DEFINING DIVERSE MECHANISMS THAT REGULATE THE ACTIVITY OF DBL-FAMILY GUANINE NUCLEOTIDE EXCHANGE FACTORS

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ABSTRACT

MARIYA KHUZEM CHHATRIWALA: Defining diverse mechanisms that regulate the activity of Dbl-family guanine nucleotide exchange factors.
(Under the direction of John Sondek)

Rho-family GTPases participate in a diverse range of critical cellular processes such as cytoskeletal rearrangement, gene transcription, and cell-fate determination; consequently, aberrant regulation of Rho-family GTPases has been implicated in diseases such as cancer and many developmental disorders. Dbl-family guanine nucleotide exchange factors (GEFs) activate Rho-family GTPases by promoting exchange of bound GDP for GTP. There have been 69 Dbl-family GEFs identified to date in the human genome, and while they vary significantly in size, domain architecture, and GTPase specificity, they all contain a Dbl-homology (DH) domain followed almost invariably by a tandem pleckstrin-homology (PH) domain. The DH domain is the core catalytic unit necessary for nucleotide exchange while the PH domain has been shown to be involved in lipid binding.

The work presented examines two mechanisms that regulate the exchange activity of Dbl-family GEFs by ultimately manipulating the ability of the DH domain to engage its cognate GTPases. We show that the N-terminal DH/PH cassette of the GEF Trio requires direct contact between the DH-associated PH domain and its cognate GTPases Rac1 and RhoG for efficient exchange, adding to a growing number of GEFs that require their PH domain for full exchange activity. In addition, we also investigate mechanisms through which Dbl-family GEFs may act as effectors of active small GTPases. These studies add to

our knowledge of the components that regulate the activity of Dbl-family GEFs.

For Husain, my little brother and best friend, and my parents who always believe in me.

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Ala, A	Alanine
ALPHAscreen	Amplified Luminescent Proximity Homogeneous Assay
aPKC	Atypical protein kinase C
Arf	ADP-ribosylation factor
Arg, R	Arginine
ARNO	ARF nucleotide binding-site opener
Arp 2/3	Actin related proteins 2 and 3
Asn, N	Asparagine
Asp, D	Aspartic acid
B-factor	Crystallographic temperature factor
Bodipy, Bod	Dipyrromethene boron difluoride
BRAG	Brefeldin-resistant Arf GEF
C2 domain	Ca2+ binding domain
C3G	Crk SH3 domain guanine nucleotide exchanger
CAAX box	Cysteine, aliphatic residue, aliphatic residue, any carboxy terminal residue
cAMP	3'-5'-cyclic adenosine monophosphate
CAPRI	Ca2+-promoted Ras inactivator
CC	Coiled-coiled
CCP4	Collaborative Computational Project Number 4 in Protein Crystallography
CCP4MG	Collaborative Computational Project Number 4, Molecular Graphics

Cdc25	Cell division cycle 25
Cdc42	Cell division cycle 42
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Chp	Cdc42 homologous protein
CM5	Carboxymethylated dextran surface plasmon resonance chip
CNS	Crystallography and NMR system
COOT	Crystallographic Object Oriented Toolkit
CR(1,2,3)	Conserved region
C-terminus	Carboxy terminus
Cys, C	Cysteine
DAG	Di-acyl glycerol
Dbl	Diffuse B-cell lymphoma
Dbs	Dbl's big sister
DENZO	An Oscillation Data Processing Program For Macro Molecular Crystallography
DH	Dbl-homology
DMEM	Dulbecco's modified Eagle's medium
DSSP	Digital shape sampling and processing
DTT	Dithiothreitol
Duet	Kalirin, RhoGEF kinase isoform 3
Duo	Kalirin, RhoGEF kinase isoform 2
E3b1	Eps8 binding protein; Abl interactor protein 1
EDTA	Ethylenediaminetetraacetic acid

EFA6	Exchange factor for ARF-6
EGFP	Enhanced green fluorescent protein
Elmo	Engulfment cell motility
Epac/cAMP-GEF 1/2	Exchange protein directly activated by cAMP
Eps8	Epidermal growth factor receptor pathway substrate 8
ERK	Extra-cellular signal-regulated kinase
FBS	Fetal bovine serum
Fbx	F-box domain; characterizes family of Arf-GEFs but also found in other proteins and often mediates interaction with a ubiquitin conjugating enzyme.
FERM	Band 4.1, ezrin, radixin, moesin
GAP	GTPase activating protein
GAP1IP4BP/RASA3	RAS p21 protein activator 3
GBF/BIG	Golgi BFA-resistance factor 1/BFA-inhibited GEF
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanosine 5′-diphosphate
GEF	Guanine nucleotide exchange factor
GEF-H1	Rho/Rac guanine nucleotide exchange factor 2, Lfc
GFP	Green Fluorescent Protein
Gln, Q	Glutamine
Glu, E	Glutamic acid
Gly, G	Glycine
GoLoco	Gαi/o-Loco
GPCR	G-protein coupled receptor

Grb2	Growth factor receptor-bound protein 2
Grp-1	GTP exchange factor for Arfs
GRP-3	Guanyl releasing protein 3
GST	Glutathione-S-transferase
GTP	Guanosine 5′-triphosphate
GTPγS	Guanosine 5'-(γ-thio)triphosphate
His, H	Histidine
HS	Horse serum
Ile, I	Isoleucine
IPTG	Isopropyl-β-D-thiogalactopyranoside
IQGAP	IQ motif containing GTPase activating protein
kDa	Kilodalton
Krit-1	Krev interaction trapped 1
LB	Luria-Bertani media
Leu, L	Leucine
Lfc	Lbc's first cousin
Lys, K	Lysine
mant	N-methylanthraniloyl
MAPK	Mitogen-activated protein kinase
MEKK	MEK kinase
MES	2-(N-Morpholino) ethanesulfonic acid 4-Morpholineethanesulfonic acid
Met, M	Methionine

Miro	Mitochondrial Rho GTPase
MR-GEF	M-Ras-regulated Rap GEF
NIH 3T3	National Institutes of Health 3T3 mouse fibroblasts
N-terminus	Amino-terminus
NuMA	Nuclear mitotic apparatus protein
N-Wasp	Wiskott-Aldrich syndrome-like
Ost	MCF.2 cell line derived transforming sequence-like isoform
PAK	p21 activated kinase
Par-3	Partitioning defective 3
Par-6	Partitioning defective 6
PBS	Phosphate buffered saline
PC-12	Rat pheochromocytoma cells
PDZ	PSD95, Discs large, ZO1
PEG	Poly(ethylene glycol)
PH	Pleckstrin homology
Phe, F	Phenylalanine
PI(4)P	Phosphatidylinositol (4) phosphate
PI3K	Phosphoinositide-3 kinase
PIP2, PI(4,5)P2	Phosphatidylinositol (4,5)-bisphosphate
PIP3, PI(3,4,5)P3	Phosphatidylinositol (3,4,5)-trisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C

P-Rex1	PI(3,4,5)P3-dependent Rac exchanger
Pro, P	Proline
RA	Ras association
RalGDS	Ral guanine nucleotide dissociation stimulator
RanGAP1	Ran GTPase activating protein
RapGRP/CalDAG-GEF	Rap/Ras guanyl releasing protein
RASAL	Ras protein activator like
RasGAP	Ras GTPase activating protein
RasGRF	Ras guanine nucleotide releasing factor
RBD	Ras/Rap binding domain
RCC1	Regulator of chromosome condensation 1
Rerg	Ras-like, estrogen-regulated, growth inhibitor
REM	Ras exchanger motif
RGS	Regulator of G-protein signaling
Rheb	Ras homolog enriched in brain
RhoBTB	Rho (broad complex, tramtrack, and bric-a-brac)
RhoGDI	Rho guanine nucleotide dissociation inhibitor
Rin	Rit-related gene expressed in neurons
Rit	Ras-like without CAAX 1
ROCK	Rho-kinase
RTK	Receptor tyrosine kinase
RU	Response units

Sar	Secretion associated and Ras-related
SDS PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
Sec14	Yeast phosphatidylinositol transfer protein
Ser, S	Serine
SER-CAT	Southeast Regional Collaborative Access Team
SH2	Src homology 2
SH3	Src homology 3
Smg GDS	Guanine nucleotide dissociation stimulator for smg p21
Sos	Son of Sevenless
SPR	Surface plasmon resonance
SRF	Serum response factor
STEF, Tiam2	SIF and Tiam1-like exchange factor, T-cell lymphoma invasion and metastasis 2
SUV	Small unilamellar vesicles
T-cell	Thymus cell
Thr, T	Threonine
Tiam1	T-cell invasion and metastasis 1
TLS	Translation, libration, screw rotation
Trio	Triple functional domain protein
Tris	Tris-hydroxymethylaminomethane
Trp, W	Tryptophan
Tyr, Y	Tyrosine
UNC-73	UNCoordinated family member 73

Wnt-1	Wingless type-1
Wrch-1	Wnt-regulated Cdc42 homolog 1
β-Pix	PAK-interacting exchange factor beta

CHAPTER 1: INTRODUCTION

Small GTPase Signaling

Overview

Large and small GTPases, which are also known as G-proteins, are guanine nucleotide binding proteins that are active when bound to GTP and inactive when GTP is hydrolyzed to GDP [1] (**see Figure 1**). Large G-proteins consist of Gα subunits that are activated by GPCRs and form a heterotrimer with Gβγ subunits until activated [2]. There are 21 different Gα subunits expressed in the human genome that fall into four major groups: the Gs, Gi/o, Gq, and $G_{12/13}$ families [3]. Once bound to GTP, Gα dissociates from Gβγ; both Gα and Gβγ are then free to bind and activate downstream effectors [2]. Small G-proteins belong to the Ras superfamily of small GTPases and are divided into five main branches: the Ran, Rab, Arf, Ras, and Rho families [4]. These small monomeric GTPases are 20 – 40 kDa in size [5], and together with the Gα subunits, comprise over 150 members in the human genome [4].

Although there are significant structural differences that distinguish the various G-proteins, they share a conserved GTPase domain originally identified in Ras isoforms, which will be discussed later in greater detail [1, 2]. The GTPase domain consists of a six-stranded β-sheet with five α-helices located on each side. The GTPase domain of small G- proteins also contains two switch regions (residues 32 – 38 and 59-67 for switch I and II, respectively,

in Ras isoforms), while large G-proteins have three switch regions. The switch regions are responsible for binding the guanine nucleotide and undergo structural rearrangement when GDP is replaced by GTP. Effectors of GTPases preferentially bind to the GTP-bound form and use the conformation of the switch regions to distinguish activation states [1]. Active small GTPases participate in signaling pathways that control critical cellular processes ranging from vesicle trafficking to neurite outgrowth [6, 7]. Consequently, aberrant regulation of the activity of small GTPases has been implicated in maladies such as tumor formation and developmental disorders [8, 9].

Most small GTPases, with the notable exceptions of Ran and Rerg, are lipid-modified to anchor them at various cellular membranes; localization of GTPases to membranes is critical for activation of downstream effectors *in vivo* [4, 10]. Additional forms of post-translational modification such as carboxy-terminal methylation, ubiquitination, and phosphorylation can also regulate sub-cellular location and function of small GTPases [11-19]. The activity of most small GTPases is further determined by three major classes of proteins. GTPase-activating proteins (GAPs) enhance the intrinsic capacity of GTPases to hydrolyze GTP to GDP; guanine nucleotide dissociation inhibitors (GDIs) shield the prenyl group on GTPases from solvent and stabilize the cytosolic, inactive, GDP-bound state; and guanine nucleotide exchange factors (GEFs) catalyze the exchange of bound GDP for GTP, thereby activating the GTPases [10]. While GAPs and GEFs have been identified for members of all five families of small GTPases, GDIs have been found only for Rab-family GTPases and Rho-family GTPases [20, 21] (**see Figure 1**).

Ran and Rab GTPase families

As previously mentioned, GTPases of the Ran family are not lipid-modified and their function does not depend on their ability to associate with a cellular membrane [4]. Only one Ran GTPase, Ran, has been identified in humans [22]. Ran is required to facilitate transport across the nuclear membrane, regulate spindle assembly, and control mitotic checkpoints [22-24]. The ability of Ran to transport cargo across the nuclear membrane is dependent on maintaining a high concentration of Ran-GDP outside the nucleus and a high concentration of Ran-GTP inside the nucleus. To maintain these separate pools of inactive and active Ran, RanGAP1 is concentrated outside of the nucleus while the Ran GEF RCC1 is abundant inside the nucleus. Active Ran binds importin-β that has entered the nucleus carrying cargo such as the microtubule organizing component, NuMA. The Ran-GTP/importin-β complex is then transported back out into the cytoplasm where RanGAP1 inactivates Ran and releases importin-β, allowing it to bind more cargo [25].

Unlike Ran GTPases, Rab-family GTPases are prenylated and function at various membranes, including the plasma membrane and endosomal membranes [26, 27]. Rab GTPases are primarily involved in regulating vesicle transport and work with tethering factors to tether vesicles to their target membranes [28]. In addition, Rab GTPases can also participate in membrane trafficking and help determine the specificity of membrane targeting [29]. To date, there have been over 60 mammalian Rab GTPases identified [26, 30]. Mis-regulation of Rab GTPases has been linked to Griscelli syndrome (characterized by dilution of hair pigmentation and uncontrolled activation of T-cells and macrophages), X-linked non-specific mental retardation, and decreased resistance to invasion by pathogens [31].

GTPases of the Ran and Rab families are critical for cellular function, but the remainder of this work will focus on the mechanisms and proteins responsible for the regulation of Rho-family GTPases. Ran and Rab GTPases are thought to cooperate with Rho-family GTPases in regulating cellular functions such as microtubule assembly [32] and cell adhesion [33]; however, the studies described subsequently do not examine these signaling pathways. Thus, there will be no further discussion of the Ran and Rab GTPase families.

Arf-family GTPases

The Arf family of GTPases is composed of Arf (ADP-ribosylation factors) proteins, Arl (Arf-like GTPases) proteins, and Sar (secretion associated and Ras-related) proteins[34, 35]; of these three sub-types, Arf GTPases are the best characterized. Arf GTPases are named for their ability to act as co-factors for cholera toxin during the catalysis of ADP-ribosylation of Gs, but are best characterized for their ability to regulate vesicular trafficking and other membrane trafficking pathways [34]. Like most other small GTPases, members of the Arf family are post-translationally modified with a lipid moiety [4, 10, 36]; however, they are myristoylated (not prenylated) on a glycine at the N-terminus and not the C-terminus [36]. Inactive Arf GTPases are soluble and cytosolic because the myristoyl moiety is buried in an internal binding pocket found in Arf GTPases themselves. Upon binding GTP, structural rearrangements expose the N-terminal lipid moiety, allowing it to insert into the plasma membrane [10, 35, 36].

The six mammalian Arf proteins identified are grouped into three families based on sequence homology and function [34, 37]. Arfs 1-3 control assembly of coat complexes into

budding vesicles, Arfs 4 and 5 have more ambiguous functions but are thought to regulate early Golgi transport, and Arf-6 likely regulates endosomal membrane trafficking and structural reorganization of membranes at the cell surface [34, 38].

Similar to the Rho-family GTPases (which will be discussed in detail later), the number of Arf GEFs exceeds the number of Arf GTPases. The mammalian genome encodes fifteen exchange factors for Arf GTPase. These GEFs are characterized by the presence of a Sec7 domain [37, 39] that consists of a cylinder composed of ten α-helices and is the core catalytic unit of the exchange factor [40, 41]. Arf GEFs can be grouped into five different families on the basis of domain architecture: the GBF/BIG, cytohesin, EFA6, BRAG, and Fbx families [37]. The cytohesin GEFs are arguably the most well defined of these families and currently consist of four members: cytohesin-1, cytohesin-2/ARNO, cytohesin-3/Grp-1, and cytohesin-4. The four cytohesin proteins are 68% identical and are composed of a coiled-coiled (CC) domain, Sec7 domain, and pleckstrin-homology (PH) domain in that order [37]. PH domains are traditionally known for binding phosphoinositides and those of the cytohesin family of GEFs generally bind phosphoinositides with relatively high affinities and specificities. Refer to the section labeled "PH domains: general introduction" for a detailed discussion of PH domain structure and function [42, 43]. The number of glycines found in the β1/β2 loop of PH domains in cytohesin GEFs significantly impacts the affinity and specificity for phosphoinositides [44]. The "3G" splice variants of the PH domains of ARNO, cytohesin-1, and Grp-1 can all bind phosphatidylinositol (3,4,5)-trisphosphate (PIP3) with sub to low micromolar affinities and are capable of membrane targeting; the "2G" splice variants bind both PIP3 and phosphatidylinositol (4,5)-bisphosphate (PIP2), but with ~30-fold lower affinity [44-47].

The substrate specificity of the cytohesin GEFs remains somewhat controversial. *In vitro* exchange data shows that although these GEFs are fairly promiscuous, they catalyze exchange most efficiently on Arf-1 [37, 48] while the EFA6 family of GEFs is more specific for Arf-6 [49]. However, other studies show that Arf-6, not Arf-1, preferentially associates with ARNO *in vivo* and that expression of ARNO results in activation of Arf-6 [50]. These two conflicting observations might be reconciled by recent data showing that while ARNO does not activate Arf-6, it will bind to active Arf-6 through its PH domain in a PIP3-dependent manner as a way of translocating to a cellular membrane where it can then activate Arf-1 [48] (**see Figure 2**).

Ras-family GTPases

The Ras family of GTPases includes (but is not limited to) the Ras, Rap, Ral, Tc21, Rheb, Rit, and Rin GTPases [4]. Ras proteins are the best characterized of these GTPases, mainly because of the high correlation between activating mutations in isoforms of Ras and the incidence of cancer. Activated mutants of Ras isoforms have been identified in >20% of human cancers [5, 51] and the original Ras isoform was identified as a retroviral oncogene from a rat sarcoma virus [52].

There are four major Ras isoforms in mammals: H-Ras, N-Ras, K-Ras(A), and K-Ras(B) [4, 51]. They are similarly and ubiquitously expressed, with the exception of K-Ras(A) whose expression levels are ~20 fold less than K-Ras(B) (personal communication with Adrienne Cox) [51]. They are also highly conserved in sequence except in their hyper-variable C-terminal regions, which undergo several post-translational modifications that dictate sub-cellular location and associated function. More specifically, these Ras isoforms

contain a C-terminal “CAAX” (C, cysteine; A, an aliphatic residue; X, any amino acid) motif that is proteolyzed to remove the last three residues prior to farnesylation or geranylgeranylation and carboxymethylation of the new C-terminal cysteine. In addition to isoprenylation, several Ras isoforms also require adjacent poly-basic residues in the hyper-variable region or further palmitoylation of this region for proper membrane targeting [5, 51, 53, 54]. Mutations that impair post-translational modification of Ras isoforms significantly impact function and often abrogate transformation potential [9, 51, 53]. Unfortunately, therapeutic agents that target lipid modification of Ras isoforms currently suffer from a lack of efficacy and specificity and often target Ras-superfamily GTPases.

The activity of Ras isoforms is also tightly controlled by GAPs and GEFs which modulate their nucleotide bound state. To date, there have been approximately nine GEFs and eight GAPs identified which directly regulate the activity of Ras isoforms [51].

GEFs for Ras GTPases are characterized by an ~ 250 amino acid Cdc25 domain (named after the yeast homolog of Ras isoforms) and an N-terminally adjacent 50 amino acid Ras exchanger motif (REM) domain [55-57]. Sos1 and -2, together with Ras-GRF1 and -2, and Ras-GRPs, comprise the three major families of exchange factors for Ras isoforms [55, 56]. Activation of Sos1 and -2 is regulated through association with receptor tyrosine kinases (RTKs) via interaction with Grb2 or possibly through allosteric modulation by phospholipids; Ras-GRF1 and -2 and Ras-GRPs respond to calcium-dependent association with calmodulin or interaction with DAG, respectively [55, 58, 59]. Interestingly, Sos1 and -2 and Ras-GRF1 and -2 also contain a DH/PH cassette, which is the catalytic exchange unit for Rho-family GTPases, and can activate the Rho-family GTPase Rac1 [8, 55, 60, 61].

Thus, Sos1 and -2 and RasGRF1 and -2 provide a direct link between the activation of Ras isoforms and Rac1.

In comparison to Ras GEFs, signaling pathways regulating GAPs for Ras isoforms are less defined. All known Ras GAPs are characterized by a 250 amino acid RasGAP domain, but do not share significant sequence homology outside of this region [55]. The tumor suppressor neurofibromin and p120 Ras-GAP are the two most extensively studied Ras GAPs [55, 62]. The neurofibromin gene is deleted in the genetic disorder neurofibromatosis [63] and p120 Ras-GAP is thought to associate with activated RTKs through its SH2 domain [64, 65], but little else is known about how they regulate activation of Ras under homeostasis. Other Ras GAPs such as RASAL, GAP1IP4BP, and CAPRI, may be modulated by regulation of phosphoinositide or calcium levels as many of them contain either PH or C2 domains [55, 66].

Activation of Ras isoforms regulates signaling events that modulate gene transcription [4, 67] and occurs in response to several different types of stimuli, including the activation of RTKs and GPCRs [67-70]. The mitogen-activated protein kinase (MAPK) pathway (Ras → Raf → MEK → ERK) is the canonical signaling pathway associated with activation of Ras GTPases [4, 67, 70], but other effectors such as PI3K, RalGDS, Tiam1, and phospholipase C (PLC)-ε have also been identified and are critical for processes including tumor formation, cellular differentiation, and Schwann cell migration [67, 71-75]. The interaction between active Ras isoforms and their effectors is mediated through well characterized motifs known as Ras-binding domains (RBDs) or Ras-association (RA) domains. Solved structures of RBD/RA domains indicate a similar ubiquitin-like fold, consisting of a ββαββαβ motif; the domains are ~100 amino acids in length with vary degrees

of sequence homology [76, 77]. However, several studies now show that the presence, or predicted presence, of an RBD or RA domain does not necessarily guarantee a direct interaction with active Ras isoforms or closely related Rap isoforms [78]. For instance, although Tiam1 was shown to co-immunoprecipitate with active H-Ras from cells [73, 78], efforts to show a direct interaction between the RBD domain of Tiam1 and H-Ras-GTP using purified components have failed (personal communication with John Sondek).

After Ras GTPases, the Rap GTPases comprise the most studied members of the Ras-family of small GTPases. Rap GTPases are involved in mediating cell adhesion, cellular differentiation, cell polarity, proliferation, neurite outgrowth, and synaptic function [79, 80]. Five closely related isoforms of Rap (Rap1a, Rap1b, Rap2a, Rap2b, Rap2c) have been identified in the human genome; of these, Rap1a and Rap1b are the best characterized [4]. Like Ras GTPases, Rap1a and -1b isoforms are lipid modified and membrane associated. They are found associated with the Golgi, endosomes, and the plasma membrane, but generally translocate to the plasma membrane upon activation [11, 81, 82]. Rap1a and -1b are >90% identical, with most differences within the C-terminal polybasic tail, and little progress has been made in elucidating specific roles for the different isoforms of Rap1 [83-85]. However, there is some evidence that Rap1a may be specific for T-cell proliferation and adhesion of lymphoid cells while Rap1b is required for platelet aggregation and is localized to the tips of neurites [85-87].

Rap1a was originally identified as a gene that could counter transformation mediated by K-Ras(A) [88]. Both Rap1 isoforms, which each share approximately 50% sequence identity with Ras isoforms, are virtually identical to Ras GTPases in the switch regions with the notable exception of glutamine 61. Residue 61 is a threonine residue in all Rap GTPases

[83]. As the switch regions mediate interactions with effectors, active Rap1 isoforms can also bind to the RBD domain of many of the same effectors as Ras GTPases. These effectors include Raf and RalGDS, which are critical for mediating transformation downstream of Ras isoforms [4]. Both active Ras and Rap1 GTPases have also been shown to interact with Tiam1 in cells, but at different binding sites (**see Figure 3**) [73, 74, 89-91]. The Q61T substitution in Rap1a and -1b renders them inefficient substrates for most Ras GAPs [92], and it is thought Rap1 isoforms counteract the transforming effects of Ras GTPases by remaining active and sequestering its effectors at the cell membrane [4, 93]. However while active Rap1 isoforms may compete with Ras isoforms for effectors, Rap1 GTPases participate in signaling pathways distinct from Ras GTPases and have their own specific effectors such as Krit-1 [68, 79, 80, 94].

There are seven known GEFs for Rap isoforms in humans: C3G, MR-GEF, PDZ-GEF, Epac/cAMP-GEF 1/2, and GRP3, RapGRP/CalDAG-GEF [93, 95-97]. In addition, the Cdc25 domain of PLC-ε may also activate Rap1a and -1b [98, 99]. The core catalytic unit of the Rap GEFs is conserved from Ras GEFs and is termed the Cdc25-homology domain (Cdc25-HD) [95, 100]; Rap GEFs also possess an REM domain which is critical for allosteric regulation of several Ras GEFs [101]. However, except for GRP3 and RapGRP, which can also catalyze nucleotide exchange on Ras GTPases, RapGEFs are specific for Rap-family members [55]. Most Rap GEFs catalyze exchange on both Rap1 and Rap2 isoforms [95-97, 102]. In addition to the catalytic unit necessary for exchange on Rap, GEFs for Rap contain other domains that allow for precise spatio-temporal regulation of their activity in response to stimulation by Ca^{2+}, cAMP, DAG, and association with RTK-

interacting proteins [93, 95-97, 102]. Many Rap GEFs also have RA domains and this may allow them to function as signaling nodes connecting Rap and Ras GTPase activity [95].

The mechanisms that regulate the activity and localization of the eleven Rap GAPs, are less well defined than those for Rap GEFs [92]. Putative GoLoco motifs, which bind to and can act as GDIs for large G-protein α-subunits [103], have been identified in three Rap GAPs, but recent work has shown that only one of these actually interacts with $G\alpha_i$ and functions as a true GoLoco motif [104-106]. Other Rap GAPs have C-terminal PDZ domains which can interact with various cellular membranes [92]. The recently solved structure of the 340 amino acid catalytic region of Rap1aGAP has revealed that Rap GAPs use mechanisms distinct from those of Ras GAPs and Rho GAPs to catalyze GTP hydrolysis [107]. As previously mentioned, Rho-family and Ras GTPases have a glutamine residue at position 61 (in Ras isoforms) which helps position a water molecule necessary for nucleophilic attack on the gamma phosphate of GTP. GAPs for these GTPases provide a catalytic arginine which further helps stabilize the nucleophilic water [108]. In the case of Rap GTPases, which have a threonine at position 61, the associated GAPs inserts a catalytic asparagine residue whose carboxyamide group fulfills the role of the glutamine 61 by stabilizing the water molecule [107].

Rho-family GTPases

The *rho* gene is the founding member of the Rho-family GTPases and was originally identified as a homolog of the *ras* gene in *Aplysia.* It is evolutionarily conserved in yeast, *C. elegans*, *Drosophila*, rat, and humans [109]. The proteins encoded by the *rho* gene in *Aplysia* are closely related to the Rho-family GTPases RhoB and RhoC and are 35% similar

to H-Ras. Subsequent structures solved of RhoA and other Rho-family GTPases indicate they are structurally distinguishable from Ras isoforms by a "Rho insert" that lies between β5 and α4 [110]. However, many of the residues that regulate nucleotide binding and interactions with effectors are conserved between Ras isoforms and Rho-family GTPases. For instance, mutation of positions 12 or 61 to a valine or leucine, respectively, renders Ras isoforms constitutively active, while mutation of residue 17 in Ras isoforms causes them to act as a dominant negative by irreversibly interacting with GEFs. Analogous mutations in Rho-family GTPases have the same effects [111-116]. In addition, most Rho-family GTPases are also targeted to various cellular membranes through lipid modification of the C-terminus at the CAAX motif, similar to isoforms of Ras [21, 110].

Twenty-three genes, which can encode 26 Rho-family GTPases, have been identified in humans [110, 117]. These GTPases can be activated as part of signaling pathways downstream of plexins, RTK, GPCRs, Eph receptors, and pathogen invasion, and thus are subject to multiple modes of regulation [118]. Rho-family GTPase are best known for regulating rearrangements of the actin cytoskeleton necessary for diverse cellular processes including: cell polarization, vesicle trafficking, cell cycle progression, cellular differentiation, host-pathogen interaction, and neurite outgrowth [117, 119-121]. Consequently, aberrant regulation of the activation of Rho-family GTPases has been implicated in a host of diseases, including cancer and neurodegenerative disorders [122, 123]. The Rho-family GTPases can be divided into six major branches: Rho, Rac, Cdc42, Rnd, RhoBTB, and Miro [110, 117]. Of these, the Rho, Rac, and Cdc42 branches are the best characterized, and will be the focus of this section.

RhoA, -B, and -C isoforms are 85% identical in sequence, comprise the Rho subgroup of Rho-family GTPases, and generally share the same GEFs and effectors [110, 117, 124, 125]. However RhoA, -B, and -C often reside in distinct sub-cellular locations, dictated by post-translational modification [125]. In addition, while both RhoA and RhoC can mediate transformation, increased expression of RhoC is more strongly associated with oncogenesis. In contrast, RhoB may function as a tumor suppressor [110, 125]. The major cell function attributed to RhoA, -B, and -C activation is regulation of contractile actin and myosin filaments necessary for formation of stress fibers [113]. Well characterized effectors of Rho isoforms include Rho-associated kinase (ROCK), citron kinase, diaphanous, rhotekin, and PLC-ε [126].

The Rac branch of Rho-family GTPases encompasses Rac1, Rac2, Rac3, and RhoG. The three Rac isoforms are 88% identical, while RhoG is 72% identical to Rac1 [110] (**see Figure 4**). Similar to RhoA, -B, and -C, most of the variation in sequence of Rac isoforms occurs at the C-terminus, but the functional differences can be attributed to expression patterns instead of differences in sub-cellular location. Rac1 is ubiquitously expressed, Rac2 is primarily hematopoietic, and Rac3 is enriched in the brain [110]. Rac proteins regulate the formation of lamellipodia, but their function is also critical for neurite extension and formation of tight junctions [114, 120]. Common effectors of Rac1, -2, and -3 include p21-activated kinases (PAKs) 1, 2, and 3, PI3K, MEKK1/4, IQGAP, and the WAVE/SCAR complex [126]. RhoG often signals in parallel with Rac isoforms and can be activated by some of the same GEFs [127, 128] but signals through different effectors [129]. The two major effectors identified to date for RhoG are kinectin, which is a scaffold protein involved

in microtubule assembly, and Elmo, which is one component of the bipartite Dock180/Elmo GEF for Rac isoforms [130, 131].

The Cdc42 branch of Rho-family GTPases consists of Cdc42, Tc10, Tcl, Wrch-1, and Chp [110, 117]. All of the Cdc42 family members are functionally similar in that they mediate the formation of filopodia [132], but some members signal through different effectors and are structurally distinct from the founding member, Cdc42. N-WASP, a canonical effector of Cdc42 [132], activates actin polymerization through the Arp2/3 complex [133]. Tc10 and Tcl also signal through N-WASP, but induce longer filopodia than Cdc42 and mediate distinct signaling pathways [134, 135]. Chp and its close relative Wrch-1 are functionally similar to Cdc42, but contain an auto-inhibitory, polyproline-rich N-terminal extension that likely interacts with adaptor proteins such as Grb2 through their SH3 domains to relieve auto-inhibition [136-139]. In addition, both Chp and Wrch-1 use palmitoylation and not prenylation for membrane targeting [139, 140]. Finally, unlike the other Cdc42-like GTPases, Wrch-1 does not interact with N-WASP but instead is responsive to Wnt-1 and signals through activation of the PAK kinases [137, 138, 141]. In addition to regulating filopodia formation, Cdc42 has well established roles in maintaining cell polarity via the Par-3 polarity complex and is critical for cell-cycle progression [120, 142]. Interestingly, Cdc42 shares many important effectors with Rac1, -2, and -3, such as the PAKs, MEKK1/4, PI3K, and IQGAP [126].

As previously mentioned, aberrant activation of Rho-family GTPases has been implicated in a host of diseases including cardiovascular disorders, neurological illnesses, and cancer [7, 122, 123, 143]. Thus, under normal conditions, their activation state is precisely controlled by GAPs, GDIs, and GEFs [10]. While mutated forms of Ras isoforms

have been identified in human cancers, this is not the case for activating mutants of RhoA, -B, or -C, the Rac branch of Rho-family GTPases, or Cdc42-like GTPases. Instead, it is the changes in expression or activity of regulators of Rho-family GTPases that are associated with various diseases [8, 66, 92, 144].

GAPs and GDIs both attenuate the activity of Rho-family GTPases, but do so using different mechanisms. GAPs accelerate the relatively slow intrinsic GTP hydrolysis rate of Rho-family GTPases by supplying a catalytic arginine which establishes contacts with the main-chain carbonyl of glycine 12 (in Rac isoforms) and helps stabilize the GTP-hydrolysis transition state [145]. GDIs sequester already inactive Rho-family GTPases in the cytosol by shielding the prenyl group in a hydrophobic binding pocket and preventing translocation to various cellular membranes, which is critical for activation [146]. The mechanisms responsible for releasing Rho-family GTPases from GDIs remain poorly defined [8, 144]. Thus far, only three human GDIs for Rho-family GTPases have been identified: RhoGDI, D4GDI, and RhoGDIγ. RhoGDI is expressed ubiquitously, while D4GDI is exclusively hematopoietic and RhoGDIγ is expressed in lung, brain, and testis [144]. In contrast to the GDIs, between 60 and 70 proteins that contain a RhoGAP domain are expressed in humans, and they vary in GTPase specificity, size, and domain architecture [92, 147, 148]. The substrate specificity for most Rho GAPs has only been tested on RhoA, Rac1, or Cdc42, but it is possible that they function on atypical Rho-family GTPases as well [147]. The different domains present in Rho GAPs allow them to function as signaling nodes or scaffolds that integrate several signals with the inactivation of Rho-family GTPases.

Similar to Rho GAPs, the number of Rho GEFs greatly exceeds the number of Rho-family GTPases [8]. Typically, GEFs are defined as proteins that interact preferentially with

the nucleotide-free form of the GTPase, in addition to catalyzing nucleotide exchange. A more detailed explanation of the basic mechanism used by Rho GEFs to catalyze exchange is provided in the next section. Several classes of Rho GEFs have been identified including GEFs related to either SmgGDS, Dock180, or Dbl [149, 150]. Of these, the Dbl-family GEFs are the best characterized biologically and structurally.

Dbl-family GEFs

Overview

The first Dbl-family GEF was identified in 1985 as an oncogene from the DNA of human diffuse B-cell lymphoma (Dbl). When transfected in to NIH 3T3 cells, this gene was able to transform the cells and produce foci [151]. In addition to transforming cells, the Dbl oncogene also functions as an exchange factor for RhoA and Cdc42 [152, 153]. The exchange activity of Dbl is dependent on the integrity of an ~200 amino acid domain known as a Dbl-homology, or DH domain [153]. In the past fifteen years, numerous evolutionarily conserved proteins that possess a DH domain and are generally capable of transforming NIH 3T3 cells have been discovered. These proteins comprise the large and biologically significant Dbl-family GEFs [154, 155].

To date, 69 Dbl-family GEFs [8] possessing diverse domain architectures, varying tissue distributions, and various GTPases specificities and sub-cellular locations have been identified in the human genome [156, 157] (**see Figure 5**). The exchange activity of the majority of Dbl-family GEFs is restricted to RhoA, -B, and -C, Rac1, -2, -3, RhoG, and Cdc42 [8]. One notable exception is P-Rex1 which can catalyze exchange on Tc10 (personal communication, Dr. Stephanie Hicks). Although most Rho-family GTPases are expressed

ubiquitously, expression of Dbl-family GEFs is more cell and tissue specific [154, 157]. Their number and variability makes Dbl-family GEFs similar to Rho GAPs in that they can integrate diverse signals, resulting in precise spatio-temporal activation of Rho-family GTPases in response to different stimuli.

Dbl-family GEFs are defined by a DH domain, followed almost invariably by a tandem PH domain [8]. DH/PH cassettes comprise the core catalytic units of these exchange factors and are generally the minimum fragments necessary for cellular transformation [158]. The DH domain forms the majority of the catalytic interface with the GTPase and is often sufficient for exchange *in vitro* [157]. The invariant position of the PH domain and observations that it is required for exchange *in vivo* and/or *in vitro*, suggest it also has a conserved function [8, 154, 159-161]. However, roles of DH-associated PH domains are not as well defined as those of DH domains and will be discussed in greater detail later. The diverse domains surrounding the DH/PH cassette provide an additional level of regulation specific to each GEF and can participate in functions such as determining sub-cellular localization, auto-inhibiting the GEF by preventing access to the DH domain, and acting as a scaffold for various activators or effectors [8, 101, 162-164].

DH domains

The first structure of a DH domain solved in 1998 was that of β-Pix and revealed a long bundle of six alpha helices [165, 166]. Structures of DH domains solved since then show that this domain structure is highly conserved in other Dbl-family GEFs [60, 158, 166-170]. There is also significant sequence conservation through α1a, α2b-d, and α5a-b. These helices form the core of the DH domain and are denoted as conserved region (CR) 1, 2, and

3. The nomenclature used here was defined by Rossman. *et al*. [166]. Residues in CR1 and CR3 mediate the majority of the interactions with the cognate GTPase which help stabilize the switch regions in a conformation that disrupts interaction with both the bound nucleotide and Mg^{2+}. When Rho-family GTPases are bound to GTP, the Mg^{2+} ion helps stabilize the bound nucleotide by interacting with the terminal gamma-phosphate on GTP [158].

The functional importance of the interface between DH domains and their cognate GTPase has been verified through several mutagenesis studies [158, 161, 165]. Mutation of residues in α1, α5 (CR1, CR3) and α6 that make direct contacts with the cognate GTPase often significantly abrogate exchange activity without destabilizing the structure of the DH domain [166]. In addition, several residues in α5 are critical for determining substrate specificity. An elegant study by Snyder *et al.* illustrates how rational mutation of these residues can switch GTPase specificity [167].

PH domains: general introduction

PH domains are the 11th most common domain in the human genome and are found in a wide variety of proteins including kinases, phospholipases, and exchange factors [43, 171]. The name pleckstrin homology domain is derived from the observation that PH domains display homology to the protein kinase C (PKC) substrate, pleckstrin [172]. PH domains can have remarkably low sequence conservation yet retain high structural similarity [43]. They consist of a core seven strand β-sandwich with a C-terminal helix and three interstrand loops between β1 and β2, β3 and β4, and β6 and β7 that participate in the majority of interactions with lipid and protein binding partners [171, 172].

As previously mentioned, PH domains have been characterized as phosphoinositide-binding modules [173, 174]. Structures of various PH domains reveal that they are generally electrostatically polarized, with a net concentration of positive charge. It was hypothesized that this polarization could facilitate binding to negatively charged phosphoinositides and help translocate proteins to the plasma membrane [172, 173]. This claim was supported by the observation that several PH domains, including those of PLC-δ and Grp-1, bind specific phosphoinositides with high affinities in the low to sub-micromolar ranges and can function as membrane anchors and targeting signals [42, 175].

However, the PH domains of PLC-δ and Grp-1 are notable exceptions to the rule, as most PH domains bind phosphoinositides with low affinities (>10 μM) and would be unable to independently target proteins to membranes [171, 176]. Instead, many PH domains that participate in low-affinity interactions with phosphoinositides also interact with other proteins [166, 171, 177]. The binding motifs for PH domains are not as conserved as they are for SH2 or SH3 domains, for example [171, 178, 179], but collectively these interactions show that PH domains can also mediate diverse protein/protein interactions. Optimal exchange activity of Dbl-family GEFs often depends on integrating both the lipid binding and protein interaction capabilities of DH-associated PH domains.

Functions of DH-associated PH domains

Regulation of exchange activity through phosphoinositide binding

Given that the PH domains of PLC-δ and Grp-1 are capable of translocating and anchoring these proteins to the plasma membrane through interactions with phosphoinositide headgroups [43, 180], numerous studies have examined if DH-associated PH domains carry

out a similar function. As Dbl-family GEFs also function at various cellular membranes in order to activate membrane bound GTPases [159, 181], loss of this localization would explain why truncation of the PH domain abolishes transformation ability. However, individually, the majority of DH-associated PH domains (with the exception of the Sos1-PH domain) bind phosphoinositides with low affinities (>10 μM) and thus would be unable to target the exchange factor to the plasma membrane without assistance [171, 176, 182, 183].

Data suggest that some Dbl-family GEFs require DH-associated PH domains for efficient translocation to the plasma membrane, but do not support a general and exclusive role for DH-associated PH domains as membrane anchors. Replacing the PH domain of Lfc with a membrane targeting signal restores transformation activity that is lost when cells are transfected with the DH domain alone [184], but similar experiments using Vav1 DH yield contrasting results and do not restore its transformation potential [185]. In the case of Dbs, targeting its DH domain to membranes using a CAAX motif restores its ability to cause transformation, but does so only partially [186]. However, these studies did not specifically examine sub-cellular localization and several groups have shown that the DH/PH fragments of Dbs, Tiam1, and Sos1 retain their capacity to localize to the plasma membrane even when the PH domain is mutated to prevent binding of phosphoinositides [159, 160, 182]. Other domains with these GEFs may be

In contrast to Dbs, Tiam1, and Sos1, two studies using immunofluorescence microscopy found that mutations introduced into the DH-associated PH domain of Dbl to prevent phosphoinositide binding also abrogate the membrane localization of Dbl [187, 188]. However, differences in sub-cellular localization between wild-type Dbl and Dbl mutants unable to bind phosphoinositides are not as pronounced as in similar experiments

performed with PLC-δ [189]. Recent studies investigating the role of the DH-associated PH domain of Asef saw much more dramatic changes in sub-cellular localization when the PH domain was removed [190]. Deletion of an entire domain, though, often has detrimental effects on protein stability and it is possible that truncation mutants of Asef lacking the PH domain were mis-folded and therefore, mis-localized. Finally, new evidence suggests that the PH domain of Dbs may be able to translocate the GEF to the plasma membrane, but only through dimerization of the DH/PH cassette. Thus, although individual DH-associated PH domains do not bind phosphoinositides with high affinity, they may be able to target Dbl-family GEFs to the plasma membrane cooperatively [191, 192].

Phosphoinositide-binding through the PH domain may not be the sole determinant of translocation to membranes, but it remains critical for exchange activity *in vivo*. Although the phosphoinositide-binding deficient mutants of Dbs and Tiam1 are localized to cellular membranes, they are no longer able to cause cellular transformation [159, 160]. Similarly, Vav1 DH artificially targeted to the plasma membrane still lacks transformation potential [185]. Thus, DH-associated PH domains likely perform functions that require phosphoinositide binding, but are not restricted to dictating sub-cellular localization.

In addition to translocating Dbl-family GEFs to the plasma membrane, it has been suggested that DH-associated PH domains act as sites for allosteric regulation by phosphoinositides. As several GEFs, including Vav1, -2, and -3 and Sos1, are activated downstream of PI3K [193, 194], it was hypothesized that PI3K could regulate the activities of these GEFs by influencing the ratio of PIP2 to PIP3 in the cell. Reports have claimed that interaction of PIP2 with the PH domains of Vav isoforms and Sos1 restricts access by the GTPase substrate to the active site while interaction with PIP3 has a positive effect on

exchange [193-195]. However, subsequent studies examining the structure of full-length Vav3 indicate that the GEF is unlikely to adopt a conformation in which the DH-associated PH domain blocks access to the DH domain [196]. Thus, phosphoinositides may regulate the exchange activities of Vav isoforms and Sos1, but not through allosteric modulation of the DH/PH cassette. It has also been suggested that Tiam1 is allosterically regulated by phosphatidylinositol (4) phosphate (PI(4)P) or PIP2 through either its N-terminal PH domain, DH-associated PH domain, or both [197, 198]. Similarly, P-Rex1 was also found to be activated through PIP3 *in vitro* [199]. These results, though, are contradicted by experiments in which abrogation of phospholipid binding has no affect on the exchange activity of purified DH/PH fragments of Dbs, Tiam1 or intersectin in the presence of soluble phosphoinositide head groups or small unilamellar vesicles (SUVs) [176].

See **Table 1** for a brief summary of studies that have examined regulation of Dbl-family GEF activity that is dependent on interaction with phosphoinositides through the DH-associated PH domain.

Auto-inhibition of Dbl-family GEFs

While DH-associated PH domains augment the activity of most-Dbl-family GEFs, there are several notable cases in which DH-associated PH domains restrict access to the DH domain and keep the GEF in an auto-inhibited state under basal conditions. Biochemical data shows that p63RhoGEF and the C-terminal DH/PH cassette of Trio (Trio-C) are both significantly less active than the DH domain alone [200, 201]. The structure of the DH/PH cassette of Sos1 also shows the PH domain occluding the active site on the DH domain, and explains why this fragment has no exchange activity in solution [60]. The auto-inhibition of

both Trio-C DH/PH and p63RhoGEF DH/PH is relieved through an interaction between active Gαq and a conserved region directly C-terminal to the PH domain [201, 202]. The molecular mechanisms that relieve the auto-inhibition of Sos1 DH/PH remain unclear, but some evidence suggests that the E3b1/Eps8 complex activates Sos1 downstream of H-Ras and PI3K [203, 204]. These aforementioned GEFs provide important examples of how DH-associated PH domains can regulate the exchange activity by mediating protein-protein interactions (**see Table 2**).

Nucleotide exchange assisted by PH domains

As previously stated, DH domains are followed almost invariably by adjacent PH domains. While DH domains are often sufficient for the catalysis of nucleotide exchange, the structures of DH domains from β-Pix and the N-terminal DH/PH cassette of Trio (Trio-N) have a disordered C-terminal (α6) helix [161, 165]. Subsequently solved structures of DH/PH cassettes reveal extensive interactions between portions of the PH domain and the C-terminal helix [158, 166-168, 170]. Residues in the C-terminal helix of DH domains form functionally relevant contacts with the cognate GTPase and DH-associated PH domains likely stabilize this helix to ensure efficient nucleotide exchange.

In addition to stabilizing DH domains, DH-associated PH domains can also participate directly in the nucleotide exchange reaction. The structures of Dbs DH/PH in complex with nucleotide-free Cdc42 or RhoA show direct contacts between the DH-associated PH domain of Dbs and the cognate GTPases [166, 167]. The majority of the residues contributing to the interface between the PH domain and the cognate GTPase are found in the β3/β4 loop and the switch II region of the respective proteins. Disrupting these

contacts results in loss of exchange activity both *in vitro* and *in vivo* SRF activation Rho signaling. The structures of the DH/PH cassettes of LARG and PDZ-RhoGEF in complex with nucleotide-free RhoA also reveal contacts between their DH-associated PH domains and RhoA, albeit through residues in the C-terminal helix of the PH domain instead of the β3/β4 loop [168, 205]. Biochemical data and sequence analysis suggest that other Dbl-family GEFs may use their DH-associated PH domain similarly to Dbs, LARG, and PDZ-RhoGEF. The residues in Dbs responsible for mediating the interaction between the DH-associated PH domain and Cdc42 or RhoA are conserved in several GEFs, including Trio-N, Dbl, and obscurin [166]. In addition, removal of the DH-associated PH domain of Trio-N results in at least a four-fold reduction in exchange activity [161, 200] (**see Table 2**).

In contrast to Dbs, LARG, and PDZ-RhoGEF; Tiam1, intersectin, and collybistin in complex with their cognate GTPases show little or no contact between the PH domain and the GTPase [158, 167, 168, 205]. However, these structures do not account for the role of membranes in facilitating nucleotide exchange. Furthermore, the region linking DH and PH domains has a high degree of conformational flexibility that might allow contact between the PH domain and the GTPase under more biological conditions [158, 166, 206] (**see Figure 6**).

One possible scenario is that, at a membrane, binding to a phosphoinositide headgroup enables flexible DH/PH cassettes to adopt a conformation allowing contact between the PH domain and the membrane-bound GTPases [160]. Thus, effective exchange *in vivo* would require DH-associated PH domains to engage the cognate GTPase and bind phosphoinositides; several studies show that residues in Dbs that bind phosphoinositides do not overlap with the residues that engage cognate GTPases [160, 176]. This model would also allow Dbl-family GEFs to use phosphoinositide levels to fine-tune their activity.

Dbl-family GEFs as effectors of small GTPases

Guanine nucleotide exchange factors as effectors of active GTPases

As previously mentioned, over 150 small and large G-proteins have been found in the human genome [2, 4]. They often participate in overlapping signaling networks and can directly modulate the activity of other GTPases. For example, activation of RhoA downstream of various Gα subunits is well documented [207-210]. Cdc42 can also activate Rac1 in several different systems, while Rac isoforms can activate RhoA [211]. In addition, instances have also been identified of Rac1 acting downstream of RhoG or various Arf GTPases [50, 131, 212]. Finally, several studies show that isoforms of Ras can also activate Rac1 and other Rho-family GTPases, and activation of these Rho-family GTPases is required for transformation [73, 74, 123].

The signaling components that facilitate the activation of one GTPase by another are complex and not completely elucidated, but exchange factors are logical mediators of these signaling pathways and there are several examples in the literature of exchange factors acting as a direct link between two GTPases. For example, the Dbl-family GEFs Sos1 and -2 and RasGrf1 and -2 are also exchange factors for Ras isoforms, and thus couple signaling by Rac1 and Ras isoforms through mechanisms that are not completely understood [58, 213]. H-Ras and Sos1 also participate in a positive feedback loop that regulates activation of H-Ras. The exchange activity of Sos1 towards H-Ras-GDP is augmented when H-Ras-GTP binds to a site distal to the Cdc25 domain [101] (**see Figure 7**). The Arf-1 GEFs ARNO and Grp-1 are activated downstream of active Arf-6 [46, 48]. Structural analysis shows that active Arf-6 can bind to the PH domain of Grp-1 and relieve auto-inhibition of the Sec-7

domain by a helical region C-terminal to the PH domain [46]. The interaction between Arf-6 and Grp-1 is enhanced by PIP3. Although exact structural details are not available, active Arf-6 likely interacts similarly with ARNO (**see Figure 2**). Finally, ARNO can control the activation of Rac1 through interaction with the Dbl-family GEF β-Pix [212]. Thus there are numerous examples of exchange factors, including Dbl-family GEFs, acting as effectors of active GTPases. In doing so, they facilitate cross-talk between different GTPases and provide an additional level of regulation.

Dbl-family GEFs as direct effectors of heterotrimeric G-proteins

It is well established that RhoA can be activated downstream of $G\alpha_{12/13}$ by the RGS-domain containing family of Dbl-family GEFs, which consists of LARG, p115RhoGEF, and PDZRhoGEF [207-210]. All three of these GEFs possess an RGS domain which allows them to bind to $G\alpha_{12}$ and $G\alpha_{13}$ [210]. The RGS domain enhances the intrinsic GTP hydrolysis activity of $G\alpha_{12}$ and $G\alpha_{13}$ subunits [214-216], but also allows active Gα subunits to regulate exchange activity of RGS-family Dbl-family GEFs either allosterically or by translocating them to the plasma membrane [217]. Evidence suggests that p115RhoGEF interacts with $G\alpha_{13}$ through its DH/PH cassette as well, and this interaction is also required for efficient activation of RhoA [218]. The mechanisms by which these interactions enhance activation of RhoA are not fully understood.

Gαq also activates RhoA, but does so through the Dbl-family GEFs p63RhoGEF and Trio-C DH/PH [201, 202, 219, 220]. As previously mentioned, the binding site for Gαq lies in the highly conserved region just C-terminal to the PH domain and is alpha-helical [201, 202]. Several groups have shown that the direct interaction between Gαq and p63RhoGEF or

Trio-C relieves auto-inhibition of the GEF by moving the PH domain away from the active site of the DH domain and is sufficient for enhancing exchange on RhoA [201, 202].

Dbl-family GEFs as effectors of small GTPases

Recent data suggests that Dbs can interact with active Rac1 through its DH-associated PH domain (**see Figure 8**). The interaction between Rac1 and Dbs is nucleotide dependent and co-transfection of constitutively active Rac1 with the N-terminally truncated oncogenic form of Dbs augments transformation of NIH 3T3 cells by Dbs [221]. In addition, mutation of residues in the DH-associated PH domain of Dbs known to mediate interaction with its cognate GTPases or phosphoinositides do to affect interaction with active Rac1 [221]. Interactions similar to those between active Rac1 and Dbs are seen between Scambio and active Rac1, -2, and -3 and Cdc42 [222]. Scambio and Dbs are evolutionarily related and belong to the same subset of Dbl-family GEF. The PH domains of Dbs and Scambio also share significant sequence homology [159, 222] (**see Figure 9)**. Thus, it is possible the interactions mediating the interface between Dbs and active Rac1 are conserved in the interface between Scambio and active Rac isoforms.

Several Ras-family small GTPases can also modulate the activation of Rac1 through Dbl-family GEFs. Active Rap1a co-immunoprecipitates with Tiam1 and Vav2 and binds to their DH-associated PH domains [89, 90]. Vav2 and Tiam1 catalyze nucleotide exchange on isoforms of Rac, and this interaction is thought to ensure proper sub-cellular localization of these exchange factors during Rac1-mediated the activation of cell-spreading [89]. Tiam1 is also activated by Rap1a-GTP as part of the signaling cascade that activates the Par-3 polarity complex in neurons and epithelial cells [90, 223, 224] (**see Figure 10**). Sequence analysis of

Tiam1 reveals the presence of a putative RBD domain and it is possible that active Rap1 may also interact with Tiam1 through its canonical effector binding region [73]. Finally, H-Ras may interact with Tiam1 through its RBD domain in a PI3K-independent manner; several reports have now shown that the interaction between Tiam1 and H-Ras is necessary for efficient activation of Rac1 in various cell types [73, 74].

Mechanisms used by active GTPases to activate GEFs

Active GTPases activate GEFs through two major mechanisms, often used in combination: allosteric modulation of exchange activity or ensuring proper sub-cellular localization. In the case of interactions between active H-Ras and the Cdc25 domain of Sos1 or interactions between active Gαq and the p63RhoGEF or Trio-C, the active GTPases allosterically modulate the activity of the GEFs and activation of Trio-C and p63RhoGEF can be seen *in vitro* using purified components [101, 201]. In contrast, as previously mentioned, both Tiam1 and Vav2 are activated by Rap1a-GTP *in vivo* through regulation of sub-cellular localization [89]. However, these data do not exclude the possibility that active Rap1a can allosterically modulate the exchange activity of either Tiam1 or Vav2. Finally, active Arf-6 activates ARNO both by translocating it to a cellular membrane and by relieving auto-inhibition [46, 48]. The mechanisms by which active Rac1 activates Dbs are unknown.

Concluding remarks

Dbl-family GEFs are controlled by many different regulatory mechanisms to ensure the precise spatio-temporal activation of Rho-family GTPases. This body of work will focus on two important components of this regulation. Chapter two of this thesis further examines

the ubiquity of using DH-associated PH domains as direct participants in the nucleotide exchange reactions by determining the role of the N-terminal DH-associated PH domain of Trio in regulating activation of its cognate GTPases.

The third and fourth chapters of this thesis examine the role of Dbl-family GEFs as effectors of small GTPases. Specifically, we investigate the nucleotide dependent interactions between active Rac1 and Dbs and active Rap1a and -1b and Tiam1. A major caveat of the studies initially characterizing the interactions between active Rac1 and Dbs and active Rap1 and Tiam1 is that the interactions were examined using co-immunoprecipitation assays [89, 90, 221]. These studies strongly suggested that Rac1 and Rap1 directly influenced the activity of Dbs and Tiam1, respectively, but it is unknown if these interactions are direct or mediated by other components in the cell; thus we further examine these interactions using purified components. Together, these studies provide a greater understanding of the complex and varied mechanisms used to control Rho-family GTPase activity through their exchange factors.

GEF	Type of phosphoinositide -dependent regulation	Phosphoinositide-binding specificity and affinity	References
Lfc/GEF-H1	Membrane-translocation[1] Membrane anchor[1]	Unknown	[184]
Asef	Membrane-translocation	PI(3,4,5)P3 Affinity unknown	[190]
Dbs	Membrane-translocation[2] Translocation-independent activity at membrane	PI(4,5)P2; 11 μM PI(3,4,5)P3; > 50 μM PI(3)P; > 50 μM	[160, 176, 191, 225]
Dbl	Allosteric Membrane-translocation Translocation-independent activity at membrane	PI(4,5)P2 PI(3,4,5)P3 Affinities unknown	[187, 188]
Vav 1, 2, 3	Translocation-independent activity at membrane Allosteric[3]	PI(4,5)P2; 3-4 μM PI(3,4,5)P3; unknown	[185, 193, 194]
Tiam1	Translocation-independent activity at membrane Allosteric[4]	PI(4,5)P2; >50 μM PI(3,4,5)P3; > 50 μM PI(3)P; 10 μM	[159, 176, 197, 226]
Sos1	Allosteric	PI(4,5)P2; 1.5 μM PI(3,4,5)P2; 0.5 μM	[182, 193, 195, 227]
Intersectin	Unknown	PI(4,5)P2; 4.2 μM PI(3,4,5)P3; > 50 μM PI(3)P; > 50 μM	[176]
P-Rex1	Allosteric Membrane-translocation	PI(3,4,5)P3 Affinity unknown	[199, 228]

Table 1: GEFs that are regulated through interaction between the DH-associated PH domain and phosphoinositides.

1. Conflicting studies suggest that the DH-associated PH domain of Lfc/GEF-H1 does not bind any phospholipids [229].
2. Conflicting studies suggest phosphoinositide binding does not influence sub-cellular localization [160].
3. Recent studies suggest that the domain architecture of Vav does not support a role for allosteric regulation by phosphoinositides [196].
4. Results showing allosteric regulation of Tiam1 DH/PH by phosphoinositides are contradicted by other studies [176].

GEF	Cognate GTPases	Role of DH-associated PH Domain.	References
Asef	Cdc42	Inhibitory; interacts with N-terminal SH3 domain	[164]
Trio-N	Rac1, RhoG	Assists nucleotide exchange; Interacts directly with cognate GTPase	[230]
Dbs	Cdc42, RhoA, RhoG	Assists nucleotide exchange; Interacts directly with cognate GTPase	[166]
p63RhoGEF	RhoA, B, C	Inhibitory; blocks active site on DH domain	[201, 202]
Trio-C	RhoA	Inhibitory; blocks active site on DH domain	[201]
Kalirin	Rac1, RhoG	Inhibitory; blocks active site on DH domain	[201]
PDZRhoGEF	RhoA	Assists nucleotide exchange; Interacts directly with cognate GTPase	[205]
LARG	RhoA	Assists nucleotide exchange; Interacts directly with cognate GTPase	[168]
Sos1	Rac1	Inhibitory; blocks active site on DH domain	[60]
Tiam1	Rac1, 2, 3	Not involved in exchange	[158]
Intersectin	Cdc42	Not involved in exchange	[167]
Collybistin	Cdc42	Not involved in exchange	[170]

Table 2: DH-associated PH domains that regulate exchange *in vitro* either through intramolecular interactions or through direct interaction with cognate GTPases.

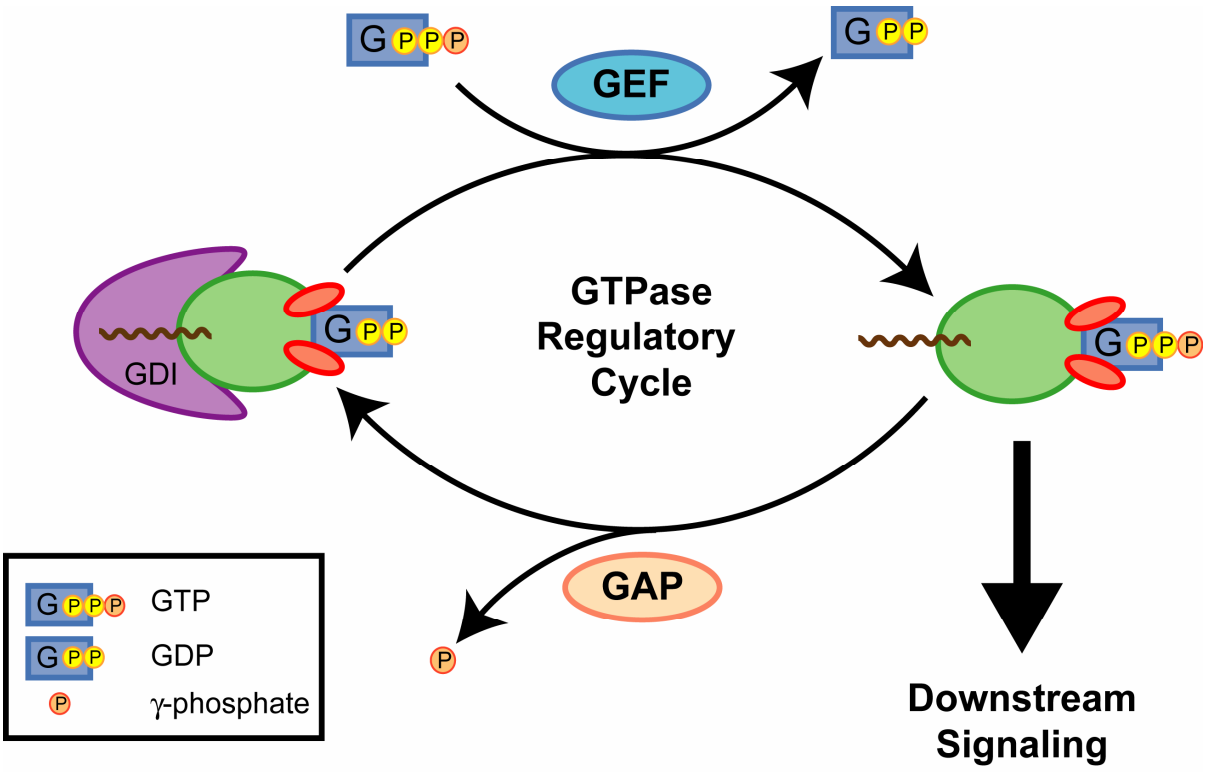

Figure 1: GTPase regulatory cycle.

Both large and small GTPases (green) act as molecular switches that cycle between inactive states (left) bound to GDP (blue square with phosphates in yellow) and actives states (right) bound to GTP. GTPase accelerating proteins (GAPs) enhance the intrinsic GTPase activity of GTPases to inactive them, while guanine nucleotide exchange factors (GEFs) activate GTPases by exchanging bound GDP for GTP. Rho-family GTPases and Rab GTPases are also regulated by guanine nucleotide dissociation inhibitors (GDIs) which sequester inactive GTPases in the cytosol by providing a hydrophobic binding pocket for the C-terminal prenyl moiety (brown wavy line) that is required for membrane localization [1]. Switch regions that change conformation based on the state of bound nucleotide are depicted in red. For sake of simplicity, intermediate steps in the regulatory cycle have been omitted.

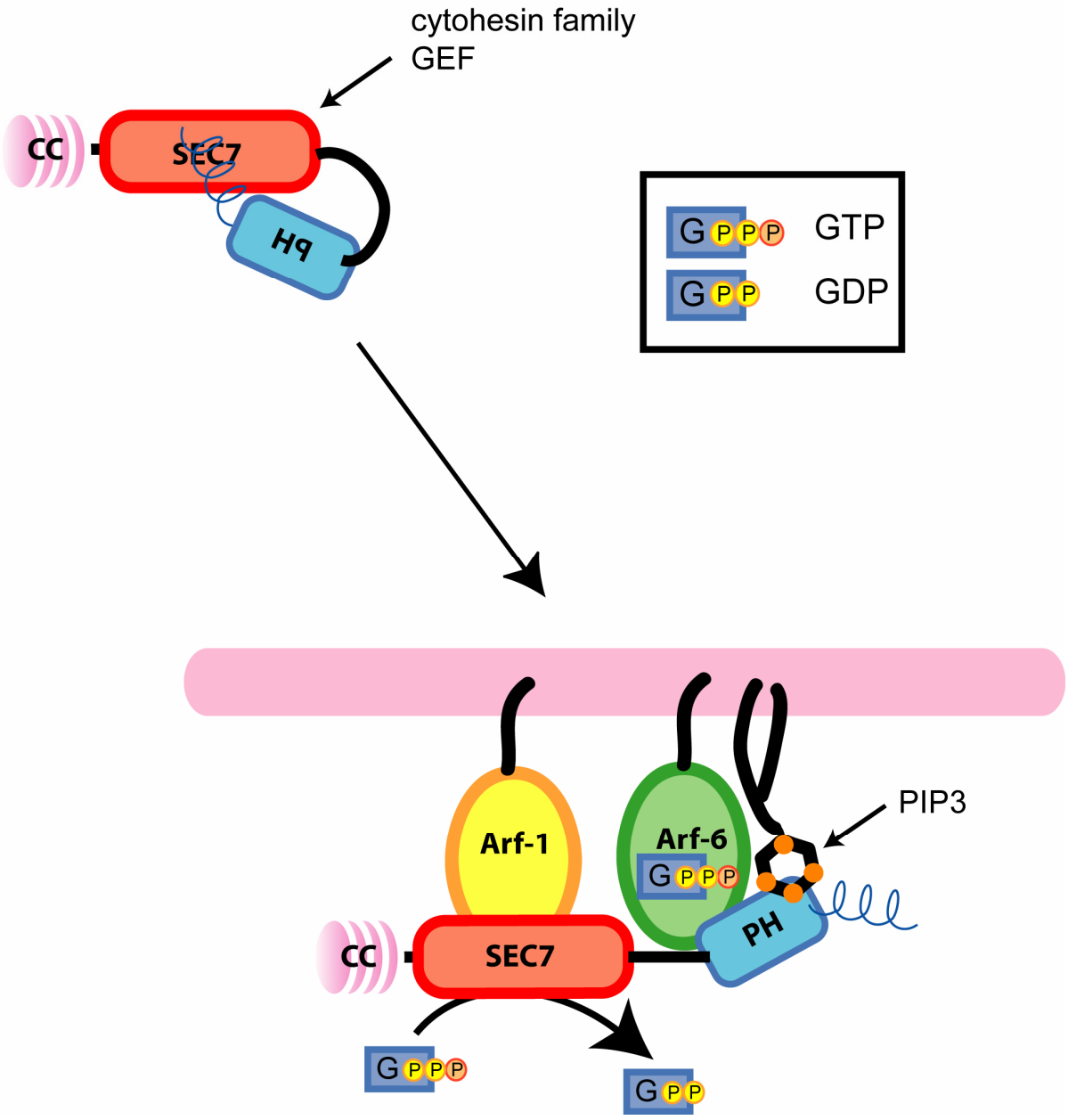

Figure 2: Active Arf-6 recruits GEFs for Arf-1 to the plasma membrane.

The cytohesin GEFs, ARNO and Grp-1, are basally auto-inhibited (upper left) and recruited to plasma membranes (pink bar) through interaction with active Arf-6 and PIP3. Recruitment releases auto-inhibition to allow ARNO and Grp-1 to activate Arf-1. The domain architectures of ARNO and Grp-1 are identical and consist of an N-terminal coiled-coil (CC); an exchange-competent SEC7 domain associated with a pleckstrin homology (PH) domain; and a C-terminal helical region necessary for auto-inhibition [46, 48].

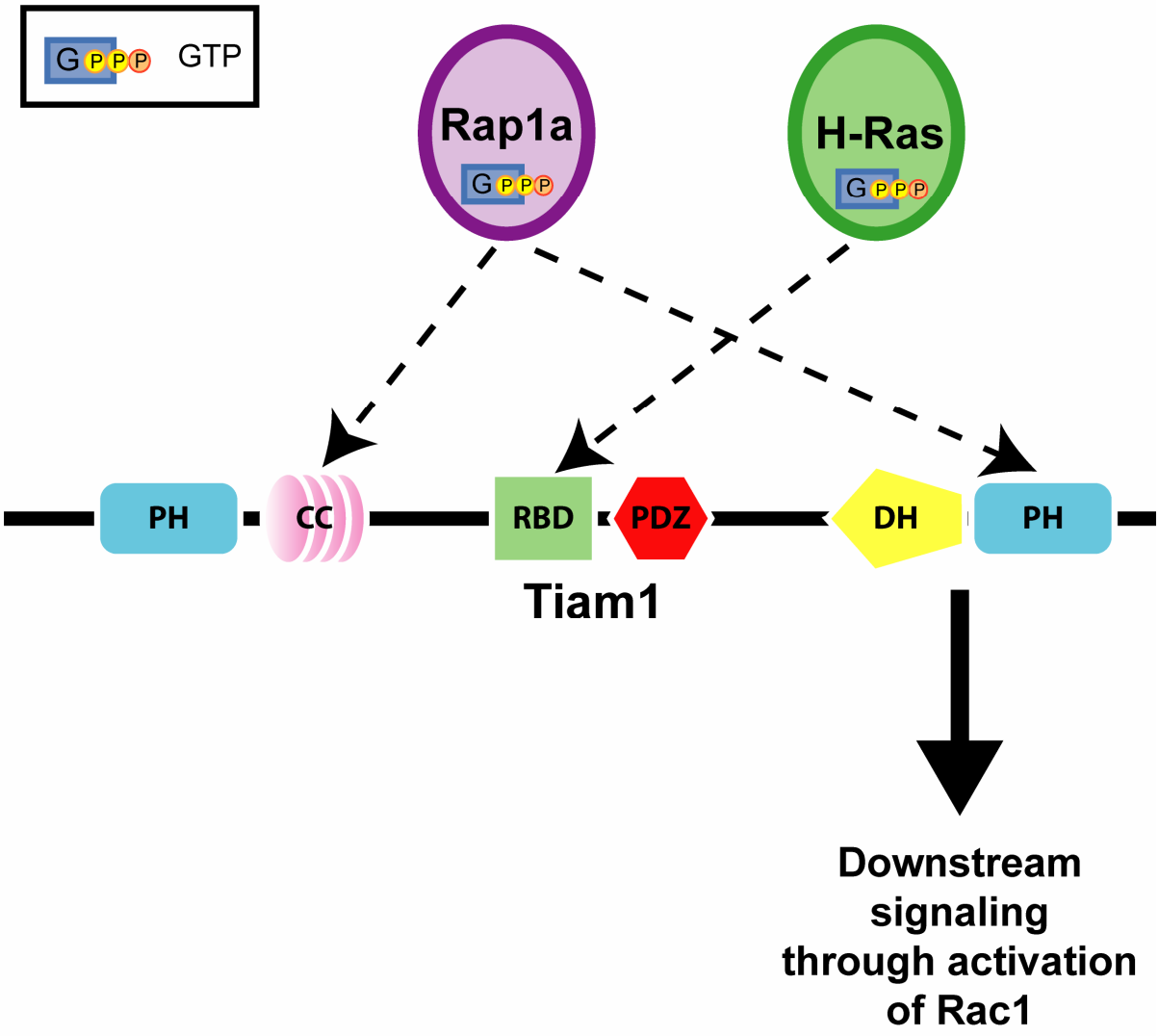

Figure 3: Tiam1 functions downstream of both H-Ras and Rap1a.

Both active Ras and Rap1a activate Tiam1. Co-immunoprecipitation experiments indicate that H-Ras interacts with Tiam1 through its canonical effector binding region, the RBD domain [73]. Active Rap1a has been shown to bind either the DH-associated PH domain or the coiled-coiled (CC) region [89, 91].

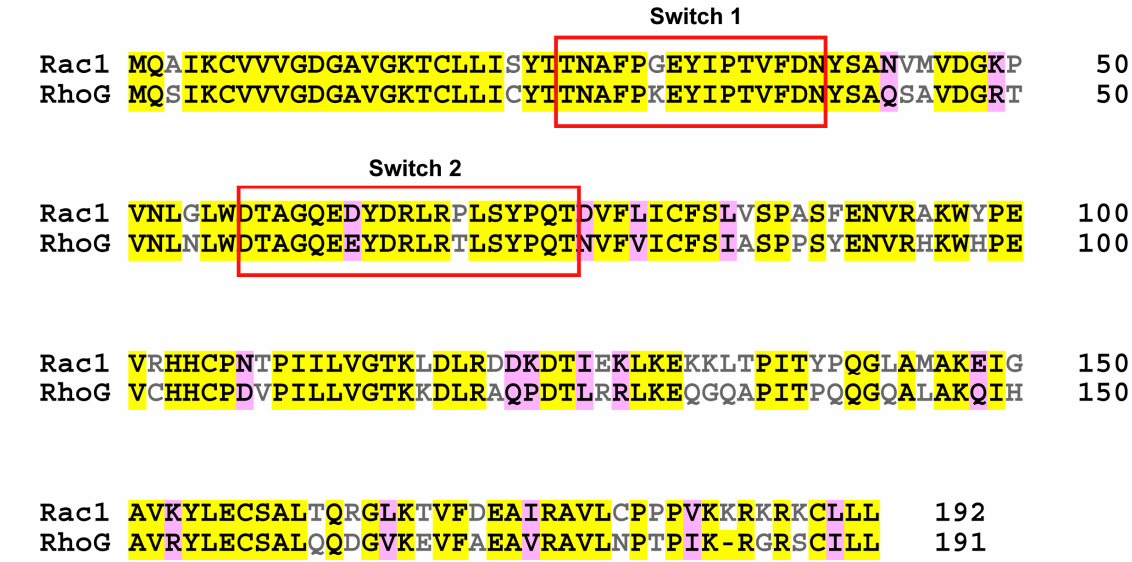

Figure 4: Sequence alignment of Rac1 and RhoG.

Identical residues between Rac1 and RhoG are highlighted in yellow, while similar residues are highlighted in pink. The switch regions are indicated by red boxes.

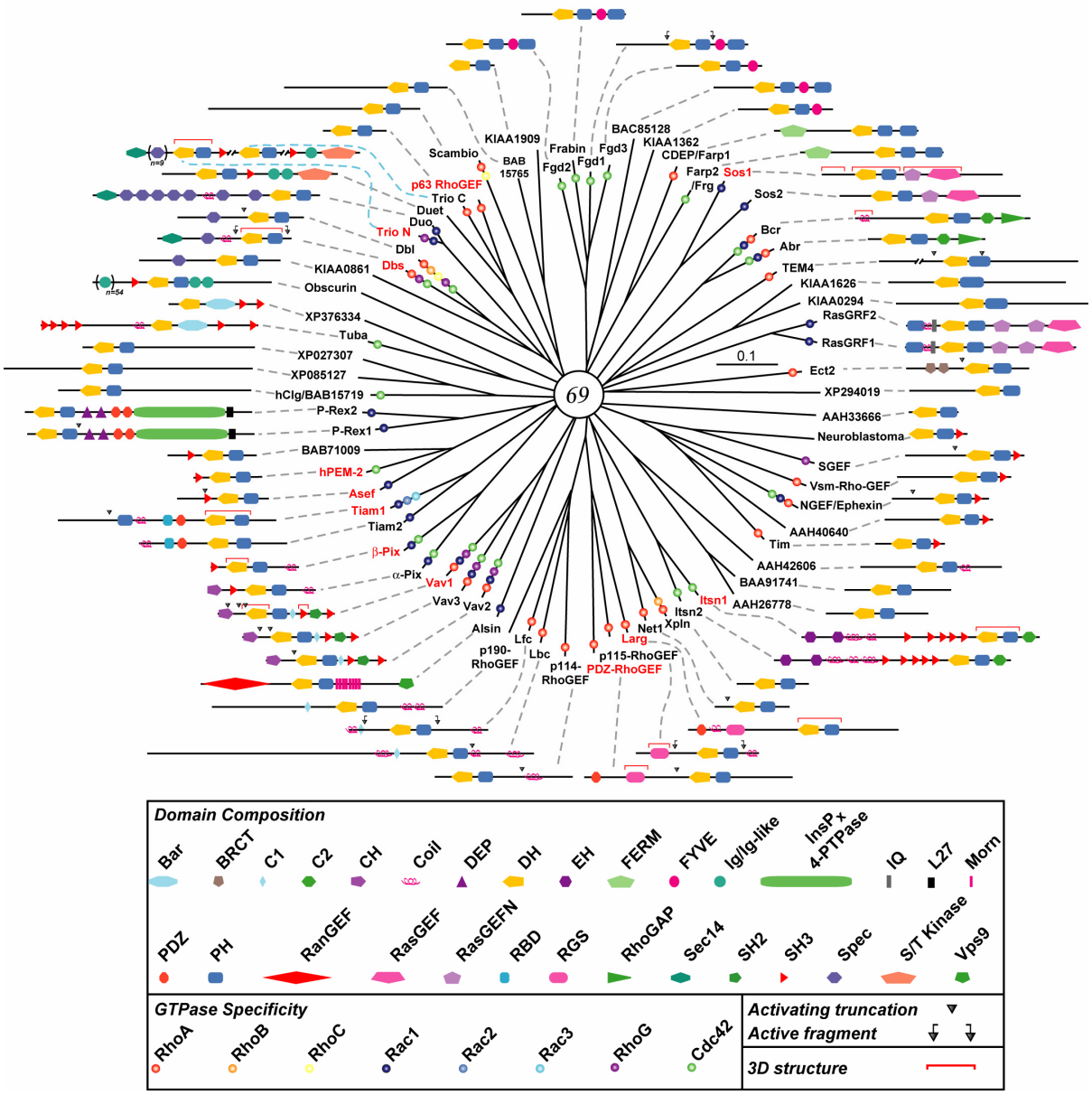

Figure 5: Human Dbl-family GEF tree.

This dendogram shows all of the 69 Dbl-family GEFs that have been identified in the human genome to date. Proteins are grouped according to sequence homology. Dbl-family GEFs that have had all or portions of their DH/PH cassette structurally characterized are high-lighted in red. (*Adapted from Rossman et al.* [8])

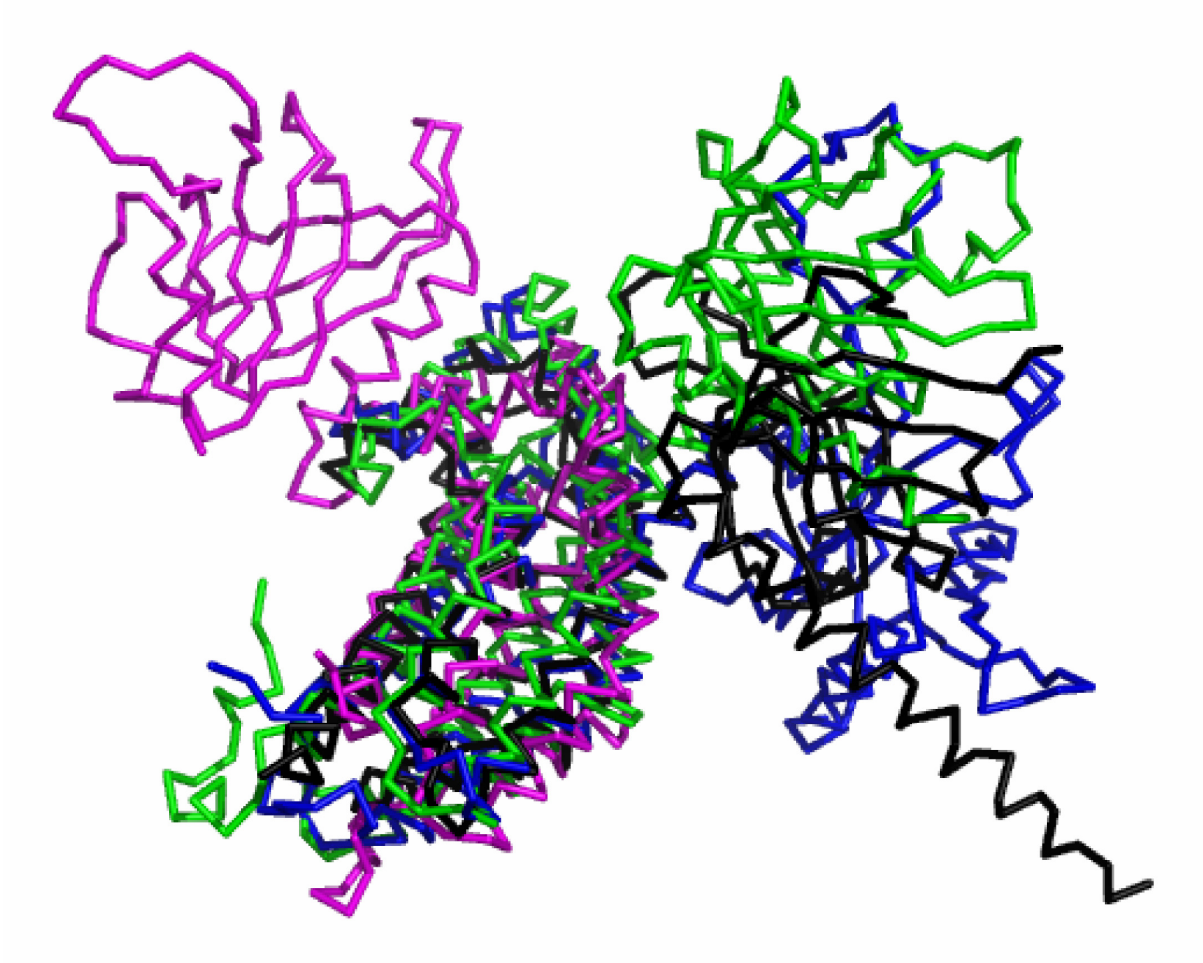

Figure 6: The DH/PH cassette has a high degree of conformational flexibility.

The DH/PH cassettes of Dbs (black), LARG (green), Tiam1 (blue) and Sos1 (magenta) were superimposed using the DH domains. Except for Sos1, all fragments also contain bound GTPase (not shown for clarity). PDB entry codes are: Dbs, 1KZ7; LARG, 1X86; Tiam1, 1FOE; and Sos1, 1DBH.

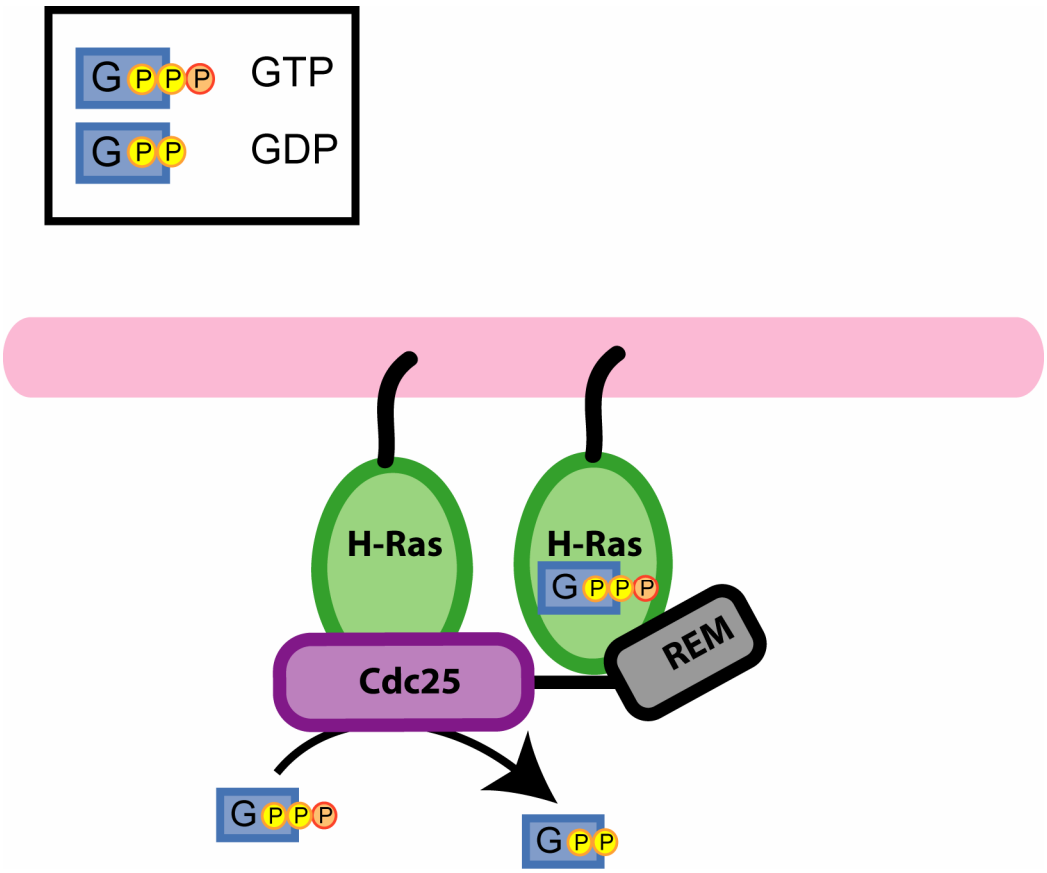

Figure 7: Active Ras interacts with the REM domain of Sos1 to increase exchange on H-Ras-GDP.
In a classic positive feedback loop, H-Ras-GTP binds to the REM domain of a fragment of Sos1 to enhance the capacity of the Cdc25 domain of Sos1 to activate H-Ras. The two sites of H-Ras engagement are distant from each other and linked by conformational changes [101, 231]. The fragment of Sos1 depicted comprised only the Cdc25 and REM domains and is sufficient for exchange on H-Ras.

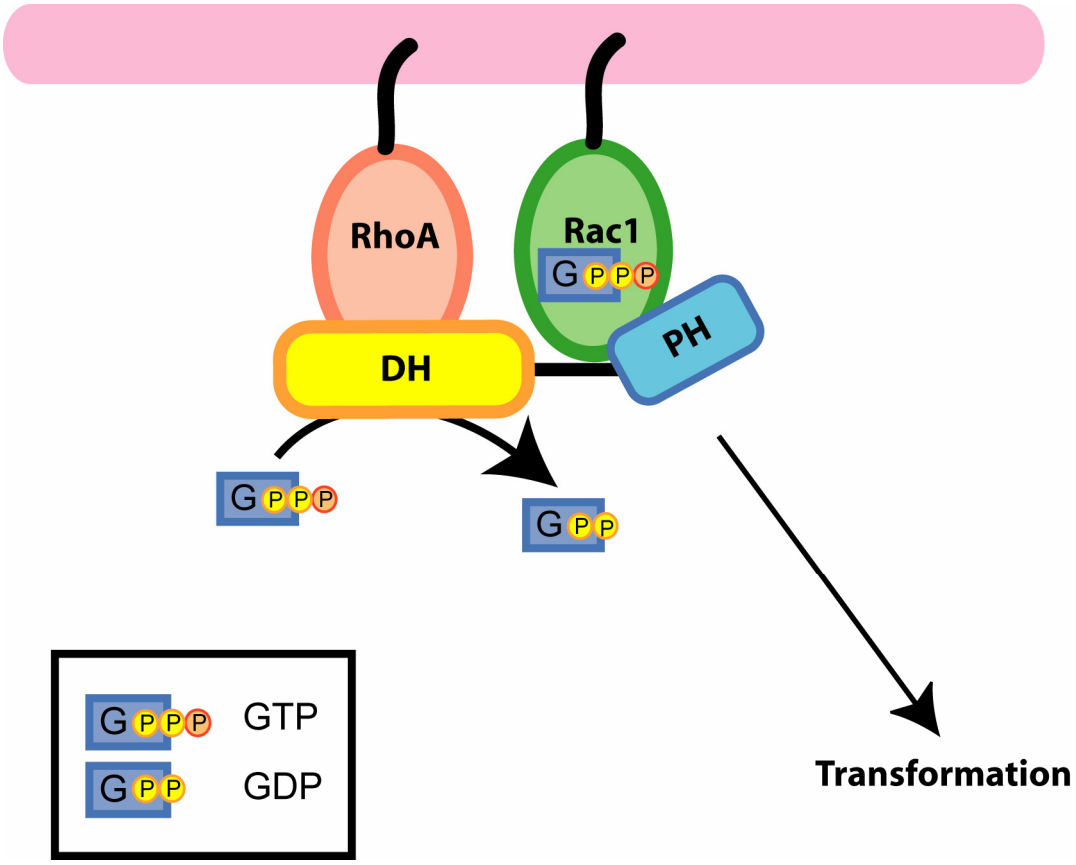

Figure 8: Activated Rac1 interacts with Dbl-family GEFs Dbs and Scambio to augment activation of RhoA.

Rac1-GTP interacts with Dbs and Scambio most likely through their DH-associated PH domain. The DH/PH cassette represents a fragment of either Dbs or Scambio. The interaction between active Rac1 and either GEF augments its ability to activate RhoA, but the mechanism is unclear. This figure was adapted from Cheng *et al.* [206, 221].

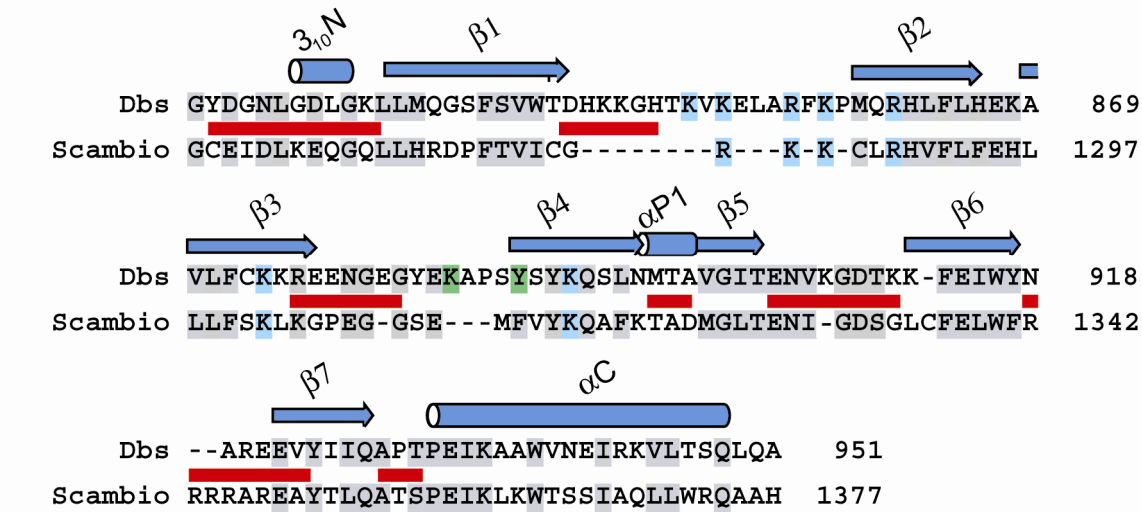

Figure 9: Sequence alignment of DH-associated PH domains of Dbs and Scambio.

The DH-associated PH domains of Dbs (residues 820 – 951) and Scambio (residues1261 – 1377) were aligned using Clustal X. Conserved or identical residues are highlighted in grey. The secondary structure elements of Dbs as defined by various structures of Dbs are highlighted as blue rods (helices) and arrows (strands) [166, 206]. In addition, residues that bind phosphoinositides are shaded cyan (putative for Scambio) [160] and residues that participate directly in exchange on RhoA and Cdc42 are shaded in green [166]. Mutation of these residues has no effect on the ability of activated Rac1 to interact with Dbs. By excluding the residues that bind phosphoinositides, RhoA, and Cdc42; and excluding the regions that are not solvent accessible when Dbs is in complex with nucleotide-free RhoA and Cdc42, I have determined the regions most likely to interact with activated Rac1 (highlighted by a thick red line in between the sequences of the Dbl-family GEFs) .

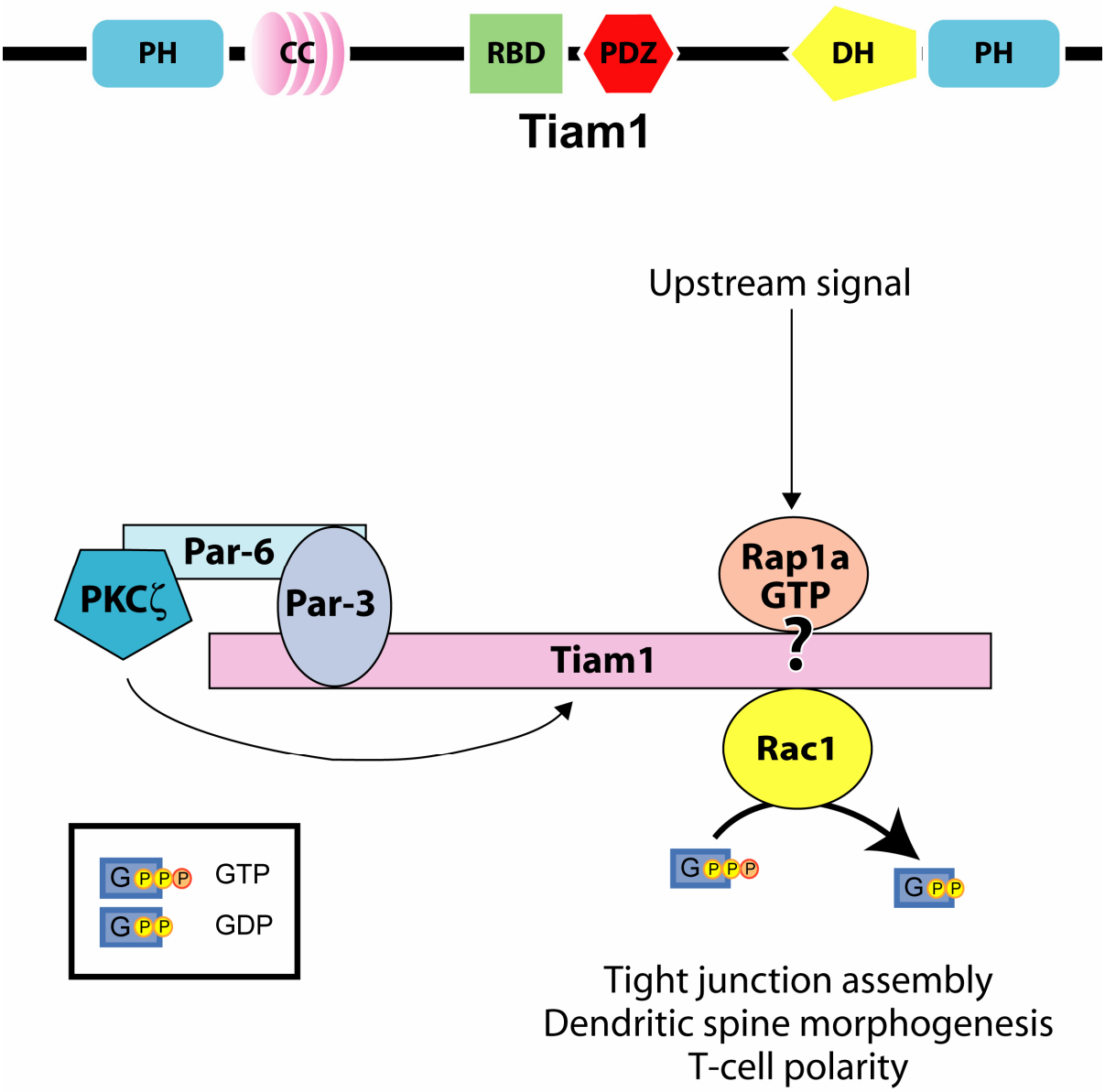

Figure 10: Active Rap1a activates Tiam1 as part of the signaling cascade involving the Par-3 polarity complex.

The domain architecture of full-length Tiam1 is shown that the top of the figure. Par-3 interacts with Tiam1 through the N-terminal PH domain and tandem coiled-coiled region (CC) of Tiam1 [232]. Tiam1 also interacts with active Rap1a and -1b, but the interaction site on Tiam1 is not definitively determined. Previous studies suggest that Rap1a augments the activity of Tiam1 *in vivo* by ensuring proper sub-cellular localization [89].

CHAPTER 2: THE DH AND PH DOMAINS OF TRIO COORDINATELY ENGAGE RHO-FAMILY GTPASES FOR THEIR EFFICIENT ACTIVATION

Introduction

Rho-family GTPases act as molecular switches that cycle between inactive GDP-bound states and active GTP-bound states [120, 156, 162]. Once activated, Rho-family GTPases bind to their effectors to elicit a variety of downstream signaling responses, including: cytoskeletal reorganization, gene expression, cell cycle progression, membrane trafficking, cell adhesion, and cell migration [120, 233]. Given their involvement in many critical cellular processes, it is not surprising that aberrant regulation of Rho-family GTPases contributes to various diseases such as cancer [123], hypertension [143], and mental retardation [234]. It is generally appreciated that delineating the mechanisms involved in the activation of Rho-family GTPases might guide treatment regimens for a variety of human diseases.

The activity of Rho-family GTPases is regulated mainly by three classes of proteins: 1) GTPase activating proteins (GAPs) accelerate the hydrolysis of bound GTP; 2) guanine nucleotide dissociation inhibitors (GDIs) sequester prenyl groups added post-translationally to Rho-family GTPases and thus stabilize cytosolic, inactive forms of the GTPases; and 3) Dbl-family guanine nucleotide exchange factors (GEFs) catalyze the exchange of bound GDP for GTP, thereby activating the GTPases [1, 8, 122, 156].

Not including splice variants, the human genome encodes 69 Dbl-family GEFs [8] of varying domain architecture, size, and GTPase specificity [8, 156]. All Dbl-family members contain a conserved Dbl-homology (DH) domain, followed almost invariably by a tandem pleckstrin-homology (PH) domain [8, 162]. The DH domain forms the majority of the interface with the GTPase and is often sufficient to catalyze nucleotide exchange [8, 157, 158] and dictate GTPase specificity [8, 157, 167]. The PH domain is necessary for regulating exchange *in vivo* [58, 154, 159, 160, 235], and in many cases, *in vitro* [161, 167, 168, 205, 236], but its exact functions remain unclear.

DH-associated PH domains, like most PH domains, are traditionally characterized as phosphoinositide binding modules; but the ability of phosphoinositides to allosterically regulate the exchange activities of Dbl-family GEFs is, at best, controversial [159, 160, 176, 194, 225]. In addition, in several instances, DH-associated PH domains are not necessary for recruiting GEFs to cellular membranes where they normally operate on membrane-resident GTPases. This is true even though PH domain mutations abrogating phosphoinositide binding diminish the ability of Dbl-family GEFs to activate their cognate GTPases *in vivo* [159, 160, 191, 237]. Another possibility that finds growing support is that DH-associated PH domains facilitate exchange through direct interactions with cognate GTPases [166-168, 205, 237]. For example, structures of Dbs DH/PH in complex with nucleotide-depleted Cdc42 or RhoA (henceforth, Dbs•Cdc42 or Dbs•RhoA, respectively) show direct contacts between the β3/β4 loop of the PH domain and its cognate GTPases [166, 167]. Mutational analysis has confirmed the catalytic importance of this interface both *in vitro* and *in vivo* and has identified His814, Gln834, and Tyr889 of Dbs as functionally significant for guanine nucleotide exchange [160, 166]. Given the highly conserved position of PH domains directly

adjacent to DH domains, it is likely that other Dbl-family GEFs also use their DH-associated PH domains to directly engage their cognate GTPases to facilitate guanine nucleotide exchange. In particular, published data on the Dbl-family GEF Trio [161, 200] and the *C. elegans* ortholog of Trio, UNC-73 [237], provide strong circumstantial support for this hypothesis.

Trio is a large, evolutionarily conserved Dbl-family GEF that is best characterized for its role in regulating neurite outgrowth [238-245]. Unlike most Dbl-family GEFs, Trio has two DH/PH cassettes (**see Figure 11(a)**); the first DH/PH cassette catalyzes exchange on Rac1 and RhoG [127, 246-248], while the second DH/PH cassette is specific for RhoA [246, 247]. Physiological functions of the C-terminal DH/PH cassette remain relatively unclear, but the N-terminal DH/PH cassette is critical and often sufficient for regulating neuronal development through activation of RhoG, and possibly Rac1 [239, 243, 244]. Interestingly, although there is some discrepancy over the relative rates of exchange, the isolated N-terminal DH domain of Trio does not exchange as effectively as the corresponding DH/PH cassette *in vitro* [161, 200]. Furthermore, deletion of the PH domain from the N-terminal DH/PH cassette of full-length Trio reduces its ability to induce neurite outgrowth in PC-12 cells through activation of RhoG [200]. Finally, residues in the PH domain of Dbs that are functionally significant for exchange on its cognate GTPases are conserved in Trio (**see Figure 11(b)**). Together, these data strongly suggest that the N-terminal DH/PH cassette of Trio might use its PH domain similarly to Dbs when catalyzing exchange on its cognate GTPases Rac1 and RhoG.

Thus, to better understand the possible functional interplay between the N-terminal DH and PH domains of Trio specifically, and between DH and PH domains in general, we

determined the crystal structure of the N-terminal DH/PH cassette of Trio in complex with nucleotide-depleted Rac1 (henceforth, Trio•Rac1). The complex recapitulates many of the interactions involving the PH domain previously seen in the Dbs / GTPase structures that are required for efficient guanine nucleotide exchange by Dbs. Mutation of this interface in Trio confirms the necessary involvement of the N-terminal PH domain of Trio in directly engaging Rac1 and RhoG for their efficient activation *in vitro* and *in vivo*. These studies further support the general capacity of DH-associated PH domains to be active participants in the exchange process and suggest a coherent model of guanine nucleotide exchange catalyzed by Dbl-family proteins that requires the direct and cooperative engagement of DH domains, as well as their associated PH domains, by Rho-family GTPases for their efficient activation.

Experimental procedures

Sequence alignment

The sequences of Dbs (**NP_079255**), Trio (**NP_009049**), Duo (**NP_003938**), Dbl (**NP_005360**), Duet (**NP_008995**), PRex1 (**NP_065871**), p63RhoGEF (**NP_891992**), Lfc (**NP_004714**), neuroblastoma (**NP_005263**), and obscurin (**NP_443075**) shown in **Figure 1(b)** were aligned using Clustal X [249]. The NCBI accession numbers are given in parentheses.

Protein preparation for guanine nucleotide exchange assays

Human Trio DH/PH (residues 1226 – 1535) (kindly provided by Dr. Yi Zheng, Cincinnati Children's Hospital Medical Center), was encoded as a fusion with an N-terminal

His_6-tag in pET15a (Novagen), expressed in the BL21 (DE3) *E. coli* strain, and purified similarly to published protocols [166, 250]. Briefly, transformed cells were grown in LB media containing 0.1 mg/ml ampicillin at 37°C to an OD_{600} of 0.7 (mid-log phase) and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 27°C for 5 hours. Harvested cells were resuspended in 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl (buffer A), and 5mM imidazole and lysed with an Emulsi-Flex C5 (Avestin). Lysate was clarified by centrifugation at > 125,000 x *g* for 30 minutes prior to loading the supernatant onto a Ni^{2+}-Sepharose affinity column (GE Healthcare) equilibrated in buffer A containing 5 mM imidazole. The column was washed with buffer A containing 55 mM imidazole and Trio eluted with buffer A containing 400 mM imidazole. The protein was further purified using a 26/60 Sephacryl-200 size exclusion column (GE Healthcare) equilibrated with 50 mM Tris, pH 8.0, 2 mM DTT, 2mM EDTA, 150 mM NaCl, and 5% glycerol. Fractions containing Trio were pooled, concentrated, and stored at -80°C.

Human Rac1 (residues 1-189, C189S) was expressed from pET21a (Novagen) in the BL21 (DE3) *E. coli* strain. Transformed cells were grown and induced similarly to cells expressing Trio. Harvested cells were resuspended in 10 mM MES, pH 6.0, 2 mM DTT, 10% glycerol, 1 mM $MgCl_2$ (buffer B) and 10 mM NaCl prior to lysis and clarification as described above. Supernatant was loaded onto an SP-Sepharose Fast Flow 26/10 column (GE Healthcare). Rac1 was eluted from the column using buffer B with an increasing gradient of NaCl from 10 mM to 600 mM and further purified using a 26/60 Sephacryl-200 size exclusion column (GE Healthcare) equilibrated with 50 mM Tris, pH 8.0, 2 mM DTT, 2mM $MgCl_2$, 150 mM NaCl, and 5% glycerol. Fractions containing Rac1 were pooled, concentrated, and stored at -80°C.

Human RhoG (residues 1-188, C188S) was expressed from the pGEX4TEV2 vector [251] with an N-terminal GST-tag in the BL21 (DE3) *E. coli* strain. Transformed cells were grown in LB media containing 0.1 mg/ml ampicillin at 37°C to an OD_{600} of 0.7 (mid-log phase) and induced with 1 mM IPTG at 20ºC for 16 hours to 18 hours. Cells were resuspended in buffer C (150 mM NaCl, 20 mM Tris, pH 8.0, 2 mM DTT, 1 mM $MgCl_2$, 10 μM GDP, and 5% glycerol) prior to lysis and clarification as described above. Supernatant was loaded onto a GST-Sepharose affinity column (GE Healthcare) equilibrated in buffer C; the column was washed with buffer C, and RhoG eluted with buffer C containing 10 mM glutathione (reduced). The GST tag was removed by cutting with TEV while dialyzing against buffer C overnight. The protein was further dialyzed against buffer D (10 mM MES, pH 6.0, 2 mM DTT, 2 mM $MgCl_2$, 10 μM GDP, and 5% glycerol) and applied to a Source-S 16/10 column (GE Healthcare) equilibrated in buffer D. RhoG was eluted with buffer D containing an increasing concentration of NaCl from 5 mM to 180 mM. The fractions containing RhoG were then loaded onto a GST-Sepharose affinity column equilibrated in buffer C to remove residual amounts of GST-tagged protein. The flow-through from the GST column was buffer exchanged into buffer C, concentrated, and stored at -80ºC.

Mutations in Trio DH/PH and Rac1 were made using the Quikchange site directed mutagenesis kit (Stratagene) following manufacturer's instructions. Sequences were verified using automated sequencing. Mutants of Trio and Rac1 were purified as described above for wild-type Trio and Rac1, respectively.

Guanine nucleotide exchange assays

Nucleotide exchange was measured using a fluorescence based assay, similar to published protocols [166, 252], in which *N*-methylanthraniloyl (mant)-GTP was loaded onto the GTPase. Spectroscopic analysis was carried out using a Perkin-Elmer LS 55 spectrometer at 20°C. The exchange assay mixture containing 20 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μM mant-GTP, and 2 μM GTPase was allowed to equilibrate with constant stirring. Trio was then added at 50 or 400 nM for exchange assays with RhoG or Rac1, respectively, and nucleotide exchange was measured by monitoring the decrease in intrinsic tryptophan fluorescence (λ_{ex}=295 nm, λ_{em}=335 nm) of the GTPase due to the binding of mant-GTP. The data were fit to one phase exponential decay curves using the program GraphPad Prism™ in order to determine the rate of nucleotide exchange.

Protein preparation for formation of Trio/Rac1 complex

Human Trio DH/PH (residues 1226 – 1536) was cloned in a pPROEX-HTa vector (Invitrogen) using NcoI and XhoI cleavage sites, expressed as a fusion protein with an N-terminal His_6-tag in BL21 (DE3) *E. coli* strain, and eluted from a Ni^{2+}-Sepharose affinity column (GE Healthcare) as described above. The His_6-tag was removed by cleavage with TEV while dialyzing (16 hrs) against a buffer containing 50 mM Tris, pH 8.0, 2 mM DTT, 2mM EDTA, 150 mM NaCl, and 5% glycerol. The removal of the His_6-tag was confirmed using SDS-PAGE. The protein was then stored at 4ºC until it was used to form a complex with Rac1.

Rac177 was expressed from the pET15b vector (Novagen) in BL21 (DE3)s *E .coli* strain and protein was purified according to published protocols [158]. Minor variations

include that the pellet precipitated after saturation with 45% ammonium sulfate was resuspended in 20 mM Tris, pH 8.0, 2 mM DTT, 1 mM $MgCl_2$, and 10 μM GDP and dialyzed extensively against buffer E (20 mM Tris pH 8.0, 10 mM NaCl, 2 mM DTT, 1 mM $MgCl_2$, and 10 μM GDP) to remove the ammonium sulfate and increase the pH. The resuspended pellet was loaded onto a Q-Sepharose Fast Flow 26/10 column (GE Healthcare) instead of a size-exclusion column. Rac177 was eluted from the column using buffer E with increasing amounts NaCl from 10 mM to 600 mM. The fractions containing Rac177 were pooled together and stored at 4ºC before being used to form a complex with Trio.

Crystallization of the Trio/Rac177 complex

The Trio/Rac177 complex was formed in the presence of an excess of nucleotide-free Rac177 in 20 mM Tris, pH 8.0, 2 mM DTT, 4mM EDTA, 200 mM NaCl, and 5% glycerol. The complex was purified on a 26/60 Sephacryl-200 size exclusion column equilibrated with 20 mM Tris, pH 8.0, 2 mM DTT, 4 mM EDTA, 200 mM NaCl, and 5% glycerol. Fractions containing purified complex were pooled together, dialyzed into a buffer containing 50 mM NaCl, 10 mM Tris, 2 mM EDTA, and 2 mM DTT, concentrated to ~21 mg/ml, and stored at -80ºC.

Trio/Rac177 crystals were obtained by vapor diffusion at 18 °C. Drops were formed by combining equal volumes of protein complex and reservoir solution (100 mM sodium cacodylate pH 5.5 – 6.5, 14 – 18% (w/v) PEG 8000 (FLUKA), and 300-500 mM calcium acetate). Crystals typically appeared after 2 – 3 days and grew to final dimensions of 0.1 x 0.1 x 0.05 mm after several days. Crystals were cryoprotected by increasing the glycerol concentration of the drop to 21% (v/v) in 3% increments. Cryoprotected crystals were then

suspended in a rayon loop (Hampton Research) and snap frozen in liquid nitrogen. The crystals belong to the space group $P2_12_12$ with unit cell parameters a = 97.492 Å, b = 108.558 Å, and c = 53.416 Å.

Data collection and structure determination

A native data set was collected using a single frozen crystal at the SER-CAT beamline (ID-22, Advanced Photon Source). Data were integrated and scaled using DENZO and SCALEPACK [253]. Phases were calculated by molecular replacement using the Trio DH/PH [169] and Rac1 [158] structures. The calculations were performed using both AMORE[254] and PHASER[255] from the CCP4 suite of programs [256], which yielded identical solutions.

Model building and structure refinement

Electron density maps were calculated using CNS [257]. The interactive graphics program O was used for the majority of the model building [258], but the molecular graphics program COOT [259], was also used to add water molecules. Most of the subsequent refinement was performed using CNS [257]; however, due the conformational mobility of the PH domain, CNS refinement was unable to produce clear density for this region of the molecule. Thus, TLS refinement from the CCP4 program Refmac5 [260, 261] was used to improve the quality of the electron density maps. Using Rac1, the DH domain, the PH domain, and the waters as TLS "groups" and employing tight geometrical restraints (matrix diagonal weighing term = 0.06), a final model with R_{work} = 22.3% and R_{free} = 24.9% was produced. In addition, the torsion angles for every non-glycine residue are within the most

favored or allowed regions of the Ramachandran diagram, as determined by PROCHECK [262]. Please refer to Table 1 for refinement statistics.

The simulated annealing omit map used to validate the interactions seen at the interface between Rac1 and the PH domain of Trio was generated from the final coordinates after omitting residues involved in this interface (residues 64 – 68, and 102 – 104 from Rac1 and residues 1405 – 1411, 1429 – 1431, and 1469 – 1473 from Trio).

All images of protein structures, with the exception of **Figure 13**, were generated using Pymol [263]. The images in **Figure 13** displaying the theoretical anisotropic motion of the atoms [264] were generated using CCP4MG [265, 266]. Pymol [263] was also used to calculate buried surface areas.

Protein Data Bank accession codes

The atomic coordinates and structure factors (code **2NZ8**) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ.

Cell culture and transfection

To introduce point mutations (H1410A, Q1430A, and Y1472F) into EGFP-Trio FL wild-type (human) (generously donated by Dr. Anne Debant, Centre de Recherche en Biochimie Macromoléculaire, Montpellier, France), a section of Trio containing the N-terminal DH/PH cassette (Trio DH/PH-long) was sub-cloned into the pMCSG7 vector [267]. Mutations were introduced using the Quikchange site directed mutagenesis kit (Stratagene) following manufacturer's instructions and verified by automated sequencing. The DH/PH

cassette of Trio was then digested from Trio DH/PH-long and sub-cloned into EGFP-Trio FL.

PC-12 cells were cultured in DMEM containing 10% horse serum (HS), 5% fetal bovine serum (FBS), and 1x penicillin/streptomycin (100 units/ml penicillin; 0.1 mg/ml streptomycin) at 37˚C in the presence of 5% CO_2. For transfection, cells were seeded at a density of 500,000 cells per well on glass coverslips coated with rat-tail collagen type I (BD Biosciences) in 6-well dishes. Cells were cultured for 18 hrs and then transfected with 1 μg of EGFP-C2 vector (Clonetech), EGFP-RhoG wild-type (human) (generously donated by Dr. Keith Burridge, UNC-Chapel Hill), or EGFP-Trio FL (wild-type and mutant forms) per well using Lipofectamine plus (Invitrogen) according to the manufacturer's instructions. Cells were fixed 48 to 72 hours post transfection for 10 minutes with 3.7% (v/v) paraformaldehyde in PBS, permeabilized with 0.3% Triton X-100, stained for actin with Alexa Fluor 546 phalloidin (Invitrogen), washed, and mounted using Fluorsave Reagent (Calbiochem) according the manufacturer's instructions.

Image collection and data processing for neurite outgrowth assays

Fixed cells were observed using an Olympus Fluoview 300 laser scanning confocal microscope with a 60X PL APO oil immersion objective when scoring for neurite positive cells. Neurite outgrowth activity was determined by positively scoring transfected cells displaying one or more neurites greater than one cell body in length. Cells found in large clumps (> 10 cells) were excluded from analysis. Displayed results represent the average of 3 independent experiments in which at least 100 cells per condition were counted for each

experiment. In addition, the studies were blinded to conceal which slides contained cells that had been transfected with wild-type and mutant versions of Trio.

Images were obtained using an Olympus Fluoview 1000 laser scanning confocal microscope with 60x PL APO oil immersion objective in 20 – 30 micron XYZ stacks. Final images were processed in ImageJ [268] and represent a standard deviation projection of the compiled stacks.

Acknowledgments

We would like to acknowledge Dr. Ashutosh Tripathy at the UNC-Chapel Hill Macromolecular Interactions Facility for his assistance with circular dichroism spectroscopy. We would also like to acknowledge Dr. Alex Singer for his technical assistance with model building and refinement. This work was supported by National Institutes of Health Grant GM62299 and GM65533.

Results

Structure of Trio in complex with nucleotide-depleted Rac1

A complex between the N-terminal DH/PH cassette of Trio (residues 1226 – 1536) and residues 1-189 (C189S) of Rac1 yielded poorly diffracting crystals. Since several structures of Rho-family GTPases indicate that the C-terminal polybasic tail is not typically well-structured, nor directly involved in binding GEFs [166-168, 170], a truncated version of Rac1 lacking this region and ending at residue 177 (Rac177) was crystallized with the N-terminal DH/PH cassette of Trio and used for structure determination of the Trio•Rac1

complex. Previous work from this lab has shown that full-length non-prenylated Rac1 and Rac177

exhibited identical capacities to bind guanine nucleotides and to be activated by the DH/PH cassette of Tiam1 *in vitro* [158]. Phases were determined for a native data set collected at the SER-CAT beamline (ID-22, Advanced Photon Source) using Trio DH/PH [169] and Rac1 [158] as models for molecular replacement. The final structure has an R_{work} = 22.3% and an R_{free} = 24.9% and incorporates data from 19.4Å– 2.0 Å (see Table 1 for data collection and refinement statistics).

The overall domain architecture of the Trio•Rac1 structure is similar to that seen in other complexes of Dbl-family GEFs and their GTPases; the DH domain of Trio consists of a bundle of six α-helices while the PH domain consists of a core seven strand β-sandwich with three inter-strand loops and a capping C-terminal helix [8, 166, 167, 170, 171, 206, 269] (**see Figures 12(a) and 14**). The majority of the interface between Rac1 and Trio, which buries ~2500 $Å^2$ of surface area, is mediated by the DH domain. However, the complex also shows significant interactions between Rac1 and the PH domain of Trio that occur predominantly through residues in the β3/β4 loop and His1410 (**see Figures 12(a) and (b)**), similar to the interactions seen between Dbs and its cognate GTPases [166, 167].

There is excellent electron density for Rac1, the DH domain, and the parts of the PH domain that contact Rac1 (**see Figure 12(c) and Table 3**). In contrast, electron density is poor for the remainder of the PH domain, especially the β1/β2 and β6/β7 loops, which have not been modeled. The unusually high average B-factor for the PH domain (**see Table 3**) suggests that it is inherently mobile. To illustrate the predicted motion of the PH domain, we have displayed the thermal ellipses for each atom of the Trio•Rac1 structure (**see Figure 13**).

These ellipses represent the anisotropic B-values obtained after TLS refinement [260, 261] and indicate that a portion of the PH domain is highly mobile. This observation is consistent with several reports that indicate many DH-associated PH domains have significant conformational mobility and are often difficult to model when crystallized [166, 170, 206]. One notable exception to this generalization is the structure of Trio crystallized without bound GTPase [169]. However, in this case, the PH domain is locked into place by several crystal contacts.

Interestingly, the anisotropic B-values and the corresponding thermal ellipses associated with residues of the PH domain that mediate the interface with Rac1, including those in the β3/β4 loop, are comparable to those for atoms in the DH domain and Rac1 (**see Figure 13 and data not shown**). While the majority of the PH domain has few stabilizing interactions, the interaction of the β3/β4 loop with Rac1 appears to restrict its motion. Furthermore, a simulated annealing omit map generated from the final model after omitting residues in the interface between the PH domain and Rac1 and the final $2F_o$-F_c map both show clear density for residues mediating this interface (**see Figure 12(c)**). Thus, the interactions between the DH-associated PH domain of Trio and Rac1 illustrated in **Figure 12(b)** are not modeling artifacts

Comparison of Trio•Rac1 to Dbs•Cdc42

Superimposition of the structures of Trio•Rac1 with the corresponding DH/PH portion of Trio in isolation indicates that an ~10° rotation of the PH domain with respect to the DH domain (toward Rac1) occurs upon complex formation. This shift mimics a similar rearrangement within the DH/PH portion of Dbs upon engagement of Cdc42 or RhoA[166,

167] (**see Figures 15(a) and (b)**). Consistent with sequence conservation between Dbs and Trio, the molecular interactions between the PH domain of Trio and Rac1 are analogous to those that mediate the interface between the PH domain of Dbs and Cdc42 (**see Figures 15(c) and (d)**). Specifically, the interactions between Asp65 (Rac1), His1410 (Trio, DH domain) and Tyr1472 (Trio, PH domain) mimic the interactions between Asp65 (Cdc42), His814 (Dbs, DH domain), and Tyr889 (Dbs, PH domain). In addition, interactions between Asp65 (Rac1), Gln1430 (Trio, PH domain), and a bridging water molecule are recapitulated in the Dbs•Cdc42 complex. Other similar interactions also occur within both structures, *i.e.*, Ser1470 within the PH domain of Trio interacts with His103 of Rac1 while Lys885 of the PH domain of Dbs interacts with His103 of Cdc42.

Functional analysis of the interface between the PH domain of Trio and Rac1

To test the functional significance of the interactions between Rac1 and the PH domain of Trio, mutations designed to disrupt these interactions were introduced into the DH/PH fragment used for crystallization (**see Figure 16**) and purified mutant proteins were assayed for exchange activity on soluble wild-type Rac1 (residues 1-189, C189S) (**see Figure 16(a)**). Relative to the equivalent wild-type DH/PH fragment of Trio, mutation of either His1410 to Ala, Gln1430 to Ala, or Tyr1472 to Phe significantly decreases exchange activity. Similar to Dbs,[166] mutation of Tyr1472 to Phe produces the largest reduction in exchange activity. Substitution of Tyr 1472 to Phe was previously assessed under similar conditions[169] and the two sets of measurements are consistent. Unsurprisingly, complementary mutations within Rac1 designed to disrupt interaction with the PH domain of Trio reduced exchange by the wild-type fragment of Trio (**see Figure 5(c)**), further

confirming the functional importance of engaging Rac1 through the PH domain for catalyzed exchange. The D65A and R66A mutations introduced in Rac1 lie within the switch region responsible for binding nucleotide. However, rates of spontaneous exchange for these mutant forms of Rac1 were no more than two-fold higher than wild-type Rac1 (data not shown) and these small differences cannot account for the relatively large decrement in the capacity of Trio to activate them. The spontaneous rate of exchange for Rac1 H103A was essentially identical to wild-type Rac1.

In this study, Trio was crystallized in complex with nucleotide-depleted Rac1, but Trio also exchanges on RhoG both *in vitro*[169, 200] and *in vivo* [127, 200, 239, 248], and does so approximately three times more efficiently *in vitro* [169] RhoG is 80% homologous to Rac1, 65% identical, and the residues in nucleotide-depleted Rac1 that are buried in the interface with Trio are 100% identical in RhoG (data not shown). Consequently, mutants of Trio that have reduced exchange activity on Rac1 were also tested for activity on soluble, wild-type RhoG (**see Figure 16 (b)**). Predictably, mutations within the PH domain of Trio also reduced its exchange activity on RhoG, with Trio Y1472F exchanging the least efficiently. These data strongly suggest that the interface between Trio and RhoG is highly similar, if not identical, to its interface with Rac1. Circular dichroism spectroscopy (**see Figure 16(d)**) and gel filtration chromatography (data not shown) confirmed that all mutant forms of Trio were properly folded and monodisperse.

Wild-type Trio robustly activates RhoG through its N-terminal DH/PH cassette to promote neurite outgrowth in PC-12 cells [239]. Furthermore, mutant forms of Trio lacking the N-terminal PH domain exhibit a reduced capacity to both activate RhoG *in vitro* and induce neurite formation in PC-12 cells [200, 239]. While these studies indicate the

importance of the N-terminal PH domain of Trio for guanine nucleotide exchange of its cognate GTPases and the attendant morphological consequences, they do not address the underlying mechanistic causes for these results. Therefore, to test the effects of disrupting specific interactions between the N-terminal PH domain of Trio and its cognate GTPases, the single substitutions described above were introduced into full-length Trio (3038 amino acids) and the capacity of these substituted forms of Trio to induce neurite outgrowth of PC-12 cells was quantified (**see Figure 17**). Consistent with the inability of these mutant forms of Trio to activate Rac1 and RhoG efficiently *in vitro*, these singly-substituted forms of full-length Trio are also significantly compromised in their capacity to induce neurite outgrowth in PC-12 cells. Since these substitutions do not perturb the overall fold of the isolated DH/PH fragment of Trio, it is highly unlikely that they do so within the context of full-length Trio.

Discussion

The work presented here describes specific interactions between the N-terminal PH domain of Trio and Rac1 or RhoG that are essential for productive activation of these GTPases. These interactions strikingly mimic the coordinate engagement of RhoA or Cdc42 by the DH and PH domains of Dbs [166, 167]. Moreover, PH domain-mediated interactions between both Trio and Dbs, and their cognate GTPases, are required for efficient guanine nucleotide exchange *in vitro* and *in vivo* and indicate that the coordinate engagement of cognate GTPases by DH and PH domains necessary for efficient guanine nucleotide exchange might be more wide-spread than currently appreciated.

For example, as predicted by sequence homology (**see Figure 11(b)**), the residues in the PH domain of Trio that mediate its interface with Rac1 are identical to the residues in the

PH domain of Dbs that mediate its interface with Cdc42 and RhoA [166, 167]. Interestingly, these residues are conserved in other GEFs, including: Duo, Dbl, neuroblastoma, and obscurin (**see Figure 11(b)**); and research has shown that Dbl also requires its DH-associated PH domain to catalyze exchange with maximal efficiency [153]. Therefore, while it is not conclusive that these GEFs also require direct engagement of their cognate GTPases by their DH-associated PH domains for full exchange potential, sequence conservation shared among this set of GEFs strongly suggests this possibility.

While the residues of Dbs and Trio that mediate the interface between their PH domains and cognate GTPase are conserved only in a subset of Dbl-family GEFs, recent data shows that more distantly related Dbl-family GEFs also use their DH-associated PH domain to engage their cognate GTPases, albeit through different interactions. For example, the DH domains of both LARG and PDZ-RhoGEF exchange less efficiently on RhoA than their respective DH/PH cassette [168, 205]. Not surprisingly, a comparison of the structure of LARG DH/PH to the structure of LARG DH/PH bound to nucleotide free RhoA (henceforth LARG•RhoA) reveals an ~30° rotation of the PH domain, allowing contact between the PH domain and the GTPase [168]. This rotation is similar to that seen with both Trio and Dbs upon engaging the nucleotide-free GTPase [206]. However, instead of allowing contact between the cognate GTPases and the β3/β4 loop of the respective PH domains, the LARG•RhoA structure and the structure of PDZ-RhoGEF bound to RhoA both reveal contacts between RhoA and residues in the αC helix of the PH domains [168, 205]. In the case of LARG•RhoA, mutational analysis has shown that these contacts are functionally significant. The structure of LARG•RhoA and accompanying biochemical analyses also reveal functionally significant contacts between the GTPase and the β1 strand of the PH

domain which are mediated through Arg 986 in the αN helix of the PH domain [168]. The αN helix extends from the C-terminus of the α6 helix of the DH domain [168], and interestingly, similar contacts are seen between the GTPase and C-terminus of the α6 helix of Trio, Dbs, and PDZ-RhoGEF [166, 205]. In the cases of PDZ-RhoGEF and LARG, the *in vivo* relevance of contacts between the PH domain and the GTPase has not been pursued. LARG and PDZ-RhoGEF belong to a subset of Dbl-family GEFs that include Lfc and p114-RhoGEF; further analysis is needed to determine if other members of this subset also require their DH-associated PH domains for maximal guanine nucleotide exchange.

In contrast to Dbs, Trio, LARG, and PDZ-RhoGEF, crystals structures of the DH/PH cassettes of the Dbl-family GEFs Tiam1 [158], intersectin [167], and collybistin [170], in complex with their cognate GTPases show little or no contact between their PH domains and their cognate GTPase. While these structures might indicate that not all Dbl-family GEFs use their DH-associated PH domain to engage cognate GTPases for effective exchange, it has been suggested that these structures might not precisely reflect the molecular details of the exchange process *in vivo* [159, 160, 166, 235]. Specifically, Dbl-family GEFs operate on membrane-resident GTPases and it has been proposed that biological membranes impose additional constraints on the conformational flexibility of DH/PH cassettes such that specific conformers are favored that could promote direct interactions between GTPases and DH-associated PH domains necessary for full exchange activity [159, 160, 166]. Indeed, many DH-associated PH domains have been shown to bind various phosphoinositides with micromolar affinities [176, 226], and while these protein-lipid interactions are not normally considered sufficient to drive sub-cellular re-localization [171], they might provide points of membrane attachment that would favor conformers or orientations of DH/PH cassettes that

stabilize interactions between DH-associated PH domains and GTPases necessary for full catalytic exchange. Studies of Tiam1 [159] and Dbs [160, 191] provide substantial support for this scenario.

There is evidence that due to the conformational flexibility within the DH/PH cassettes, structural studies provide only a partial description of the exchange process. For example, there are four independent copies of the DH/PH cassette of Dbs in the crystal structure of this fragment without bound GTPase [206]. In each of the four molecules, the position of the PH domain is different relative to the DH domain. Much of this conformational flexibility is lost when Dbs engages a cognate GTPase as evidenced in the crystal structures of Dbs bound to either RhoA [167] or Cdc42 [166]. Similarly, the structure of collybistin in complex with Cdc42 shows two conformers of the DH/PH cassette within the asymmetric unit that differ by an ~35 degree rotation of the PH domain with respect to the DH domain [170]. Sos1 presents an extreme example. In this case, the PH domain occludes the GTPase-binding site on the DH domain [60, 61], and the DH/PH fragment alone is incapable of activating GTPases *in vitro* (S. Soisson, personal communication). However, Sos1 does activate Rac1 *in vivo* [58], and for this to occur, the PH domain must undergo a dramatic rearrangement from its position relative to the DH domain to allow binding and activation of Rac1. Some evidence suggests that the E3b1/Eps8 complex activates Sos1 downstream of H-Ras and PI3K [203, 204], but the molecular details of the Sops DH/PH rearrangement remain unclear. In the case of Trio, we show here that portions of the PH domain that are not in direct contact with Rac1 are highly mobile in the crystal structure of the Trio•Rac1 complex. This mobility does not manifest in the crystal structure of the

DH/PH fragment of Trio in isolation. However, in this case, lattice contacts within the crystal clearly limit possible motion.

The studies presented here support earlier indications that the N-terminal PH domain of Trio is required for the activation of its cognate GTPases, and together with previous studies of Dbs [160, 166, 167], provide detailed mechanistic information regarding roles of DH-associated PH domains in directly engaging GTPases for their activation *in vivo*. PH domains associated with DH domains might function in a variety of contexts to support activation of GTPases. However, an attractive and parsimonious model posits that both of the two domains must engage cognate GTPases for their effective and regulated activation *in vivo*. The inherent flexibility between DH and PH domains would be restricted under controlled cellular conditions, *i.e.*, at membranes and upon the binding of specific phosphoinositides to the PH domain, which would favor productive engagement of both portions of the cassette with cognate GTPases. Under extreme conditions, such as Sos1, the PH domain would move off the surface of the DH domain to allow access by GTPases [60, 61] and this movement might also be controlled by membranes and phosphoinositide-binding to the DH-associated PH domain. Alternatively, the conformation of the DH/PH cassette might be altered by interaction with currently unknown protein activators. In all cases though, the DH and PH domains would act cooperatively to integrate various cellular inputs leading to Rho-family GTPases activation; the PH domain would not be a simple membrane localization device, but more properly thought of as an intrinsic component of the exchange process carried out by Dbl-family proteins reacting to various cellular conditions.

Data collection	
Wavelength (Å)	1.0712
Resolution (Å)	19.4 – 2.0
Total observations	213,443
Unique reflections	39,416
Completeness[1] (%)	99.6 (99.8)
I/σ[1,2]	38.1 (3.5)
*R*sym[1,3] (%)	6.9 (52.4)

Refinement statistics	
Resolution (Å)	19.4 – 2.0
Reflections (working/test)	36932/1968
R_{work}[4] (%)	22.3
R_{free}[5] (%)	24.9
R.m.s. deviations	
Bond distances (Å)	0.009
Bond angles (°)	1.138
Average *B*-factor (Å^2)	
Molecule	61.1
Rac1	39.4
DH domain	39.1
PH domain	143.6
Waters	51.4
Ramachandran Statistics	
Favorable (%)	99.8
Allowed (%)	0.2
Disallowed (%)	0.00

Table 3: Data collection and refinement statistics for Trio•Rac1

[1] Values for the highest resolution shell are given in parentheses.

[2] I/σ is the mean signal to noise ratio, where I is the integrated intensity for a measured reflection and σ is the estimated error in the measurement.

[3] $Rsym = 100 \times \sum | I - \langle I \rangle | / \sum I$, where I is the integrated intensity for a measured reflection.

[4] $R_{work} = \sum | F_o - F_c | / \sum F_o$, where F_o and F_c are the observed and calculated structure factor amplitudes, respectively

[5] R_{free} is calculated similarly to R_{work} using test set reflections.

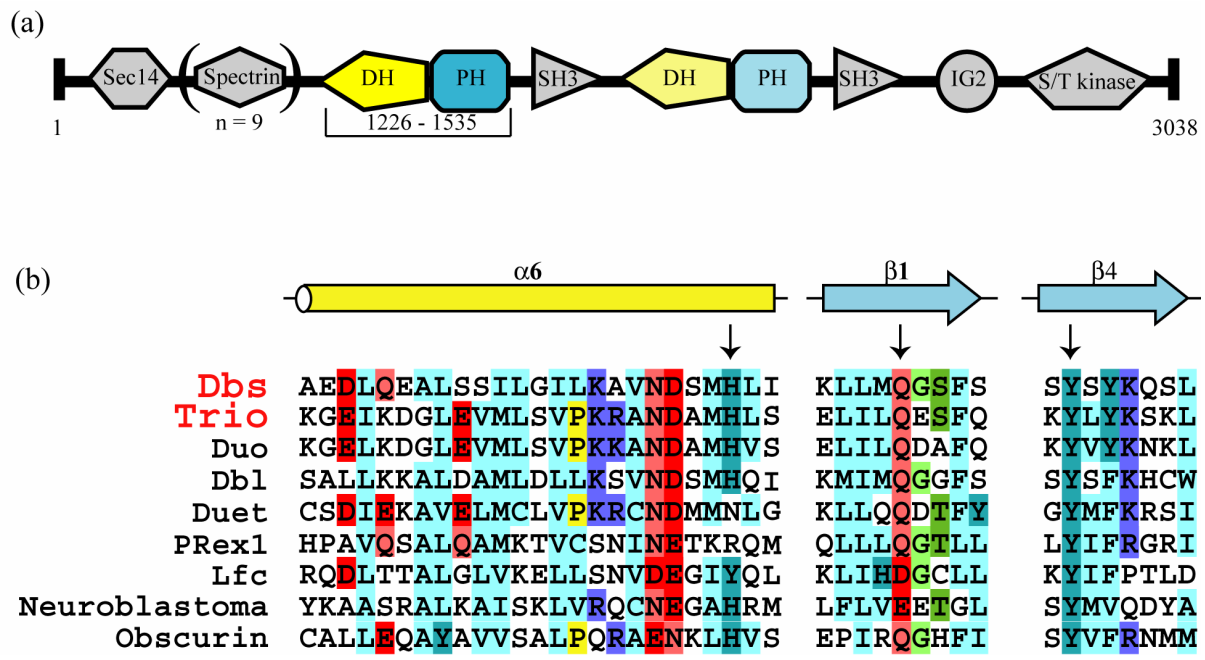

Figure 11: Trio-related GEFs.

(a) Crystallized fragment of Trio is highlighted within the domain architecture of full-length Trio. (b) Residues of Dbs (arrows) that mediate contacts between its N-terminal PH domain and cognate GTPases are conserved in other Dbl-family GEFs, including Trio. The relative position of these residues in context of the DH/PH cassette is indicated in Figure 3.

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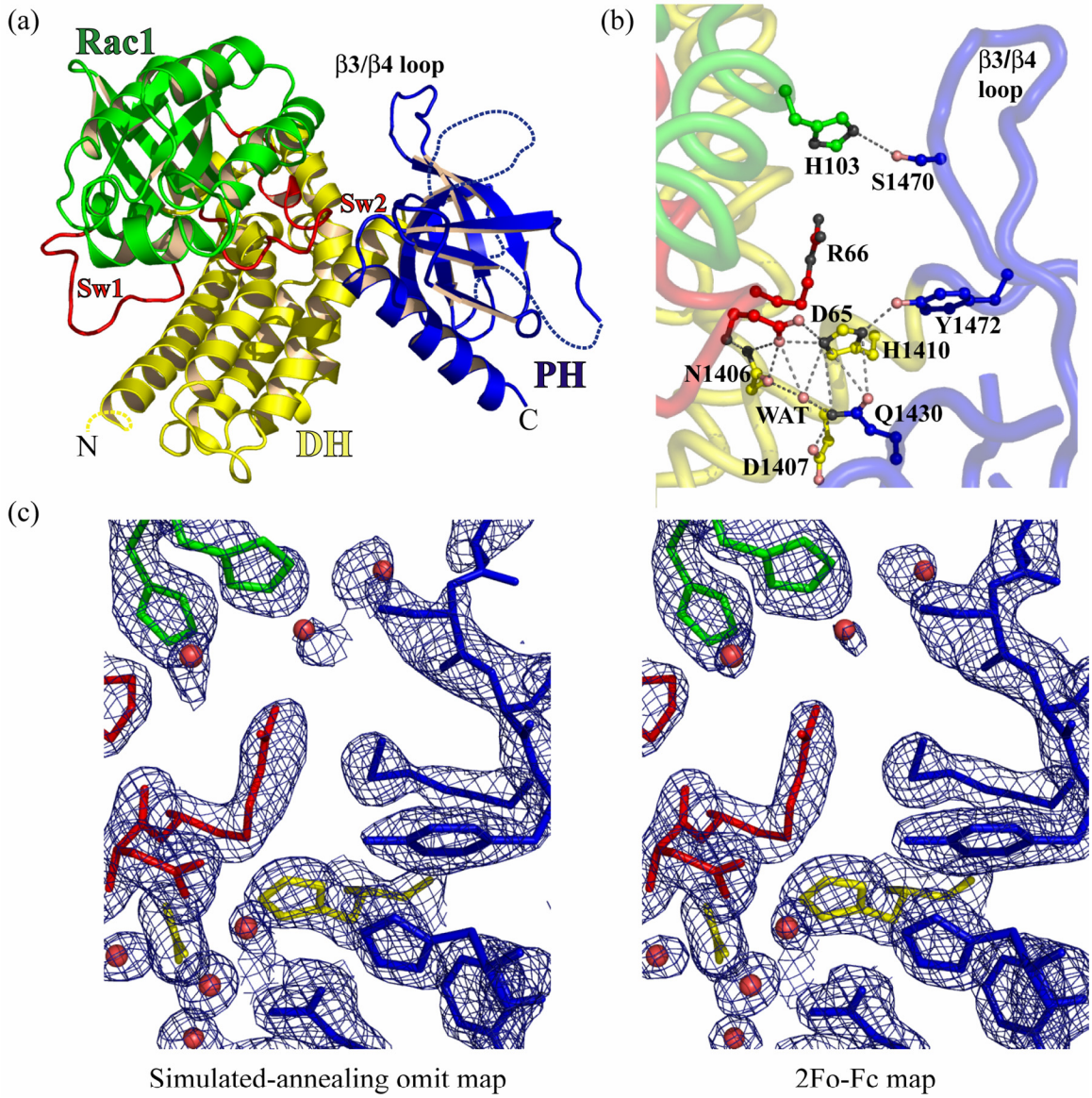


Figure 12: Crystal structure of the DH/PH fragment of Trio bound to nucleotide-free Rac1.

(a) The N-terminal DH (yellow) and PH (blue) domains of Trio are bound to nucleotide-depleted Rac1 (green with switch regions in red). Disordered regions are indicated with dotted lines. (b) Atomic details of the interface between Rac1 and the PH domain of Trio. Hydrogen bonds (2.6 – 4.0 Å) are indicated with dotted lines (c) A simulated annealing omit map (left) contoured at 1.0σ and a 2Fo-Fc map (right) contoured at 1.2σ generated using the final coordinates highlight the electron density at the interface between Rac1 and the PH domain.

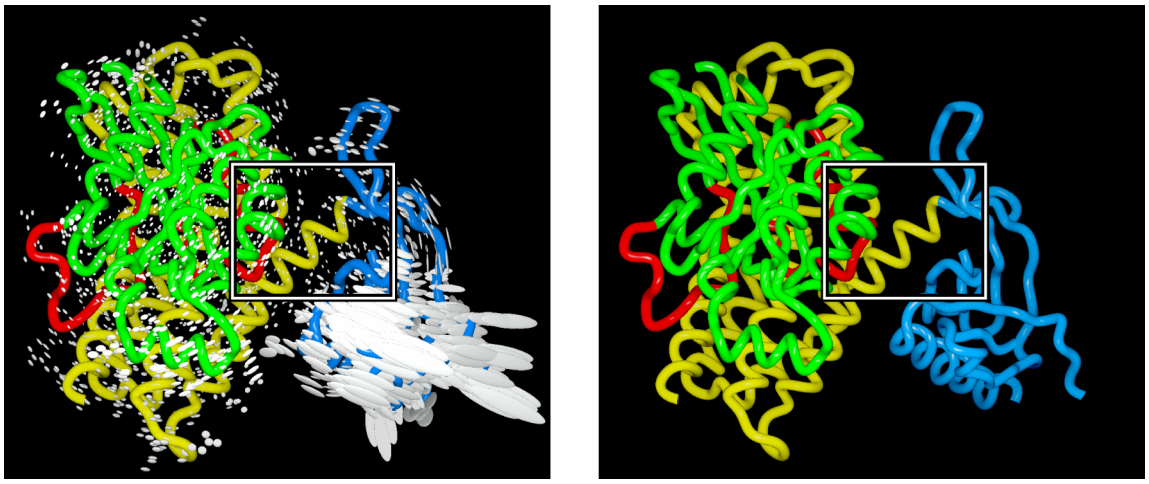


Figure 13: The DH-associated PH domain of Trio is inherently mobile

The anisotropic motion of each atom is displayed as a thermal ellipse (left). An identical image without the thermal ellipses is shown as a reference (right). The interface between Rac1 and the PH domain of Trio, also depicted in **Figures 12(b) and 12(c**), is highlighted by the box.

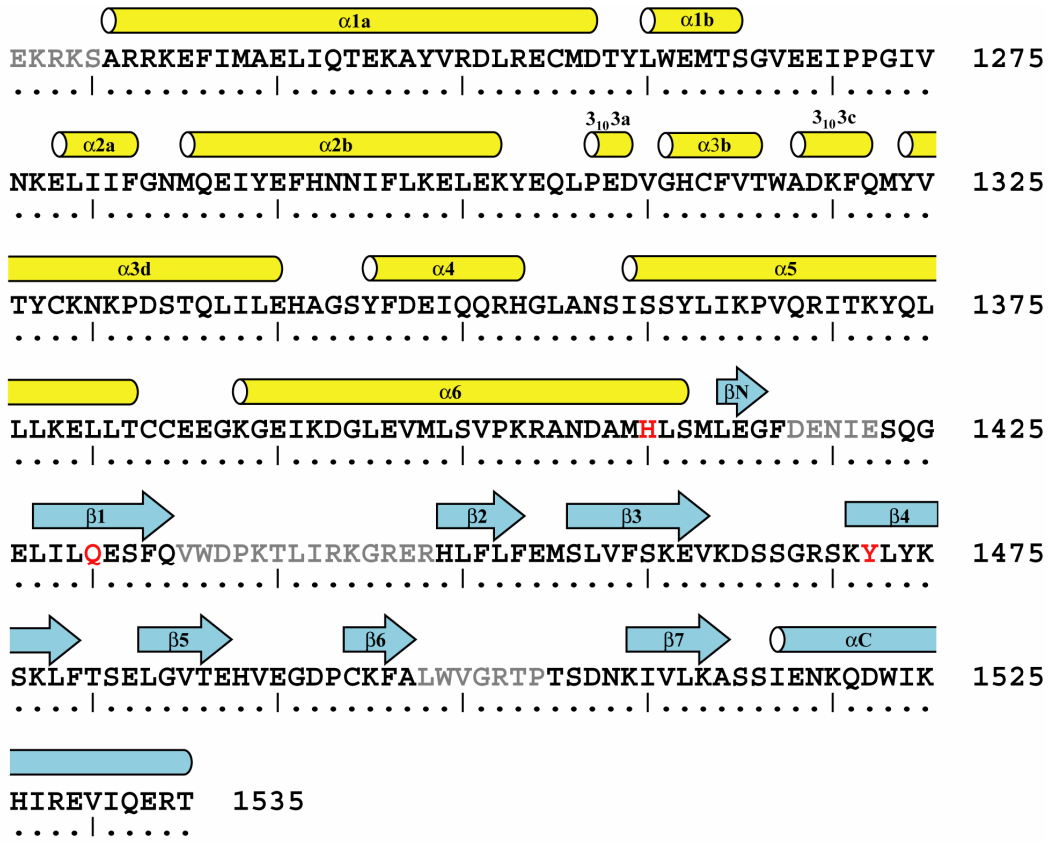


Figure 14: Secondary structure of the DH/PH fragment of Trio.

The crystal structure of the Trio fragment bound to Rac1 was used to define α-helices and β-strands according to nomenclature standardized for DH/PH cassettes [158]. Secondary structure assignments were made using the program DSSP [270], coupled with visual assessment. Residues highlighted in red contribute to the interface between the PH domain of Trio and Rac1. Residues in gray are disordered and were not modeled.

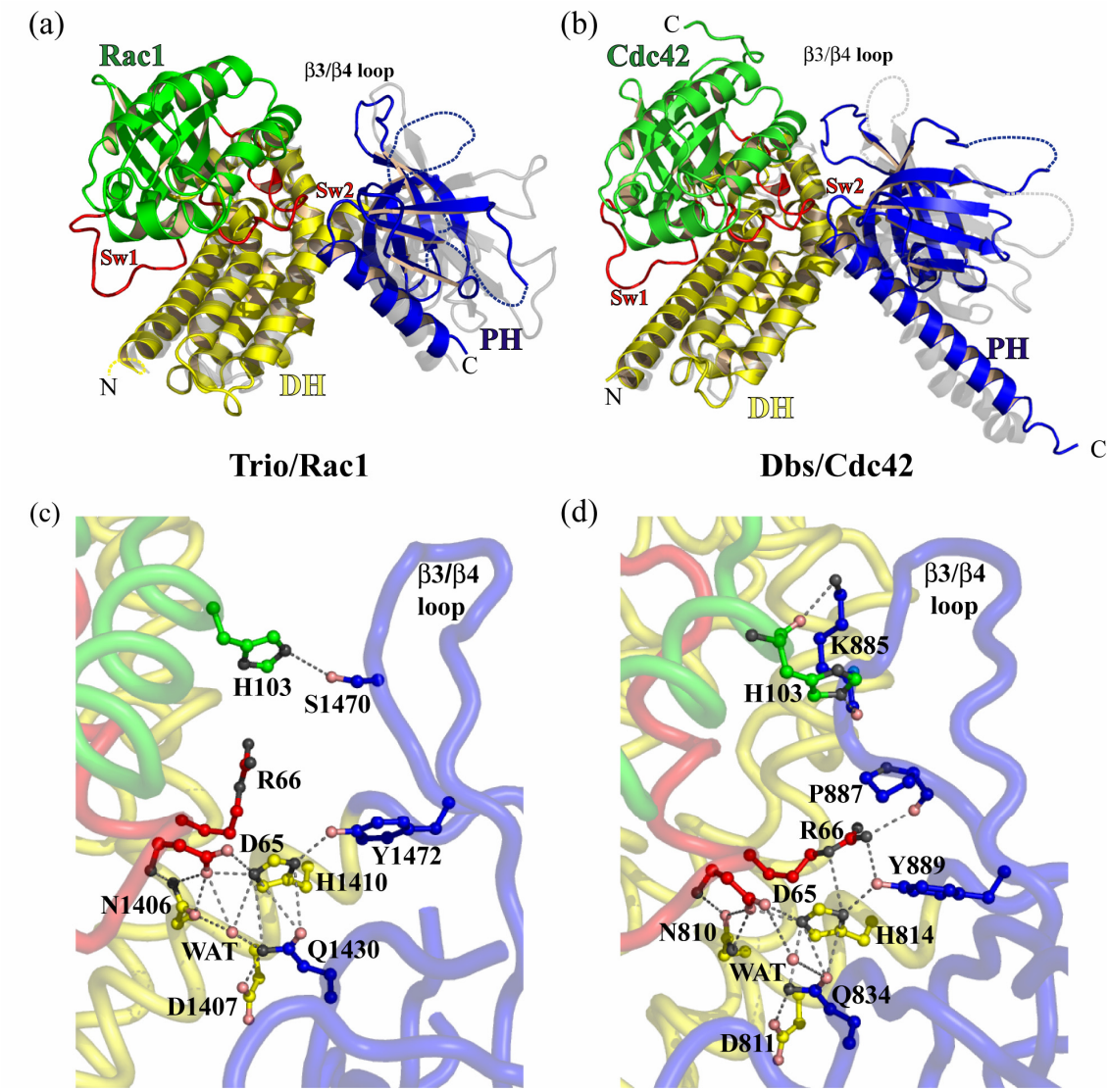


Figure 15: The PH domains of Trio and Dbs interact similarly with their cognate GTPases.

DH/PH fragments of Trio (a) and Dbs (b) in complex with their cognate GTPases have been superimposed upon equivalent, unbound fragments (gray) using the DH domains. (c and d) Lower panels highlight conserved interactions found in both GEF/GTPase complexes that require specific residues involving the PH domains. Color scheme is maintained from **Figure 12**.

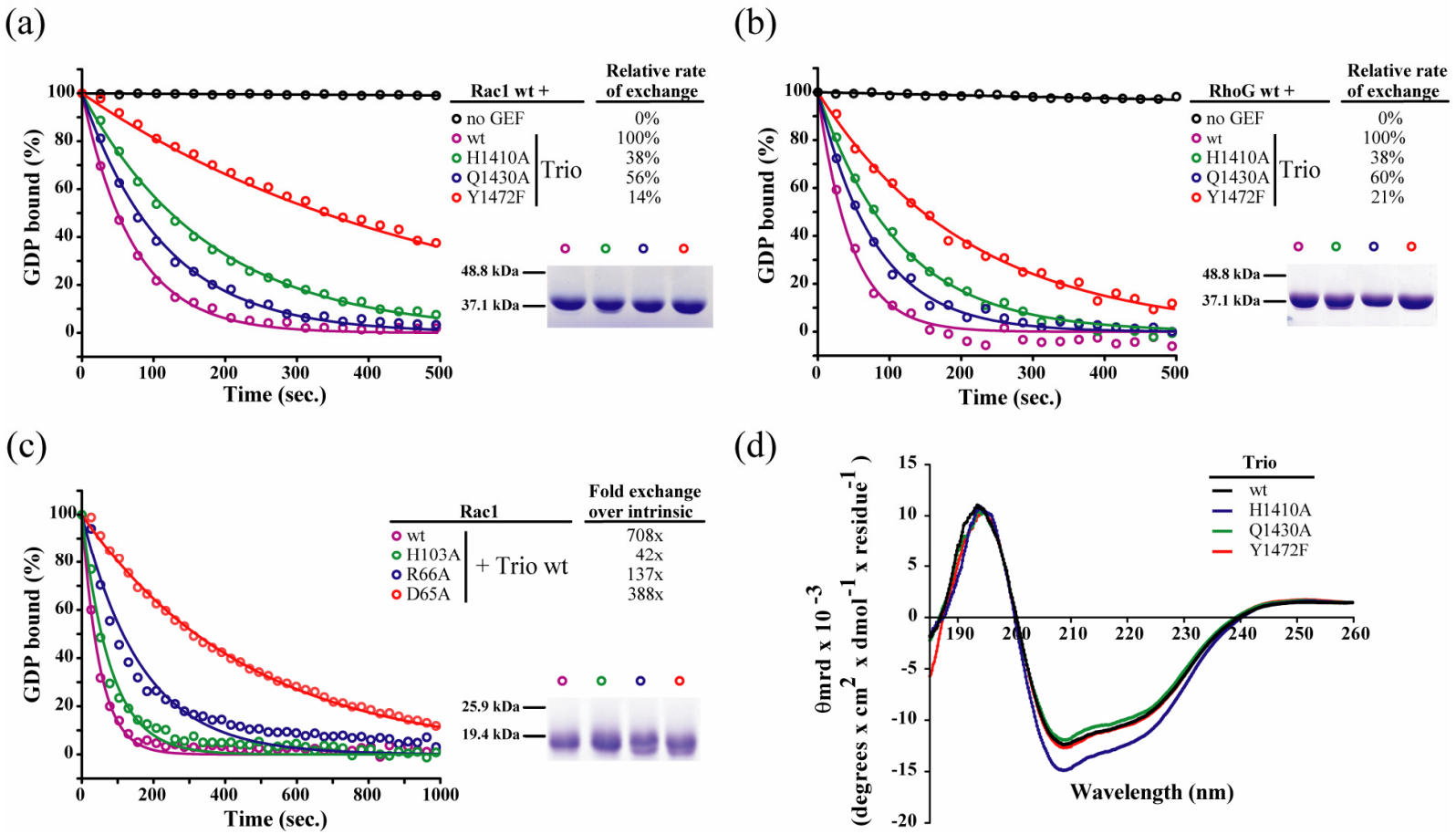


Figure 16: Mutations that disrupt the interface between the PH domain of Trio and Rac1 diminish nucleotide exchange.

Residues within, or supported by, the PH domain of Trio that form the interface with Rac1 were mutated and the exchange activities of the mutants were measured on both Rac1 (a) and RhoG (b). (c) Residues of Rac1 that interact directly with the PH domain of Trio were also analyzed. Exchange assays (n=2) were carried out as described in Methods. Exchange rates are reported as a percentage of the exchange rate of wild-type Trio (a and b) or as fold exchange over the intrinsic exchange rate of the respective mutant of Rac1 (c). Proteins (5 µg) were subjected to SDS-PAGE and stained with Coomassie Blue (insets) to verify purity and concentration. (d) Circular dichroism spectroscopy confirmed the proper folding of Trio DH/PH fragments.

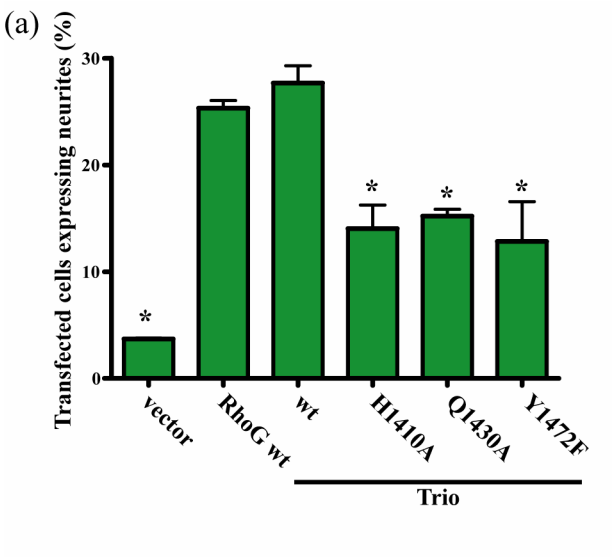
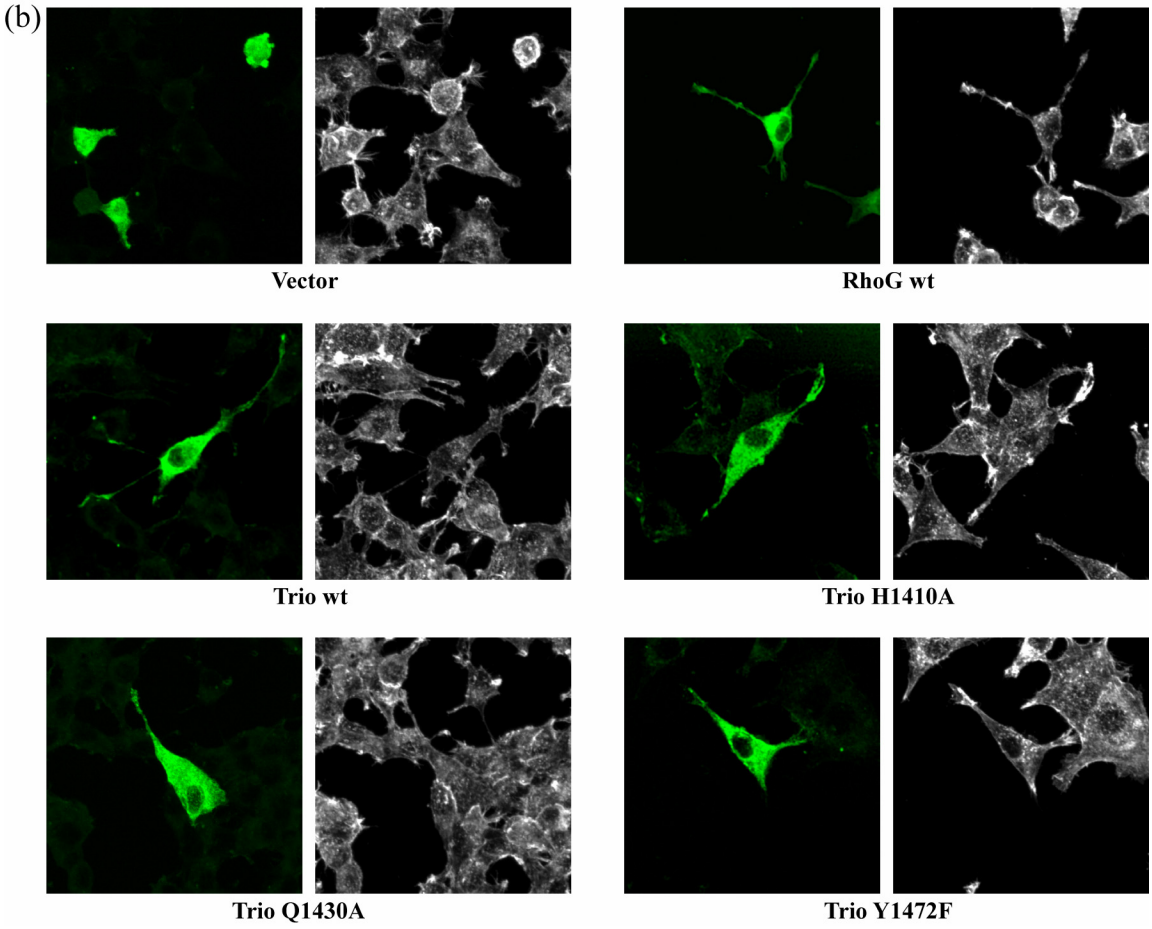


Figure 17: Mutations within, or supported by, the PH domain of Trio that reduce GTPase activation in vitro also reduce the capacity of full-length Trio to induce neurite outgrowth in PC-12 cells.

(a) Neurite outgrowth in transfected PC-12 cells was assessed as described in Methods (*; p-values < 0.05 in comparison to wild-type Trio using student's T-test). (b) Representative images of transfected PC-12 cells show both GFP fluorescence (left) and filamentous actin stained with Alexa Fluor 546 phalloidin (right). All constructs were GFP-tagged at the N-terminus.



CHAPTER 3: ACTIVATION OF DBS BY ACTIVE RAC1

Introduction

Dbl-family GEFs are the major class of proteins responsible for activating the canonical Rho-family GTPases RhoA, -B, and -C, Rac1, -2, and -3, and Cdc42. The 69 Dbl-family GEFs found in the human genome are all characterized by the presence of a DH domain followed almost invariably by a tandem PH domain. However, most also contain a variety of other regulatory domains such as coiled-coiled regions, SH3 domains, and FERM domains. This variability makes Dbl-family GEFs key in integrating signals that result in the precise spatiotemporal activation of Rho-family GTPases [8].

Dbs (Dbl's big sister) is a Dbl-family GEF originally identified as part of a screen to identify proteins that cause transformation of NIH 3T3 cells, as is the case for many Dbl-family GEFs [271]. In addition to its DH/PH cassette, it contains a yeast phosphatidylinositol transfer protein (Sec14) domain at the N-terminus which binds lipids, two spectrin repeats, and an SH3 domain that is found C-terminal to the PH domain [272]. While the roles of the spectrin repeats and SH3 domain remain relatively uncharacterized, the Sec14 domain and DH/PH cassette have well defined functions. The Sec14 domain interacts with the PH domain of Dbs and auto-inhibits its activity by blocking access by the cognate GTPase to the catalytic DH domain. The Sec14 domain also influences sub-cellular localization by binding to perinuclear structures near the Golgi apparatus [272]. Extensive

work on the DH/PH cassette of Dbs shows that it exchanges on RhoA, Cdc42, and possibly RhoG [129, 166, 221, 273]. Like most Dbl-family GEFs, the DH domain of Dbs provides the majority of its catalytic interface with its cognate GTPases, but the PH domain also participates directly in the exchange reaction [166, 167]. The PH domain of Dbs also weakly binds PIP2 and can cooperatively target Dbs to the plasma membrane [176, 191]. In addition, its ability to bind phosphoinositides is critical for exchange activity once at the plasma membrane [160].

Recently, the PH domain of Dbs was shown to interact with active Rac1 using both co-immunoprecipitation and immunofluorescence assays [221]. Dbs interacts with the active form of Rac1, strongly suggesting that it functions as an effector of the GTPase. This interaction is specific to active Rac1, and not Cdc42 or RhoA [221]. Furthermore, these results build on earlier studies which show that the PH domain of Ost, the rat homolog of Dbs, also interacts with active Rac1 [274]. Cheng *et al.* were also able to show that co-transfection of active Rac1 with an oncogenic version of Dbs increases the ability of Dbs to transform NIH 3T3 cells [221]. Transformation by Dbs has been shown to be mediated through activation of RhoA [273]; however, transfection with constitutively activated RhoA does not result in transformation as robust as co-transfection of Dbs with active Rac1 [221]. Thus, active Rac1 is cooperating with Dbs to enhance activation of RhoA.

The interaction between active Rac1 and Dbs has been shown only when using co-immunoprecipitation or immunofluorescence, leaving open the possibility that their interaction is mediated via some other protein or molecule. It is also unknown if active Rac1 allosterically modulates the activity of Dbs or Scambio, or if it increases activation *in vivo* by regulating sub-cellular localization. The structure of PLC-β2 in complex with active Rac1

shows a direct interaction between Rac1-GTPγS and the PH domain of PLC-β2 [177], suggesting that such an interaction is also possible between active Rac1 and the PH domain of Dbs. In the case of PLC-β2, active Rac1 functions to translocate PLC-β2 to the plasma membrane, thus activating it by determining sub-cellular localization [177].

The ability of Dbl-family GEFs to act as effectors of some Rho-family GTPases while activating others indicates another important level of regulation in the complex signaling cascades involving Rho-family GTPases. However, the molecular details behind the activation of Dbl-family GEFs by active Rho-family GTPases are not well understood. The goal of these studies is to examine these interactions by using the association between activated Rac1 and the Dbl-family GEF Dbs as a model system.

Experimental Procedures

Protein purification for surface plasmon resonance and ALPHA screen

Murine Dbs (623 – 956, wt or E639A) was expressed from the pGEX4TEV2 vector [251] with an N-terminal GST-tag in the BL21 (DE3) *E. coli* strain. Transformed cells were grown in LB media containing 0.1 mg/ml ampicillin at 37°C to an OD_{600} of 0.7 (mid-log phase) and induced with 1 mM IPTG at 20ºC for 16 hours to 18 hours. Cells were resuspended in buffer A (200 mM NaCl, 20 mM Tris, pH 7.5, 2 mM DTT and 10% glycerol) and lysed with an Emulsi-Flex C5 (Avestin). Lysate was clarified by centrifugation at > 125,000 x *g* for 30 minutes and the supernatant was loaded onto a GST-Sepharose affinity column (GE Healthcare) equilibrated in buffer A; the column was washed with buffer A, and Dbs eluted in buffer A containing 10 mM glutathione (reduced). The fractions containing

Dbs were then buffer exchanged extensively into buffer A to remove the glutathione, concentrated, frozen, and stored at -80ºC.

Human Rac1 (residues 1 – 177) was purified exactly as previously described [158]. Tagless human Rac1 (residues 1-189, C189S) was expressed from pET21a (Novagen) in the BL21 (DE3) *E. coli* strain. Transformed cells were grown in LB media containing 0.1 mg/ml ampicillin at 37°C to an OD_{600} of 0.7 (mid-log phase) and induced with 1 mM IPTG at 27ºC for 5 hours. Harvested cells were resuspended in buffer B (10 mM MES, pH 6.0, 2 mM DTT, 10% glycerol, 1 mM $MgCl_2$) containing 10 mM NaCl prior to lysis and clarification as described above. Supernatant was loaded onto an SP-Sepharose Fast Flow 26/10 column (GE Healthcare). Rac1 was eluted from the column using buffer B with an increasing gradient of NaCl from 10 mM to 600 mM and further purified using a 26/60 Sephacryl-200 size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0, 2 mM DTT, 2mM $MgCl_2$, 150 mM NaCl, and 5% glycerol. Fractions containing Rac1 were pooled, concentrated, frozen, and stored at -80°C.

Human RhoA (residues 1 – 190, C190S) was expressed from pProExHTb in BL21 (DE3) *E. coli* strain as previously described [167] with minor variations. Cells were grown in Zym-5052 [275] self-inducing media for 3 hours at 37ºC and then for an additional 16 – 18 hours at 20ºC. Self-inducing media does not require the addition of IPTG to start protein expression. Harvested cells were resuspended in buffer C (20 mM Tris, pH 8.0, 200 mM NaCl) containing 10 mM imidazole. Cells were lysed and clarified as described above prior to loading the supernatant onto a Ni^{2+}-Sepharose affinity column (GE Healthcare) equilibrated in buffer C containing 10 mM imidazole. The column was washed with buffer C containing 55 mM imidazole and RhoA eluted with buffer C containing 400 mM imidazole.

Fractions containing RhoA were dialyzed against 20 mM Tris, pH 8.0, 2 mM DTT, 2 mM EDTA, 200 mM NaCl, and 10% glycerol while cleaving with tobacco etch virus (TEV) protease to remove the N-terminal His_6-tag. The protein was further purified using a 26/60 Sephacryl-200 size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0, 2 mM DTT, 2 mM $MgCl_2$, 200 mM NaCl, and 10% glycerol. Fractions containing RhoA were pooled, concentrated, frozen, and stored at -80°C.

His_6 – tagged human Rac1 (residues 1-189, C189S) was expressed from pProExHTb and purified exactly as described above for RhoA except that the His_6-tag was not removed by cleavage with TEV protease. Protein was concentrated and stored at -80ºC.

Human PAK (residues 70 – 132) was expressed from the pGEX4TEV2 vector [251] in Zym-5052 media as described above for RhoA and purified as detailed above for Dbs with minor variations. Fractions from the GST-Sepharose affinity column (GE Healthcare) containing PAK were pooled together and dialyzed extensively into buffer D (20 mM Tris, pH 8.0, 2 mM DTT, and 10% glycerol) with 10 mM NaCl. Dialyzed fractions were then pooled and loaded onto a Source-Q 16/10 column (GE Healthcare) equilibrated in buffer D with 10 mM NaCl and washed extensively in same buffer. Protein was then eluted in with buffer D using an increasing gradient of NaCl from 10 mM to 600 mM. Fractions containing PAK were pooled, concentrated, frozen, and stored at -80°C.

GST was expressed from the pGEX4TEV2 vector [251] in Zym-5052 media as described above for RhoA and purified as detailed above for Dbs with minor variations. Briefly, fractions from the GST-Sepharose affinity column (GE Healthcare) containing GST were pooled together and further purified using a 26/60 Sephacryl-200 size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0, 2 mM DTT, 2 mM $MgCl_2$, 200 mM

NaCl, and 10% glycerol to remove excess glutathione. Fractions containing GST were pooled, concentrated, and stored at -80°C.

Protein purification for guanine nucleotide exchange assays and determination of stable complex between active Rac1 and Dbs

Tagless human RhoA (residues 1 – 190, C190S or residues 1 – 178) and tagless human Rac1 (residues 1 – 189, C189S) were purified exactly as described above.

Dbs DH/PH (residues 623 – 967) with a C-terminal His_6-tag was expressed from pET28a as described previously with minor changes [166]. Briefly, cells were grown in Zym-5052 self-inducing media containing 0.05 mg/mL kanamycin, instead of 0.1 mg/mL ampicillin, as described above for RhoA. Harvested cells were resuspended in buffer E (20 mM Tris, pH 7.5, 300 mM NaCl) containing 5 mM imidazole and were also lysed and clarified as detailed above. The supernatant was applied to a Ni^{2+}-Sepharose affinity column (GE Healthcare) equilibrated in buffer E containing 5 mM imidazole and eluted as described above for RhoA. The protein was further purified using a 26/60 Sephacryl-200 size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0, 2 mM DTT, 1 mM EDTA, 200 mM NaCl, and 5% glycerol. Fractions containing Dbs were pooled together, concentrated, and frozen at -80ºC.

To purify a complex between Dbs DH/PH and RhoA (residues 1 – 178), Dbs was incubated with a four-fold molar excess of RhoA in 20 mM Tris, pH 8.0, 2 mM DTT, 4 mM EDTA, 150 mM NaCl, and 5% glycerol. The complex was concentrated and separated from excess RhoA over a 26/60 Sephacryl-200 size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0, 2 mM DTT, 1 mM EDTA, 150 mM NaCl, and 5%

glycerol. Fractions containing the complex were pooled and concentrated before being flash frozen and stored at -80ºC.

Loading of various GTPases with guanine nucleotides

Purified GTPases were incubated in buffer G (1 mM $MgCl_2$, 150 mM NaCl, and 20 mM Hepes, pH 7.5) with 7.5 mM EDTA in the presence of a 10 mM excess of GDP or GTPγS for 40 minutes at room temperature. $MgCl_2$ (20 mM) was added to stop the exchange reaction. For use in guanine nucleotide exchange assays or ALPHA screens, a HiTrap Desalting column (GE Healthcare) was used to remove excess nucleotide and buffer exchange the protein into buffer G (for the guanine nucleotide exchange assays) or 2 mM $MgCl_2$, 200 mM NaCl, 20 mM Hepes, pH 7.5, and 2mM DTT (for the ALPHA screens) before concentrating and freezing for storage at -80ºC.

Radioligand binding assay to check efficiency of guanine nucleotide loading

Various amounts of purified GTPases were loaded with GTPγS^{35} (Perkin Elmer) at a 10-fold molar excess of the highest amount of protein used. Guanine nucleotide loading reactions were carried out in triplicate at a final volume of 50 µL as described above. Reactions were diluted in 4 mL of buffer H (150 mM NaCl, 25 mM $MgCl_2$, and 20 mM Tris, pH 7.5) before being applied nitrocellulose filters. Filters were washed twice with buffer H and soaked in scintillation fluid before counting samples to determine nucleotide loading.

Surface plasmon resonance

GST-tagged proteins were immobilized on an anti-GST antibody covalently coupled to a CM5 sensor chip (Biacore), generating approx. 1000 RUs of surface. Rac1, RhoA, and Cdc42 (data not shown) were loaded with GDP or GTPγS and buffer exchanged into 150 mM NaCl, 20 mM Tris, pH 7.5, and 2mM $MgCl_2$ (except where indicated). The sensor chip was equilibrated in the same buffer (except where indicated). GTPases at various concentrations were flowed over the GST-ligand surface for the indicated times, and allowed to dissociate for 60 s. Curves were normalized by subtracting binding to the negative control of GST.

Guanine nucleotide exchange assays

The exchange assays measuring activation of RhoA by Dbs were performed as described previously with minor variations [166, 201, 236]. RhoA was loaded with Bodipy fluorescein (FL)-conjugated GDP (Bod-GDP, Molecular Probes) as described above and buffer exchanged into buffer J (20 mM Tris, pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 5% glycerol, and 2 mM DTT). Bod-GDP has a high level of fluorescence when bound to a GTPase. For the exchange reactions, the indicated concentration of RhoA – Bod-GDP was allowed to equilibrate with 10 μM cold GDP before adding indicated amounts of the exchange factor. The rate of decrease in the fluorescence of the Bod-GDP (λ_{ex} = 500 nm, λ_{em} = 511 nm) is indicative of the rate of loading of GDP in place of Bod-GDP. These assays were performed with a Perkin-Elmer LS 55 spectrometer. Rates of exchange were determined by fitting the data to a single exponential decay curve using the program

GraphPad Prizm, and reported as a multiple of basal GDP loading in the absence of an exchange factor.

Determination of a stable complex between Dbs or Dbs/RhoA (residues 1- 178) and active Rac1

All proteins were buffer exchanged into buffer K (150 mM NaCl, 20 mM Tris, pH 7.5, 2 mM DTT, 2 mM $MgCl_2$) using a HiTrap desalting column (GE Healthcare). Normalized protein amounts were applied to a Superdex-75 analytical gel filtration column (GE Healthcare) equilibrated in buffer K and eluted in same buffer.

ALPHAscreen

The His_6-tagged binding partner and GST-tagged binding partner were incubated together (at the indicated concentrations) for 30 minutes in 20 mM Hepes, pH 7.5, 1 mM $MgCl_2$, 5% glycerol, and 150 mM NaCl at room temperature. Donor beads conjugated to glutathione (final concentration 20 μg/mL) and accepter nickel chelate beads (final concentration 20 μg/mL) (Perkin Elmer) were incubated with the protein interaction partners for another 30 minutes in the absence of light. Protein interaction was assessed by measuring the emission at 580 nM after excitation at 670 nM in the Pherastar microplate reader.

Results

Surface plasmon resonance detects a weak interaction between Rac1-GTPγS and Dbs DH/PH

Surface plasmon resonance was used to test if Dbs interacts specifically with Rac1 in a nucleotide dependent manner. We found that the DH/PH cassette of Dbs interacts with GTPγS-loaded Rac1 (and not Rac1-GDP), but only at high concentrations of Rac1-GTPγS (**see Figure 18**). When investigating the interaction between active Rac1 and Dbs DH/PH, we noticed a high level of non-specific interaction between Rac1 and the negative control, GST. We were unable to reduce this non-specific interaction, while still maintaining the interaction between Rac1-GTPγS and Dbs DH/PH, by varying the running buffer (**see Figure 19**). We also removed the polybasic tail of Rac1, purifying only residues 1-177 and verified that this region was not responsible for interacting non-specifically with the unconjugated carboxy methyl dextran matrix on the surface plasmon resonance chip (**see Figure 20**). Radioligand binding assays were used to verify that both Rac1 (residues 1-189) and Rac1 (residues 1-177) were loaded with GTPγS with > 95% efficiency (data not shown).

Dbs and Rac1-GTPγS do not form a stable complex

Rac1-GTPγS incubated with equimolar amounts of either Dbs DH/PH or Dbs DH/PH•RhoA was applied to a size exclusion column to determine if the proteins would elute as a stable complex. We found that Rac1-GTPγS does not form a stable complex with either Dbs DH/PH or Dbs DH/PH•RhoA (**see Figure 21**). Rac1-GTPγS, Dbs DH/PH, and Dbs DH/PH•RhoA were analyzed alone to establish retention times for these proteins alone.

Rac1-GTPγS does not modulate the guanine nucleotide exchange activity of Dbs or Scambio in vitro

We wanted to determine if Rac1-GTPγS could allosterically modulate the exchange activity of Dbs DH/PH on RhoA *in vitro*, analogous to the modulation of p63RhoGEF by Gαq, despite the weak binding affinity between Dbs DH/PH and Rac1-GTPγS. We compared the level of exchange by Dbs DH/PH on RhoA in the absence and presence of increasing concentrations of Rac1-GTPγS and saw no significant differences **(see Figure 22**).

The ALPHAscreen does not detect an interaction between Dbs and Rac1-GTPγS

The ALPHAscreen (Amplified Luminescent Proximity Homogeneous Assay) (PerkinElmer) was also used to determine if purified Dbs interacts with Rac1-GTPγS. The interaction between active Rac1 and PAK was used as a positive control and proof of principle for the ALPHAscreen. We were unable to see an interaction between Dbs DH/PH and active Rac1 using the ALPHAscreen (**see Figure 23**)

Co-immunoprecipitation of Dbs with active Rac1 is not reproducible

Attempts to co-immunoprecipitate active Rac1, 2, or 3 specifically with the DH-associated PH domain of Dbs were not successful (data not shown).

Discussion

Previously published co-immunoprecipitation data and transformation assays strongly suggest that active Rac1 can interact with Dbs to positively regulate its exchange activity on RhoA [221, 222]. However, attempts to reproduce and examine the physical interaction

between Dbs and active Rac1 using purified proteins have been unsuccessful, and our data would suggest that while Dbs may interact with active RhoA, the interaction would have to be mediated by some other component in cells. This component may be phosphoinositides, as is the case with active Arf-6 and ARNO. However, the interaction may also require some other unidentified component or be too transient to allow physical characterization.

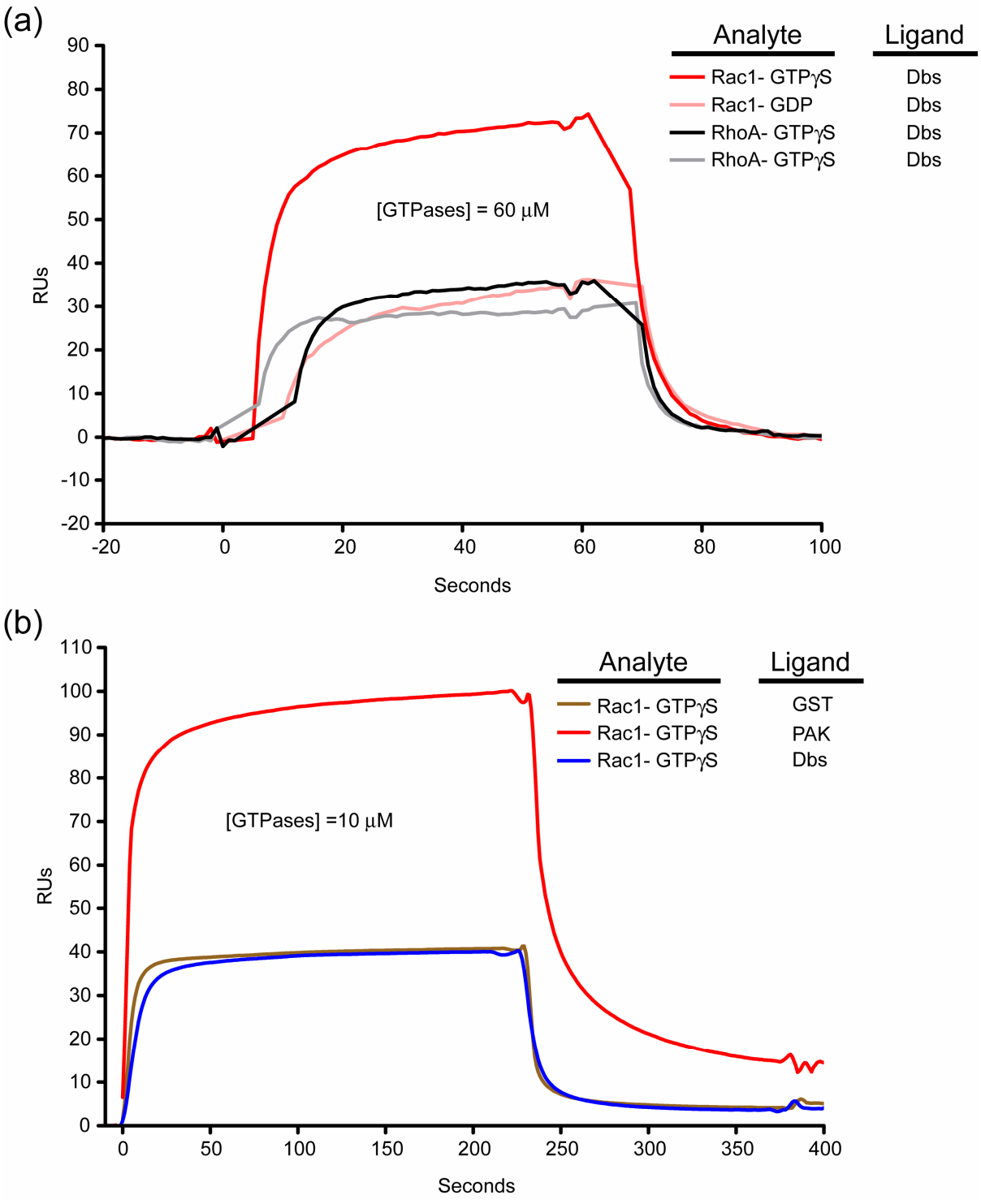


Figure 18: Rac1-GTPγS interacts specifically but weakly with wild-type Dbs DH/PH.
(a) Sensograms showing the interaction between Rac1 or RhoA (pre-loaded with GDP or GTPγS) and Dbs DH/PH wild-type. (b) Sensograms showing the interaction between Rac1-GTPγS and GST (negative control), PAK (positive control), or Dbs DH/PH wild-type. Concentrations of the GTPases are indicated.

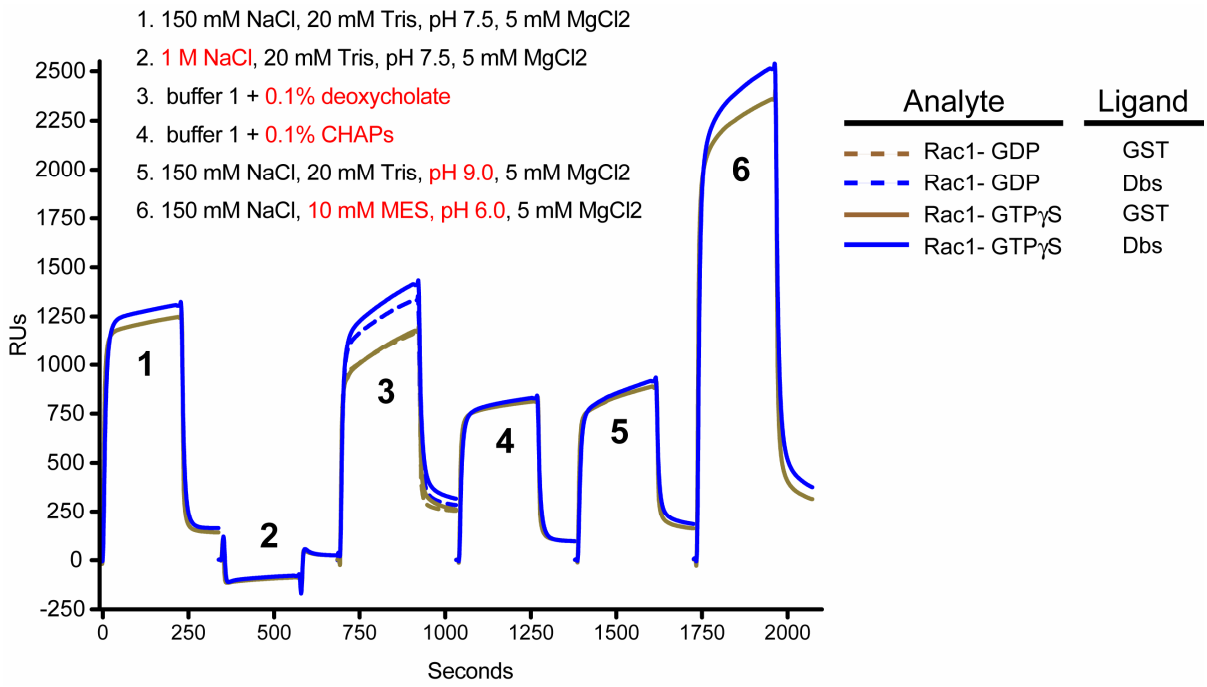


Figure 19: Optimizing buffer conditions to reduce non-specific binding to GST by Rac1-GTPγS.
Sensograms showing the interaction between Rac1 (loaded with GTPγS or GDP where indicated) and Dbs DH/PH wild-type. The buffer in condition 1 was varied (variations indicated in red) to try and reduce non-specific binding by Rac1 to GST.

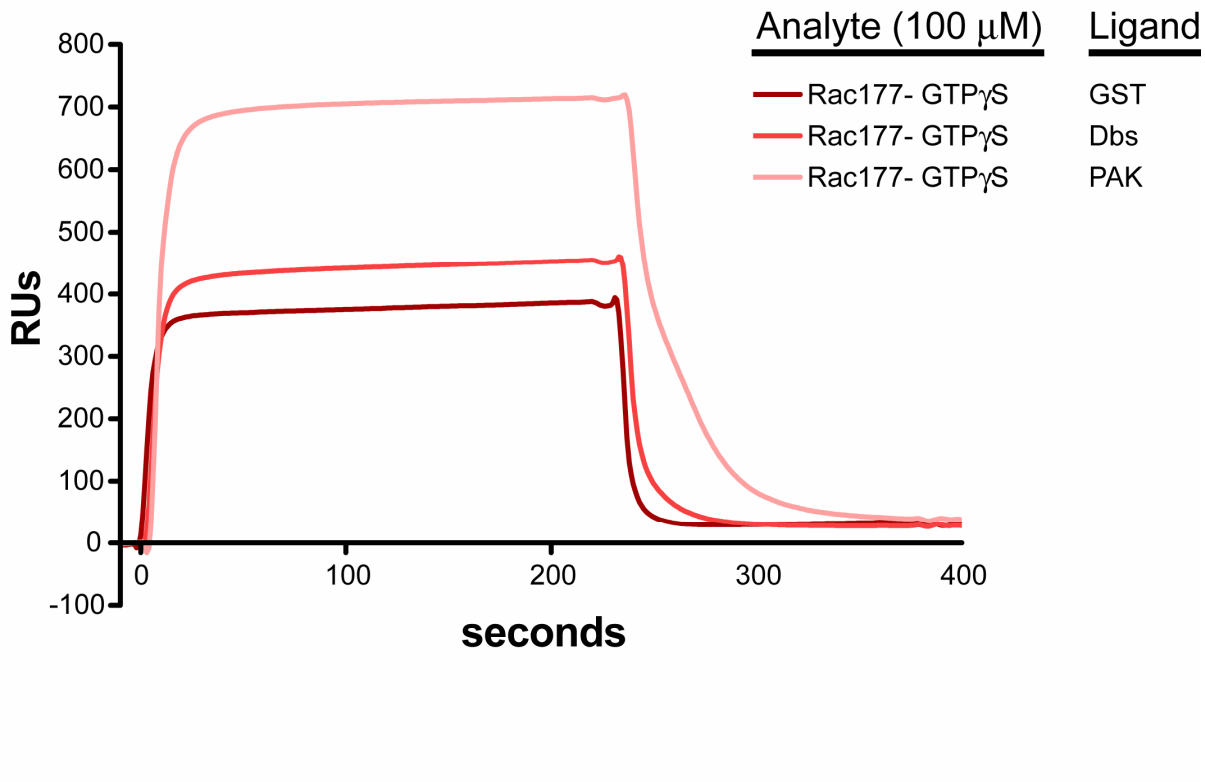


Figure 20: Removal of the polybasic region from Rac1 does not affect interaction between Rac1-GTPγS and GST or Dbs DH/PH.

Sensograms showing the interaction between Rac1 (residues 1-177) loaded with GTPγS and the indicated ligands as described in the methods section.

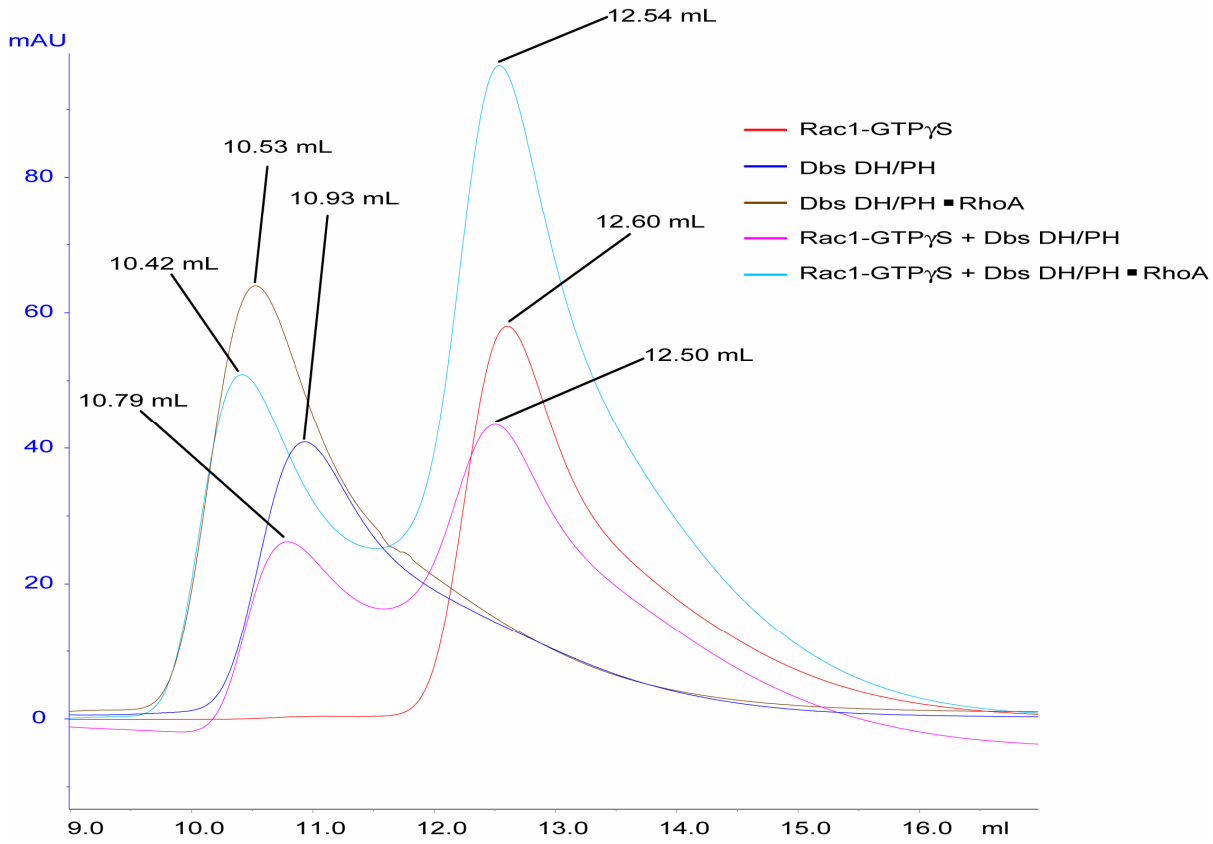


Figure 21: Rac1-GTPγS does not form a stable complex with Dbs DH/PH or Dbs DH/PH•RhoA

Various protein combinations were applied to a Superdex-75 analytical gel filtration column (GE Healthcare) as described in the methods section. The retention times for the various peaks resulting from the different loaded samples are labeled.

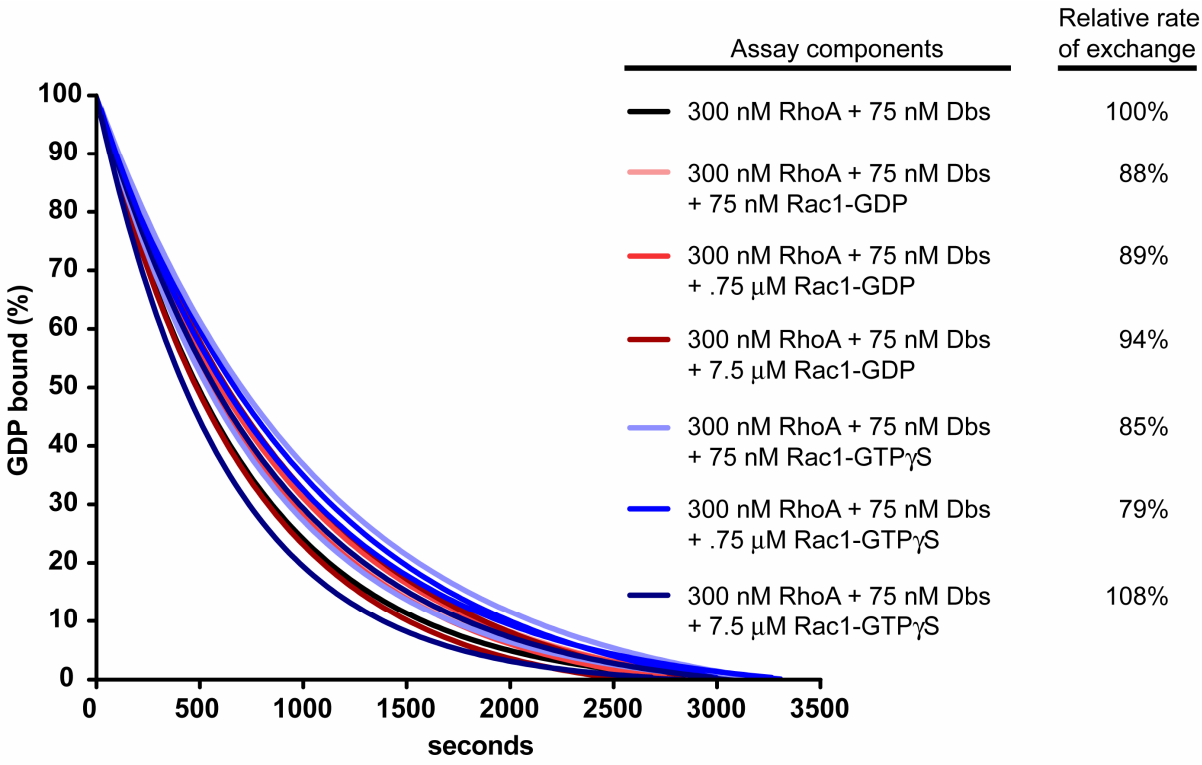


Figure 22: Modulation of the exchange activity of Dbs on RhoA by active Rac1.

Exchange by Dbs on RhoA was measured in the presence of increasing concentrations of Rac1-GDP or Rac1-GTPγS as described in the methods section. Rates of exchange are measured relative to exchange by wild-type Dbs DH/PH on Bod-RhoA.

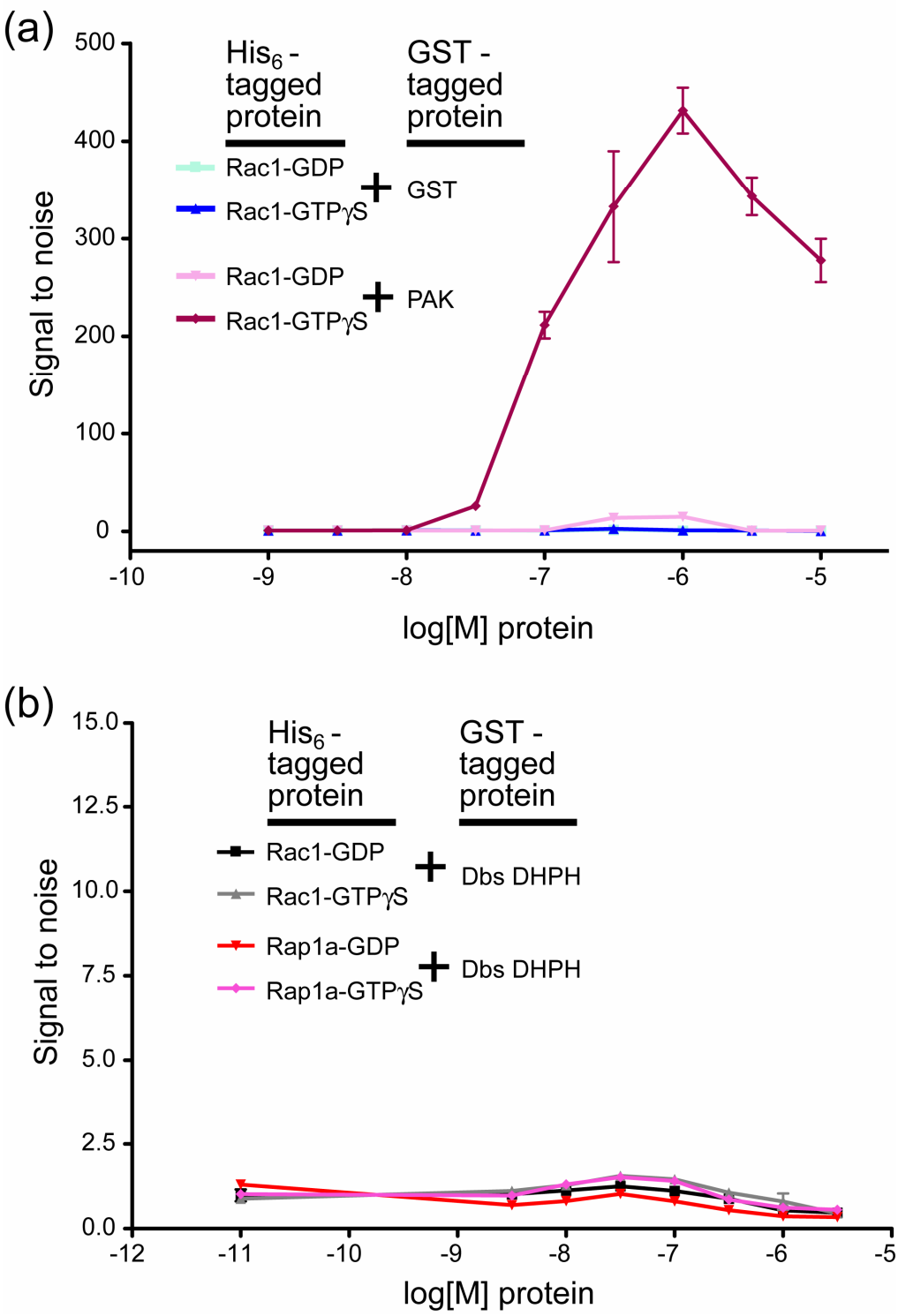


Figure 23: The ALPHA screen does not detect a direct interaction between active Rac1 and Dbs DH/PH.
(a) The ALPHA screen was used to show an interaction between active Rac1 and PAK as a proof of principle. (b) The ALPHA screen did not detect any interaction between active Rac1 and Dbs DH/PH. In both cases, the concentration of both binding partners was varied at the same time so that equimolar amounts of each protein were used for every assay point.

CHAPTER 4: ACTIVATION OF TIAM1 BY ACTIVE RAP1A

Introduction

The Par-3 polarity complex, which consists of Par-3, Par-6, and PKC-ζ, regulates critical cellular processes such as dendritic spine morphogenesis, polarization of T-cells, and formation of tight junctions [90, 223, 224]. Several studies have now shown that activation of the Par-3 polarity complex results in robust activation of Rac1 by the Dbl-family GEF Tiam1 [90, 223, 224, 232].

Par-3, like Par-6, belongs to the *partitioning defective* class of proteins that are evolutionarily conserved from *C. elegans* to humans [276]. Par-3 is a large protein of ~1300 amino acids that has several splice variants. The protein is characterized by the presence of three PDZ domains used to mediate protein-protein interactions. The PDZ domains are flanked by a conserved region at the N-terminus whose function is relatively unknown and an atypical PKC (aPKC) binding region followed by coiled-coiled regions at the C-terminus [223, 232, 276]. The first PDZ domain of Par-3 interacts with Par-6, while the kinase domain of PKC-ζ interacts with Par-3 through the aPKC binding region [232]. Finally, co-immunoprecipitation studies have also shown that Par-3 interacts with Tiam1; the binding interface is mediated through the C-terminal region of Par-3 (third PDZ domain to the C-terminus) and the N-terminal PH domain and tandem coiled-coiled region of Tiam1 [90, 223, 232] (**see Figure 24**). Signaling cues responsible for activating the Par-3 polarity complex

are not completely elucidated, but several studies have shown that active Cdc42 interacts with the polarity complex through Par-6 and is necessary for the signaling activity of the complex [90, 232, 276].

Recently, studies have also identified the Ras-related GTPases Rap1a and -1b as key regulators of the Par-3 polarity complex. Active Rap1 isoforms mediate the activity of the Par-3 polarity complex by interacting with Tiam1 [90], which is an effector of H-Ras [73]. Activation of Rap1 is known to be critical for several processes that the Par-3 polarity complex is also involved in, such as neuronal polarization, T-cell polarization, and formation of tight junctions [79, 80, 90]; thus, its interaction with the Par-3 polarity complex is not surprising. Two separate labs have now been able to use co-immunoprecipitation to show an interaction between active Rap1a or -1b and Tiam1; the interaction is nucleotide dependent, suggesting that Tiam1 functions as an effector of Rap1 isoforms [89, 90].

The mechanisms by which active Rap1 isoforms activate Tiam1 are not completely elucidated, but deletion analysis has suggested that Rap1a and -1b interact with Tiam1 through the DH/PH cassette of the Dbl-family GEF. This interaction is thought to mediate proper sub-cellular localization of Tiam1 and ensure tight regulation of the activity of Rac1 in the context of cell spreading [89]. Interestingly, the Par-3 polarity complex also exacts precise spatio-temporal control on the activation of Tiam1 by influencing sub-cellular localization and likely activating Rac1 in the presence of appropriate effectors [224].

The goal of these studies is to determine if active Rap1a interacts directly with Tiam1 as a *bona fide* effector. If Tiam1 does interact directly with active Rap1a, we will also delineate the regions of Tiam1 responsible for mediating this interaction and further investigate the mechanisms used by active Rap1a to activate Tiam1. Several examples now

exist in the literature of GEFs acting as effectors of GTPases, and the results of these studies will provide a better understanding of the mechanisms that link the activation of two GTPases via a Dbl-family GEF (**see Figures 2, 7, 8, and 10**).

Experimental Procedures

Cloning of GST-tagged constructs of Tiam1

The following constructs of Tiam1 were cloned into pMCSG7 containing an N-terminal GST-tag followed by a TEV cleavage site (henceforth referred to as pLIC-GST) using standard cloning techniques: DH/PH (residues 1022 – 1406), PH to PH (residues 421 – 1406), PH to CC (residue 421 – 595), PH to CCext (residues 421 – 772), PH to RBD (residue 421 – 842), RBD to PH (residues 755 – 1406), and PDZ to PH (residues 834 – 1406)..

Protein purification

GST-Tiam1 DH/PH was amplified from pLIC-GST in the BL21 (DE3) *E. coli* strain. Transformed cells were grown in Zym-5052 [275] self-inducing media supplemented with 0.1 mg/mL ampicillin for 3 hours at 37ºC and then for an additional 16 – 18 hours at 20ºC. Cells were resuspended in buffer A (200 mM NaCl, 20 mM Tris, pH 8.0, 2 mM DTT and 10% glycerol) and lysed with an Emulsi-Flex C5 (Avestin). Lysate was clarified by centrifugation at > 125,000 x *g* for 30 minutes and the supernatant was loaded onto a GST-Sepharose affinity column (GE Healthcare) equilibrated in buffer A. The column was washed with buffer A, and Tiam1 was eluted with buffer A containing 10 mM glutathione (reduced). The fractions containing Tiam1 were further purified using a 26/60 Sephacryl-200 size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0, 2 mM

DTT, 200 mM NaCl, and 10% glycerol. Fractions containing Tiam1 were pooled, concentrated, and stored at -80°C.

GST-Tiam1 PH to PH was also expressed from pLIC-GST in the BL21 (DE3) *E. coli* strain. Transformed cells were grown exactly as described for GST-Tiam1 DH/PH. Harvested cells were resuspended in buffer B (1 M NaCl, 20 mM Tris, pH 8.0, 2 mM DTT, 10% glycerol) and lysed and clarified as described above. The supernatant was treated with 0.5% PEI for 30 minutes on ice to remove excess DNA bound to the GST-Tiam1 PH to PH and the lysate was clarified again by ultracentrifugation as described above. The resulting supernatant was applied to a GST-Sepharose affinity column (GE Healthcare) equilibrated in buffer B. The column was washed with buffer B, and Tiam1 was eluted with buffer B containing 10 mM glutathione (reduced). The fractions containing Tiam1 were further purified using a 26/60 Sephacryl-300 size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0, 2 mM DTT, 200 mM NaCl, and 10% glycerol. Fractions containing Tiam1 were pooled, concentrated, and stored at -80°C.

GST-Tiam1 PH to CC, PH to CCext, PH to RBD, RBD to PH, and PDZ to PH were all expressed from pLIC-GST in the BL21 (DE3) *E. coli* strain. Transformed cells were grown in LB media containing 0.1 mg/ml ampicillin at 37°C to an OD_{600} of 0.7 (mid-log phase) and induced with 0.1 mM IPTG at 27ºC for 5 hours (PH to CC and PH to CCext) or at 19ºC for 16-18 hours (PH to RBD, RBD to PH, and PDZ to PH). Harvested cells were resuspended in buffer C (2 mM DTT, 20 mM Tris, pH 8.0, 10% glycerol, and 200 mM NaCl for the PH to CC construct or 1M NaCl for all of the other constructs) and lysed and clarified as described above for GST-Tiam1 DH/PH. The supernatant was applied to a GST-Sepharose affinity column (GE Healthcare) equilibrated in buffer C. The column was

washed with buffer C, and the various pieces of Tiam1 were eluted with buffer C containing 10 mM glutathione (reduced). The fractions containing Tiam1 were further purified using a 26/60 Sephacryl-200 size exclusion column (PH to CC, PH to CCext, and PH to RBD) or 26/60 Sephacryl-300 size exclusion column (RBD to PH and PDZ to PH) equilibrated with 20 mM Hepes, pH 7.5, 2 mM DTT, 200 mM NaCl, and 10% glycerol. Fractions containing Tiam1 were pooled, concentrated, and stored at -80°C.

His_6-tagged Tiam1 PH to PH was expressed from pPROEX-HTb in the BL21 (DE3) *E. coli* strain. Transformed cells were grown in LB media containing 0.1 mg/ml ampicillin at 37°C to an OD_{600} of 0.7 (mid-log phase) and induced with 0.1 mM IPTG at 27ºC for 5 hours. Harvested cells were resuspended in buffer D (300 mM NaCl, 10% glycerol, 20 mM Tris, pH 8.0) with 10 mM imidazole and then lysed and clarified as described above. The supernatant was treated with 0.5% PEI for 30 minutes on ice to remove excess DNA bound to the protein and the lysate was clarified again by ultracentrifugation as described above. The resulting supernatant was applied to a Ni^{2+}-Sepharose affinity column (GE Healthcare) equilibrated in buffer D containing 10 mM imidazole. The column was washed with buffer D containing 55 mM imidazole and His_6-tagged Tiam1 PH to PH was eluted with buffer D containing 400 mM imidazole. Fractions containing Tiam1 were pooled and dialyzed into buffer E (200 mM NaCl, 10% glycerol, 20 mM Hepes, pH 7.0, and 1 mM DTT) before being applied to a SP-Sepharose Fast Flow 26/10 column (GE Healthcare). Tiam1 PH to PH was eluted from the column using buffer E with an increasing gradient of NaCl and further purified using a 26/60 Sephacryl-300 size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0, 2 mM DTT, 200 mM NaCl, and 10% glycerol. Fractions containing Tiam1 PH to PH were pooled, concentrated, flash-frozen, and stored at -80°C.

Human Rap1a and Rap1b (residue 1-181, C181S) (henceforth soluble Rap1) were both expressed from pPROEX-HTa in the BL21 (DE3) *E. coli* strain. Transformed cells were grown in LB media containing 0.1 mg/ml ampicillin at 37°C to an OD_{600} of 0.7 (mid-log phase) and induced with 1 mM IPTG at 27ºC for 5 hours. Harvested cells were resuspended in buffer F (200 mM NaCl, 10% glycerol, and 20 mM Tris, pH 8.0) with 5 mM imidazole. The supernatant from lysed and clarified cells (as described above) was applied to a Ni^{2+}-Sepharose affinity column (GE Healthcare) equilibrated in buffer F containing 5 mM imidazole. His_6-Rap1 was eluted analogously to His_6-Tiam1 PH to PH. Fractions containing soluble Rap1a were further purified using a 26/60 Sephacryl-200 size exclusion column (GE Healthcare) equilibrated with 20 mM Tris, pH 8.0, 2 mM DTT, 200 mM NaCl, 2 mM $MgCl_2$ and 10% glycerol. Fractions containing soluble Rap1a were pooled, concentrated, and stored at -80°C.

RalGDS RBD (generously provided by Dr. Leslie Parise, UNC-Chapel Hill) was expressed from pGEX2T in the BL21 (DE3) *E. coli* strain. Transformed cells were grown in Zym-5052 and purified exactly as described for GST-Tiam1 DH/PH above.

Loading of Rap1a and -1b with guanine nucleotides

Purified GTPases were incubated in buffer G (1 mM $MgCl_2$, 150 mM NaCl, and 20 mM Hepes, pH 7.5) with 10 mM EDTA in the presence of a 10-fold molar excess of GDP or GTPγS for 1 hour at room temperature. $MgCl_2$ (20 mM) was added to stop the exchange reaction. For use in guanine nucleotide exchange assays or ALPHA screens, a HiTrap Desalting column (GE Healthcare) was used to remove excess nucleotide and buffer exchange the protein into buffer G for the guanine nucleotide exchange assays or 2 mM

$MgCl_2$, 200 mM NaCl, 20 mM Hepes, pH 7.5, 5% glycerol, and 2mM DTT for ALPHA screens before concentrating and freezing for storage at -80ºC. Efficiency of the loading reaction was verified by checking binding of Rap1a and -1b with its *bona fide* effector RalGDS RBD on a native gel.

ALPHA screens

His_6-tagged binding partner and GST-tagged binding partner were incubated together (at the indicated concentrations) for 30 minutes in 20 mM Hepes, pH 7.5, 1 mM $MgCl_2$, 5% glycerol, and 150 mM NaCl at room temperature. Donor beads conjugated to glutathione (final concentration 20 µg/mL) and accepter nickel chelate beads (final concentration 20 µg/mL) (Perkin Elmer) were incubated with the protein interaction partners for another 30 minutes in the absence of light. Protein interaction was assessed by measuring the emission at 580 nM after excitation at 670 nM in the Pherastar microplate reader.

Native gels

Proteins were applied to native gels (PHASTgel, GE Healthcare) in the indicated amounts according to the manufacturer's protocol.

Results

The ALPHA screen does not detect a direct interaction between active Rap1a and Tiam1

Previous studies have suggested that Tiam1 interacts with Rap1a in a nucleotide dependent manner [89, 90]. The binding interface for this interaction has been mapped to the DH-associated PH domain of Tiam1 [89]; however, Tiam1 also contains an RBD domain

that might interact with active Rap1a. To test if the interaction between active Rap1a and Tiam1 is direct, we purified soluble Rap1a with an N- terminal His_6-tag and loaded it with GTPγS to mimic the active state. The functionality of the GTPγS-loaded Rap1a was tested by confirming its ability to interact with RalGDS RBD in the ALPHA screen (**see Figure 25**). We also purified various GST-tagged fragments of Tiam1 encompassing the region from the N-terminal PH domain to the C-terminal PH domain (**see Figure 26**). Although previous studies have been able to co-immunoprecipitate the PH to PH form of Tiam1 (full-length Tiam1) with active Rap1, evidence suggests that full-length Tiam1 might be auto-inhibited (unpublished data, Sondek Lab), potentially masking the binding site for Rap1a. Thus, we chose to use several fragments of Tiam1 to maximize the possibility of interacting with active Rap1a and potentially further delineate the region of Tiam1 responsible for this interaction. However, the ALPHA screens performed as described in the experimental procedures section are unable to detect an interaction between any of the fragments of Tiam1 and Rap1a loaded with GTPγS. The weak interaction seen between fragments of Tiam1 containing the N-terminal PH domain (except for the PH to PH fragment) and what appears to be Rap1a-GTPγS is actually an interaction between the fragments of Tiam1 and the nickel chelate acceptor beads (**see Figure 27**).

Full-length Tiam1 and Rap1a do not interact in a nucleotide dependent manner, as assessed by native gel analysis

Although we do not detect an interaction between active Rap1a and Tiam1 in the ALPHA screens, we wanted to confirm the lack of a specific, nucleotide-dependent

interaction using a native gel. As with the ALPHA screens, the nucleotide bound state of Rap1 was confirmed by measuring a nucleotide-dependent interaction with the RBD domain of RalGDS (**see Figure 28**). Again, we are unable to detect a specific, nucleotide dependent interaction between full-length Tiam1 and active Rap1a (**see Figure 29**). Tiam1 PH to PH is unable to enter the resolving buffer of the native gel, but does appear to interact with some Rap1a-GTPγS and prevent it from entering the resolving buffer; however, the same effect is seen with Rap1a-GDP, suggesting that the interaction between Tiam1 and Rap1a is non-specific.

Discussion

Our data conclusively shows that Tiam1 does not interact directly with active Rap1a, despite previous data suggesting otherwise and the presence of a putative RBD domain. Interestingly, these data support recent biochemical studies which also detect no interaction between the RBD domain of Tiam1 and either active Rap1a and -1b or Ras isoforms [78, 91].

While Tiam1 and active Rap1a may interact in a cellular context, the interaction is not direct and may be mediated by other components within the cell. Since active Rap1a and Tiam1 interact as part of the Par-3 polarity complex, an attractive possibility is that while Rap1a does not interact directly with Tiam1, it might bind to another component of the Par-3 polarity complex such as Par-3, Par-6, PKC-ζ, Cdc42, or Wrch-1. Thus far, this possibility has not been tested.

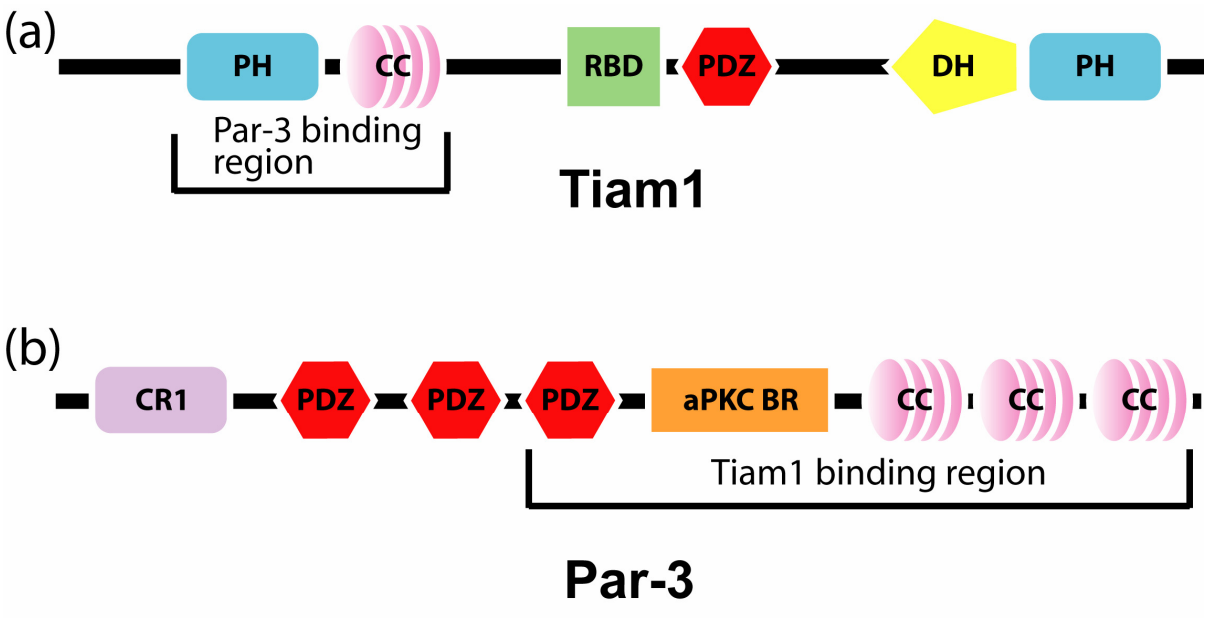


Figure 24: Putative binding interface between Par-3 and Tiam1.

(a) Domain architecture of Tiam1. Region thought to interact with Par-3 is indicated. (b) Domain architecture of Par-3. Region thought to interact with Tiam1 is indicated.

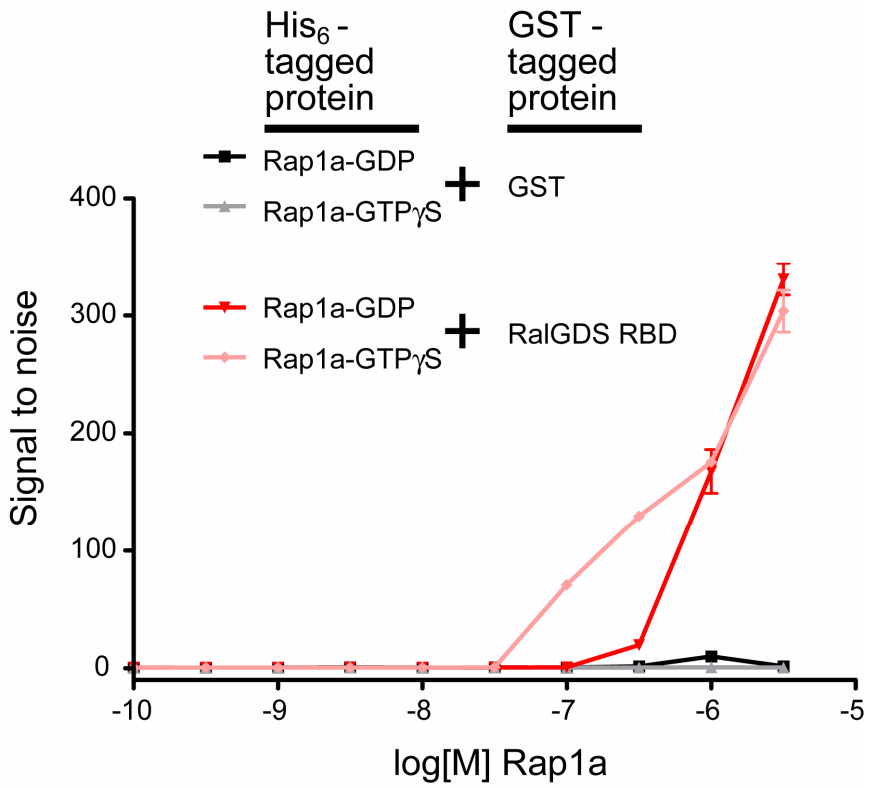


Figure 25: RalGDS RBD interacts with Rap1a-GTPγS with a higher affinity than Rap1a-GDP.
The ALPHA screen was used to ensure that GTPγS-loaded Rap1a had a higher affinity for RalGDS RBD than Rap1a-GDP. The concentrations of GST and RalGDS RBD were held fixed at 1 μM.

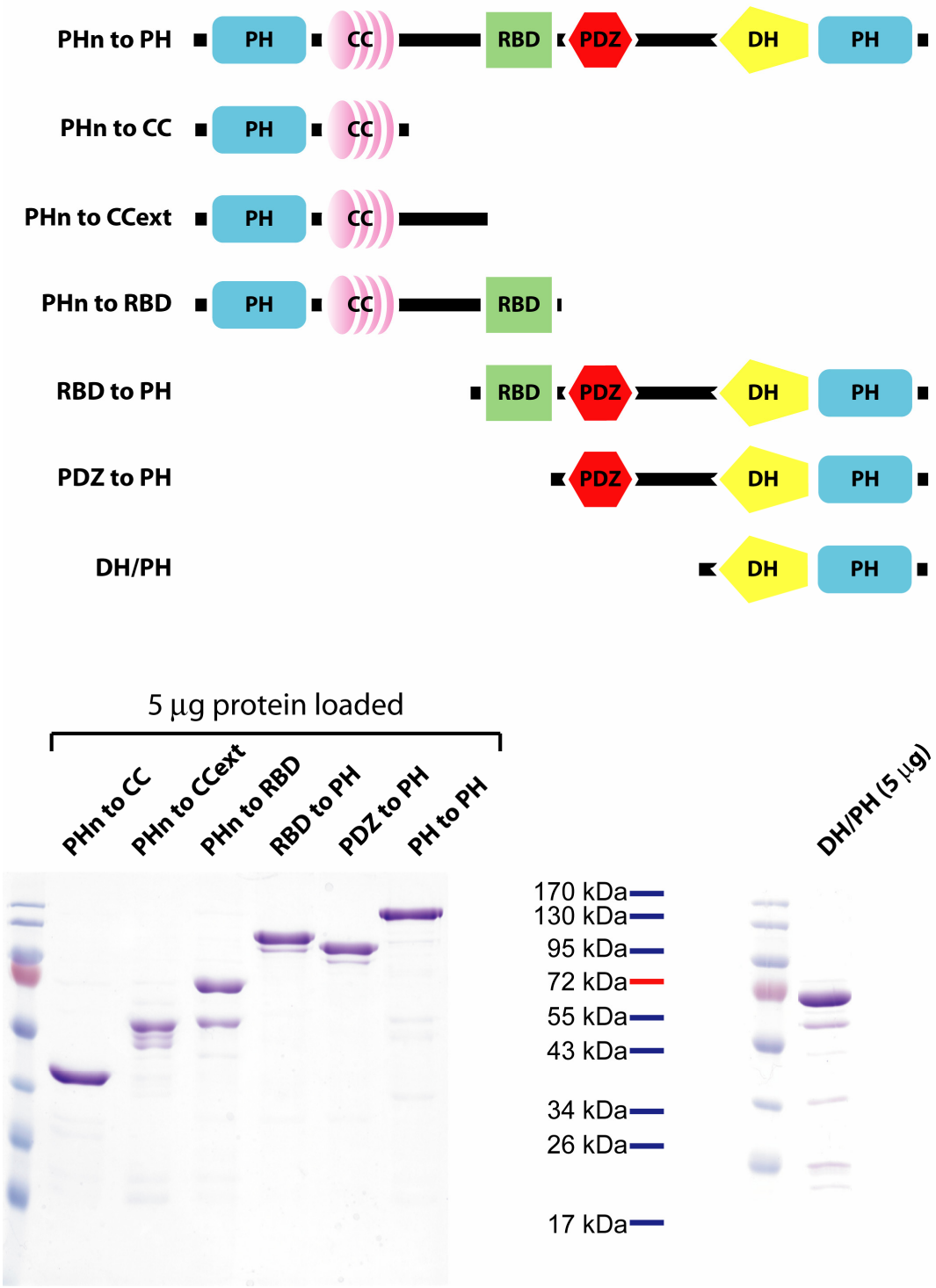


Figure 26: Fragments of Tiam1 tested for interaction with Rap1a-GTPγS using the ALPHA screen.

The domain architecture of the various GST-tagged fragments of Tiam1 tested for interaction with active Rap1a is depicted above. The GST tag is N-terminal in all cases. All fragments have been purified to near homogeneity (below).

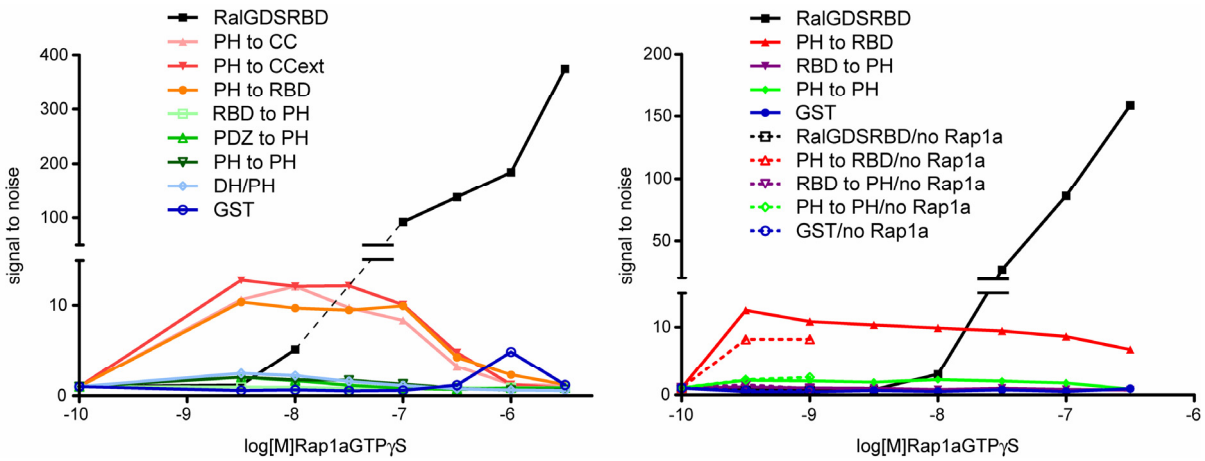


Figure 27: Rap1a-GTP does not interact with Tiam1.

The ALPHA screen is used to test if various fragments of Tiam1 interact with Rap1a-GTPγS. All fragments of Tiam1 are tagged with GST, as is the RalGDS RBD. The concentration of the GST tagged proteins was held fixed at 1 μM. Fragments of Tiam1 that contain the N-terminal PH domain (with the exception of PH to PH) interact weakly with the nickel chelate acceptor beads. Rap1-GTPγS is functional as it interacts robustly with RalGDS RBD. The ALPHA screen was carried out as described in experimental procedures.

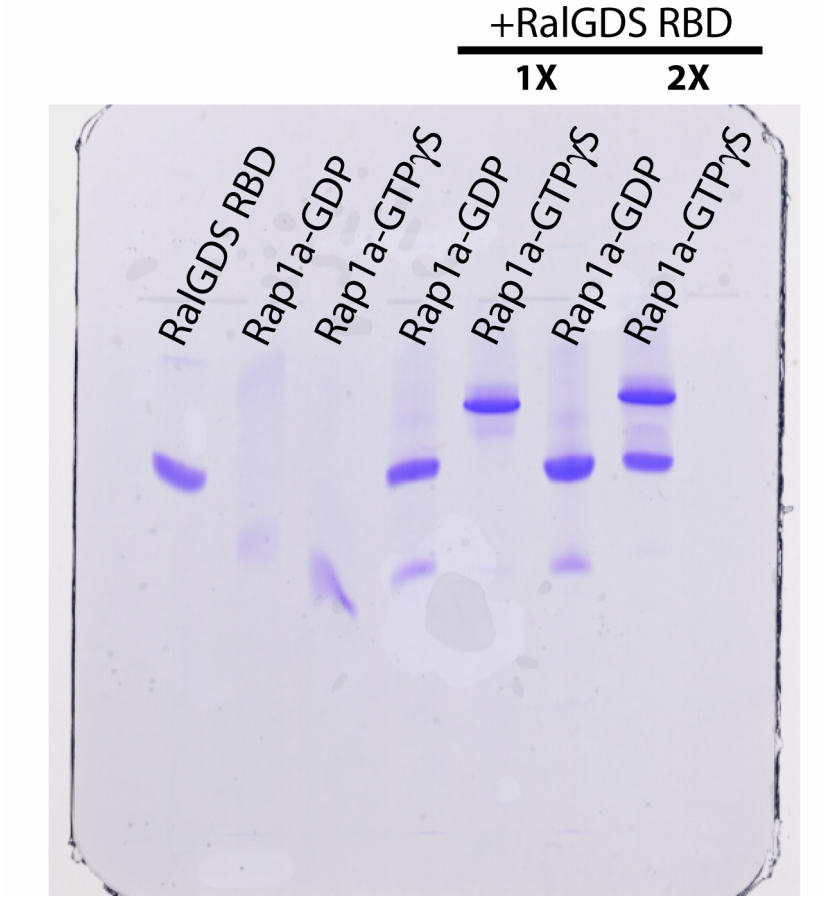


Figure 28: RalGDS RBD interacts specifically with the GTPγS-loaded form of Rap1a.

Both Rap1a-GDP and Rap1a-GTPγS (0.2 nmoles) were incubated with 1X or 2X RalGDS RBD (molar concentrations) for 20 minutes at room temperature in the following buffer: 1 mM $MgCl_2$, 150 mM NaCl, 20 mM Hepes, pH 7.5, and 10% glycerol. A gel-shift, indicating an interaction, is visible when RalGDS RBD is incubated with Rap1a-GTPγS but not Rap1a-GDP.

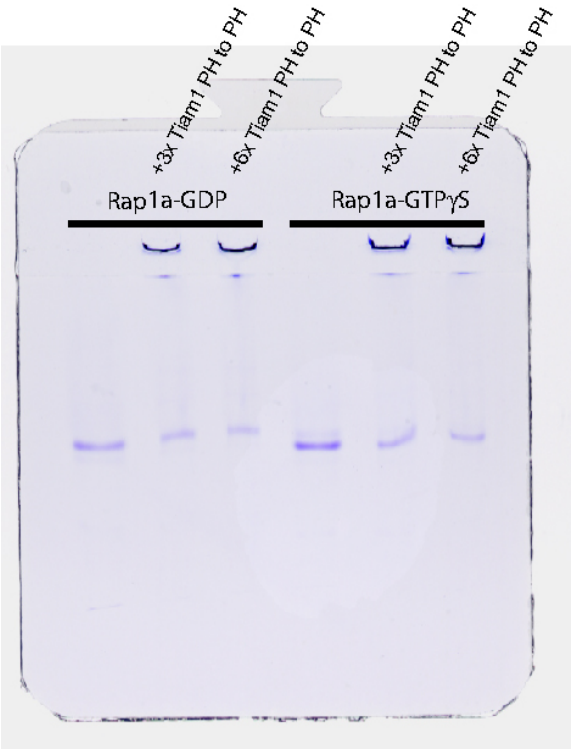


Figure 29: Tiam1 PH to PH does not interact specifically with Rap1a-GTPγS.

Rap1a (0.2 nmoles) bound to GDP or GTPγS was incubated with the indicated amounts of Tiam1 PH to PH overnight at 4ºC before loading the proteins on a native gel. Proteins were incubated in the following buffer: 1 mM $MgCl_2$, 150 mM NaCl, 20 mM Hepes, pH 7.5, and 10% glycerol.

CONCLUSIONS AND FUTURE DIRECTIONS

Nucleotide exchange assisted by PH domains

Despite the conserved position of DH-associated PH domains, past work examining their function has been unable to assign them a conserved role. DH-associated PH domains have been shown to function as membrane anchors, assist in translocation of Dbl-family GEFs to cellular membranes, serve as sites for allosteric modulation by phosphoinositides, and mediate interaction with other proteins within the cell [8, 184, 191, 193]. This work examines another emerging role for DH-associated PH domains: participating directly in guanine nucleotide exchange by forming direct contacts with the cognate GTPase. Functional characterization of the N-terminal DH-associated PH domain of Trio builds on earlier studies with Dbs, LARG, and PDZRhoGEF that show DH-associated PH domains can stabilize the interaction between the DH domain and the cognate GTPase by also contributing to functional interactions with the cognate GTPase [166, 168, 205].

In the cases of Dbs and Trio-N, mutational analysis has shown that the interface between the DH-associated PH domain and the cognate GTPase is functionally significant both *in vitro* and *in vivo* [160]. In contrast, studies have confirmed the functional significance of the DH-associated PH domains of LARG and PDZRhoGEF only *in vitro* [168, 205]. Both LARG and PDZRhoGEF directly link stimulation of G-protein-coupled receptors by agonists such as endothelin-1, angiotensin II, thromboxane 2A, and thrombin to

activation of RhoA and subsequent regulation of Ca^{2+} levels [205, 219, 277-279]. As alluded to, RhoA is critical for cardiac function and linked to functions such as Ca^{2+}- independent contractility of the vascular smooth muscle [205, 280] and expression of endothelin-1, a potent vasoconstrictor [281]. Unregulated increases in contraction can result in hypertension and coronary spasms [143] while vasoconstrictors affect vascular tone and remodeling [143]. Mutations to the DH-associated PH domain that affect nucleotide exchange by LARG and PDZRhoGEF *in vitro* most likely affect regulation of RhoA activation downstream of GPCRs *in vivo*. In order to test this hypothesis one could assay for SRF activation, which has been used previously to test activation of LARG downstream of $G\alpha_{12}$ and $G\alpha_{13}$ [209].

Trio-N and Dbs are part of a larger subset of Dbl-family GEFs that include Dbl, Duo, Duet, and Obscurin. Residues participating in the interactions between the PH domains of Dbs and Trio and their cognate GTPases are conserved in these Dbl-family GEFs (**see Figure 11**). One could use mutational analysis, coupled with *in vitro* exchange assays and transformation assays to determine if sequence analysis can be used to delineate the ubiquity of using DH-associated PH domains as direct participants in catalyzing nucleotide exchange. Similar analysis would be informative for other Dbl-family GEFs closely related to PDZRhoGEF and LARG.

Unlike Dbs, Trio-N, LARG, and PDZRhoGEF, the crystal structures of several other DH/PH cassettes in complex with their cognate GTPases show no interaction between the DH-associated PH domain and the GTPase [158, 167, 170]. However, while the solved structures of Dbl-family GEFs in complex with cognate GTPases provide valuable information about the mechanisms regulating catalytic exchange, they do not take into account the contribution of cellular membranes to exchange activity. Dbs DH/PH shows

contacts between the cognate GTPase and the DH-associated PH domain *in vitro* while Tiam1 DH/PH does not. However, neither GEF requires its PH domain for proper sub-cellular localization, even though both require the ability to bind phosphoinositides for function *in vivo* [159, 160]. This suggests that their DH-associated PH domains may play a similar role *in vivo* that is not apparent *in vitro.* In addition, structural analysis has shown that DH/PH cassettes are highly flexible (**see Figure 6**) and can adopt a variety of conformations that would allow contact between the PH domain and the GTPase under the proper conditions [158, 166, 206].

One possible model that is supported by all of the previously discussed data, and would function as a conserved mechanism of exchange for Dbl-family GEFs in a physiologically relevant setting, is depicted in **Figure 30**. In this model, binding to a phosphoinositide headgroup would enable the flexible DH/PH cassette to adopt a conformation allowing contact between the PH domain and the membrane-bound GTPases [160]. Thus, effective exchange would require DH-associated PH domains to bind phosphoinositides and interact with cognate GTPases. As DH-associated PH domains have different phosphoinositide binding specificities [176], this system would also provide a means of regulating exchange activity through fluctuating phosphoinositide levels.

An intriguing study by Robbe *et al.* shows that Tiam1 DH/PH has a higher exchange activity on prenylated Rac1 inserted into lipid vesicles than it does on soluble Rac1 in the presence of lipid vesicles, supporting the model suggested in **Figure 30**. However, as DH-associated PH domains can bind phosphoinositides, albeit with low affinity, it is possible that the increase in exchange activity by Tiam1 on prenylated Rac1 in lipid vesicles is due to an increase in the effective concentration of Tiam1 near lipid vesicles. To address this issue, the

Dbl-family GEFs can be physically attached to the membrane in biotinylated lipid vesicles via a streptavidin/biotin interaction (**see Figure 31**). A flexible linker in between the streptavidin tag and the Dbl-family GEF would restrict localization without severely restricting the conformation of the DH/PH cassette and remove any variation in its effective concentration. The streptavidin/biotin interaction is extremely tight ($K_d \approx 10^{-15}$ M) [282] and it is unlikely that the streptavidin-tagged Dbl-family GEF will dissociate back into solution once bound to the vesicle. Mutations can then be introduced that abrogate phosphoinositide binding by the PH domain before using exchange assays to measure if these mutations diminish exchange activity on prenylated GTPases

Although this system is novel to the study of exchange by Dbl-family GEFs, similar systems have been used previously to study the role of membranes in regulating the stimulation of $G\alpha_s$, which stimulates adenylate cyclase, by β-adrenergic receptors, and in regulating the GAP activity of RGS proteins [283, 284]. Two obvious GEFs that lend themselves to analysis using this system are Dbs and Tiam1 as they represent two classes of Dbl-family GEFs: those that use their PH domains to catalyze exchange as determined by structural analyses and those that do not.

As previously mentioned, both intersectin and collybistin in complex with their cognate GTPase Cdc42 show no contacts between their DH-associated PH domains and Cdc42. If the PH domain does play a conserved role in regulating nucleotide exchange *in vivo* by interacting with phosphoinositides at the membrane, both intersectin and collybistin should behave analogously to Tiam1. More specifically, one would not expect to see mutations abrogating phosphoinositide binding also have an effect on the sub-cellular

localization of these GEFs. However, one would expect to see reduced exchange activity *in vivo* as assessed by transformation assays or a similar functional readout.

Activation of Dbs by active Rac1

Although we do not see an interaction between purified Dbs DH/PH and Rac1-GTPγS, it is still possible that the two proteins interact but require another cellular component. One possibility is that active Rac1 binds the DH-associated PH domain of Dbs only in the presence of phosphoinositides, similar to the interaction between active Arf-6 and ARNO [46, 48]. Thus, it would be beneficial to repeat the SPR or ALPHA screen experiments detailed in Chapter 3 to test an interaction between active Rac1 and Dbs DH/PH in the presence of PI(4,5)P2 (**see Table 1**).

Regulation of Tiam1 and Par-3 polarity complex by active Rap1a

While active Rap1a does not interact directly with Tiam1, it is possible that the interaction is mediated either by a portion of Par-3 or another member of the Par-3 polarity complex. We are currently in the process of cloning and optimizing purification protocols for sections of Par-3 and will test them for interaction with both active Rap1 and Tiam1.

Interestingly, recent data has emerged showing an interaction between active Rap1 and STEF (also known as Tiam2). Like Tiam1, STEF possesses an N-terminal PH domain followed by a coiled-coiled region, RBD domain, PDZ domain, and DH/PH cassette. STEF has also been shown to be regulated by the Par-3 polarity complex. Co-immunoprecipitation experiments suggest that the interaction between STEF and active Rap1a is mediated through the coiled-coiled region following the PH domain. This interaction is not reproducible with

Tiam1 despite STEF and Tiam1 being closely related [91]. The studies that initially characterized an interaction between isoforms of active Rap1 and endogenous Tiam1 may have used antibodies that showed cross-reactivity with STEF. A sequence alignment of the coiled-coiled regions of STEF and Tiam1 (**see Figure 32**) indicates that the regions are highly homologous, but not completely identical. Thus, the residues in the coiled-coiled domain of STEF may be able to mediate an interaction with active Rap1a, unlike Tiam1. We would like to use purified components to test if STEF interacts directly with active Rap1a.

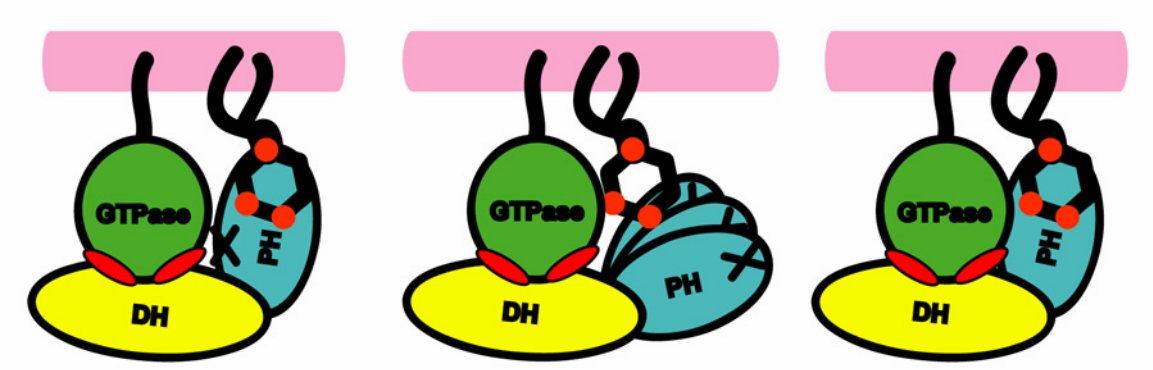


Figure 30: Proposed model of nucleotide exchange.

In the proposed model of nucleotide exchange, binding to phoshoinositides at a membrane locks the DH/PH cassette into a conformation that allows contact between the PH domain and the cognate GTPase (right). Mutations in the PH domain that abrogate binding to phosphoinositides (center) or the GTPase (left) both compromise exchange activity. While most GEFs contain other regulatory domains, only the DH/PH cassette has been depicted. Model adapted from Rossman et al [160].

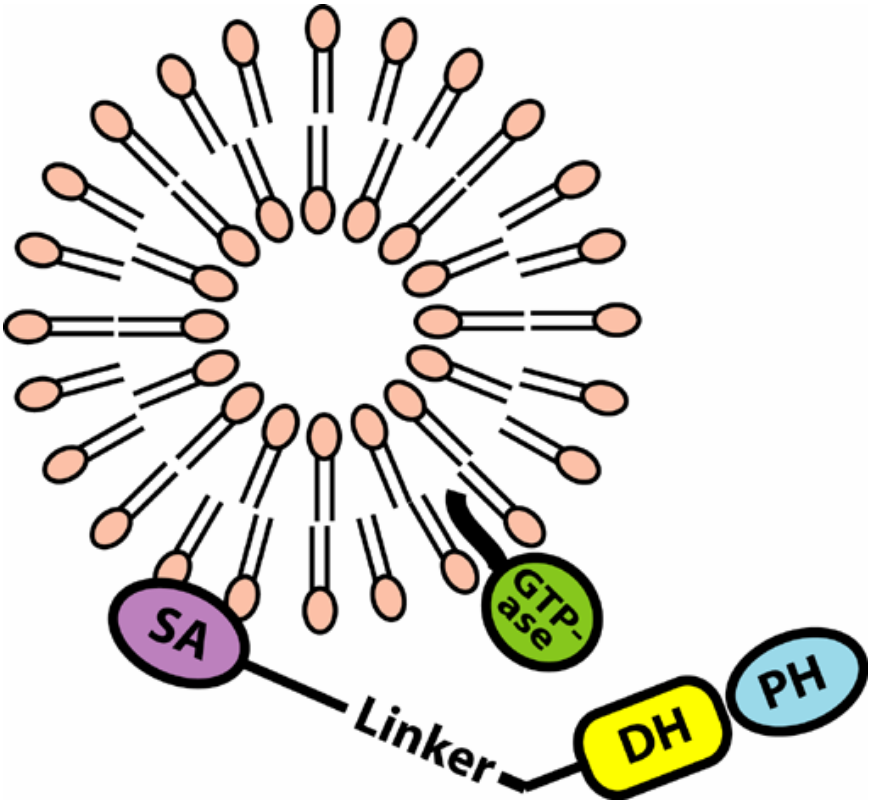


Figure 31: In vitro system for measuring exchange at a membrane.
Prenylated GTPases will be reconstituted in phosphoinositide containing biotinylated vesicles. DH/PH cassettes of Dbl-family GEFs linked to a streptavidin tag (purple) will be attached to the lipid vesicle via a streptavidin/biotin interaction.

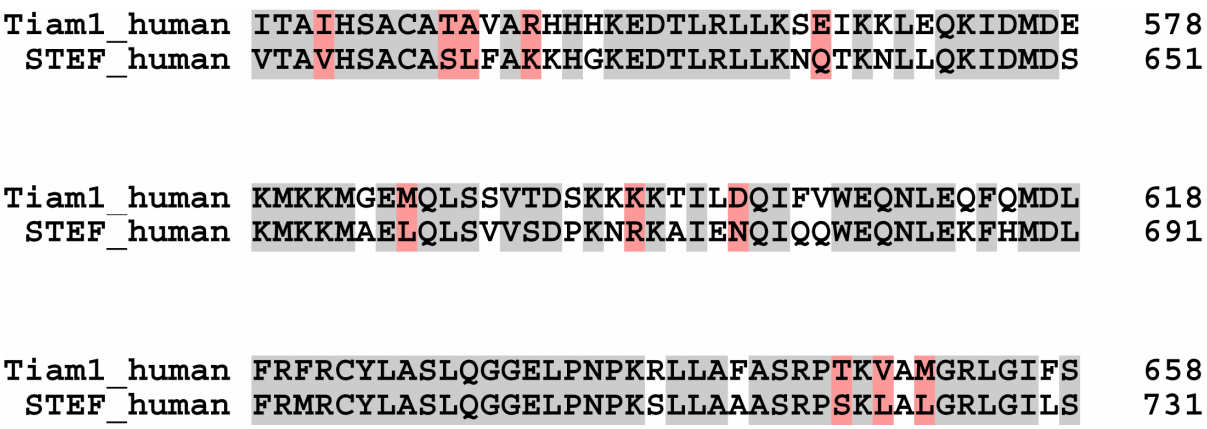


Figure 32: Sequence alignment of the coiled-coiled regions of STEF and Tiam1.

The coiled-coiled regions that follow the N-terminal PH domain of Tiam1 and STEF (residues 539 – 658 and residues 612 – 731, respectively) were aligned using Clustal X. Identical residues are highlighted in grey, while similar residues are highlighted in pink.

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