAG-GEFI. *J. Biol. Chem.* 10.1074/jbc.RA118.002712

number of platelet defects, including a marked reduction in integrin activation in response to agonist stimulation (1).

Small GTPase activity is modulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The most abundant Rap-GEF and Rap-GAP in platelets are calcium- and diacylglycerol-regulated guanine nucleotide exchange factor I, (CalDAG-GEFI, RasGRP2) and Ras p21 protein activator 3, (RASA3, GAP1<sup>IP4bp</sup>), respectively (17, 40). CalDAG-GEFI contains a Ras exchange motif (REM) domain with no known function, a catalytic Cdc25 domain, a pair of calcium binding EF hands, and an atypical C1 domain with no known function. Studies in mice, dogs, and humans lacking functional CalDAG-GEFI, demonstrate that CalDAG-GEFI/Rap1B signaling is crucial for rapid activation of  $\alpha$ IIB $\beta$ 3 required for platelet adhesion at sites of vascular injury (17, 103-105).

EF hands are composed of pairs of helices that typically bind calcium using acid residues within the intervening loop. These residues are defined by their relative positions (1, 3, 5, and 12) and in particular position 12 is a highly conserved glutamate that coordinates calcium through bidentate interactions. Mutating this glutamate cripples calcium binding (51, 56). Calcium binding generally leads to major conformational changes in the EF hands and other regions of the protein (53).

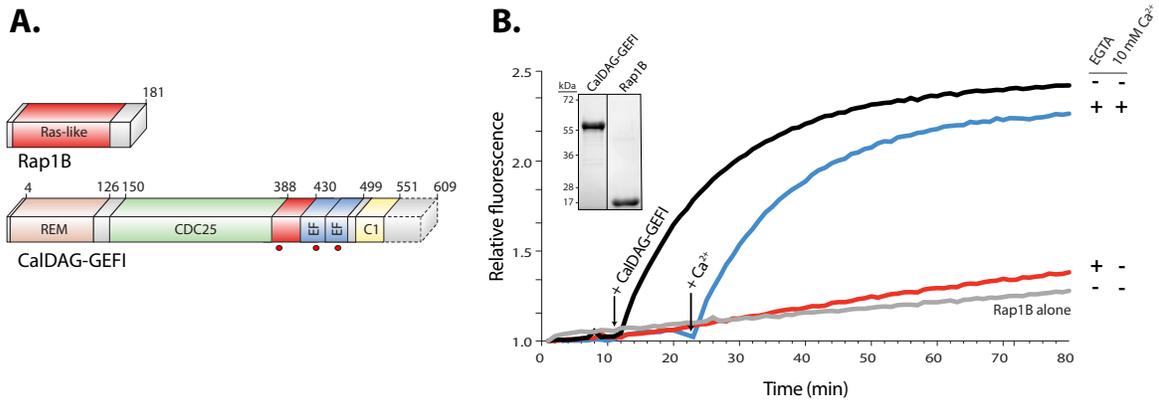
Our functional studies in platelets suggested calcium is involved the regulation of CalDAG-GEFI activity (10). In this work, we present biochemical evidence that both EF hands contribute to CalDAG-GEFI activation and that activity require calcium. Hydrogen-deuterium exchange mass spectrometry studies further suggest that calcium binding induces global conformational changes within CalDAG-GEFI, most prominently in the EF hands and a putative autoinhibitory linker sequence connecting the EF hands and Cdc25 domain. Analysis of different

mutant forms of CalDAG-GEFI confirms the linker is important for autoinhibiting CalDAG-GEFI in the absence of calcium. Thus, our work provides the first evidence that (1) CalDAG-GEFI activity is directly regulated by calcium and (2) release of autoinhibition as the molecular mechanism underlying CalDAG-GEFI activation.

## Results

### **CalDAG-GEFI activation is calcium-dependent.**

Previous studies using isothermal titration calorimetry determined the isolated EF hands of CalDAG-GEFI (amino acid residues 417-495) bind calcium with very high affinity ( $K_d \sim 80$  nM) (54). To determine if calcium affects catalytic activity in full length CalDAG-GEFI, we established a cell-free nucleotide exchange assay using purified human CalDAG-GEFI and Rap1B proteins. Purified CalDAG-GEFI (amino acid residues 1-551, WT) was slightly truncated at the C-terminus to increase protein stability. Rap1B (amino acid residues 1-181) was purified with a substitution, cysteine 181 to serine, to increase solubility (Fig. 7A). Purification was performed using affinity and size exclusion chromatography. Proteins were >95% pure as confirmed by SDS-PAGE (Fig. 1B, inset). Proteins were stable at 4°C for up to 15 hours. Compared to Rap1B alone, exchange activity increased by ~16-fold in samples containing Rap1B and CalDAG-GEFI. Of note, exchange activity was not altered by the addition of free calcium (not shown). However, treatment of CalDAG-GEFI with a 20mM ethylene glycol-bis ( $\beta$ -aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA), a calcium-selective chelator, dramatically reduced catalytic activity. Importantly, exchange activity was restored upon addition of 10 mM free calcium, demonstrating that the loss of activity in EGTA-treated samples was not due to protein instability (Fig. 7B).

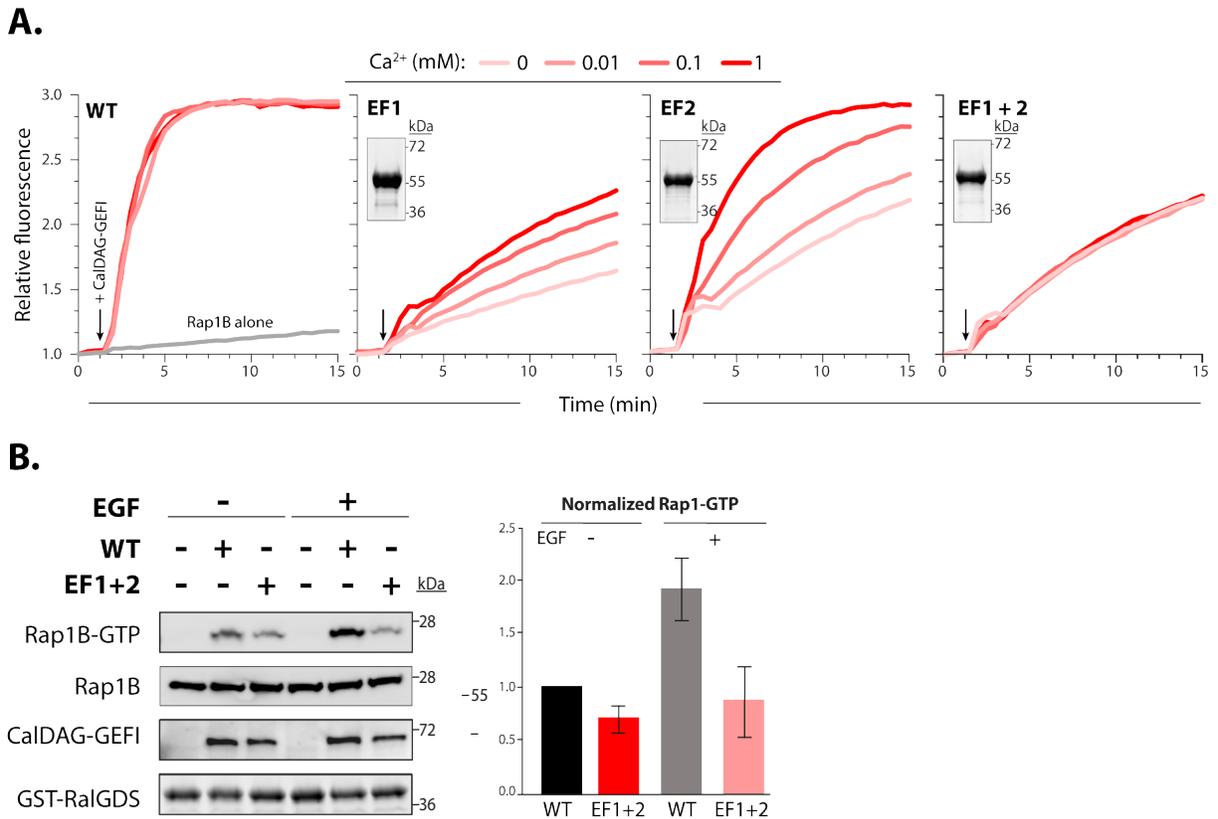


**Figure 7. CalDAG-GEFI requires calcium to activate Rap1B.**

A. Domain architecture. Rap1B is primarily composed of a Ras-like domain (red) while CalDAG-GEFI is a multi-domain protein consisting of a Ras exchange motif (REM) domain (salmon), catalytic Cdc25 domain (green), a putative autoinhibitory linker (red), two calcium-binding EF hands (blue), and an atypical C1 domain (yellow). CalDAG-GEFI was truncated (dotted lines) at residue 551 for purification. Truncated residues are not conserved and do not impact the capacity of CalDAG-GEFI to activate Rap1B. Substitutions in CalDAG-GEFI used in this paper are marked (red circles) below its domain architecture. B. Activation of Rap1B by CalDAG-GEFI monitored by the increased fluorescence of BODIPY FL GDP loaded onto Rap1B. Nucleotide (100 nM) and GTPase (1  $\mu$ M) were incubated in four wells monitored simultaneously ( $\lambda_{\text{ex/em}} = 480/520$ ). Select reactions also included 10 mM EGTA as indicated. At 12 min (left arrow), 400 nM CalDAG-GEFI was added to all reactions except the one marked “Rap1B alone”. Addition of 10 mM free Ca<sup>2+</sup> (right arrow) reconstituted exchange activity. Inset: purified CalDAG-GEFI and Rap1B (2  $\mu$ g) used in nucleotide exchange reactions; stained gel after SDS-PAGE.

**Both EF hands are critical for regulating CalDAG-GEFI exchange activity.**

To determine if and how the individual EF hands regulate CalDAG-GEFI activity, we mutated a key residue in the calcium binding loops (Fig. 8A). Substitutions of glutamic acid at position 12 in the calcium binding loop of EF hands has been shown to reduce the binding affinity for calcium by over 100-fold (106, 107). Glutamic acid substitution for alanine at position 450 in the N-terminal EF hand (EF1) or position 479 in the C-terminal EF hand (EF2) markedly decreased nucleotide exchange towards Rap1B. Catalytic activity was restored by adding increasing concentrations of calcium in EF2, while only a partial recovery was observed in the EF1. Catalytic activity in the double EF hand mutant (EF1+2) could not be restored (Fig. 8A). These data provide strong evidence that calcium binding to EF1 is essential for exchange activity in CalDAG-GEFI, while EF2 is important but not essential in this process. To validate our in vitro findings in a cellular context, we studied agonist-induced Rap1 activation in HEK293T cells expressing WT or EF1+2 CalDAG-GEFI (Figure 8B). Cellular stimulation with epidermal growth factor (EGF) led to a significant increase in Rap1-GTP levels in cells expressing WT CalDAG-GEFI. In contrast, Rap1 activation was not observed in EGF-stimulated cells expressing EF1+2 CalDAG-GEFI.



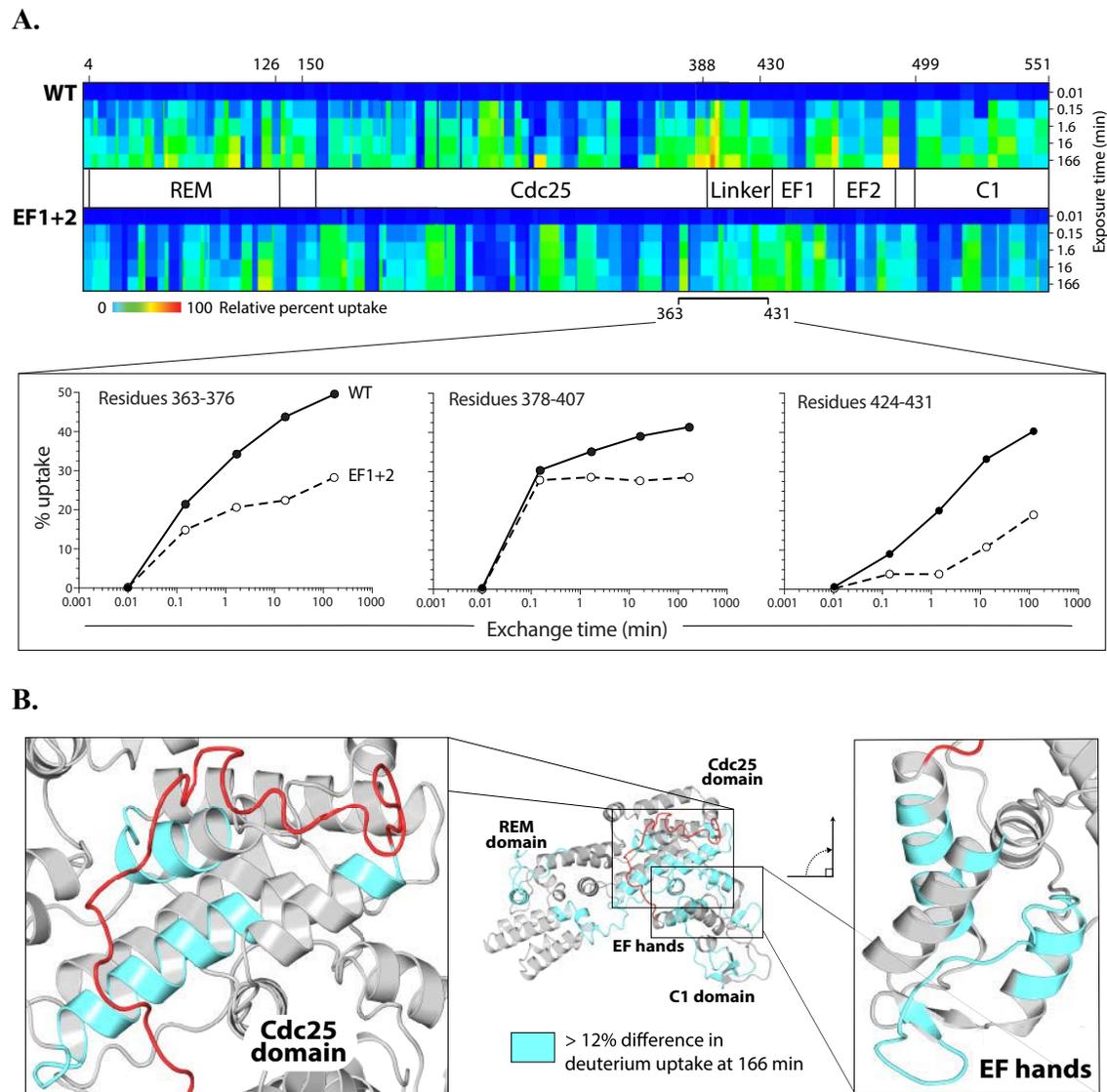
**Figure 8. Mutations in the calcium-binding EF hands reduce the capacity of CalDAG-GEFI to active Rap1B.**

A. Nucleotide exchange was monitored as described in Figure 1. EF1 and EF2 indicate mutant forms of CalDAG-GEFI substituted (E450A and E479A) at equivalent positions within the N- and C-terminal EF hands, respectively; EF1+2 indicates the double mutant. Insets: stained gels of purified, mutants of CalDAG-GEFI (2  $\mu$ g) after SDS-PAGE. B., left panel: stained gel for GST-RBD pull down of active Rap1B from HEK-293T cells expressing WT or EF1+2 proteins after SDS-PAGE and Western blot analysis. Right panel (representative of three independent experiments). Cells were incubated for 5 minutes in the presence and absence of 100ng/ml epidermal growth factor (EGF) before lysis. Right panel: Quantification of Rap1-GTP levels (mean  $\pm$  SD).

## **Calcium binding to CalDAG-GEFI induces conformational rearrangements required for its activity.**

We next performed hydrogen-deuterium exchange mass spectrometry experiments (HDX-MS) to determine the differences in deuterium uptake between WT and EF1+2 proteins. Measuring the rate of deuterium uptake defines the stability of hydrogen bond networks between residues stabilizing secondary structure in proteins as well as residues that are more solvent accessible (108, 109). The most stable hydrogen bonds have the slowest exchange while more dynamic regions exchange faster. WT and EF1+2 proteins were exposed to deuterated water for a designated amount of time and then quenched and digested for mass spectrometric analysis. We recovered 326 peptides from the WT sample and 329 from the EF1+2 sample, with complete coverage of both samples.

The relative difference in deuterium uptake was represented in a heat map to highlight the regions with slowest (dark blue) and fastest (red) deuterium exchange (Fig. 9A). WT and EF1+2 showed unique deuterium exchange signatures, suggesting conformational differences between the active and the apo form of the protein. A greater than 5% difference in uptake between two samples is considered significant (109). Deuterium uptake in a putative autoinhibitory region linking the Cdc25 domain and EF1 was markedly higher in the WT sample (23% and 25%, respectively) (Fig. 9A). Different regions of the WT sample had greater than 12% higher rates of exchange in WT compared to EF1+2 (Fig. 9B), including a small portion of the REM domain (19%), the Cdc25-Rap1B interface (20%), and the EF hands (17%) Displacement of the linker in response to calcium binding would provide a likely explanation for the very fast deuterium exchange rates measured for the residues comprising the Cdc25 domain-Rap1B interface in the WT protein.

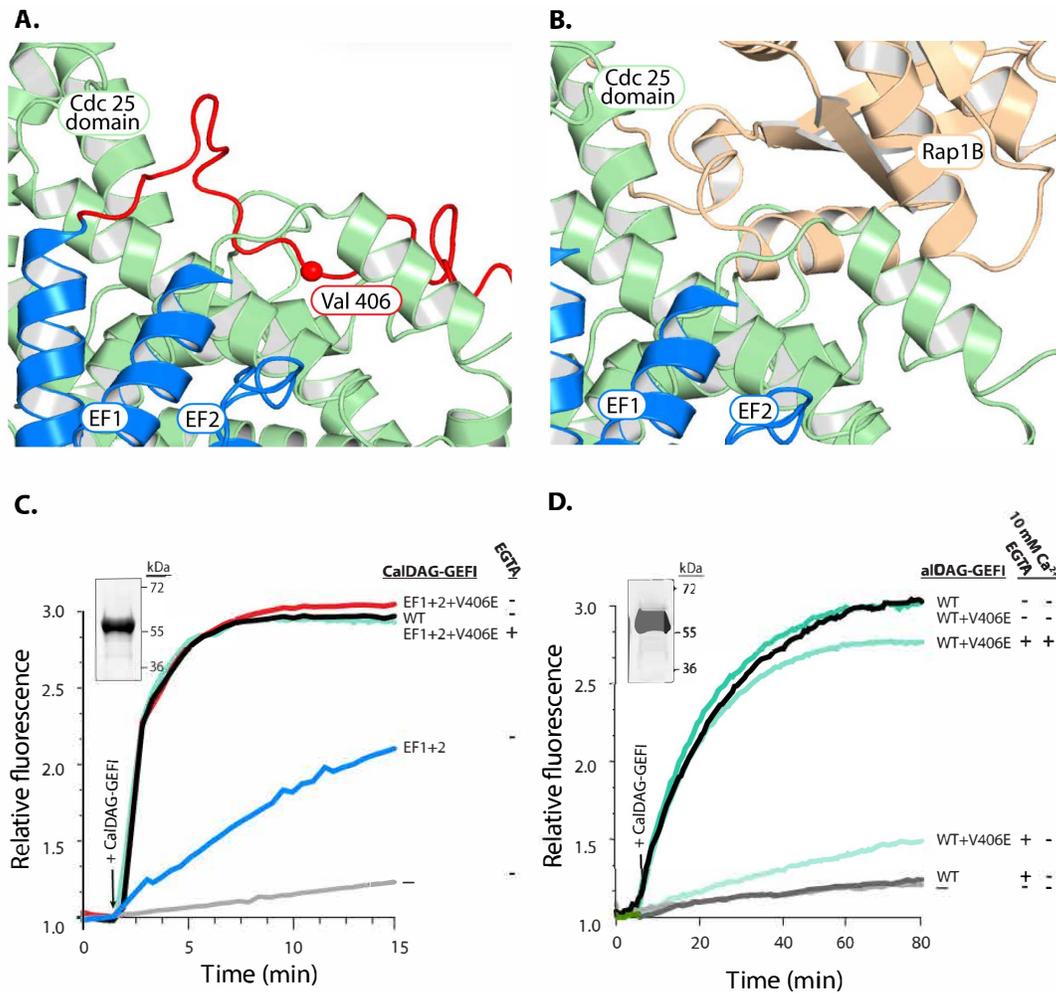


**Figure 9. Differential hydrogen-deuterium exchange between CalDAG-GEFI WT and EF1+2.**

A. Heat maps of deuterium uptake for CalDAG-GEFI and EF1+2. Proteins were incubated in deuterated water for indicated times prior to measurements of deuterium uptake using mass spectrometry. Largest differences in uptake between the two proteins span the putative autoinhibitory linker and EF hands shown for individual peptides below the heat maps. B. Homology model of CalDAG-GEFI. Blue regions map enhanced deuterium uptake (> 12%; excluding the linker) of CalDAG-GEFI relative to EF1+2. The majority of these regions cluster within the binding site for Rap1B and EF hands shown in expanded views

### **Valine 406 contributes to maintain CalDAG-GEFI in an autoinhibited state.**

We next determined critical residues within the putative autoinhibitory linker region using a homology model of inactive CalDAG-GEFI, based on predictions by the Iterative Threading ASSEMBLY Refinement (I-TASSER) server (for more details see Material and Methods section) (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>). We identified amino acids 406-410 (VLEEW) as the residues that insert directly into the Rap1B binding groove (Fig. 10A, B). This region of the linker is fully conserved in all mammals. To test whether displacement of this linker is sufficient to activate CalDAG-GEFI, we purified both WT and EF1+2 protein with an additional substitution, a valine 406 for glutamic acid and tested the capacity of these proteins for nucleotide exchange towards Rap1B. We selected to substitute valine 406 as our homology model suggested a key role for this residue in anchoring the autoinhibitory linker in the Rap1 binding groove. Consistent with this conclusion, the V406E mutation completely restored catalytic activity in EF1+2 CalDAG-GEFI, suggesting that the autoinhibitory linker was displaced in this mutant in absence of bound calcium (Fig. 10C). The V406E mutation had a weaker effect in the WT protein, where nucleotide exchange activity was still markedly impaired in the presence of EGTA (Fig. 10D). These studies suggest that the interaction of V406 with the Cdc25 domain contributes to the autoinhibited state of CalDAG-GEFI.



**Figure 10. Substitution of a conserved valine fully restores the exchange activity of EF1+2.**

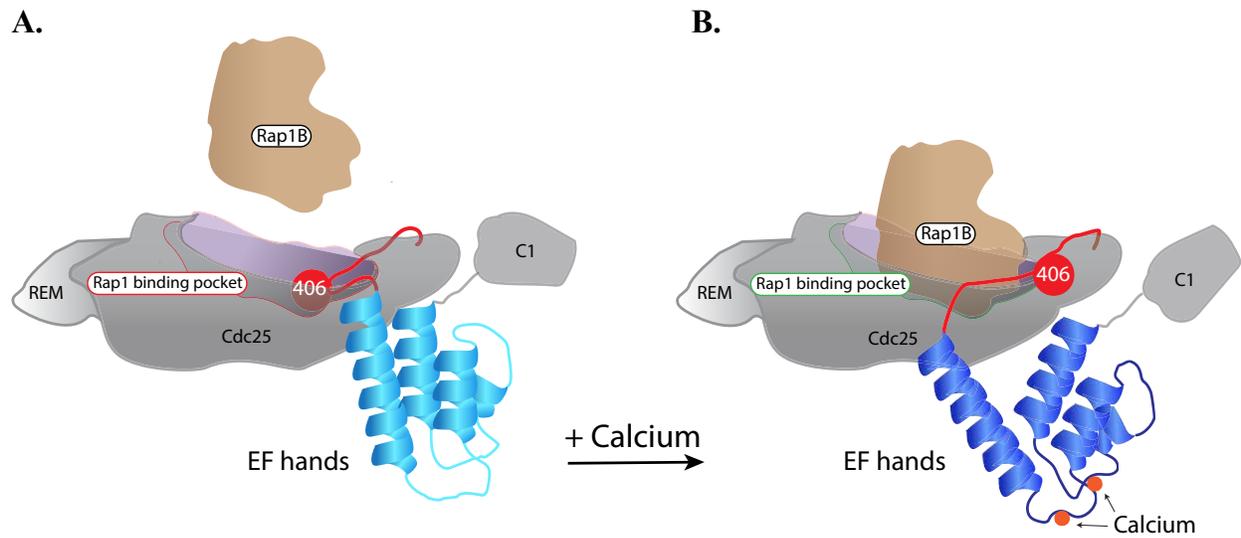
A. Homology model of CalDAG-GEFI with putative autoinhibitory linker highlighted in red. B. Equivalent homology model with linker removed and Rap1B docked onto the structure. C. Substitution of valine 406 (V406E) fully restores the exchange capacity of EF1+2. Inset: stained gel of purified EF1+2+V406E after SDS-PAGE. D. Exchange activity of CalDAG-GEFI WT+V406E, both in the presence and absence of EGTA and exogenous calcium. Inset: stained gel of purified WT+V406E after SDS-PAGE.

## *Discussion*

The four members of the CalDAG-GEF (RasGRP) family are critical for the proper function of different blood cell types (94). They all possess a characteristic domain structure with an N-terminal REM/Cdc25 catalytic domain and a C-terminal regulatory domain consistent of a pair of EF hands and a C1 domain. In this work, we investigated the mechanistic details by which calcium affects nucleotide exchange activity in CalDAG-GEFI, a key regulator of Rap1 signaling in platelets. Compared to CalDAG-GEFII (RasGRP1), the best studied family member, CalDAG-GEFI shows significant differences in the Cdc25 catalytic domain and the EF hand and C1 regulatory domains. CalDAG-GEFII is a RasGEF that exists as a dimer in solution. Binding of diacylglycerol (DAG) to its C1 domain is critical for dimer release and thus CalDAG-GEFII function. In contrast, CalDAG-GEFI is primarily a Rap-GEF, does not dimerize, and does not contain a typical, DAG binding C1 domain. There are also significant differences with regard to the EF hand regulatory domain; while CalDAG-GEFII contains only one active, low affinity ( $K_D > 1\mu\text{M}$ ) EF hand, CalDAG-GEFI contains two fully functional EF hands with high affinity for calcium ( $K_D < 100\text{nM}$ ) (54). The high affinity for calcium is consistent with the documented role of CalDAG-GEFI in the rapid, calcium-dependent activation of Rap1 and integrin  $\alpha\text{IIb}\beta_3$  that is required for platelet adhesion under shear stress conditions (10). Using biochemical and biophysical approaches, we demonstrate that both EF hands are critical for CalDAG-GEFI function and we provide evidence that calcium binding induces global conformational changes in CalDAG-GEFI, most prominently in an autoinhibitory linker region that prevents Rap1 binding to the Cdc25 domain in absence of calcium.

Our data suggest a straightforward model for the regulation of CalDAG-GEFI exchange activity (Fig. 5). In circulating platelets with low intracellular levels of calcium, CalDAG-GEFI

is in an autoinhibited state stabilized by the linker region between the Cdc25 domain and the EF hands. This linker blocks the catalytic surface of the Cdc25 domain so that it cannot engage Rap1B. Upon an external stimulus that raises intracellular levels of calcium, the EF hands bind calcium and change conformation. These conformational changes are coupled to movement of the autoinhibitory linker that reveals the catalytic surface needed to engage Rap1B. After Rap1B binding and GTP for GDP exchange, the complex dissociates freeing GTP-bound Rap1B to engage downstream effectors (Figure 11).



**Figure 11. Model for calcium-dependent rearrangements within CalDAG-GEFI required for its engagement and activation of Rap1B**

At low concentrations of intracellular calcium (left), the linker encompassing valine 406 blocks the surface of the Cdc25 domain that engages Rap1B. As calcium levels rise (right), the EF hands bind calcium and change conformation. This rearrangement moves the linker, liberating the surface of CalDAG-GEFI needed to bind Rap1B.

Based on isothermal titration calorimetry experiments, the individual EF hands of CalDAG-GEFI bind one calcium ion with high affinity ( $K_d \sim 80$  nM) (54). This result strongly suggests that the two EF hand domains may act in concert to activate CalDAG-GEFI. Indeed, disabling either EF hand markedly impaired the exchange activity. Addition of free calcium in a dose-dependent manner restored exchange activity in the EF2 mutant. In contrast, only a partial recovery of function was observed for the EF1 mutant. Disabling both EF hands in CalDAG-GEFI rendered CalDAG-GEFI unresponsive to calcium. These studies suggest that EF2 is critical for high affinity calcium binding, while EF1 is important for inducing conformational changes required for CalDAG-GEFI catalytic activity.

Regulating activity via high affinity binding to calcium explains perfectly why CalDAG-GEFI plays such a crucial role in platelet function. Platelets circulate at high velocity in the blood stream, patrolling the vasculature for breaches in the endothelial lining. To fulfill their hemostatic function, platelets need to be able to sense minute changes in their environment and to rapidly change from an anti-adhesive to a pro-adhesive state. Even weak platelet agonists such as ADP cause rapid and significant changes in cytoplasmic calcium. CalDAG-GEFI can quickly integrate this calcium signal and mediate a near-immediate activation of Rap1 and  $\alpha$ IIb $\beta$ 3 integrin.

However, such high sensitivity to calcium, also poses a challenge, as unwanted CalDAG-GEFI activation could lead to premature platelet activation and thrombosis. In fact, one would expect that, if CalDAG-GEFI binds calcium at such high affinity, a large pool of CalDAG-GEFI would be activated in resting, circulating platelets. Thus, additional regulatory mechanisms must be in play to prevent unwanted platelet activation. One important mechanism that would offset CalDAG-GEFI signals is via negative regulation by RASA3, a Rap-GAP highly expressed in the

platelet membrane. In mice, loss of RASA3 function leads to platelet pre-activation, rapid platelet clearance and severe thrombocytopenia due to unbalanced CalDAG-GEFI signaling (40).

It is also possible that CalDAG-GEFI function depends on more than just calcium binding to its EF hands. For example, the C1-like domain may provide additional regulatory activity. Within the CalDAG-GEF family, CalDAG-GEFI is the only member that contains an atypical C1 domain with very low affinity for diacylglycerol ( $K_d \sim 2 \mu\text{M}$  vs.  $K_d \sim 5 \text{nM}$  for the other family members) (64). Structural studies by Iwig et al. demonstrated that DAG binding to the C1 domain in CalDAG-GEFII is important to release C1 domain dimerization, causing protein translocation to the membrane (54). In contrast, CalDAG-GEFI is a monomer, and platelets lacking CalDAG-GEFI aggregate normally in response to stimulation with phorbol ester (17), a DAG mimetic that engages typical C1 domains. However, platelets expressing a truncated CalDAG-GEFI, lacking the C1 domain, are defective in their integrin activation and aggregation responses (36). Thus, the C1 domain clearly plays a role in CalDAG-GEFI function, but further studies will be required to unravel the underlying molecular mechanism(s).

The comparison of hydrogen-deuterium exchange profiles for active (wild-type) and inactive (EF1+2) CalDAG-GEFI provides important structural insights on how GEF activity is regulated by binding of calcium. Central to this regulation are the autoinhibitory linker and the EF hands. The autoinhibitory linker is bracketed by residues with some of the largest deuterium uptake differentials between the active and crippled forms of CalDAG-GEFI. Furthermore, the regions of the Cdc25 domain critical for GEF activity, based on the homology model, also showed markedly increased deuterium uptake in the active protein when compared to the EF1+2 mutant. These differences support the contention that the autoinhibitory linker moves away from the catalytic surface of the Cdc25 domain upon engagement of the EF hands by calcium. The

fact that substitution of valine 406, centered within the autoinhibitory linker, is sufficient to fully rescue the exchange capacity of the EF1+2 mutant strongly supports our interpretation of the HDX data. Our studies with the WT V406E mutant further suggest that residues in the vicinity of valine 406 contribute to stabilizing the autoinhibitory linker in the Rap1 binding groove. At this point, we can only speculate why the EF1+2 mutant is more sensitive to the introduction of the V406E mutant. We observed that, in the presence of EGTA, the EF1+2 mutant has a much higher exchange activity relative to the WT protein (not shown). This finding may suggest that the point mutations in the EF hands have a minimal effect on the structure of the protein, slightly increasing exchange activity. Introduction of the V406E mutation then shifts the protein into a conformation that allows full activity in our cell-free assay. In the WT protein, introduction of the V406E mutation has a milder effect.

It is interesting that additional regions of CalDAG-GEFI also exhibit differential hydrogen-deuterium exchange. These regions include portions of the REM domain, the region between REM and Cdc25 domains, and portions of the C1 domain. Presumably, the calcium-dependent activation of CalDAG-GEFI is initiated by structural alterations within the EF hands, but these alterations consequently propagate throughout the protein. The functional impact of these additional alterations should be determined in future work, as there is precedent in Sos1 that the REM and Cdc25 domains cooperate to activate Ras GTPase (76). Intriguingly, the autoinhibitory linker might be expected to contain the highest differences in hydrogen-deuterium exchange based on the proposed model. However, this is not the case, suggesting that it is not completely disordered upon calcium-dependent activation. Instead, it seems likely that this region adopts an alternative, stable conformation upon calcium binding perhaps interacting with the EF hands themselves.

Taken together, our data provide mechanistic insight on the functional consequences of calcium binding to the EF hands of CalDAG-GEFI and the structural rearrangements required for GEF activity to occur. These findings will be important in our understanding of how mutations in patients impair CalDAG-GEFI function and in efforts to design inhibitors of CalDAG-GEFI signaling.

### *Experimental procedures*

Reagents. HRP-conjugated secondary antibodies, His-trap HP affinity, and size exclusion columns were purchased from GE Healthcare, Marlborough, MA. FLAG M2 (catalog no. F3165-.2MG) monoclonal antibody purchased from Sigma-Aldrich (St. Louis, MO). Epidermal growth factor (EGF) (catalog no. E9644), GDP-BODIPY (catalog no. G22360), and EGTA (catalog no. E1219) were purchased from Gibco/Thermo Fisher Scientific, Waltham, MA.

#### Protein purification.

A plasmid containing the gene coding for human CalDAG-GEFI was purchased from Harvard University's Human ORFeome v5.1. The gene was PCR-amplified using primers introducing a stop at position 1653 in the nucleotide sequence. The cDNA was subcloned into a p15LIC2 bacterial expression vector. The expressed protein, CalDAG-GEFI (1-551), contained all functional domains plus an N-terminal 6x histidine tag required for purification. Cloning was performed according to ligation-independent cloning protocol. CalDAG-GEFI (1-551) EF hand mutant (EF1) was made by substituting glutamic acid to alanine at position 450. The EF hand mutant (EF2) was made by substituting glutamic acid to alanine at position 479. The EF hand double mutant (EF1+2) was made by substituting glutamic acid to alanine at positions 450 and 479. The EF hand, linker mutant (EF1+2+V406) was made by substituting valine to glutamic

acid at position 406 using EF1+2 cDNA for PCR template. All mutations were carried out using Quikchange site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol.

CalDAG-GEFI proteins were expressed in a Rosetta strain of *Escherichia coli* (*E. coli*) (Novagen). Cell cultures were grown at 37°C in Lysogeny broth, (LB), 0.5 μM ZnSO<sub>4</sub>, 50 μg/ml ampicillin and chloramphenicol. Protein expression was induced by 500 μM isopropyl β-D-1-thiogalactopyranoside then incubated for 15 hours at 20°C. Cell pellets were resuspended in 20 mM PIPES pH 6.8, 300 mM NaCl, 0.5 μM ZnSO<sub>4</sub>, 10 mM β-mercaptoethanol, 10 mM imidazole, 5% glycerol, and 1x protease-inhibitors (Roche). Cell pellets were lysed using Emulsiflex C5 cell homogenizer (Avestin) and clarified by ultracentrifugation at 45,000 rpm for 45 minutes at 4°C. Filtered supernatant was applied to Hi-Trap HP affinity column (GE healthcare) equilibrated with buffer A (20 mM PIPES pH 6.8, 300 mM NaCl, 8 mM imidazole, and 5% glycerol). Protein was eluted from the column with 400 mM imidazole using buffer B (20 mM PIPES pH 6.8, 300 mM NaCl, 1M imidazole, and 5% glycerol). Peak fractions were collected and treated with Tobacco etch virus (TEV) protease overnight at 4°C and dialyzed in buffer A, less imidazole. A second pass over the His-Trap HP affinity column removed the cleaved His-tag and TEV protease from the sample. Flow-through fractions were concentrated using vivaspin column (Sartoris) with a 10,000 molecular weight cutoff filter then loaded onto Superdex 200 10/300 size exclusion column (GE Healthcare) equilibrated with S200 size exclusion buffer containing 20 mM PIPES pH 6.8, 300 mM NaCl, and 4 mM DTT. Final protein product was 95% pure, monomeric and concentrated to 20 mg/ml. Aliquots were flash frozen in liquid nitrogen and stored at -80°C.

Human Rap1B (1-181) C181S was cloned into pProEXHtb vector and purified from *E. coli*. 500 μM isopropyl β-D-1-thiogalactopyranoside was used to induce protein expression and

cultures were incubated for 8 hours at 20°C. Protein purification was done using the same chromatography steps as CalDAG-GEFI proteins listed above with buffer modifications substituting the PIPES buffer with Tris-HCl pH 7.0, 1  $\mu$ M ZnSO<sub>4</sub> with 5 mM MgCl<sub>2</sub> and adding 50  $\mu$ M GDP to protein lysis buffer.

#### Nucleotide exchange assay.

Nucleotide exchange on Rap1B was measured using a fluorescence-based assay as previously described (93). Reaction volume was 100  $\mu$ l containing 1  $\mu$ M of Rap1B, between 300-500 nM CalDAG-GEFI, and 100 nM BODIPY-GDP (life technologies). Reaction buffer contained 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 5% glycerol, and 0.004% NP-40. Reactions were performed in a black bottom 96 well plate (Corning). Fluorescent signal was measured using a Pherastar microplate reader (BMG LABTECH). (Wavelength excitation/emission = 480/520 nm, and 1 nm slits). For calcium chelation experiments, 100  $\mu$ M CalDAG-GEFI was treated with 10 mM EGTA for 2 hours at 4°C. Sample was spun at 4,000 RPM for 30 seconds then transferred to a new tube containing reaction buffer, diluting calcium-free CalDAG-GEFI to 400 nM. Samples were treated with 10 mM calcium where indicated. Rap1B pull down assays. Rap1B pull-down experiments were performed as described recently (10).

#### Hydrogen-deuterium exchange mass spectrometry (HDX-MS).

HDX-MS experiments on WT CalDAG-GEFI and EF1+2 were performed using a Waters UPLC HDX system coupled with a Q-ToF Premier mass spectrometer (Waters Corp, Milford, MA) as previously described (110). Briefly, each protein sample was buffer-exchanged into PBS then diluted to 1.5 mg/ml. 1  $\mu$ l of sample was diluted 1:7 (v/v) in 10 mM phosphate, 99.9 % D<sub>2</sub>O, pD 7.0 at 20°C using a robotic autosampler to initiate hydrogen-deuterium exchange. Exchange

reactions were quenched after 0.01, 0.15, 1.6, 16, and 166 minutes by placing them at 1°C and adding equal volume of pre-cooled quenching buffer (100 mM phosphate, 0.5 M TCEP, 0.8% formic acid, 2% acetonitrile, pH 2.5) Each sample and each time point were done in replicates of six. Samples from each time point were fragmented through a Waters Enzymate BEH pepsin column, and the peptic fragments were separated on an in-line C18 HPLC column then analyzed by mass spectrometry. Mass assignment for each peptide without HDX was inspected manually; any assignment with a mass deviation >0.2 Da was removed. The extent of deuterium incorporation in each peptide was calculated and tabulated using the Waters software. The relative fractional deuterium uptake was represented as a heat map and is measured for each peptide by calculating the number of residues that exchange an amide proton for deuterium over the total number of protons that are able to exchange (peptide length -1) and averaged for each time point.

#### Homology model.

The homology model of CalDAG-GEFI was built using the Iterative Threading ASSEMBLY Refinement, (I-TASSER) server available to academic researchers through the Zhang Lab at The University of Michigan, (<https://zhanglab.ccmb.med.umich.edu/I-TASSER/>). I-TASSER generated our model by comparing the protein sequence of CalDAG-GEFI to protein sequences of all proteins that have crystal or CryoEM structures deposited in the Protein Data Bank, (PDB). These structures were used as templates to predict secondary structures in CalDAG-GEFI based on sequence homology to the template sequence. I-TASSER predicted five homology models of CalDAG-GEFI ranked in order from highest to lowest probability; we chose the highest probable model.

## **CHAPTER 6: GENERAL CONCLUSIONS AND FUTURE DIRECTIONS**

Activated receptors on the surface of platelets bind ligands and transmit signals across the membrane to activate proteins at the inner leaflet. Regulating intracellular proteins and the signals transmitted by these activated proteins requires three events: 1) activated proteins directly bind to effector proteins, 2) the activities of effector proteins are altered, and 3) the activities of proteins and effectors return to their basal level. The temporal and spatial regulation of proteins involved in signaling pathways are often difficult to determine. Biochemical data from experiments using isolated platelets can depict regulation of their intracellular proteins as simple, binary interactions between proteins in a linear path, and can be misleading.

These studies determined how the activity of CalDAG-GEFI is regulated in platelets. Our studies were based on biochemical data from previous work which showed that: a) CalDAG-GEFI proteins become active when the intracellular calcium concentration in platelets increases, b) CalDAG-GEFI is required to activate Rap isozymes, and c) Rap isozymes are required for platelet adhesion (1, 2, 111). To define the biochemical role of calcium for CalDAG-GEFI we used purified, full length CalDAG-GEFI and Rap1B proteins in a nucleotide exchange assay to measure the activation of Rap1B by CalDAG-GEFI, recapitulating physiological fluctuations in calcium concentrations measured in platelets and by mutational analysis to determine specific residues in CalDAG-GEFI coupled to its activity. Hydrogen-deuterium exchange mass spectrometry (HDX-MS) experiments strongly suggest that calcium binding to the EF hands in CalDAG-GEFI induces long-range conformational rearrangements including the Cdc25 domain, auto-inhibitory linker, and EF hands.

Serendipitously, while working out the mechanistic details for how calcium regulates CalDAG-GEFI, multiple independent groups identified patients with an idiopathic bleeding phenotype due to mutations in CalDAG-GEFI (105, 112). Not surprisingly, many of them occur in the catalytic Cdc25 domain leaving little doubt about their effect on the ability for CalDAG-GEFI to interact with Rap1B. For two of the mutations identified, we purified those proteins and tested their activity toward Rap1B. We determined that the S341F mutation destabilized CalDAG-GEFI based on chromatographic data, and that CalDAG-GEFI with G248W mutation was stable but unable to exchange nucleotide on Rap1B (unpublished). Furthermore, a homology models indicate that serine 341 is a residue buried in the core of the Cdc25 domain that stabilizes an elaborate hydrogen bond network. The phenylalanine mutation at this position most likely alters CalDAG-GEFI's secondary structure causing the instability. In contrast, glycine 248 is not in the core of the protein but in a small loop region between two helices at the base of the Rap1B binding pocket. When the tryptophan mutation is modeled, it protrudes up into that binding pocket most likely, displacing Rap1B even with CalDAG-GEFI bound by calcium.

It is clear from the previous example that the structural data we determined in this study and our robust functional assay has opened the door to many future studies that will answer outstanding questions about the mechanisms regulating CalDAG-GEFI function. Subramanian et al., provided biochemical evidence that CalDAG-GEFI is phosphorylated by PKA at serine 587, and minimally at serine 116 and 117 leads to impaired Rap1 activation. (100). However, it is unclear how these phosphorylation events affect the function of CalDAG-GEFI. Using our models and functional assay, we can introduce mutations that mimic phosphorylation (ser116, 117, 587Asp) and measure their direct effects on nucleotide exchange activity. If there is an effect, we could then compare the rate of deuterium exchange between WT and phosphorylated-

CalDAG-GEFI, and we could determine if phosphorylation and calcium regulate CalDAG-GEFI antagonistically.

Our efforts to determine the crystal structure reached an impasse after 3.5 years. During that time, we systematically screened over 10,000 crystal growing conditions using various full length and truncated forms of CalDAG-GEFI alone and in complex with Rap1B but got no crystals. Despite this major setback in our ability to address our specific aim, it gave us a wealth of information about the chemical behavior of CalDAG-GEFI which provided data to support that hydrogen/deuterium exchange-mass spectrometry would be a viable tool for determining the structural consequence of calcium binding. Utilizing the crystal structure of CalDAG-GEFII we built a model of autoinhibited CalDAG-GEFI. Using this model, we determined regions that must undergo rearrangement in order for CalDAG-GEFI to engage Rap1B. Thus, we measured the structural dynamics of calcium binding CalDAG-GEFI using HDX-MS. HDX-MS determined the regions in CalDAG-GEFI that become inaccessible or exposed to the solvent when calcium binds to its EF hands. While processing our HDX-MS data, a crystal structure of CalDAG-GEFI in complex with Rap1B was published (113) (PDB: 6AXF). However, this work, could not address any of the same mechanisms we addressed in our work. Instead they used the crystal structure to highlight histidine 212 that serves as a pH sensing mechanism activating CalDAG-GEFI when the histidine was protonated; a mechanism that regulates CalDAG-GEFI activity when the pH inside the cell is low (113).

In many ways the HDX-MS data provided more information than we would have gained from a crystal structure. In particular, the conformational space sampled by CalDAG-GEFI in the active or inactive state and defined regions of the protein that were very dynamic, information that would have been lost in crystallographic data. Of particular interest, a small region in the

REM domain that showed a dramatic increase in deuterium exchange when CalDAG-GEFI is in the active state. This is interesting because there is no function associated with the REM domain. Other REM domains in Cdc25 domain GEFs have a function associated with allosteric activation of Ras, as well as membrane recruitment. (78, 81). Future studies could determine how the residues in the REM domain that show calcium-dependent alterations in deuterium uptake affect protein stability, catalytic function, or the ability for CalDAG-GEFI to respond to calcium.

One such study would define regulatory mechanism related to the REM domain much like we determined the function of the EF hands. Another interesting project would be to determine the role membranes have on the regulation of CalDAG-GEFI. Rap1B is geranylgeranylated and sequestered to the membrane. Thus, CalDAG-GEFI has to be recruited to the membrane which may alter its catalytic activity. Of note, some preliminary data from the work of us and others suggest that the C1 domain of CalDAG-GEFI has lipid-binding properties and this is important for membrane association. In our nucleotide exchange assay, the C1 domain had no impact on exchange activity, but this of course could be different in the context of the membrane. Another important question is how exactly the EF hands contribute to CalDAG-GEFI activity. My data shows that EF1 is tightly coupled to CalDAG-GEFI but we still do not understand how the inter-domain interactions between EF1 and EF2 are doing to promote the conformational rearrangements in CalDAG-GEFI. To determine the interdomain interaction we would perform extensive mutagenesis analysis. By making combinatorial mutational analysis we would determine the residues within the interface of EF1 and EF2 that provide the coupling energy required to displace the linker by EF hand movement. Perhaps it is the unique coupling between EF hands that has evolved with platelets giving them one of the most specialized functions of any cell in our body.

## REFERENCES

1. Chrzanowska-Wodnicka, M., Smyth, S. S., Schoenwaelder, S. M., Fischer, T. H., and White, G. C. (2005) Rap1b is required for normal platelet function and hemostasis in mice. *J. Clin. Invest.* **115**, 680–687
2. Stefanini, L., and Bergmeier, W. (2016) RAP1-GTPase signaling and platelet function. *J. Mol. Med.* **94**, 13–19
3. Bergmeier, W., and Hynes, R. O. (2012) Extracellular matrix proteins in hemostasis and thrombosis. *Cold Spring Harb Perspect Biol.* 10.1101/cshperspect.a005132
4. Shattil, S. J., Kim, C., and Ginsberg, M. H. (2010) The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol.* **11**, 288–300
5. Stefanini, L., Ye, F., Snider, A. K., Sarabakhsh, K., Piatt, R., Paul, D. S., Bergmeier, W., and Petrich, B. G. (2014) A talin mutant that impairs talin-integrin binding in platelets decelerates  $\alpha$ IIb $\beta$ 3 activation without pathological bleeding. *Blood.* **123**, 2722–2731
6. Nesbitt, W. S., Giuliano, S., Kulkarni, S., Dopheide, S. M., Harper, I. S., and Jackson, S. P. (2003) Intercellular calcium communication regulates platelet aggregation and thrombus growth. *The Journal of Cell Biology.* **160**, 1151–1161
7. Juška, A. (2011) Calcium fluxes into and out of cytosol in human platelets: analysis of experimental data. *Biochemical and Biophysical Research Communications.* **412**, 537–542
8. Bergmeier, W., Weidinger, C., Zee, I., and Feske, S. (2013) Emerging roles of store-operated  $\text{Ca}^{2+}$  entry through STIM and ORAI proteins in immunity, hemostasis and cancer. *Channels (Austin).* **7**, 379–391
9. Stegner, D., and Nieswandt, B. (2011) Platelet receptor signaling in thrombus formation. *J. Mol. Med.* **89**, 109–121
10. Stefanini, L., Roden, R. C., and Bergmeier, W. (2009) CalDAG-GEFI is at the nexus of calcium-dependent platelet activation. *Blood.* **114**, 2506–2514
11. Zufferey, A., Fontana, P., Reny, J.-L., Nolli, S., and Sanchez, J.-C. (2011) Platelet proteomics. *Mass Spectrom. Rev.* **31**, 331–351
12. Guidetti, G. F., and Torti, M. (2012) The Small GTPase Rap1b: A Bidirectional Regulator of Platelet Adhesion Receptors. *Journal of Signal Transduction.* **2012**, 412089
13. Bos, J. L., Rehmann, H., and Wittinghofer, A. (2007) GEFs and GAPs: Critical Elements in the Control of Small G Proteins. *Cell.* **129**, 865–877

14. Rossman, K. L., Der, C. J., and Sondek, J. (2005) GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol.* **6**, 167–180
15. Kuiperij, H. B., Rehmann, H., and Zwartkruis, F. J. T. (2006) Biochemistry of the Rap-specific guanine nucleotide exchange factors PDZ-GEF1 and -2. *Methods in enzymology.* **407**, 174–186
16. Popovic, M., Leeuw, M. R.-D., and Rehmann, H. (2013) Selectivity of CDC25 Homology Domain-Containing Guanine Nucleotide Exchange Factors. *Journal of Molecular Biology.* **425**, 2782–2794
17. Crittenden, J. R., Bergmeier, W., Zhang, Y., Piffath, C. L., Liang, Y., wagner, D. D., Housman, D. E., and Graybiel, A. M. (2004) CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. *Nature Medicine.* **10**, 982–986
18. Stefanini, L., and Bergmeier, W. (2010) CalDAG-GEFI and platelet activation. *Platelets.* **21**, 239–243
19. Italiano, J., Jr. (2013) Unraveling Mechanisms that Control Platelet Production. *Semin Thromb Hemost.* **39**, 015–024
20. Jackson, S. P., Nesbitt, W. S., and Westein, E. (2009) Dynamics of platelet thrombus formation. *Journal of thrombosis and haemostasis : JTH.* **7 Suppl 1**, 17–20
21. Rivera, J., Lozano, M. L., Navarro-Nunez, L., and Vicente, V. (2009) Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica.* **94**, 700–711
22. Li, Z., Delaney, M. K., O'Brien, K. A., and Du, X. (2010) Signaling during platelet adhesion and activation. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **30**, 2341–2349
23. Ruggeri, Z. M., Orje, J. N., Habermann, R., Federici, A. B., and Reininger, A. J. (2006) Activation-independent platelet adhesion and aggregation under elevated shear stress. *Blood.* **108**, 1903–1910
24. Yuan, Y., Kulkarni, S., Ulsemer, P., Cranmer, S. L., Yap, C. L., Nesbitt, W. S., Harper, I., Mistry, N., Dopheide, S. M., Hughan, S. C., Williamson, D., la Salle, de, C., Salem, H. H., Lanza, F., and Jackson, S. P. (1999) The von Willebrand factor-glycoprotein Ib/V/IX interaction induces actin polymerization and cytoskeletal reorganization in rolling platelets and glycoprotein Ib/V/IX-transfected cells. *J. Biol. Chem.* **274**, 36241–36251
25. Heemserk, J. W. M., Mattheij, N. J. A., and Cosemans, J. M. E. M. (2013) Platelet-based coagulation: different populations, different functions. *Journal of Thrombosis and Haemostasis.* **11**, 2–16

26. Marjoram, R. J., Li, Z., He, L., Tollefsen, D. M., Kunicki, T. J., Dickeson, S. K., Santoro, S. A., and Zutter, M. M. (2014)  $\alpha 2\beta 1$  integrin, GPVI receptor, and common Fc $\gamma$  chain on mouse platelets mediate distinct responses to collagen in models of thrombosis. *PLoS ONE*. **9**, e114035
27. Poulter, N. S., Pollitt, A. Y., Owen, D. M., Gardiner, E. E., Andrews, R. K., Shimizu, H., Ishikawa, D., Bihan, D., Farndale, R. W., Moroi, M., Watson, S. P., and Jung, S. M. (2017) Clustering of glycoprotein VI (GPVI) dimers upon adhesion to collagen as a mechanism to regulate GPVI signaling in platelets. *Journal of thrombosis and haemostasis : JTH*. **15**, 549–564
28. Thiere, M., Kliche, S., Müller, B., Teuber, J., Nold, I., and Stork, O. (2016) Integrin Activation Through the Hematopoietic Adapter Molecule ADAP Regulates Dendritic Development of Hippocampal Neurons. *Front Mol Neurosci*. **9**, 91
29. Goult, B. T., Zacharchenko, T., Bate, N., Tsang, R., Hey, F., Gingras, A. R., Elliott, P. R., Roberts, G. C. K., Ballestrem, C., Critchley, D. R., and Barsukov, I. L. (2013) RIAM and vinculin binding to talin are mutually exclusive and regulate adhesion assembly and turnover. *J. Biol. Chem*. **288**, 8238–8249
30. Morse, E. M., Brahme, N. N., and Calderwood, D. A. (2014) Integrin Cytoplasmic Tail Interactions. *Biochemistry*. **53**, 810–820
31. Faull, R. J., and Ginsberg, M. H. (1996) Inside-out signaling through integrins. *J. Am. Soc. Nephrol*. **7**, 1091–1097
32. Lee, H. S., Anekal, P., Lim, C. J., Liu, C. C., and Ginsberg, M. H. (2013) Two modes of integrin activation form a binary molecular switch in adhesion maturation. *Molecular Biology of the Cell*. **24**, 1354–1362
33. Heemskerk, J. W., Willems, G. M., Rook, M. B., and Sage, S. O. (2001) Ragged spiking of free calcium in ADP-stimulated human platelets: regulation of puff-like calcium signals in vitro and ex vivo. *J. Physiol. (Lond.)*. **535**, 625–635
34. Reiter, N., Eichelberger, B., Kaider, A., and Panzer, S. (2009) Thrombin-inducibile platelet adhesion and regulation of the platelet seven-transmembrane thrombin receptor-1 (PAR-1): effects of unfractionated heparin and lepirudin. *Platelets*. **20**, 582–587
35. Stalker, T. J., Newman, D. K., Ma, P., Wannemacher, K. M., and Brass, L. F. (2012) Platelet signaling. *Handb Exp Pharmacol*. 10.1007/978-3-642-29423-5\_3
36. Stolla, M., Stefanini, L., Roden, R. C., Chavez, M., Hirsch, J., Greene, T., Ouellette, T. D., Maloney, S. F., Diamond, S. L., Poncz, M., Woulfe, D. S., and Bergmeier, W. (2011) The kinetics of  $\alpha$ IIb $\beta$ 3 activation determines the size and stability of thrombi in mice: implications for antiplatelet therapy. *Blood*. **117**, 1005–1013

37. Stefanini, L., Boulaftali, Y., Ouellette, T. D., Holinstat, M., Desire, L., Leblond, B., Andre, P., Conley, P. B., and Bergmeier, W. (2012) Rap1-Rac1 Circuits Potentiate Platelet Activation. *Arteriosclerosis, Thrombosis, and Vascular Biology*. **32**, 434–441
38. Purvis, J. E., Chatterjee, M. S., Brass, L. F., and Diamond, S. L. (2008) A molecular signaling model of platelet phosphoinositide and calcium regulation during homeostasis and P2Y1 activation. *Blood*. **112**, 4069–4079
39. Karaźniewicz-Łada, M., Danielak, D., Burchardt, P., Kruszyna, Ł., Komosa, A., Lesiak, M., and Główska, F. (2013) Clinical Pharmacokinetics of Clopidogrel and Its Metabolites in Patients with Cardiovascular Diseases. *Clin Pharmacokinet*. **53**, 155–164
40. Stefanini, L., Paul, D. S., Robledo, R. F., Chan, E. R., Getz, T. M., Campbell, R. A., Kechele, D. O., Casari, C., Piatt, R., Caron, K. M., Mackman, N., Weyrich, A. S., Parrott, M. C., Boulaftali, Y., Adams, M. D., Peters, L. L., and Bergmeier, W. (2015) RASA3 is a critical inhibitor of RAP1-dependent platelet activation. *J. Clin. Invest*. **125**, 1419–1432
41. Bellido-Martín, L., Chen, V., Jasuja, R., Furie, B., and Furie, B. C. (2011) Imaging fibrin formation and platelet and endothelial cell activation in vivo. *Thromb Haemost*. **105**, 776–782
42. Dickson, E. J., Falkenburger, B. H., and Hille, B. (2013) Quantitative properties and receptor reserve of the IP<sub>3</sub> and calcium branch of G<sub>q</sub>-coupled receptor signaling. *J Gen Physiol*. **141**, 521–535
43. Chen, X.-F., Li, C.-X., Wang, P.-Y., Li, M., and Wang, W.-C. (2008) Dynamic simulation of the effect of calcium-release activated calcium channel on cytoplasmic Ca<sup>2+</sup> oscillation. *Biophysical Chemistry*. **136**, 87–95
44. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol*. **4**, 517–529
45. Ahmad, F., Boulaftali, Y., Greene, T. K., Ouellette, T. D., Poncz, M., Feske, S., and Bergmeier, W. (2011) Relative contributions of stromal interaction molecule 1 and CalDAG-GEFI to calcium-dependent platelet activation and thrombosis. *Journal of thrombosis and haemostasis : JTH*. **9**, 2077–2086
46. Liao, Y., Plummer, N. W., George, M. D., Abramowitz, J., Zhu, M. X., and Birnbaumer, L. (2009) A role for Orai in TRPC-mediated Ca<sup>2+</sup> entry suggests that a TRPC:Orai complex may mediate store and receptor operated Ca<sup>2+</sup> entry. *Proc Natl Acad Sci USA*. **106**, 3202–3206
47. Rosado, J. A. (2016) Calcium Entry Pathways in Non-excitabile Cells. Preface. *Adv. Exp. Med. Biol*. **898**, vii–viii

48. Salido, G. M., Sage, S. O., and Rosado, J. A. (2009) *Biochimica et Biophysica Acta. BBA - Molecular Cell Research.* **1793**, 223–230
49. Gao, Z. H., Krebs, J., VanBerkum, M. F., Tang, W. J., Maune, J. F., Means, A. R., Stull, J. T., and Beckingham, K. (1993) Activation of four enzymes by two series of calmodulin mutants with point mutations in individual Ca<sup>2+</sup> binding sites. *J. Biol. Chem.* **268**, 20096–20104
50. Moncrief, N. D., Kretsinger, R. H., and Goodman, M. (1990) Evolution of EF-hand calcium-modulated proteins. I. Relationships based on amino acid sequences. *J. Mol. Evol.* **30**, 522–562
51. Kumar, S., Ahmad, E., Kumar, S., Khan, R. H., and Gourinath, S. (2012) Flexibility of EF-hand motifs: structural and thermodynamic studies of Calcium Binding Protein-1 from *Entamoeba histolytica* with Pb. *BMC Biophysics.* **5**, 1–1
52. Zhou, Y., Yang, W., Kirberger, M., Lee, H.-W., Ayalasomayajula, G., and Yang, J. J. (2006) Prediction of EF-hand calcium-binding proteins and analysis of bacterial EF-hand proteins. *Proteins.* **65**, 643–655
53. Lewit-Bentley, A., and Réty, S. (2000) EF-hand calcium-binding proteins. *Current Opinion in Structural Biology.* **10**, 637–643
54. Iwig, J. S., Vercoulen, Y., Das, R., Barros, T., Limnander, A., Che, Y., Pelton, J. G., Wemmer, D. E., Roose, J. P., and Kuriyan, J. (2013) Structural analysis of autoinhibition in the Ras-specific exchange factor RasGRP1. *eLife.* **2**, e00813
55. Kawasaki, H., and Kretsinger, R. H. (2012) Analysis of the movements of helices in EF-hands. *Proteins.* **80**, 2592–2600
56. Declercq, J.-P., Evrard, C., Lamzin, V., and Parello, J. (1999) Crystal structure of the EF-hand parvalbumin at atomic resolution (0.91 Å) and at low temperature (100 K). Evidence for conformational multistates within the hydrophobic core. *Protein Science*
57. Zhang, M., Tanaka, T., and Ikura, M. (1995) Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nat. Struct. Biol.* **2**, 758–767
58. Torti, M., Ramaschi, G., Sinigaglia, F., Lapetina, E. G., and Balduini, C. (1993) Association of the low molecular weight GTP-binding protein rap2B with the cytoskeleton during platelet aggregation. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7553–7557
59. Vetter, I. R. (2001) The Guanine Nucleotide-Binding Switch in Three Dimensions. *Science.* **294**, 1299–1304
60. Díez, D., Sánchez-Jiménez, F., and Ranea, J. A. G. (2011) Evolutionary expansion of the Ras switch regulatory module in eukaryotes. *Nucleic Acids Res.* **39**, 5526–5537

61. Szamałek, M., and Baer-Dubowska, W. (2007) RasGRP proteins--Ras-activating factors. *Postepy Biochemii*. **53**, 112–120
62. Reuther, G. W., Lambert, Q. T., Rebhun, J. F., Caligiuri, M. A., Quilliam, L. A., and Der, C. J. (2002) RasGRP4 is a novel Ras activator isolated from acute myeloid leukemia. *J. Biol. Chem.* **277**, 30508–30514
63. Hartzell, C., Ksionda, O., Lemmens, E., Coakley, K., Yang, M., Dail, M., Harvey, R. C., Govern, C., Bakker, J., Lenstra, T. L., Ammon, K., Boeter, A., Winter, S. S., Loh, M., Shannon, K., Chakraborty, A. K., Wabl, M., and Roose, J. P. (2013) Dysregulated RasGRP1 responds to cytokine receptor input in T cell leukemogenesis. *Science Signaling*. **6**, ra21–ra21
64. Czikora, A., Lundberg, D. J., Abramovitz, A., Lewin, N. E., Kedei, N., Peach, M. L., Zhou, X., Merritt, R. C., Jr., Craft, E. A., Braun, D. C., and Blumberg, P. M. (2016) Structural Basis for the Failure of the C1 Domain of Ras Guanine Nucleotide Releasing Protein 2 (RasGRP2) to Bind Phorbol Ester with High Affinity. *J. Biol. Chem.* **291**, 11133–11147
65. Yamashita, S., Mochizuki, N., Ohba, Y., Tobiume, M., Okada, Y., Sawa, H., Nagashima, K., and Matsuda, M. (2000) CalDAG-GEFIII activation of Ras, R-ras, and Rap1. *J. Biol. Chem.* **275**, 25488–25493
66. Ohba, Y., Mochizuki, N., Yamashita, S., Chan, A. M., Schrader, J. W., Hattori, S., Nagashima, K., and Matsuda, M. (2000) Regulatory proteins of R-Ras, TC21/R-Ras2, and M-Ras/R-Ras3. *J. Biol. Chem.* **275**, 20020–20026
67. Lambert, Q. T., and Reuther, G. W. (2006) Activation of Ras proteins by Ras guanine nucleotide releasing protein family members. *Methods in enzymology*. **407**, 82–98
68. Yang, Y., Li, L., Wong, G. W., Krilis, S. A., Madhusudhan, M. S., Sali, A., and Stevens, R. L. (2002) RasGRP4, a new mast cell-restricted Ras guanine nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Identification of defective variants of this signaling protein in asthma, mastocytosis, and mast cell leukemia patients and demonstration of the importance of RasGRP4 in mast cell development and function. *J. Biol. Chem.* **277**, 25756–25774
69. Neel, N. F., Martin, T. D., Stratford, J. K., Zand, T. P., Reiner, D. J., and Der, C. J. (2011) The RalGEF-Ral Effector Signaling Network: The Road Less Traveled for Anti-Ras Drug Discovery. *Genes & Cancer*. **2**, 275–287
70. Feig, L. A., Urano, T., and Cantor, S. (1996) Evidence for a Ras/Ral signaling cascade. *Trends Biochem. Sci.* **21**, 438–441
71. Bodemann, B. O., and White, M. A. (2008) Ral GTPases and cancer: linchpin support of the tumorigenic platform. *Nat Rev Cancer*. **8**, 133–140

72. Smith, S. C., and Theodorescu, D. (2009) The Ral GTPase pathway in metastatic bladder cancer: key mediator and therapeutic target. *Urol. Oncol.* **27**, 42–47
73. Esser, D., Bauer, B., Wolthuis, R. M., Wittinghofer, A., Cool, R. H., and Bayer, P. (1998) Structure determination of the Ras-binding domain of the Ral-specific guanine nucleotide exchange factor Rlf. *Biochemistry.* **37**, 13453–13462
74. Popovic, M., Schouten, A., Rensen-de Leeuw, M., and Rehmann, H. (2016) The structure of the Guanine Nucleotide Exchange Factor Rlf in complex with the small G-protein Ral identifies conformational intermediates of the exchange reaction and the basis for the selectivity. *J. Struct. Biol.* **193**, 106–114
75. Popovic, M., Jakobi, A. J., Rensen-de Leeuw, M., and Rehmann, H. (2013) The guanine nucleotide exchange factor Rlf interacts with SH3 domain-containing proteins via a binding site with a preselected conformation. *J. Struct. Biol.* **183**, 312–319
76. Margarit, S. M., Sondermann, H., Hall, B. E., Nagar, B., Hoelz, A., Pirruccello, M., Bar-Sagi, D., and Kuriyan, J. (2017) Structural Evidence for Feedback Activation by Ras·GTP of the Ras-Specific Nucleotide Exchange Factor SOS. *Cell.* **112**, 685–695
77. Gureasko, J., Galush, W. J., Boykevisch, S., Sondermann, H., Bar-Sagi, D., Groves, J. T., and Kuriyan, J. (2008) Membrane-dependent signal integration by the Ras activator Son of sevenless. *Nat. Struct. Mol. Biol.* **15**, 452–461
78. Sondermann, H., Soisson, S. M., Boykevisch, S., Yang, S.-S., Bar-Sagi, D., and Kuriyan, J. (2004) Structural analysis of autoinhibition in the Ras activator Son of sevenless. *Cell.* **119**, 393–405
79. Freedman, T. S., Sondermann, H., Friedland, G. D., Kortemme, T., Bar-Sagi, D., Marqusee, S., and Kuriyan, J. (2006) A Ras-induced conformational switch in the Ras activator Son of sevenless. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 16692–16697
80. de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Cool, R. H., Nijman, S. M., Wittinghofer, A., and Bos, J. L. (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature.* **396**, 474–477
81. Sugawara, K., Shibasaki, T., Takahashi, H., and Seino, S. (2016) Structure and functional roles of Epac2 (Rapgef4). *Gene.* **575**, 577–583
82. Takahashi, H., Shibasaki, T., Park, J.-H., Hidaka, S., Takahashi, T., Ono, A., Song, D.-K., and Seino, S. (2015) Role of Epac2A/Rap1 signaling in interplay between incretin and sulfonylurea in insulin secretion. *Diabetes.* **64**, 1262–1272
83. Hwang, M., Go, Y., Park, J.-H., Shin, S.-K., Song, S. E., Oh, B.-C., Im, S.-S., Hwang, I., Jeon, Y. H., Lee, I.-K., Seino, S., and Song, D.-K. (2017) Epac2a-null mice exhibit obesity-prone nature more susceptible to leptin resistance. *Nature Publishing Group.* **41**, 279–288

84. Rehmann, H., Arias-Palomo, E., Hadders, M. A., Schwede, F., Llorca, O., and Bos, J. L. (2008) Structure of Epac2 in complex with a cyclic AMP analogue and RAP1B. *Nature*. **455**, 124–127
85. VanSchouwen, B., Selvaratnam, R., Fogolari, F., and Melacini, G. (2011) Role of dynamics in the autoinhibition and activation of the exchange protein directly activated by cyclic AMP (EPAC). *J. Biol. Chem.* **286**, 42655–42669
86. Tsalkova, T., Blumenthal, D. K., Mei, F. C., White, M. A., and Cheng, X. (2009) Mechanism of Epac activation: structural and functional analyses of Epac2 hinge mutants with constitutive and reduced activities. *J. Biol. Chem.* **284**, 23644–23651
87. Dhillon, A. S., Hagan, S., Rath, O., and Kolch, W. (2007) MAP kinase signalling pathways in cancer. *Oncogene*. **26**, 3279–3290
88. Shin, S. Y., Rath, O., Choo, S. M., Fee, F., McFerran, B., Kolch, W., and Cho, K. H. (2009) Positive- and negative-feedback regulations coordinate the dynamic behavior of the Ras-Raf-MEK-ERK signal transduction pathway. *Journal of Cell Science*. **122**, 425–435
89. Kamioka, Y., Yasuda, S., Fujita, Y., Aoki, K., and Matsuda, M. (2010) Multiple decisive phosphorylation sites for the negative feedback regulation of SOS1 via ERK. *J. Biol. Chem.* **285**, 33540–33548
90. Matallanas, D., Birtwistle, M., Romano, D., Zebisch, A., Rauch, J., Kriegsheim, von, A., and Kolch, W. (2011) Raf family kinases: old dogs have learned new tricks. *Genes & Cancer*. **2**, 232–260
91. Waters, S. B., Holt, K. H., Ross, S. E., Syu, L. J., Guan, K. L., Saltiel, A. R., Koretzky, G. A., and Pessin, J. E. (1995) Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex. *J. Biol. Chem.* **270**, 20883–20886
92. Stork, P. J. S., and Dillon, T. J. (2005) Multiple roles of Rap1 in hematopoietic cells: complementary versus antagonistic functions. *Blood*. **106**, 2952–2961
93. Ren, J., Cook, A. A., Bergmeier, W., and Sondek, J. (2016) A negative-feedback loop regulating ERK1/2 activation and mediated by RasGPR2 phosphorylation. *Biochemical and Biophysical Research Communications*. **474**, 193–198
94. Stone, J. C. (2011) Regulation and Function of the RasGRP Family of Ras Activators in Blood Cells. *Genes & Cancer*. **2**, 320–334
95. Mertins, P., Qiao, J. W., Patel, J., Udeshi, N. D., Clauser, K. R., Mani, D. R., Burgess, M. W., Gillette, M. A., Jaffe, J. D., and Carr, S. A. (2013) Integrated proteomic analysis of post-translational modifications by serial enrichment. *Nat Meth.* **10**, 634–637

96. Huttlin, E. L., Jedrychowski, M. P., Elias, J. E., Goswami, T., Rad, R., Beausoleil, S. A., Villén, J., Haas, W., Sowa, M. E., and Gygi, S. P. (2010) A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell*. **143**, 1174–1189
97. Sharma, K., D'Souza, R. C. J., Tyanova, S., Schaab, C., Wiśniewski, J. R., Cox, J., and Mann, M. (2014) Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling. *Cell Rep*. **8**, 1583–1594
98. Wu, X., Tian, L., Li, J., Zhang, Y., Han, V., Li, Y., Xu, X., Li, H., Chen, X., Chen, J., Jin, W., Xie, Y., Han, J., and Zhong, C.-Q. (2012) Investigation of receptor interacting protein (RIP3)-dependent protein phosphorylation by quantitative phosphoproteomics. *Molecular & Cellular Proteomics*. **11**, 1640–1651
99. Guidetti, G. F., Manganaro, D., Consonni, A., Canobbio, I., Balduini, C., and Torti, M. (2013) Phosphorylation of the guanine-nucleotide-exchange factor CalDAG-GEFI by protein kinase A regulates Ca<sup>2+</sup>-dependent activation of platelet Rap1b GTPase. *Biochem. J*. **453**, 115–123
100. Subramanian, H., Zahedi, R. P., Sickmann, A., Walter, U., and Gambaryan, S. (2013) Phosphorylation of CalDAG-GEFI by protein kinase A prevents Rap1b activation. *Journal of thrombosis and haemostasis : JTH*. **11**, 1574–1582
101. Nagai, T., Nakamuta, S., Kuroda, K., Nakauchi, S., Nishioka, T., Takano, T., Zhang, X., Tsuboi, D., Funahashi, Y., Nakano, T., Yoshimoto, J., Kobayashi, K., Uchigashima, M., Watanabe, M., Miura, M., Nishi, A., Kobayashi, K., Yamada, K., Amano, M., and Kaibuchi, K. (2016) Phosphoproteomics of the Dopamine Pathway Enables Discovery of Rap1 Activation as a Reward Signal In Vivo. *Neuron*. **89**, 550–565
102. Cook, A. A., Deng, W., Ren, J., Li, R., Sondek, J., and Bergmeier, W. (2018) Calcium-induced structural rearrangements release autoinhibition in the Rap-GEF, CalDAG-GEFI. *J. Biol. Chem.* 10.1074/jbc.RA118.002712
103. Westbury, S. K., Canault, M., Greene, D., Bermejo, E., Hanlon, K., Lambert, M. P., Millar, C. M., Nurden, P., Obaji, S. G., Revel-Vilk, S., Van Geet, C., Downes, K., Papadia, S., Tuna, S., Watt, C., NIHR BioResource–Rare Diseases Consortium, Freson, K., Laffan, M. A., Ouwehand, W. H., Alessi, M.-C., Turro, E., and Mumford, A. D. (2017) Expanded repertoire of RASGRP2 variants responsible for platelet dysfunction and severe bleeding. *Blood*. **130**, 1026–1030
104. Boudreaux, M. K., Catalfamo, J. L., and Klok, M. (2007) Calcium-diacylglycerol guanine nucleotide exchange factor I gene mutations associated with loss of function in canine platelets. *Transl Res*. **150**, 81–92
105. Lozano, M. L., Cook, A., Bastida, J. M., Paul, D. S., Iruin, G., Cid, A. R., Adan-Pedroso, R., Ramón González-Porrás, J., Hernández-Rivas, J. M., Fletcher, S. J., Johnson, B., Morgan, N., Ferrer-Marin, F., Vicente, V., Sondek, J., Watson, S. P., Bergmeier, W., and Rivera, J. (2016) Novel mutations in RASGRP2, which encodes

- CalDAG-GEFI, abrogate Rap1 activation, causing platelet dysfunction. *Blood*. **128**, 1282–1289
106. Cates, M. S., Berry, M. B., Ho, E. L., Li, Q., Potter, J. D., and Phillips, G. N. (1999) Metal-ion affinity and specificity in EF-hand proteins: coordination geometry and domain plasticity in parvalbumin. *Structure/Folding and Design*. **7**, 1269–1278
107. Busch, E., Hohenester, E., Timpl, R., Paulsson, M., and Maurer, P. (2000) Calcium affinity, cooperativity, and domain interactions of extracellular EF-hands present in BM-40. *J. Biol. Chem.* **275**, 25508–25515
108. Konermann, L., Pan, J., and Liu, Y.-H. (2011) Hydrogen exchange mass spectrometry for studying protein structure and dynamics. *Chem. Soc. Rev.* **40**, 1224–1234
109. Morgan, C. R., and Engen, J. R. (2010) *Investigating Solution-Phase Protein Structure and Dynamics by Hydrogen Exchange Mass Spectrometry*, Current Protocols Protein Science, Hoboken, NJ, USA, 10.1002/0471140864.ps1706s58
110. Deng, W., Wang, Y., Druzak, S. A., Healey, J. F., Syed, A. K., Lollar, P., and Li, R. (2017) A discontinuous autoinhibitory module masks the A1 domain of von Willebrand factor. *Journal of thrombosis and haemostasis : JTH*. **15**, 1867–1877
111. Bergmeier, W., and Stefanini, L. (2015) Platelet signaling - blood's great balancing act. *Oncotarget*. **6**, 19922–19923
112. Canault, M., Ghalloussi, D., Grosdidier, C., Guinier, M., Perret, C., Chelghoum, N., Germain, M., Raslova, H., Peiretti, F., Morange, P. E., Saut, N., Pillois, X., Nurden, A. T., Cambien, F., Pierres, A., van den Berg, T. K., Kuijpers, T. W., Alessi, M.-C., and Tregouet, D.-A. (2014) Human CalDAG-GEFI gene (RASGRP2) mutation affects platelet function and causes severe bleeding. *Journal of Experimental Medicine*. **211**, 1349–1362
113. Vercoulen, Y., Kondo, Y., Iwig, J. S., Janssen, A. B., White, K. A., Amini, M., Barber, D. L., Kuriyan, J., and Roose, J. P. (2017) A Histidine pH sensor regulates activation of the Ras-specific guanine nucleotide exchange factor RasGRP1. *eLife*. 10.7554/eLife.29002