THE DOCK FAMILY OF ATYPICAL GUANINE NUCLEOTIDE EXCHANGE FACTORS: REGULATION BY ELMO1 AND RHOG

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ABSTRACT

CYNTHIA P. HOLLEY: The Dock Family of Atypical Guanine Nucleotide Exchange Factors: Regulation by ELMO1 and RhoG
(Under the direction of John Sondek)

The Dock family of proteins regulates diverse biological processes including cell migration, phagocytosis and neuronal polarization. These proteins contain a unique type of guanine nucleotide exchange factor (GEF) domain, and function as GEFs for Rho-family GTPases. Several Dock-family proteins form complexes with ELMO proteins and the Dock/ELMO complex acts as a bi-partite GEF for Rac. Molecular details of how the Dock/ELMO complexes bind and exchange nucleotide on Rac are critical for our understanding of their biological effects, yet remain poorly defined.

As described here, purified Dock2/ELMO1 complex is a stable heterotetramer composed of two molecules each of Dock2 and ELMO1. This heterotetramer coordinates a *single* molecule of nucleotide-free Rac. We identify an inhibitory conformation within ELMO1 mediated through contacts between the N- and C-terminal regions of ELMO1 and describe a mechanism for relief of this inhibition through the binding of RhoG, another Rho-family GTPase. The interaction between RhoG and ELMO1 is both nucleotide-dependent, and dependent upon the C-terminal polybasic region of RhoG. These data provide fundamentally important molecular insights into the composition of the Dock/ELMO complex and regulation of nucleotide exchange via the Dock/ELMO proteins.

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LIST OF ABBREVIATIONS

3-AT	3-amino-1,2,4-triazole
A_{600}	Absorbance at 600 nm
ACG	Activated Cdc42-associated GEF
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
ADP	Adenoside 5'-diphosphate
APP	Amyloid precursor protein
ARF6	ADP-ribosylation factor 6
ArhGEF8 (Net1)	(Neuroepithelial transforming protein-1)
ARM	Armadillo
ARNO	ARF nucleotide binding site opener
BAI1	Brain-specific angiogenesis inhibitor 1
BLAST	Basic local alignment search tool
BODIPY	Dipyrromethene boron difluoride
BODIPY-FL	BODIPY fluorescein
BSA	Bovine serum albumin
C	Cysteine
C2	Ca^{2+} binding domain
$CD4^{+}$	Cluster of differentiation 4 positive
CDM	Ced-5/Dock180/Myoblast city
Ced-5	Cell death abnormality 5
CHO-K1	Chinese hamster ovary cell line K1

COS-7	African green monkey SV40 kidney fibroblast cell line
C-terminus	Carboxy-terminus
CZH-1	CDM-zizimin homology 1
CZH-2	CDM-zizimin homology 2
DAD	Diaphanous autoregulatory domain
Dbl	Diffuse B-cell lymphoma
DH	Dbl-homology
DHR-1	Dock homology region 1
DHR-2	Dock homology region 2
DNA	Deoxyribonucleic acid
Dock180	180 kDa protein downstream of Crk
DTT	Dithiothreitol
E	Glutamic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
ELMO	Engulfment and cell motility
F	Phenylalanine
FBS	Fetal bovine serum
FCS	Fetal calf serum
G	Glycine
GAP	GTPase accelerating protein
GBD	GTPase binding domain
GDI	Guanine dissociation inhibitor

GDP	Guanosine 5’-diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guanosine 5’-triphosphate
GTPase	Guanosine triphosphatase
GTPγS	Guanosine 5'-(γ-thio)triphosphate
HA	Hemagglutinin
HEK293T	Human embryonic kidney 293T cell line
HeLa	Henrietta Lacks cervical cancer cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1	Human immunodeficiency virus 1
HRP	Horseradish peroxidase
IBP	IRF-4 binding protein
IL-4Rα	Interleukin 4 receptor alpha
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITSN	Intersectin
JNK	C-Jun N-terminal kinase
kDa	Kilodalton
k_{obs}	Observed exchange rate
L	Leucine
LB	Luria-Bertani media
LIC	Ligation-independent cloning

MBC	Myoblast city
mDia1	Mammalian ortholog of Diaphanous 1
MES	2-(*N*-morpholino)ethanesulfonic acid
MFG-E8	Milk fat globule EGF factor 8
MOCA	Modifier of cell adehesion
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
N	Asparagine
NIH3T3	National Institutes of Health 3T3 cell line
NP-40	Nonidet P-40 detergent
N-terminus	Amino-terminus
Op18	Oncoprotein 18
p130Cas	P130 Crk-associated substrate
PBP	presinilin binding protein
PBR	Polybasic region
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PH	Pleckstrin homology
P_i	Inorganic phosphate
$PI(3,4,5)P_3$	Phosphatidylinositol (3,4,5)-trisphosphate
PMSF	Phenylmethanesulphonylfluoride
PRONE	Plant-specific Rop nucleotide exchanger
PS	Phosphatidylserine

PS1	Presinilin 1
Q	Glutamine
RANKL	Receptor Activator for Nuclear Factor κ B Ligand
Rop	RHO-related proteins from plants
RU	Response units
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SH2	Src-homology 2
SH3	Src-homology 3
SmgGDS	Guanine nucleotide dissociation stimulator for smg p21
SopE	Salmonella outer protein-E
Sos	Son of sevenless
SPR	Surface plasmon resonance
T	Threonine
TB	Terrific broth
TEV	Tobacco etch virus
Tiam1	T-cell invasion and metastasis factor
Tris	Tris-hydroxymethylaminomethane
TSC	Tuberous sclerosis
VCAM-1	Vascular cell adhesion molecule 1
W	Tryptophan
Wt	Wild-type
Zir	Zizimin-related

CHAPTER 1: INTRODUCTION

Small GTPases

Proteins from the Ras superfamily of small guanosine triphosphatases (GTPases) function as binary molecular switches within cellular signaling pathways. These proteins cycle from an inactive GDP-bound state to an active GTP-bound state. There are over 150 human proteins within this family, with orthologs in organisms ranging from roundworms and fruit flies to slime mold and plants [1].

Within this larger superfamily, the Rho family of small GTPases comprises one of five major branches grouped by sequence similarity [2]. Rho-family GTPases regulate cellular signaling pathways that coordinate actin reorganization and gene expression. Diverse cellular processes including phagocytosis, adhesion, migration, neurite extension and retraction, growth, survival and cell polarization are controlled through intricate regulation of these 22 Rho-family GTPases and the numerous effector proteins to which they bind [2].

Rho-family GTPases are typically located at a membrane within cells, and this association is enabled by a four-residue CAAX motif (for C = cysteine, A = aliphatic, X = any amino acid) at the C-terminus of the proteins [3]. This motif enables the lipid-modification of the protein at the cysteine residue which prompts the removal of the final three amino acids [3]. A polybasic region often precedes the CAAX motif, and can be

involved in membrane association as well, through electrostatic interactions with the generally negatively-charged phospholipid head groups of the membrane surface [4].

As the name implies, small GTPases have intrinsic GTP hydrolysis activity, and are capable of self-inactivation through hydrolysis of the gamma phosphate in GTP. The mechanism of activation involves dissociation of the bound GDP molecule, and then binding of the more prevalent GTP located within the cytosol [1]. Most small GTPases have a higher affinity for GTP compared to GDP. Two regions within the GTPases, called switch 1 and switch 2, change conformations depending upon the bound nucleotide [1]. The movement of the switch regions is what allows for binding to effector proteins in the GTP-bound state. The loading of GTP and hydrolysis of GTP occur relatively slowly, and thus, a variety of proteins act as catalysts and regulators of the GTPase cycle [1] (Fig. 1).

GTPase activating proteins, or GAPs, increase the intrinsic hydrolysis activity of the GTPase by stabilizing the GTPase active site in a conformation needed for hydrolysis [5]. The GAPs provide a system of control whereby the GTPases are rapidly inactivated, preventing overstimulation of signaling pathways. Highly transforming mutations in GTPases often disable the ability of the GTPase to hydrolyze GTP, or interfere with GAP binding [6,7].

Guanine dissociation inhibitors, or GDIs, prevent activation of GTPases by sequestering the inactive, GDP-bound form through specific binding and removal from the membrane [8]. Without proper localization to the membrane, the GTPases are unable to function in the proper signaling pathways, and therefore, signaling through those pathways is suppressed.

Guanine nucleotide exchange factors, or GEFs, catalyze the exchange of GDP for GTP, thereby activating the GTPase, enabling signaling to downstream effectors. The exchange reaction is catalyzed by the destabilization of nucleotide binding caused by the GEF, and the exchange of GDP for GTP is driven by the substantially higher ratio of GTP:GDP within the cell [9,10]. Once activated, the GTPases are able to bind to downstream effector proteins, setting off a chain-reaction-like string of events within the cell.

Guanine Nucleotide Exchange Factors

Although there are only 22 known human Rho-family members, there are approximately four times as many known human GEFs for these proteins [9]. Obviously, Rho-family GTPases can function in more than one cellular signaling pathway, and it has been suggested that exchange factors can determine in which pathway a specific GTPase will act by localization and even acting as a scaffold to bring requisite components of a specific pathway together [9].

The Dbl-family of GEFs is well-studied and the largest of the known Rho-family GEFs. Composed of 69 human members, Dbl-family GEFs can be identified by the presence of a Dbl homology (DH) domain followed by a pleckstrin homology (PH) domain [9]. Nucleotide exchange is catalyzed by the DH domain, while the purpose of the PH domain is less certain. In some cases, the PH domain participates in the GTPase binding surface, and in others it is far removed from the GTPase. Membrane localization, allosteric modulation through phospholipid binding, and protein interactions are all suggested roles for the PH domain [9-11].

The mechanism for exchange by Dbl-family GEFs involves significant change to the nucleotide-binding pocket of the GTPase caused by the binding of the exchange factor to the switch regions [9]. The remodeling of the switch regions destabilizes the required Mg^{2+} ion cofactor (which helps coordinate the nucleotide in the binding pocket) which, in turn, destabilizes the nucleotide, allowing for dissociation of GDP and subsequent association of Mg^{2+} and GTP. This general mechanism is common to the Dbl-family GEFs as well as the non-homologous bacterial toxin SopE [9,10]. This protein does not have the DH and PH domains of the Dbl-family GEFs, yet the similarity of the mechanisms shown by crystal structures would suggest a common mechanism may be seen even for other GEFs without a DH domain.

No Dbl-family GEFs have been identified for plant Rho-family GTPases, or Rops. Instead, a new family of RopGEFs, containing a domain named PRONE (for plant-specific Rop nucleotide exchanger) has been identified [12]. These GEFs do not possess any sequence homology with the Dbl-family GEFs. A structure of the PRONE domain from RopGEF8 has recently been solved both in isolation and in a complex with Rop4 [13]. Although the structure of the PRONE domain is unlike any GEF structure solved to date, the mechanism of activation as determined by these structures is similar to that seen with Dbl-family GEFs and SopE. The primary method of catalyzing nucleotide exchange involves manipulation of the switch residues of the GTPase to destabilize the nucleotide binding pocket [12-14].

Other mammalian Rho-family-specific GEFs have been identified that do not contain the DH/PH domains of the Dbl-family GEFs. SWAP-70 and related IBP are proteins that contain a PH domain, but lack a DH domain [15-17]. SWAP-70 has Rac1-

specific GEF activity and lies downstream of phosphatidylinositol 3-kinase (PI3K). A region with low homology to DH domains has been predicted to exist N-terminal to the PH domain, but that prediction is not statistically significant. Alternatively, these proteins are predicted to contain a coiled-coil region C-terminal to the PH domain. These atypical GEFs are poorly characterized [9,15-17].

SmgGDS is an atypical GEF for Rho-family GTPases. The literature on this particular GEF identifies a number of different Rho and Ras-family GTPase substrates for this exchange factor, but recent studies in our lab have determined that SmgGDS is specific for RhoA and RhoB [18]. SmgGDS is an armadillo (ARM)-repeat containing protein, and has no detectable DH or PH domains. The polybasic regions of the GTPases play an active role in the binding and exchange activity of this particular GEF [18-24].

The Dock1-related family of proteins contains 11 mammalian members, with orthologs in humans, roundworms, slime mold, fruit flies, yeast and plants, among others (referred to as the Dock family of proteins in the rest of this document) [25-29]. This family also contains no obvious homology to the Dbl-family GEFS, but are, instead, defined by two regions of homology within the family, DHR-1 and DHR-2 for Dock homology region 1 and 2. The DHR-2 region is the center of exchange activity for these atypical exchange factors for Rho-family GTPases. While these proteins have been shown to be bona fide GEFs, information on how these proteins are regulated is relatively sparse and the mechanism by which they catalyze exchange is still unknown [9,27,28]. The Dock family has been grouped into four different subfamilies (Fig. 2), based on sequence identity over the entire sequence of the proteins, with 50-65% sequence identity between subfamily members[26,27,29]. The following sections review each subfamily.

Dock-family GEFs

Dock A subfamily: Dock1, Dock2 and Dock5

The Dock A subfamily of proteins includes two of the most well-studied of the Dock-family proteins, Dock1 and Dock2. This subfamily has several features in common. The domain structure consists of the typical Dock-family DHR-1 and DHR-2 regions, along with an N-terminally located SH3 domain and a C-terminally located, variable proline and serine-rich region in most members [26-29]. Rac is the known Rho-family GTPase substrate for two of the three proteins within the Dock A subfamily [26-29].

Dock1, originally identified as Dock180 (for 180-kDa protein downstream of Crk), is a prototype member of the Dock family of proteins [30,31]. Identified as a binding partner for the SH2 and SH3-domain containing adaptor protein, Crk, Dock1 mRNA was expressed strongly in the placenta, lungs, kidneys, pancreas and ovaries than in the thymus testes and colon. In contrast, expression was not detected in blood cells, suggesting that Dock1 was expressed only in adherent cells [30]. Subsequent analysis found that Dock1 association with CrkII was stimulated by integrin in NIH3T3 cells and that this binding correlated with CrkII binding to p130Cas, a protein that localizes to focal adhesions [32]. Consequently, coexpression of p130cas and CrkII with Dock1 induced local membrane spreading and accumulation of complexes containing Dock1/CrkII/p130cas at focal adhesions. The C-terminal proline and serine-rich region of Dock1 was determined to be necessary for the Dock1-CrkII interaction [30,32].

Dock1 was found to increase active Rac1 in cells and was capable of direct interaction with Rac1, but not Cdc42 or RhoA, and Dock1-induced membrane spreading

was inhibited by dominant-negative Rac1 [33]. Two homologs of Dock1 in *Drosophila* (myoblast city, MBC) and *C. elegans* (ced-5) were identified, and were grouped into a family of proteins, CDM, for ced-5, MBC, and Dock180 [31]. This family of proteins was suggested to be an evolutionarily conserved family involved in the extension of cell surfaces. MBC was necessary for dorsal closure and myoblast fusion in *Drosophila* embryos [34], and ced-5 was necessary for cell corpse engulfment and distal tip cell migration [31]. The $\alpha_v\beta_5$ integrin was shown to mediate phagocytosis of apoptotic cells through recruitment of the Dock1/CrkII/p130cas complex, which in turn triggered Rac1 activation and phagosome formation [35]. A later study demonstrated that a secreted glycoprotein, MFG-E8 which can bind phosphatidylserine (PS) on apoptotic cells, is a ligand for the $\alpha_v\beta_5$ integrin, and can trigger activation of Rac1 through Dock1 for phagocytosis of apoptotic cells [36].

The identification of Dock1, and determination that it and its homologs participated in the activation of Rac1 and extension of cell surfaces paved the way for identification of Dock1 and homologs as Rho-family specific GEFs. The ability to directly activate Rac1 by a portion of Dock1 called the Docker domain led to the proposal that Dock1 was an atypical, non-Dbl-family Rac-specific GEF [25]. About the same time, the other mammalian members of the Dock-family of proteins were identified through homology searches, and several were shown to have direct GEF activity on Rho-family GTPases [26,28]. The Docker domain was found to be within a region of homology between the family members and was named DHR-2 [26] or CZH-2[28]. Another region of homology between the Dock-family proteins was N-terminal to the DHR-2 region, and was named DHR-1 [26], or CZH-1 [28]. Dock1 and its homolog,

MBC, can bind phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) through the DHR-1 region. This binding mediates localization of Dock1 to the membrane [37-39].

The ELMO (ced-12) family of proteins were identified as Dock1/ced-5 binding partners [25,40]. These proteins were shown to functionally cooperate with Dock1, although they had no exchange activity alone, leading to the suggestion that the Dock1/ELMO complex functioned as a bipartite GEF for Rac1 [25]. This finding was not universally accepted, as Dock1 was capable of acting as an exchange factor *in vitro* without ELMO1 [27,38]. Also, it was found by some groups that efficient activation of Rac1 by Dock1 required ELMO1 in cells [25,41], and others found the ELMO1 interaction dispensable [26].

Interactions between Dock1 and ELMO proteins include at least two direct interactions between the N-terminal 350 residues of Dock1 and the C-terminal 100 residues of ELMO1, with one of those interactions involving the Dock1 SH3 domain and the ELMO1 PxxP motif [25,41,42]. Separate from these interactions, the isolated PH domain of ELMO1, although missing the regions of ELMO1 necessary for direct interaction with Dock1, is capable of binding, *in trans*, to a complex of Dock1 and Rac1 [41]. This interaction between the PH domain and the Dock1/Rac1 complex is capable of enhancing the exchange activity of Dock1. It was suggested that the PH domain stabilizes the nucleotide-free transition state of Rac1 bound to Dock1, allowing for enhanced exchange activity[41]. The SH3 domain of Dock1 is also capable of binding to the DHR-2 region of Dock1 and competing for binding with Rac1 [42]. This SH3/DHR-2 interaction is relieved upon binding of ELMO1. The authors suggested this was an inhibitory mechanism for regulation of the Dock1 molecule, although it is unclear if the

two proteins exist separately within cells [42]. The assembly of the Dock1/ELMO complex may be regulated by the C-terminal SH3 domain of CrkII [43]. Endogenous Dock1/ELMO complex has been immunoprecipitated from HeLa and CHO-K1 nuclear extracts using an anti-Dock1 antibody [44]. The resulting Dock1/ELMO complexes contained more than one Dock1 molecule and more than one ELMO molecule per complex. The isoform of ELMO (1, 2 or 3) immunoprecipitated with Dock1 depended on the cell line. Multiple isoforms of ELMO1 were precipitated with Dock1 in each cell line [44].

Other functions for the interaction between Dock1 and ELMO proteins are evident. ELMO1 is capable of inhibiting ubiquitylation of Dock1, thereby protecting Dock1 from degradation [45]. Brain-specific angiogenesis inhibitor 1 (BAI1), a PS-sensitive receptor, can interact with the Dock1/ELMO1 complex through ELMO1 [46]. Recruitment of Dock1/ELMO1 to the BAI1 receptor mediates phagocytosis of apoptotic cells. RhoG, another Rho-family small GTPase has been shown to bind specifically to the N-terminus of ELMO1 in a GTP-dependent manner [47]. This interaction can localize Dock1/ELMO1 to the plasma membrane. Activation of Rac1-mediated cell migration is mediated by RhoG through the Dock1/ELMO1 complex. Interestingly, the interaction of Dock1 with CrkII is dispensable for this function [48]. Phagocytosis of apoptotic cells is also mediated by this interaction, and this mechanism is conserved in *C. elegans* as well as humans [49]. The interaction between RhoG and ELMO1 is exploited by the bacterial pathogen, *Shigella*. The bacterial-produced IpgB1 protein binds to ELMO1 in a manner mimicking RhoG, and activates production of membrane ruffles through the Dock1/ELMO complex, enabling bacterial entry into epithelial cells [50].

The Dock1/ELMO complex has also been implicated in other pathways promoting cell motility. A recent study implicates Dock1 in mediating attractive responses by neurons to netrin-1, an axon guidance cue [51]. It has also been reported that the Dock1/ELMO complex mediates the activation of Rac1 by ARNO, a GEF for the ADP-ribosylation factor 6 (ARF6) at the leading edge of migrating cells [52].

Despite the multitude of interacting proteins and pathways in which Dock1 is involved, it is clear that Dock1 is active in pathways that promote cell migration and phagocytosis within adherent cells. The evidence that ELMO proteins are a key component of Dock1 activity whether by direct activation or indirect activation through localization and protein binding is also clear.

Dock2 was identified through homology to Dock1, and the expression pattern of Dock2 in tissues is nearly opposite that of Dock1 [53]. While Dock1 is present in mostly adherent cells, Dock2 is expressed mainly in non-adherent, hematopoietic cells. Dock2 mRNA expression was detected in peripheral blood cells, with slight expression in spleen and thymus. Dock2 was expressed only in lymphocytes and macrophages of various organs as detected by immunostaining of human cadaver tissue [53]. Dock2 is a specific exchange factor for Rac1, and does not activate Cdc42 or RhoA and is at least capable of binding to Rac2 [26,53].

Unlike Dock1, Dock2 does not bind to CrkII. It does, however, associate with a hematopoietic-specific Crk-like protein, CrkL, which can induce activation of Rac1 [53]. Interruption of this interaction inhibited CrkL activation of Rac1. Dock2 is essential for lymphocyte chemotaxis [54]. In Dock2-deficient cells, Rac activation and actin polymerization induced by chemokines was nearly abolished.

ELMO proteins are capable of interacting with Dock2, and this interaction is critical for Dock2/ELMO1-mediated Rac1 activation[55]. For instance, expression of Dock2 in T-hybridoma cells lacking endogenous Dock2 induced Rac activation and actin polymerization. In addition, expression of a Dock2 mutant incapable of binding ELMO1 failed to effect these changes [55]. In plasmacytoma cells expressing Dock2 but not ELMO1, expression of ELMO1 induces Rac activation [55].

Dock2 has been implicated in a variety of processes critical for the survival of hematopoietic cells. For instance, Dock2 is essential for the activation of Rac1 required for the formation of immunological synapses mediated by T-cell receptors [56]. In this case, Dock2 regulates antigen-induced translocation of T-cell receptors and lipid rafts during synapse formation. Similarly, deletion of Dock2 has been shown to suppress cardiac allograft rejection by eliminating lymphocyte homing and immunological synapse formation [57]. Furthermore, chemokine-stimulated adhesion of lymphocytes under shear stress requires Dock2 for efficient attachment to VCAM-1 [58]. Vav1, a Dbl-family GEF that specifically activates Rac GTPases, is also required for this process, but the interplay between Vav1 and Dock2 is not well understood. Dock2 expression is also required for development of mouse Vα14 natural killer T cells [59], which play important roles in host defense against pathogens, immune regulation, and tumor surveillance. Dock2 is necessary for another type of T-cell differentiation in $CD4^+$ T cells. Dock2 links T-cell receptor signals to IL-4Rα downregulation to control lineage commitment of these cells [60]. Finally, spingosine-1-phosphate-mediated egress of lymphocytes from peripheral lymph nodes and interstitial mobility of lymphocytes also depend on Dock2 [61].

Considering the importance of Dock2 in hematopoietic cells as discussed above, it is unsurprising that the Dock2/ELMO1 machinery has been taken advantage of by a pathogen. The HIV-1 protein Nef, a potent virulence factor, can form a complex with Dock2/ELMO1, and this interaction is dependent upon ELMO1 [62]. Nef activates Rac in T cell lines and in primary T cells, and the authors suggest that Nef modulates multiple aspects of T cell function through the Dock2/ELMO1 complex [62].

The variety of studies showing the necessity of properly functioning Dock2 in lymphocytes serves to highlight the fact that Dock2 is critical for regulation of the actin cytoskeleton and mobility within hematopoietic cells. The specific contributions of ELMO proteins within the context of the Dock2/ELMO complex remain to be determined.

Very little is known about mammalian Dock5, as it has not yet been cloned. Out of 65 Dbl-family GEFs and 11 Dock-family GEFs tested, only Dock5 and Net1, a Dbl-family GEF, were up-regulated during RANKL-induced osteoclastogenesis [63]. In this case, silencing of Dock5 in RAW264.7 cells was found to be extremely cytotoxic, although it had no apparent effect in NIH3T3 fibroblasts. The zebrafish ortholog of Dock5 was cloned and used to design morpholino oligonucleotides to block the function of the protein in zebrafish embryos[64]. In this study, morpholino embryos for Dock5 showed defective fast-myoblast fusion. Dock1, Dock5, Crk and CrkL were all tested similarly, and when different combinations of Dock and Crk proteins were tested, the highest level of fusion suppression observed occurred in embryos injected simultaneously with Dock5 and CrkL morpholinos.

Dock B subfamily: Dock3 and Dock4

The Dock B subfamily consists of two mammalian members, Dock3 and Dock4. This branch of the family is most similar to the Dock A proteins, with Dock C and Dock D proteins further removed by sequence homology. The Dock B proteins are about 56% identical within the subfamily, but are only about 40% identical to Dock A subfamily members [26,28]. Like the Dock A members, however, Dock B proteins contain an N-terminal SH3 domain, which allows association of ELMO1.

The presinilin (PS) gene is linked to approximately 50% of familial Alzheimer's disease (AD) cases [65,66]. Dock3 (also modifier of cell adhesion – MOCA) was identified through yeast-two hybrid screens as a protein capable of interacting with presinilin1(PS1) and labeled as PBP, or presinilin binding protein [67]. This interaction was confirmed with immunoprecipitation of the two proteins from transfected mammalian cells. Following up on this interaction, the authors discovered that co-expression of Dock3 and PS1 localized primarily cytoplasmic Dock3 to a discrete, organelle-like compartment, likely the endoplasmic reticulum, where PS1 is primarily localized. Dock3 mRNA was only found in the brain and spinal cord, highly expressed in the cerebral cortex and hippocampus, and the level of Dock3 in the soluble fraction of AD brains was reduced compared to age-matched normal brains [67]. A later study found that Dock3 is associated with intracellular neurofibrillary tangles (NFT) and expression increases phosphorylation of the NFT protein, tau, suggesting that Dock3 may play a role in the AD neurodegenerative process [68]. Further study showed that expression of Dock3 decreased amyloid precursor protein (APP) and amyloid β-peptide secretion, and reduced cell-substratum adhesion, likely through direction of APP to the

proteasome for degradation [69]. Another study identified the Dock3 gene as one of two genes disrupted by a pericentric inversion on chromosome 3 associated with an attention deficit hyperactivity disorder (ADHD)-like phenotype [70].

Dock3 has been shown to bind to Rac1, to enhance Rac1 activation, and to promote Rac1-dependent cell migration [71,72], but it does not interact with RhoA or Cdc42 [72]. In addition Dock3 interacts with ELMO1, and co-expression of Dock3 and ELMO1 is necessary for the promotion of Rac1-dependent cell migration [71]. Membrane-targeted Dock3 also enhances GTP-loading of Rac1 and JNK activation in cells, and endogenous Dock3 co-localizes with F-actin at the leading edge of lamellipodia [72]. It was determined that Dock3 promotes cell-cell adhesion and neurite outgrowth mediated by N-cadherin [69].

The Dock4 gene was identified as a homozygous deletion a mouse osteosarcoma cell line, and was mutated in a subset of human cancer cell lines [73]. In this study, restoration of wild-type Dock4 in the Dock4-null osteosarcoma lines restored contact inhibition, reduced colony formation in soft agar and formed small, non-invasive tumors when injected into nude mice, as compared to the Dock4 null cell line which formed large invasive tumors. The authors also reported that cells lacking Dock4 fail to form adherens junctions and this phenotype could be rescued by expressing Dock4 or by expressing constitutively active Rap1. Expression of Dock4 and dominant negative Rap1 failed to promote the formation of adherens junctions. In addition, Dock4 was shown to promote activation of Rap1 in transfected cells [73]. Other studies, however, describe Dock4 as a Rac1 activator, and were unable to detect Rap1 activation by Dock4 [42,74]. Dock4 was able to interact with ELMO1, and expression of active RhoG induced

translocation of the Dock4/ELMO1 complex to the plasma membrane from the cytoplasm [74]. This translocation enhanced the Dock4/ELMO1-dependent Rac1 activation and cell migration, and knockdown of Dock4 expression reduced cell migration. Dock4 is expressed in multiple tissue types, with the highest expression noted in skeletal muscle, prostate and ovary [73,75]. An isoform of Dock4, alternatively spliced in the C-terminus, is a strong Rac activator and is expressed in the brain, inner ear and eye tissues [75]. This isoform interacts with harmonin and is present in the hair bundles of auditory sensory cells.

Dock C subfamily: Dock6, Dock7 and Dock8

The Dock C subfamily of the CDM proteins has the least amount of information available about its representative members. Unlike both the Dock A and Dock B subfamilies, these proteins have not been shown to interact with ELMO proteins. The domain structure for these proteins is also not well known, with the only described domains being the DHR-1 and DHR-2 regions [27,29] and a predicted coiled-coil motif in the C-terminus [9].

Dock6 is reported to activate Rac1 and Cdc42, but is otherwise poorly understood [76]. For instance, full-length Dock6 or its isolated DHR-2 region immunoprecipitated from HEK293T cells activated Rac1 and Cdc42, but not RhoA. Similarly, the DHR-2 region activated Rac1 and Cdc42 in cells and promoted associated filopodia and lamellipodia [76]. However, unlike other Dock family members, Dock6 did not bind ELMO1, ELMO2 or CrkII [76]. Dock6 might regulate neuronal processes since its expression is highly increased upon differentiation of mouse N1E-115 neuroblastoma cells and Dock6 can regulate neurite outgrowth through Rac1 and Cdc42 [76].

Initial attempts to characterize the GTPase specificity of Dock7, also named zizimin-related 2 (Zir2) failed to show significant activation of either Rac1, Cdc42 or RhoA. The protein used for these assays, however, was *in vitro* translated, and only consisted of the DHR-2 region of the protein [26]. Later results identified Dock7 as a binding partner for TSC1 or hamartin, a protein that, when mutated, causes tuberous sclerosis (TSC) – a disease characterized by development of benign tumor-like "hamartomas" in kidneys, heart, skin and brain. TSC2, or tuberin, forms high affinity dimers with hamartin, and can interact with and act as a GAP for Rheb, a Ras-family small GTPase. It was suggested that Dock7 might be a Rheb-GEF, given this linkage of interactions [77,78]. More recent studies, though, have elegantly described Dock7 as a Rac specific GEF that is highly expressed in the developing rat brain, specifically in hippocampal neurons [79]. In unpolarized hippocampal neurons, Dock7 is asymmetrically distributed, and is selectively expressed in the axon. Dock7 expression can be manipulated to affect axon formation, with over-expression mediating formation of multiple axons and knockdown inhibiting axon formation. Interestingly, Dock7 and Rac activation leads to inactivation of a microtubule destabilizing protein, stathmin/Op18, through phosphorylation [79]. Following a trend in lipid binding seen in other Dock-family proteins, $PI(3,4,5)P_3$ is produced in the developing axon, and PI 3-kinase inhibitors abrogated the ability of Dock7 to form multiple axons [79] suggesting that the DHR-1 domain of Dock7 may also be capable of interacting with phosphoinositides. Cote and Vuori [27] make an interesting point that stathmin was identified as a gene regulating border cell migration in *Drosophila* oogenesis [80]. Myoblast city, the Drosophila Dock1 homolog, is also critical in border cell

migration[81]. Perhaps regulation of microtubules could be a common effect of different Dock-family proteins.

Dock8 (Zir3) was identified in a yeast two-hybrid screen as a Cdc42-interacting protein, and subsequent northern blot tissue analysis revealed a relatively ubiquitous expression pattern including placenta, lung, kidney and pancreas, with relatively low expression in heart, brain and skeletal muscle [82]. Subsequent immunofluorescence staining of cells showed that endogenous as well as over-expressed Dock8 was present at edges undergoing lamellipodia formation. Transfection of a C-terminal fragment (1179-1701) resulted in ring-like vesicular structures that contained filamentous actin [82]. Additional yeast two-hybrid assays demonstrated binding between Dock8 residues 1044-1701, which includes the DHR-1 domain and both activated (61L) and inactive (N17) Cdc42 and Rac1, but not activated RhoA [82]. This Dock8 fragment also interacted with TCL and TC10 – Rho-family GTPases closely related to Cdc42. An assay using immunoprecipitated Dock8 1179-1701, however, failed to bind to active or inactive GST fusions of Rac1, Cdc42 or RhoA. This same fragment was tested for binding to GTPases in a filter binding assay, and no interaction was detected [82].

A homozygous deletion of the locus for Dock8 was found in a lung cancer cell line [83]. Subsequent analysis of primary lung cancers revealed 87% had reduced Dock8 expression levels compared with normal cells. Homozygous deletions of Dock8 were also found in a gastric and a breast cancer cell line. The reductions in gene expression occurred regardless of histological type, suggesting that the reductions may be caused by DNA methylation or histone deacetylation [83]. Furthermore, Takahashi, *et al.* suggested that the "down-regulation of Dock8 by epigenetic mechanisms is involved in the

development and/or progression of lung cancer" [83]. Dock8 was originally described as 1701 amino acids in length [82]; however, later work proposed a version (2099 residues) with an extended N-terminus [83] and most rece3nt work suggests that Dock8 contains 2033 residues [84]. Disruptions of the Dock8 gene have also been found in two unrelated patients with mental retardation [84].

Dock D subfamily: Dock9, Dock10 and Dock11

Proteins in the Dock D subfamily of Dock proteins contain the typical DHR-1 and DHR-2 regions, but, like the Dock C subfamily, this group does not contain the N-terminal SH3 domain of the Dock A and B subfamily members [26,28,29]. As expected with the lack of an SH3 domain, proteins in this subfamily do not interact with ELMO proteins. The Dock D proteins, however, do contain a PH domain in the N-terminal region [26,28,29]. Two of the three mammalian proteins in this subfamily have been identified with specificity for Cdc42 over other Rho-family GTPases.

Dock9, also named zizimin1, is the most-studied protein of this subfamily. Originally identified in a screen for proteins that specifically interacted with nucleotide-depleted Cdc42 and were eluted by the addition of GTPγS, Dock9 was precipitated from NIH3T3 cells, human umbilical vein endothelial cells, rat vascular smooth muscle cells and COS-7 cells [28]. Interestingly, one group discovered that Dock9 mRNA levels were highest in brain lung and kidney, while levels in the heart, liver, skeletal muscle and hematopoietic organs were low [85]. Another group discovered a slightly different mRNA distribution where levels were highest in heart and placenta, with relatively high levels in kidney, brain, lung and skeletal muscle and low levels in liver, intestine and hematopoietic tissues[28].

The protein was named zizimin1 (from the Hebrew word for spikes), reflecting its ability to induce microspike formation when over-expressed in fibroblasts, a function linked to Cdc42 activation [28]. Dock9 was shown to interact directly with Cdc42, but not Rac1 or RhoA [26,28,85] and to require the DHR-2 domain for its exchange activity. Deletion of the N-terminus of Dock9 resulted in a stronger interaction with nucleotide-depleted Cdc42 [28]. Full-length Dock9 is capable of activating Cdc42 within cells, and the isolated DHR-2 region is capable of activating Cdc42 in vitro, but is not efficient at activating Cdc42 within cells [26,28]. When cells were co-transfected with the DHR-2 region and with a cytoplasmic form of Cdc42, the DHR-2 region seemed fully active on Cdc42 [28]. The authors suggested that the N-terminal PH domain may function in membrane association, and the lack of the PH domain in the DHR-2 did not allow the protein to localize properly to activate Cdc42.

Building upon the observation that N-terminal truncations of Dock9 interacted more strongly with Cdc42, Meller and colleagues [86] found that three regions within the N-terminal portion of Dock9 are capable of interaction with the DHR-2 region. The DHR-1 region, the region N-terminal to the PH domain, and a region C-terminal to the PH domain were all capable of interaction with the DHR-2 region. The interaction between the N- and C-terminal regions of Dock9 inhibited Cdc42 binding. Analysis of Dock9 by limited proteolysis, suggested that the DHR-2 homology region might not be a complete structural domain, and that residues beyond the homology region may be a part of the GEF domain of the protein [86].

Homology between Dock-family proteins was used to identify Dock10 (also named zizimin3) [26,28]. This protein has yet to be fully cloned, but expression analysis

indicates that Dock10 is enriched in the brain, lung, spleen and thymus [85]. Additionally, the DHR-2 domain of Dock10 was cloned and expressed in COS-7 cells, and lysates were incubated with various nucleotide-depleted GTPases bound to beads [85]. The beads were washed and then bound proteins were eluted with GTP. The DHR-2 domain of Dock10 associated weakly with nucleotide-depleted Cdc42 and TCL, and even more weakly with TC10 and RhoA. There was no detectable interaction with Rac1 [85]. Dock10 was also found to be up-regulated in some aggressive, poorly differentiated papillary thyroid carcinoma specimens [87].

Dock11 (also named activated Cdc42-associated GEF (ACG) [88] and zizimin2 [28]) was identified through homology to other Dock-family members [26,28], and was later cloned as a gene expressed at higher levels in germinal center B cells than non germinal center B cells [85]. Expression was detected primarily in hematopoietic organs, with low-level expression in some non-hematopoietic tissues. The C-terminal two-thirds of Dock11, as well as the smaller DHR-2 domain were sufficient for binding to nucleotide-depleted Cdc42, but the DHR-2 domain does not interact with TC10 or TCL. Full-length Dock11 and the isolated DHR-2 domain can both activate Cdc42 in cells [85].

Immunoprecipitation assays utilizing GST-Cdc42 as a pull-down matrix identified Dock11 as a binding partner, but surprisingly, it was binding preferentially to active Cdc42 (Q61L), not nucleotide-free GTPase [88]. In contrast, the isolated DHR-2 domain of Dock11 bound preferentially to the nucleotide-depleted form of Cdc42. The full-length form of Dock11 was an active GEF for Cdc42, while the DHR-2 domain only showed weak activity. Furthermore, deletion of the N-terminal 272 amino acids of Dock11 changed the binding preference from Cdc42 (Q61L) to nucleotide-depleted

Cdc42 (T17N). The essential region for binding active Cdc42 was determined to be within residues 66-126 of Dock11, N-terminal to the PH domain [88]. These residues (10-127) were capable of binding to the DHR-2 domain, and that interaction was increased in the presence of active Cdc42, but not nucleotide-free Cdc42. A deletion of the N-terminal 126 amino acids, abrogating interaction with active Cdc42 also severely impaired GEF activity. The authors proposed a positive feedback model where the N-terminus of Dock11 binds the DHR-2 domain and this form has weak GEF activity. Activation of Cdc42 allows the active GTPase to interact with the N-terminal region and the DHR-2 domain, enhancing the activity of the GEF [88].

A second study examined the interaction between Dock11 and Cdc42 and found that while full-length Dock11 did bind GTP-Cdc42, it bound preferentially to the nucleotide-depleted Cdc42 [86]. The authors of this second paper suggest that the discrepancy between the two sets of results could arise from the differences between the Cdc42 proteins used. In the first paper, Cdc42 mutants were utilized for their activated (Q61L) and nucleotide-depleted (T17N) forms [88], while in the second paper, the Cdc42 proteins were treated with EDTA, nucleotides and $MgCl_2$ to get the desired nucleotide state of the protein [86].

Dock-family GTPase recognition

Most studies have focused on assessing the capacity of Dock-family members to directly activate specific GTPases. In contrast, there are limited studies addressing the structural determinants within Dock-family members required for the engagement and activation of specific GTPases. A recent study, however, has compared the binding properties of the Rac-specific Dock2 and Cdc42-specific Dock9 [89]. By using a

multitude of chimeras of Rac2 and Cdc42, as well as point substitutions, the authors have been able to map important residues for recognition of cognate GTPases by Dock2 and Dock9 [89]. Dbl-family GEFs can discriminate between Rac1 and Cdc42 based on the β2 and β3 strands of the GTPase [90,91]. Substitution of tryptophan 56 in Rac1 to the phenylalanine of Cdc42 and vice versa is sufficient to change the specificity of Dbl-family GEFs Tiam1 and ITSN. For example, Rac-specific Tiam1 is unable to catalyze exchange on Rac1 (W56F), but Cdc42-specific ITSN is capable of catalyzing exchange on this mutant [92]. The reverse holds true for Cdc42 (F56W), where Tiam1 is capable of catalyzing exchange but ITSN loses that ability [93]. Dock2 and Dock9 utilize the β3 strand of the GTPase for substrate recognition as well. Similar to the Dbl-family GEFS, the Dock-family GEFs gain the ability to catalyze exchange on non-cognate GTPases upon mutation of the GTPase residue 56. However, neither Dock2 nor Dock9 lose the ability to catalyze exchange on their mutated cognate GTPase [89]. This suggests that while the determinants for GTPase recognition and specificity for Dock-family GEFs overlaps to some extent with the Dbl-family GEFs, there are additional determinants for Dock-family GTPase recognition. Emphasizing this, the divergent residues at positions 27 and 30 within the switch 1 regions of Cdc42 and Rac1 are important for substrate recognition by Dock2 and Dock9, while these residues are usually not utilized for substrate discrimination by Dbl-family GEFs [89].

Concluding remarks

Identification and characterization of the relatively new Dock family of proteins is still a work in progress. As a consequence, an understanding of how these proteins

function and are regulated is very limited. What is evident is that these proteins play crucial roles in the regulation of Rho GTPases and their effectors. Identification of functional and regulatory mechanisms for the Dock-family GEFs will give us insight, both into the general mechanisms of Rho-family GTPase activation, and into the specific ways in which these Dock-family GEFs coordinate multiple signals and resultant outcomes within cells.

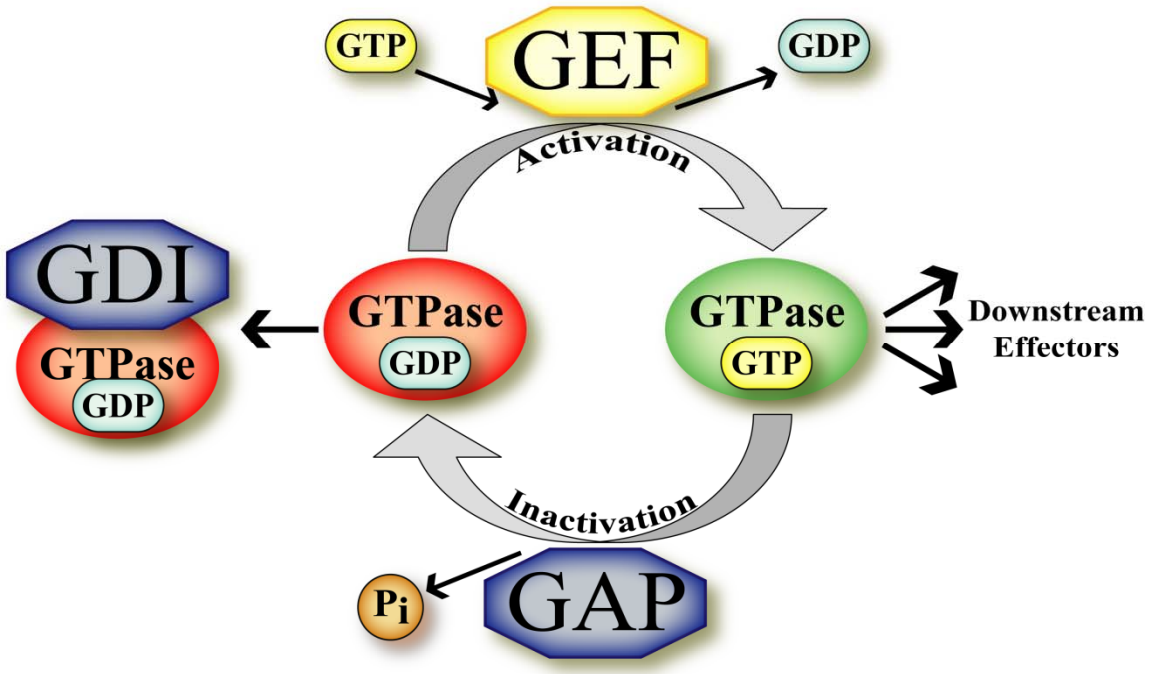


Figure 1: Overview of the Rho-family GTPase cycle.

Rho GTPases cycle from an inactive (GDP-bound) state to an active (GTP-bound) state with the help of regulatory proteins. Active GTPase is capable of binding downstream effector proteins. GEF: guanine nucleotide exchange factor, GAP: GTPase accelerating protein, GDI: guanine dissociation inhibitor, P_i: inorganic phosphate, GDP: guanosine 5'-diphosphate, GTP: guanosine 5'-triphosphate.

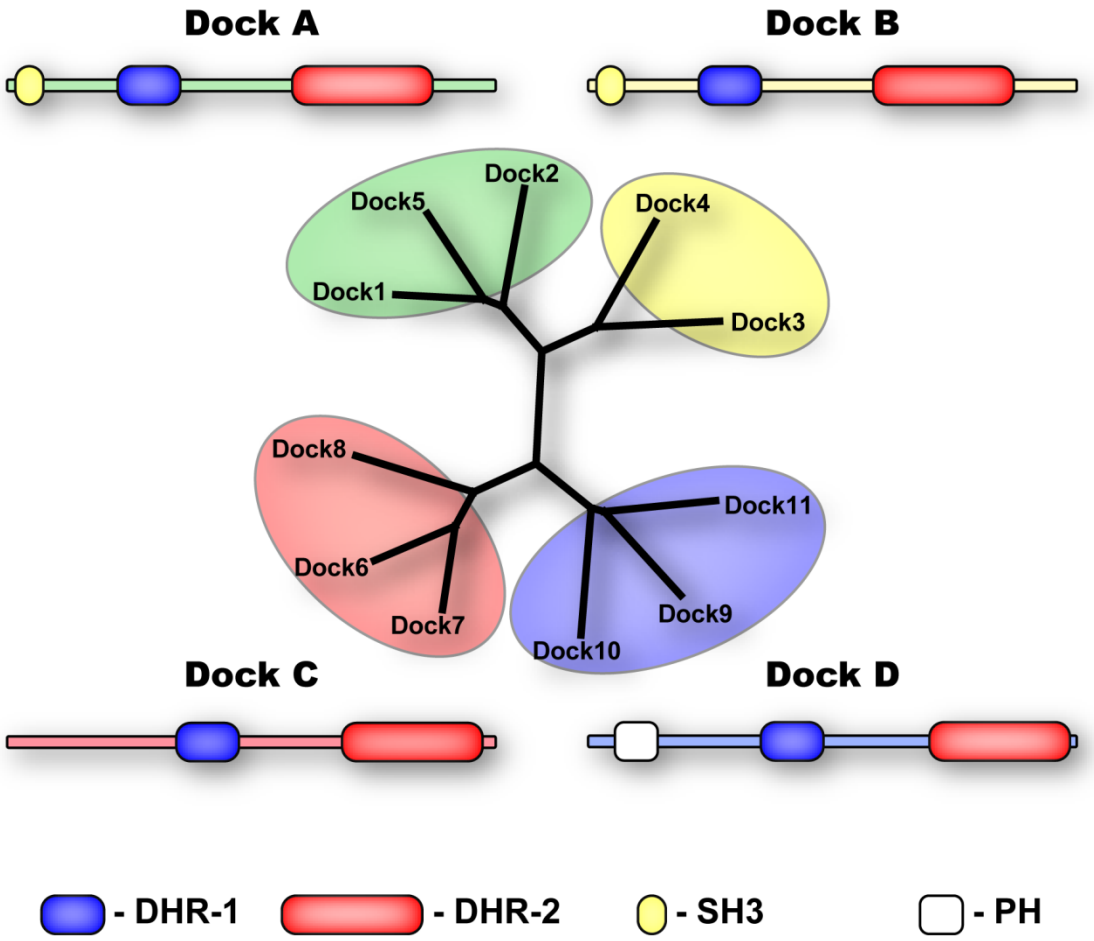


Figure 2: Overview of the Dock family of proteins (mammalian).

The Dock family of proteins can be subdivided into four groups based on sequence homology, Dock A, B, C, and D. Center: a cladogram showing the relative homology between Dock-family members. The typical domain structures for each subfamily is shown next to its grouping on the cladogram. DHR-1: Dock homology region 1, DHR-2: Dock homology region 2, SH3: Src-homology 3 domain, PH: pleckstrin homology domain.

CHAPTER 2 – DOCK2/ELMO1: MOLECULAR INTERACTIONS OF A HETEROTETRAMERIC GEF

Background

The Dock family of guanine nucleotide exchange factors (GEFs) is evolutionarily conserved in eukaryotes ranging from mammals to plants and yeast [25,29,94]. Encompassing 11 mammalian members, the Dock-family proteins are critical for a variety of biological processes. In *C. elegans*, CED-5 is essential for distal tip cell migration and clearance of apoptotic bodies [31]. Mutations within MBC, the *Drosophila* homolog, are linked to developmental defects including improper myoblast fusion and problems with thoracic and dorsal closure [34,95]. In mammals, Dock1 (also named Dock180) has an important role in apoptotic cell engulfment and cell migration [25,35,40,71], while Dock2, primarily expressed in hematopoietic cells, is essential in T-cell activation and chemotaxis [54,56], and is involved in HIV-1 infection [62]. The neuronally-expressed Dock3 regulates N-cadherin-mediated cell-cell adhesion and neurite outgrowth [69,70,72], and is potentially linked to Alzheimer's disease and attention-deficit hyperactivity disorder (ADHD) [70,96]. Dock4 was identified in a screen for genes deleted in tumor progression, and can act as a tumor suppressor [73]. The Dock C and Dock D subfamilies (Dock6 – Dock8, and Dock9 – Dock11) have less well-defined functions, although Dock7 plays a role in axon specification [27,29,79].

Dock-family proteins activate Rho-family GTPases by catalyzing the exchange of GDP for GTP; GTP-bound GTPases directly engage downstream effectors to modulate their activities. The relatively new family of Dock proteins varies significantly from the well-studied Dbl-family GEFs which utilize a tandem Dbl homology (DH) domain and pleckstrin homology (PH) domain to catalyze nucleotide exchange [9]. The Dock family of proteins, instead, relies upon a unique region, the docker-homology-region-2 (DHR-2, also named "Docker" or "CZH2") for catalytic activity [25,26,28]. This region of homology between the Dock-family members possesses no obvious sequence or predicted structural homology to the DH and PH domains of the Dbl-family GEFs and, therefore, may function in a unique manner.

Several of the Dock-family members are known to form a complex with ELMO proteins [71,74]. The interaction between Dock1 and ELMO1 is critical for phagocytosis of apoptotic cells and for cellular migration [25,41,47,49,55,71,97,98]. ELMO proteins, which exist as three mammalian isoforms (1, 2 and 3), have no GEF activity alone, but it has been proposed that together, Dock and ELMO proteins function as bipartite GEFs – with ELMO proteins aiding in the stabilization of Rac1 in a nucleotide-free transition state [25,41,71]. Understanding how Dock and ELMO proteins interact should lead to a better comprehension of the functional consequences of this association.

Of the Dock-family proteins, only the Dock A and Dock B subfamilies have been shown to interact with ELMO proteins [71,74]. Through examination of deletion constructs and mutational analysis, the interactions between Dock1 and ELMO1 are known to involve at least three separate regions: (1) the N-terminal SH3 domain of Dock1 and a polyproline (PxxP) motif in the C-terminus of ELMO1; (2) the N-terminal

357 residues of Dock1 and the C-terminal 100 residues of ELMO1, likely within the residues between the end of the PH domain and the beginning of the PxxP motif and (3) the PH domain of ELMO1 binds to a Dock1/Rac1 complex *in trans* [41,42]. Disruption of the first two interactions is necessary to prevent formation of the Dock1/ELMO1 complex. Deletion of the SH3 domain or the PxxP motif is not sufficient to eliminate the interaction between Dock1 and ELMO1. A mutation of glycine 171 to glutamic acid (G171E) in the N-terminus of Dock1 is sufficient to disrupt the second interaction. When combined, the deletion of the SH3 domain and the G171E mutation can fully eliminate the direct interaction between Dock1 and ELMO1. Alternatively, termination of ELMO1 at residue 629 prevents formation of the complex, presumably by removing the sites for both interactions [42]. Interestingly, the binding of the PH domain of ELMO1 *in trans* to a complex of Dock1 and Rac1 increases the exchange activity of Dock1. It has been proposed that the PH domain aides in the stabilization of nucleotide-free Rac1 in the transition state while associated with Dock1 and Rac1, and this stabilization is responsible for the increase in exchange activity seen with this trimeric complex [41].

ELMO proteins also play a role in proper localization of Dock-family proteins, by binding to activated RhoG, another Rho-family GTPase, which functions to bring Dock/ELMO complexes to the membrane [47-49,74]. The N-terminal region of ELMO1 is necessary and sufficient for this interaction [47,48]. Disruption of the RhoG-ELMO1 complex by mutation of the N-terminus of ELMO1 results in the failure of the Dock1/ELMO1 complex to promote phagocytosis, cell migration, formation of lamellipodia and the mutant complex is not localized to membrane ruffles [49].

Defining the mechanism by which Dock and ELMO proteins function together would provide a greater understanding of how the various biological effects of the complex are carried-out and regulated. Here we refine the predicted domain boundaries for Dock1, Dock2 and ELMO1, as well as identify new domains. We show that isolated ELMO1 exists as a monomer, and can interact with both Rac1 and RhoG. This interaction is nucleotide-dependent, and is dependent upon the presence of the polybasic region (PBR) of Rac1 and/or RhoG. We demonstrate that Dock2 and ELMO1 can be purified as a stable complex, and this Dock2/ELMO1 complex exists as an obligate heterotetramer, consisting of two molecules each of Dock2 and ELMO1. We also describe the substrate specificity of the complex using a screen of purified GTPases, and demonstrate that this tetramer binds a single molecule of Rac1.

Experimental procedures

Domain architecture prediction

Domain analysis of Dock1, Dock2 and ELMO1 was carried out using a combination of sequence alignments using ClustalX version 1.83 [99], protein-protein BLAST and psi-BLAST searches [100], secondary structure prediction programs, and tertiary structure prediction and threading programs [100-106].

Plasmids

ELMO1 constructs for insect cell expression were PCR-amplified and inserted into pFastBac1 using a modified ligation-independent cloning strategy (LIC) [107]. ELMO1 (1-727) was amplified from pGEX4T2-ELMO1 [40].

Full-length human Dock2 (1-1830) was PCR-amplified from pCXN2-Flag-Dock2, a gift of Dr. Michiyuki Matsuda [32,33], and cloned into a modified pFastBac1 vector (Invitrogen) using a LIC strategy [107]. The resulting construct encodes a non-cleavable hexahistadine tag immediately preceding the N-terminus of Dock2.

RhoG-pGEX4TEV2 and Rac1 constructs (C189S and Δ177) were as described [108,109]. HA-Rac1 (3X-HA epitope N-terminal to Rac1) was amplified from RAC010TN00 (UMR *cDNA Resource Center)*, introducing a C189S mutation and stop codon, as well as NcoI and XhoI sites, and the fragment was ligated into pET15b via the NcoI and XhoI sites. Flag-Rac1 was amplified from Rac1C189S introducing a Flag epitope at the N-terminus of Rac1, and was ligated into pET15b as above. All new constructs were verified by automated sequencing.

Protein purification

Baculoviruses encoding the Dock2 and ELMO1 constructs were generated from the pFastBac vectors using the Bac-to-Bac system (Invitrogen). High-Five insect cells were co-infected with viruses encoding Dock2 and ELMO1. Cells were harvested by low-speed centrifugation after incubation at 27°C for 48 hours with shaking at 140 rpm. Cells were resuspended in 20 mM Tris, 1 M NaCl, 5 mM imidazole, 10% glycerol, pH 8.0 (buffer N1). Cells were lysed with an Emusiflex-C5 homogenizer (Avestin), and lysate was cleared by centrifugation at 265,000 x *g* for 45 minutes at 4°C. Cleared lysate was passed through a 0.45 μm filter before being loaded onto a 5 ml nickel-charged metal chelating column (GE Healthcare) equilibrated with buffer N1. The protein bound to the column was washed with buffer N1 and buffer N1 containing 50 mM imidazole before being eluted with buffer N1 containing 400 mM imidazole. Fractions containing

Dock2/ELMO1 were pooled and concentrated to 10 ml in a 10K MWCO Vivaspin (Vivascience) 20 ml concentrator before being loaded onto a 26/60 Sephracryl S300 size exclusion column (GE Healthcare) equilibrated in 20 mM Tris, 300 mM NaCl, 10% glycerol, 2 mM DTT, pH 8.0. Fractions containing Dock2/ELMO1 were pooled and dialyzed versus 20 mM Tris, 100 mM NaCl, 2 mM DTT, pH 8.0, concentrated as above, snap frozen in liquid N_2, and stored at -80°C.

Bacterially-produced ELMO1 and ELMO1ΔN were expressed as GST-fusion proteins from pGEX4T2-ELMO1 and pGEX4T2-ELMO1 532-727 in the BL21(DE3) *E. coli* strain. Cells expressing ELMO1 were grown at 37°C in Terrific Broth (TB) until they reached an absorbance at 600 nm (A_{600}) of ~0.8, then protein expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were allowed to grow ~12 hours at 25°C before harvesting by low-speed centrifugation. Cells expressing ELMO1ΔN were grown at 37°C in self-inducing ZYM-5052 media [110] until they reached an A_{600} of ~0.6, then cells were allowed to grow ~15 hours at 20°C before harvesting by low-speed centrifugation. Cell pellets were resuspended in 20 mM Tris pH 8.0, 150 mM NaCl, 2 mM DTT, and 10% glycerol (GST buffer), lysed using an Emulsiflex-C5 homogenizer (Avestin), and clarified by ultracentrifugation at ~150,000 x *g*. Clarified supernatant was loaded onto a 5 ml GSTrap FF column (GE Healthcare) pre-equilibrated with GST buffer, and eluted with GST buffer supplemented with 10 mM reduced glutathione. Fractions containing GST-ELMO1 or GST-ELMO1ΔN were pooled and the GST tag was cleaved overnight with thrombin. ELMO1 was dialyzed into 20 mM Tris pH 8.0, 10 mM NaCl, 2 mM DTT and 10% glycerol (buffer Q) and loaded onto an anion-exchange chromatography column pre-equilibrated with buffer Q, then

eluted with a linear gradient of 10-300 mM NaCl. Fractions containing ELMO1 were pooled and dialyzed vs. GST buffer, and remaining fusion protein was removed using a 5 ml GSTrap FF column. ELMO1ΔN was dialyzed into 20 mM HEPES pH 7.0, 10 mM NaCl, 2 mM DTT and 10% glycerol (buffer S1) and loaded onto a cation-exchange chromatography column pre-equilibrated with buffer S1, then eluted with a linear gradient of 10-300 mM NaCl. Fractions containing ELMO1ΔN were pooled and dialyzed vs. GST buffer, and remaining fusion protein was removed using a 5 ml GSTrap FF column. ELMO1 and ELMO1ΔN were concentrated using 10K MWCO Vivaspin 20 ml concentrators, snap frozen in liquid N_2, and stored at -80°C.

GST-ELMO2 was expressed from pGEX4T2 as for ELMO1, and purified essentially as described above for ELMO1 through the first affinity chromatography step. GST-ELMO2 was then concentrated, snap frozen and stored at -80°C.

ELMO1 was also produced in insect cells. The protein was expressed and purified as above for the Dock2/ELMO1 complex with a few changes. Less virus encoding Dock2 relative to ELMO1 was used, and ELMO1 contained an N-terminal hexa-histidine tag. A small amount of excess ELMO1 was purified away from the Dock2/ELMO1 complex during the size exclusion chromatography step and then stored at -80°C for subsequent analysis.

The GST-RhoG expression construct was expressed in the BL21(DE3) *E. coli* strain. Cells were grown at 37° in enriched media (24 g yeast extract and 12g tryptone per liter, pH 7.4) supplemented with 0.1 mg/ml ampicillin until they reached an A_{600} of ~0.8, then protein expression was induced with 0.1 mM IPTG. Cells were allowed to

grow ~15 hours at 20°C before harvesting by low-speed centrifugation. RhoG was essentially purified as described [108].

Rac1 proteins (residues 1-189 C189S and Δ177) were expressed and purified essentially as described [108,109]. HA-Rac1 and Flag-Rac1 were purified as described for Rac1 C189S [108].

Size and oligomeric state determination

ELMO1 purified from bacteria and from insect cells was subjected to size exclusion chromatography on a Superdex 200 10/300 GL (GE Healthcare) analytical column. The molecular weight was estimated by comparing the peak elution volume to the peak elution volumes of a set of globular proteins of known molecular weight applied to the same column under similar conditions.

Light scattering measurements were made with a Wyatt DAWN EOS light scattering instrument (Wyatt Optilab refractometer, and Wyatt dynamic light scattering module) interfaced to an AKTA FPLC (GE Healthcare). 100 µl of the Dock2/ELMO1 complexes at 2 mg/ml were loaded onto a Superose 6 10/300 GL column (GE Healthcare) pre-equilibrated in 20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM DTT and 0.02% NaN_3 at 25°C. For ELMO1 samples, 100 µl at 4.5 mg/ml was loaded onto a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated with the same buffer as above. Data collection and analysis was performed with ASTRA software version 4.90.08 (Wyatt technologies). The refractive increment (dn/dc) was set at 0.185 for each protein in the molecular mass calculations, based on the premise that dn/dc is constant for unmodified proteins [111]. Experiments were repeated 3-5 times.

Native PAGE assays

GTPases were pre-loaded with the indicated nucleotide (or lack thereof) by incubating with EDTA at 2 times the concentration of $MgCl_2$ in the protein buffer (usually 2 mM EDTA) and 10-fold molar excess of desired nucleotide for 30 minutes at room temperature. Loading reactions were then stopped by addition of 2.5-fold excess of $MgCl_2$ over the concentration of EDTA (usually 5 mM $MgCl_2$), except in the case of the nucleotide-depleted forms, where buffer containing 2 mM EDTA and no $MgCl_2$ was used.

GTPases were incubated at indicated concentrations with ELMO1 for 15 minutes at room temperature, then were put on ice and loaded onto a 12.5% or 20% PhastGel (GE Healthcare) in combination with Native gel buffer strips (GE Healthcare), using a 6-well comb which loads 4µl of sample per well. Gels were run with the manufacturer's suggested protocols for 12.5% or 20% native gels. Proteins were visualized by Coomassie brilliant blue staining.

Surface plasmon resonance assays

Utilizing a BIACORE 3000 surface plasmon resonance (SPR) instrument (GE Healthcare), anti-GST antibody was coupled to a CM5 chip per the manufacturer's instructions (GE Healthcare). Equal response units of indicated, GST-fusion ligand proteins were loaded onto the chip by brief injections of protein over the antibody surface. Interactions of Rho GTPases at a concentration of 10 µM were analyzed using the KINJECT command. Graphs of the response units are plotted with curves for the ligand protein of interest (ELMO1 or ELMO2) overlaid on the corresponding curve for the GST-only ligand surface to identify interactions.

Exchange assays – loading

Exchange assays monitoring loading of GTPase with fluorescent nucleotide was carried out in 20 mM HEPES, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 nM BODIPY-FL-GDP and 10% glycerol at 10°C. GTPases were added to 2 μM and 100 nM Dock2/ELMO1 complex or 20 mM EDTA were added as exchange factors. Loading of BODIPY-FL-GDP onto the GTPase was monitored by an increase in fluorescence by a Perkin-Elmer LS-55 fluorimeter with excitation and emission wavelengths of 503 and 511, respectively. Addition of EDTA was used to confirm the GTPase was active. Rac and RhoG purifications are described above. All other GTPases were purified as described [112].

Rac-binding immunoprecipitation

Purified Dock2/ELMO1, Flag-Rac1, HA-Rac1 and Rac1Δ177 were added as indicated to appropriate reactions. 450 pmoles Dock2/ELMO1 was used per reaction, and 450 pmoles of the tagged Rac1 construct was used per reaction. 900 pmoles of Rac1Δ177 was used per reaction. Proteins were diluted to equivalent concentrations in 20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA, and allowed to incubate with each other, as indicated, for 15-30 minutes at 25°C. Anti-HA antibody-coupled beads (Roche) or Anti-Flag M2 antibody-coupled beads (Sigma) were washed with wash buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.25% NP-40, 100 μg/ml BSA) and 25 μl of appropriate beads were used per reaction. Wash buffer was added to reactions to bring the volume up to 500 ml and the tubes were incubated at 4°C on a rotator for 90 minutes. Beads were collected by centrifugation at 6000 x *g* for 30 seconds, and supernatant was removed by aspiration. Beads were washed 3 times with 1 ml wash buffer and protein

was eluted by boiling in 30 µl SDS-PAGE gel-loading dye. Samples were loaded onto an SDS-PAGE gel, and bound proteins were visualized by staining with Coomassie brilliant blue.

Results

Dock1, Dock2 and ELMO1 domain architecture

To determine if there are additional predicted domains within the sequences of Dock-family members and ELMO proteins, and to help define the domain boundaries of published, predicted domains, we used a combination of multiple sequence alignments, secondary structure prediction programs, tertiary structure prediction programs and threading algorithms. Others have reported that ELMO1 contains a PH domain, a leucine-zipper motif, a PxxP motif, and Armadillo repeats. We found, through our analysis, that the PH domain boundaries predicted by SMART are reasonable, if a bit extended, and thus, we labeled the PH domain as consisting of residues 555-677 (Fig. 3B). BLAST analysis of the PH domain of ELMO1 finds the PH domain of PLC-δ to have the highest homology with that region. We did not find convincing evidence, however, for the predicted leucine zipper motif, and believe it to be unlikely to exist within the predicted PH domain of ELMO1, which is where a potential leucine zipper was identified [40]. The PxxP motif at the C-terminus is conserved amongst the different ELMO proteins (1, 2 and 3) and across species and is located within residues 707-717 of mouse ELMO1 (Fig. 3B). Reportedly, ELMO1 contains Armadillo (ARM) repeats, which are regions of about 35-50 amino acids that form three alpha helices per repeat. Each repeat stacks with others, usually forming a superhelix of helices [113,114]. We

also found evidence for the ARM repeat region in the ELMO proteins; however, we would define the boundaries of the ARM repeat region differently than published. The N-terminal region of mouse ELMO1 was repeatedly aligned with SCOP family a.118.1 (ARM repeats [115]) when using a meta-server which compiles data from a variety of prediction and threading programs (http://www.bioinfo.pl/meta). Upon examination of the models and alignments built by the threading algorithm, 3D-PSSM, and several secondary structure prediction programs, we determined that the most likely boundaries for the ARM repeat region span residues 81-515 (Fig. 3B).

We performed a similar domain analysis for Dock1 and Dock2. Previously-predicted domains for Dock proteins were largely based on homology with other family members, since only a few domains had been predicted with a high degree of confidence. The SH3 domain of Dock1 and Dock2 is readily identified by programs such as SMART, and the boundaries are labeled accordingly. The DHR-1 and DHR-2 regions have not been identified as structural domains, but as regions of homology as their names suggest (Dock-homology-region-1 or 2). The limits of the DHR-1 region are labeled based on the literature (Dock1 422-664) [26,38], and the limits of the DHR-2 region are also based on the literature, but with refinements from our own multiple sequence alignments and secondary structure predictions (Dock2 1100-1617) [25,26,28]. The proline and serine-rich region at the C-terminus of Dock1 is shown as identified from the sequence; Dock2 does not have a noticeable proline/serine rich region (Fig. 3A) [33,35,53,116].

To delineate other domains within the Dock protein sequences, we used portions of the sequences outside of defined domains to aid the prediction programs by eliminating easily-defined domains. Within these sequences, domains were often

identified with low confidence scores, but the scores could be increased by using the boundaries defined by the initial hits to refine our sequence range. A C2 domain was identified within the DHR-1 region using 3D-PSSM. As the domain limits were refined by multiple rounds of 3D-PSSM and secondary structure predictions, the confidence score increased into the 95% confidence range (Fig. 3A). This independently-predicted C2 domain was mentioned in earlier literature [26], but was more or less ignored until a later paper defining phosphoinositide-binding properties of the DHR-1 region re-examined the region [38]. This paper defines the C2 domain as residues 422-587 of Dock1, but suggests that the minimum lipid-binding site encompasses residues 422-619. Our assessment of the domain would suggest an even smaller region for the actual C2 domain, including residues 422-560 (Fig. 3A).

A similar method was used to delineate the Armadillo repeat region of Dock1 and Dock2. Similar to the ELMO1 ARM repeats, the results returned from a meta-server (http://www.bioinfo.pl/meta) identified the region from approximately residues 600-1340 as belonging to the SCOP family a.118.1, or ARM repeats. The region defined by homology to various ARM repeat proteins from the results compiled by the meta-server were submitted separately to 3D-PSSM and the resulting ARM repeat models were in the 95% confidence range. The ARM repeat region was defined by combining these results from the meta-server, the 3D PSSM results and several secondary structure predictions (615-1332, Dock1; 600-1339, Dock2; Fig 1A). Interestingly, the ARM repeat region extends well into the DHR-2 region, but does not contain the entire DHR-2 region. We were unable to detect any known domains within the latter half of the DHR-2 region, although there do seem to be secondary structure elements predicted, including both

helical and beta-sheet content, with random coil becoming much more prevalent immediately after the 1617 (Dock1) cutoff for the DHR-2 region. In fact, once past the C-terminal end of the DHR-2 region, the remaining protein sequence is predicted to be primarily random coil.

Despite the inability to predict any defined domains within the latter half of the DHR-2 region, COILS, a program which predicts coiled-coil (CC) regions within proteins [117], predicted a CC region spanning residues 1490-1510 in Dock1 and 1495-1509 in Dock2. This CC region was also predicted for Dock3, Dock4 and Dock5, and a mutation immediately preceding it (Dock1 ISP➔AAA, residues 1487-1489) is known to eliminate the binding and exchange capacity of Dock1 [25]. The contribution of this predicted CC region to activity and/or dimerization has yet to be determined.

Purified ELMO1 exists as a monomer

ELMO1, purified from either insect cells or bacteria was examined by size exclusion chromatography (SEC) to determine the oligomeric state of the protein. Since dimerization/oligomerization has been shown to be important both in a Dock1/ELMO complex and in Dock9 (zizimin1) [44,118], we wanted to determine if ELMO1 is self-associating in order to better understand the oligomerization of the complex. When analyzed over an analytical size exclusion column and compared to a range of proteins of known molecular weight run on the same column, we found that ELMO1 eluted at a volume consistent with a monomer of approximately 80 kDa (Fig. 4A). Any dimers or higher-order oligomers of the protein would have been approximately 170 kDa and larger, and would have been clearly visible as a shifted peak elution volume on the SEC column used.

In addition to the SEC analysis, purified ELMO1 applied to an analytical SEC column was analyzed by multi-angle laser light scattering (Fig. 4B-C). The protein eluted as a single, monodispersed peak with an average calculated molar mass of 87 kDa, with the standard deviation over three independent experiments being less than 1 kDa. This molecular weight is consistent with the approximate predicted value of an 84 kDa ELMO1 monomer. These data show that purified ELMO1 exists solely as a monomer in solution. This verifies that it is highly unlikely that any oligomerization of the Dock/ELMO complex originates with an ELMO-ELMO protein interaction.

ELMO1 interaction with RhoG and Rac1

Data from various papers detail an interaction between activated RhoG and the N-terminus of ELMO1. We examined this interaction using purified components to identify features of RhoG and ELMO proteins that are important to this interaction. Native, or non-denaturing, polyacrylamide gel electrophoresis (Native PAGE) separates proteins based on size, shape, and isoelectric point (pI) of the protein(s) [119]. A gel-shift assay can be used to visualize protein-protein interactions, providing that a protein complex has properties (size, shape and/or pI) which are different enough from the individual proteins to visualize on a gel.

We analyzed the ability of ELMO1 to form complexes with Rac1 and RhoG using native gels. Neither Rac1 C189S or RhoG C188S were capable of entering the gel, presumably due to their high pI values as determined by ProtParam [101], which are 8.83 and 8.46, respectively. The RhoG/Rac1 and ELMO1 complex does not enter the gel either. ELMO1, however, does enter the gel, and complex formation was monitored by depletion of the ELMO1 band in the gel caused by formation of the complex. The

ELMO1 band was depleted, indicating complex formation, when ELMO1 was incubated with RhoG in its activated state, bound to GTPγS (a non-hydrolyzable GTP analogue). ELMO1 did not interact with RhoG in the GDP-bound or nucleotide-depleted form (Fig. 5C). Surprisingly, we also saw similar, nucleotide-specific interaction with Rac1-GTPγS (Fig. 5A). We tested the ability of increasing amounts of RhoG-GTPγS and Rac1-GTPγS to deplete the ELMO1 band in the native gels, and found that, indeed, this was the case (Fig. 5B, D). Interestingly, Rac1Δ177, which has the PBR removed, is incapable of forming a complex with ELMO1 (Fig. 3E).

To further examine these interactions, we used surface plasmon resonance to measure binding of RhoG and Rac1 to ELMO1. GST-ELMO1 or GST-ELMO2 and GST were used to create ligand protein surfaces on an anti-GST antibody-coupled CM5 chip. The binding of 10 μM Rac1 or RhoG was analyzed as relative response units (RU). We compared the binding of each GTPase form to the GST surface, and found that both Rac1 and RhoG bound to ELMO1 or ELMO2 only in the activated form (GTPγS-bound) and only when the PBR of the GTPase was present (binding is interpreted as an increase over the signal seen with GST). Rac1Δ177, RhoGΔ177, Cdc42 and Ras were not capable of interacting with ELMO1, ELMO2 or GST in any nucleotide state (Fig. 6 and Fig. 7).

These results show that the interaction of RhoG and Rac1 with ELMO1 and ELMO2 is specific, nucleotide-dependent and dependent upon the polybasic region. The interaction of Rac1 with ELMO proteins was a surprise, considering that immunoprecipitation assays were able to detect the active RhoG-ELMO1 interaction, but not an interaction with active Rac1 and ELMO1. Those assays were performed using the N-terminal 362 residues of ELMO1 [47], so perhaps the interaction with Rac1 is through

the C-terminal region of the protein. Alternatively, these interactions with Rac1 could be an *in vitro* artifact due to the similarity of Rac1 and RhoG, which are 71% identical.

Dock2 and ELMO1 are stably associated through the purification process

Attempts to express and purify Dock2 in insect cells produced nearly no detectable protein, but when co-expressed when ELMO1, milligram quantities of purified complex were readily obtainable (Fig. 8B). Critical components of the Dock2/ELMO1 complex purification included protease inhibitors during lysis, clarification, and loading onto the affinity chromatography column. Also important, 1 M NaCl increased apparent binding of the complex to the affinity column, as well as decreasing non-specific binding of other proteins. Susceptibility to degradation necessitated a quick progression from lysis through the affinity and size exclusion chromatography (SEC) steps, all complete within 12 hours of lysis. Once purified over the SEC column, continued degradation of the complex was negligible. Some proteolytic fragments of Dock2 and ELMO1 typically purified with the complex and were removed by dialysis overnight into buffer containing 50 – 100 mM NaCl which promoted specific precipitation of the fragments, which were subsequently removed by centrifugation. After the addition of NaCl to 150 mM, the complex was concentrated and incubated at 37°C for 10 minutes to precipitate any further proteolytic fragments or unstable complex before being stored at -80°C. Throughout the purification process, Dock2 and ELMO1 remained tightly associated, even in the presence of salt ranging from 0.05 – 1 M NaCl, with and without the presence of glycerol. The purification yielded a highly-active complex of nearly homogeneous Dock2/ELMO1, stable at concentrations up to at least 40 mg/ml. In addition, the Dock2/ELMO1 complex was able to form a trimeric complex with purified Rac1 upon

incubation of the two proteins in the presence of EDTA. This trimeric complex was stable and excess Rac1 could be purified away by use of a SEC column (Fig. 8B).

Dock2/ELMO1 is an efficient, specific exchange factor for Rac isoforms

Dock2 is known to activate Rac1 and Rac2, and not Cdc42 or RhoA, but its activity on other GTPases has not been tested [26,53,54,56], nor has its efficiency as a GEF been evaluated. To test the exchange specificity of the Dock2/ELMO1 complex, we performed fluorescence-based guanine nucleotide exchange assays on a library of GTPases. Dock2/ELMO1 catalyzed exchange on Rac1, Rac2 and Rac3, as well as Rac1Δ177 (Fig. 8), but not on other Rho-family GTPases (Fig. 9), including RhoG, which is highly homologous to the Rac isoforms. Dock2/ELMO1 was capable of exchange on Rac1 forms expressed with epitope tags (HA and Flag), but was completely inactive on an N-terminal GST fusion of Rac1 (data not shown). Dock2/ELMO1 also failed to catalyze exchange on other GTPases belonging to the Ras superfamily (Fig. 10). When compared to Dbl-family GEFs in similar reaction conditions, the Dock2/ELMO1 complex is an efficient exchange factor, comparable to, or even more efficient than, Dbl-family GEFs that were tested (data not shown).

Dock2/ELMO1 is a heterotetramer

Several interactions between Dock and ELMO proteins have been defined, but the role that ELMO proteins play within the complex has been debated [27,94]. Zizimin1 (Dock9) dimerizes via a portion of the DHR-2 domain, and Dock1 is capable of self-association in an immunoprecipitation assay [118]. Endogenous Dock1 and ELMO proteins were also immunoprecipitated as a complex from HeLa cells, and the major

component proteins were identified using mass spectrometry as Dock1, ELMO2 and ELMO3. The size of the complex was approximated at 700 kDa via size exclusion chromatography [44]. In order to better understand the functional relationship between Dock and ELMO proteins, we determined the stoichiometry of the complex using purified components.

Dock2/ELMO1 applied to a size exclusion column eluted in a single, mono-dispersed peak with a calculated molar mass of 598 ± 23 kDa (Fig. 11A) as determined by multi-angle laser light scattering [111]. This molecular mass is within the experimental error of the calculated weight (594 kDa; Fig. 11C) of a hetero-tetramer consisting of two molecules of Dock2 and two molecules of ELMO1; no other combination is consistent with the experimentally determined molecular weight. Similar analysis of Dock2/ELMO1ΔN (Fig. 11B) yielded a molecular mass of 466 ± 7 kDa, which is very close to the predicted molecular mass (472 kDa) of a hetero-tetramer with 2:2 stoichiometry (Fig. 11C). Combined, these results strongly suggest that Dock2/ELMO1 exclusively exists as a stable 2:2 hetero-tetramer that does not require the N-terminal portion of ELMO1. Hetero-tetramer formation is most likely mediated through Dock2, since zizimin1, which does not interact with ELMO1, is also dimeric [118]. In addition, it seems unlikely that ELMO1 contributes significantly to the dimerization interface, since size exclusion chromatography and light scattering analyses indicate that isolated ELMO1 is a monomer (Fig. 4).

The Dock2/ELMO1 complex binds a single molecule of Rac

The existence of Dock2/ELMO1 exclusively as a stable heterotetramer implies a functional significance to this particular oligomer. To explore this possibility, we

examined the interaction between the Dock2/ELMO1 heterotetramer and Rac1. A stable complex between Dock2/ELMO1 and nucleotide-depleted Rac1 can be made by incubating the purified proteins in the presence of EDTA and this Dock2/ELMO1/Rac1 complex can be purified over a size exclusion column (Fig. 8B). It is important to note that Dock2/ELMO1 is capable of binding to, and is fully active upon all forms of Rac1 used in these assays (data not shown). To determine the number of Rac1 molecules that bind to each tetramer of Dock2/ELMO1, we performed an immunoprecipitation assay utilizing differentially-tagged forms of Rac1. Using purified proteins, we incubated Dock2/ELMO1 complex with either Flag-Rac1 or HA-Rac1 and non-tagged Rac1Δ177 and performed an immunoprecipitation with anti-Flag or anti-HA beads as appropriate. The beads and bound protein were washed extensively, and then the bound proteins were eluted by boiling in SDS-PAGE gel-loading buffer, and were run on an SDS-PAGE gel. If two or more molecules of Rac1 were capable of interacting with the Dock2/ELMO1 complex, both species of Rac1 would be visible on the gel. If only one molecule of Rac1 was bound to the Dock2/ELMO1 complex, then only the tagged form of Rac1 would be visible on the gel, as the Dock2/ELMO1 bound to the untagged-Rac1Δ177 would not be immunoprecipitated. Surprisingly, we found that only a single molecule of Rac1 associates with the Dock2/ELMO1 heterotetramer (Fig. 12). This result suggests that either the structural layout of the tetramer is such that only one Dock2 molecule is capable of interacting with Rac1 or that the two Dock2 molecules cooperate to form the Rac1 binding site.

Discussion

A significant body of literature describes the association of Dock and ELMO proteins, as well as the interaction between RhoG and ELMO1 (reviewed in Cote and Vuori [27]; Lu and Ravichandran [94]; and Meller, Merlot and Guda [29]). The majority of these studies have been limited to using non-purified protein components in which it can often be difficult to distinguish direct effects of the proteins studied from the contributions of endogenous proteins. We were able to use purified proteins to study the interactions between ELMO1 and RhoG, as well as the interactions and activity of the Dock2/ELMO1 complex.

In native gel shift assays and surface plasmon resonance analysis, activated RhoG bound to ELMO1 in a manner consistent with previous reports [47,48,74]. Activated Rac1 was able to bind similarly, which is an interaction that has not been detected previously. In fact the interaction between Rac1 and ELMO1 has been tested by pull-down assays [47] and yeast two-hybrid assays [40] and no binding was detected. Not only did these two Rho-family GTPases bind to ELMO1 in a GTP-dependent manner, this association also required the polybasic region (PBR) of the GTPase. Binding dependent upon the PBR is reported for other ARM repeat containing proteins, such as SmgGDS, a non-Dbl-family GEF specific for RhoA and RhoB [4,18]. Additionally, FHOS, a member of the formin family of proteins, is known to require the PBR to bind to Rac1 [120]. Formins use ARM repeats, in addition to a helical GTPase binding domain, to bind Rho-family GTPases, although in the crystal structure of mDia bound to RhoC a truncated form of RhoC missing the PBR was used [121,122].

The significance of activated Rac1 binding to ELMO1 is yet to be determined. It is possible that Rac1, being 71% identical to RhoG, is simply mimicking, *in vitro*, the biologically-relevant association of activated RhoG with the N-terminus of ELMO1. The potential for this binding of Rac1 to ELMO1 to be a bona fide interaction must also be considered. This could indicate the presence of a feed-forward loop, whereby a Dock/ELMO complex activates Rac1, which goes on to bind to ELMO1, and perhaps, increase or help sustain the exchange activity of the Dock/ELMO complex. Interactions similar to this have been reported for the Ras GEF Son of sevenless (Sos) and for the Cdc42-specific Dock11, where the activated substrate GTPases of the GEFs return to bind the exchange factor. In the case of Sos, activated Ras binds through the PH domain [123,124], and activated Cdc42 binds through the N-terminus in the case of Dock11 [88]. Future analyses of the binding between Rac1 and ELMO1 using truncation mutants of ELMO1 will be useful in determining the biological relevance of this interaction.

Although detailed data on the interactions between Dock-family proteins and ELMO proteins are available, Dock-family proteins are often treated as separate entities. Our results provide evidence that the Dock/ELMO complex is a stable heterotetramer that acts as an exchange factor upon Rac isoforms in a Dock:ELMO:Rac stoichiometry of 2:2:1. These data would support a hypothesis that Dock-family proteins in the Dock A and Dock B subfamilies exist as obligate heterotetramers with ELMO proteins.

One indication of the obligatory nature of the interaction between Dock2 and ELMO1 was the necessity of co-expressing both of these proteins in insect cells in order to purify any detectable protein. In fact, once the two proteins were co-expressed, the quantities of proteins able to be purified per liter of cell culture changed from essentially

zero to several milligrams of pure protein. When expressed alone, Dock2 was produced since an elution fraction from the initial affinity column did possess exchange activity towards Rac1. The protein, however, was not clearly visible on a gel and the elution fraction contained a large number of contaminating peptides, as determined by SDS-PAGE. The possibility that ELMO1 protects Dock2 from degradation within the insect cells is supported by a paper from Makino *et al.* [45], which shows that ELMO1 inhibits ubiquitylation of Dock1.

Extending beyond the idea of protection from proteolysis, we found that the Dock2/ELMO1 complex is very stable, even in conditions ranging from 50 mM to 1M NaCl, and incubation at 37°C. The complex stayed tightly associated through the entire purification process. The stability of the complex was emphasized by the light scattering experiments in which the single elution peak for the complex was found to be nearly 99% monodispersed. This indicates that the entire peak which eluted from the size exclusion column contained a single species. The lack of any sort of detectable dissociation by this complex supports a hypothesis that the interaction between the ELMO proteins and the Dock-family proteins (Dock A and Dock B families) is an obligatory interaction.

We also showed that the heterotetramer consisting of 2 molecules each of Dock2 and ELMO1 is a fully active, specific GTPase for Rac1, Rac2 and Rac3, but no other Rho family or Ras superfamily members that were tested. The heterotetramer functions as a single unit; only binding to one molecule of Rac1. This is in contrast to published results for Dock9 (Zizimin1), in which the Dock9 dimer is shown to bind a single molecule of Cdc42 [118]. It is unlikely that the two Dock-family proteins, although only distantly related within the family, interact with their respective substrate proteins in such a

different manner. To resolve this difference, we would propose that the data in Meller, *et al.* could be interpreted differently.

A non-Cdc42-binding mutant utilized in the paper showed a decrease in dimerization capability. The authors reported decreased binding to Cdc42 for a dimer of this mutant and wild-type Dock9 and interpreted Cdc42 binding to this mutant/wild-type dimer as evidence for one Cdc42 binding site per Dock9 molecule. We hypothesize, rather, that the impaired dimerization capability of the mutant results in a reduced binding affinity for a single molecule of Cdc42. If each Dock-family protein provides a part of the binding site for the GTPase, then a fault in dimerization could damage the ability of that molecule to bind the GTPase. Meller, *et al.* also reported that a small fragment of Dock9 with poor dimerization capabilities was sufficient to bind Cdc42, and suggested that this supported the stoichiometry of one Cdc42 molecule per Dock9 molecule. While a reasonable conclusion, we would argue that, in light of this new data with purified proteins, each of the two Dock-family proteins in the dimer/heterotetramer may provide part of the GTPase binding site. A construct of the Dock protein containing the binding site, but with sub-optimal dimerization may still interact with the substrate GTPase, but poorly. In support of this idea, we have noted a coiled-coiled region predicted within the DHR-2 region of Dock1 through Dock5 (Fig 1A). A mutation which abolishes Dock1 exchange activity (Dock1 ISP) is located immediately N-terminal to this region [25]. Examining the dimerization capabilities of this known GEF-dead mutant may provide additional evidence for this hypothesis.

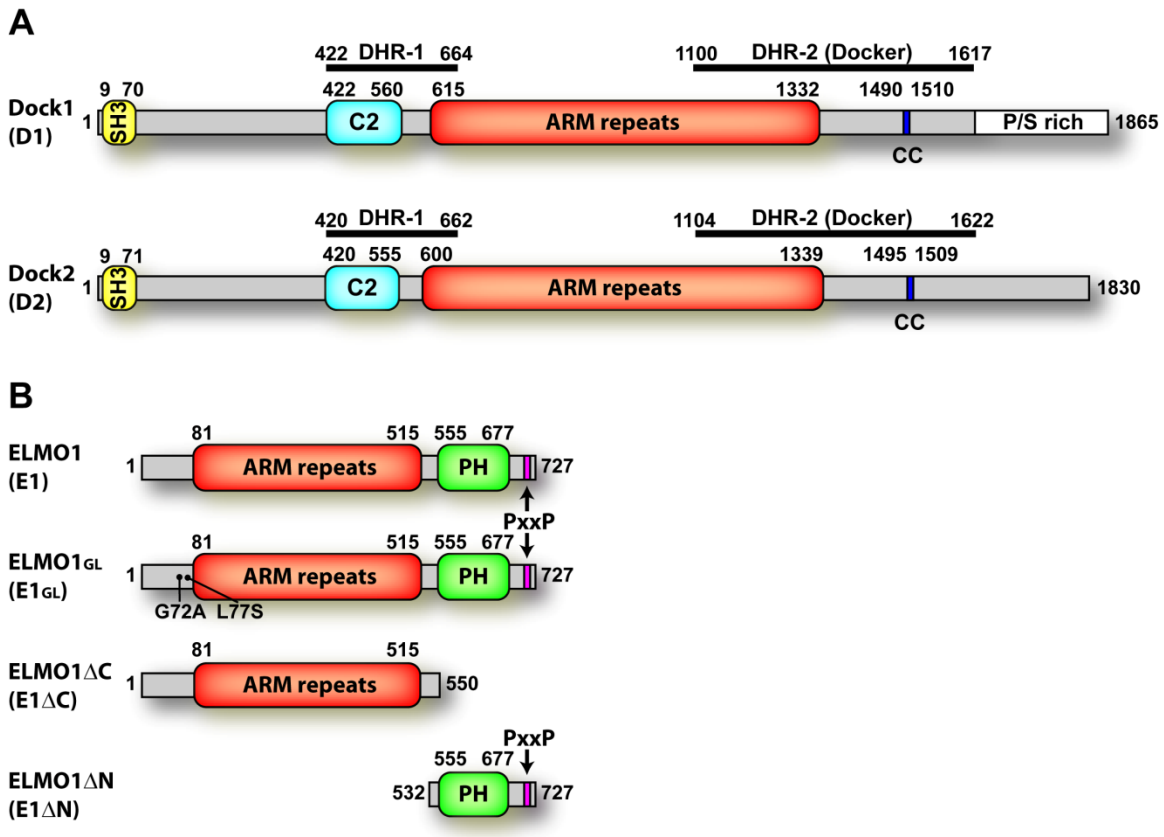


Figure 3: Schematic representation of Dock1, Dock2 and ELMO1 constructs and domains.

A) Predicted domain structure of human Dock1 (Dock180) and Dock2. Numbers denote amino acid residues for the boundaries of predicted regions. SH3 domain in yellow, C2 domain in cyan, region containing armadillo (ARM) repeats in red, coiled-coil region in blue, proline and serine-rich region (P/S rich) in white. Docker-homology region-1 (DHR-1) and docker-homology region-2 (DHR-2, or Docker domain) are represented by black bars. **B)** Predicted domain structure, constructs and mutants for mouse ELMO1. Region containing predicted ARM repeats in red, PH domain in green and PxxP motif in magenta. Numbers denote residues for beginning and end of constructs and of domains. Point mutations are labeled on the ELMO1GL construct.

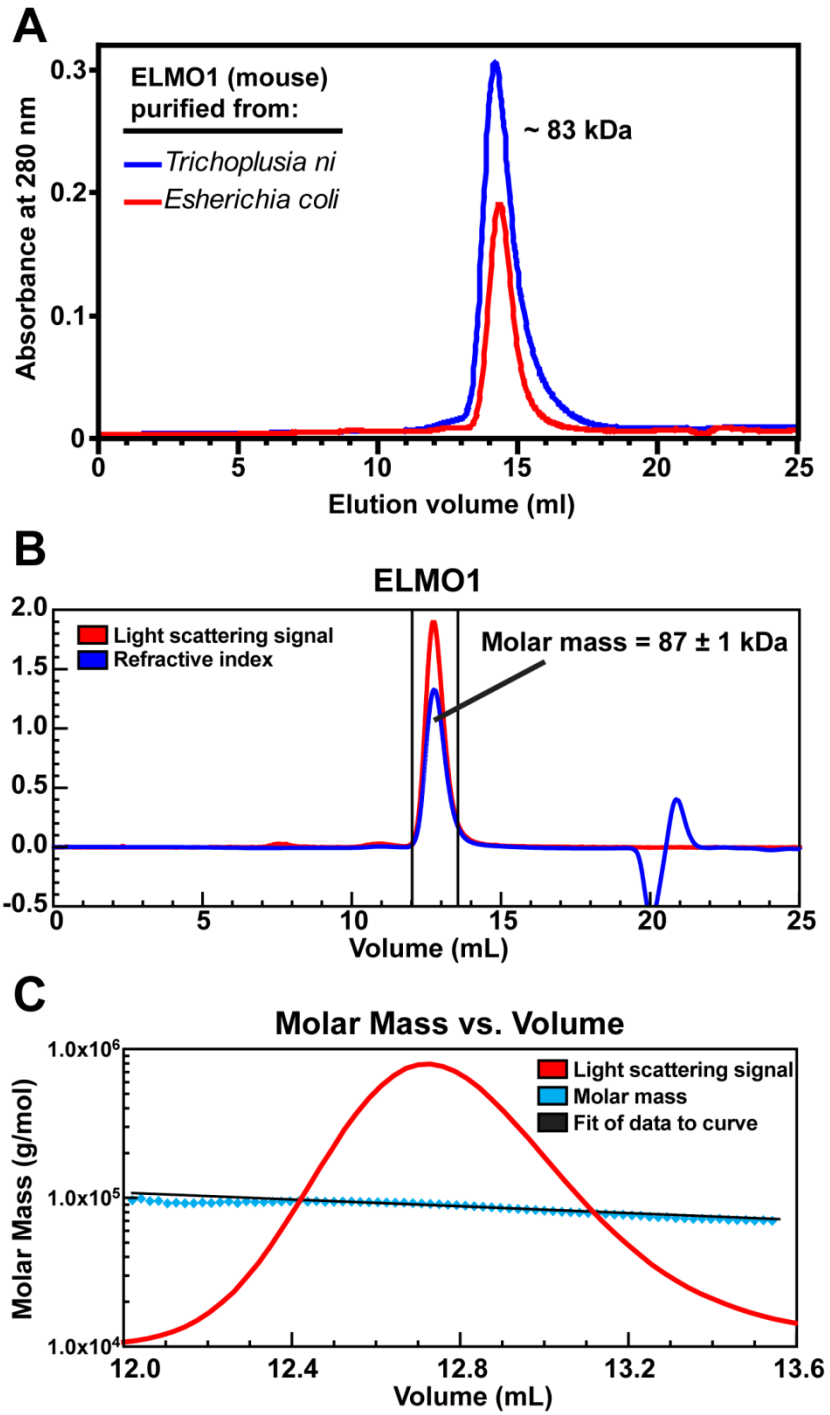


Figure 4: Purified ELMO1 is a monomer in solution.

A) ELMO1 purified from bacteria (red) and insect cells (blue) was applied to an analytical SEC column. The resulting chromatograms are overlaid. The peak elution volume was compared to the elution volumes of globular proteins of known size applied to the same column, and a molecular weight of ~83 kDa was calculated for both ELMO1 protein samples. **B)** ELMO1 was subjected to SEC-coupled multi-angle laser light scattering to determine molecular weight and eluted in a single peak with a calculated molecular weight of 87±1 kDa. **C)** Experimentally determined molar mass of ELMO1 plotted vs. elution volume of the light scattering peak. The nearly horizontal molar mass data indicates monodispersity of the sample.

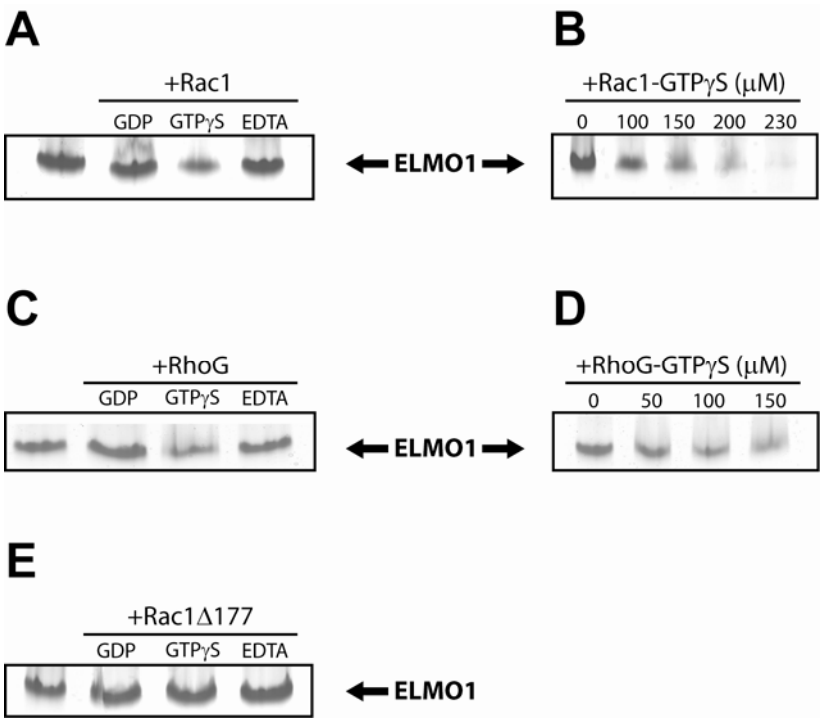


Figure 5: Binding of Rac1 and RhoG to ELMO1 is both nucleotide-dependent and PBR-dependent.
ELMO1 and RhoG/Rac1 complex formation was monitored by depletion of the unbound ELMO1 band in non-denaturing polyacrylamide (native) gels, using an elctrophoretic mobility shift assay (gel shift). Proteins were detected by Coomassie brilliant blue staining. **A)** ELMO1 (50 μM) was incubated with Rac1 (100 μM) in a GDP-bound, GTPγS-bound or nucleotide-free (EDTA) state. **B)** ELMO1 (50 μM) was incubated with increasing concentrations of Rac1-GTPγS. **C)** ELMO1 (25 μM) was incubated with RhoG (100 μM) in a GDP-bound, GTPγS-bound or nucleotide-free (EDTA) state. **D)** ELMO1 (25 μM) was incubated with increasing concentrations of Rac1-GTPγS. **E)** ELMO1 (50 μM) was incubated with Rac1Δ177 (100 μM) in a GDP-bound, GTPγS-bound or nucleotide-free (EDTA) state.

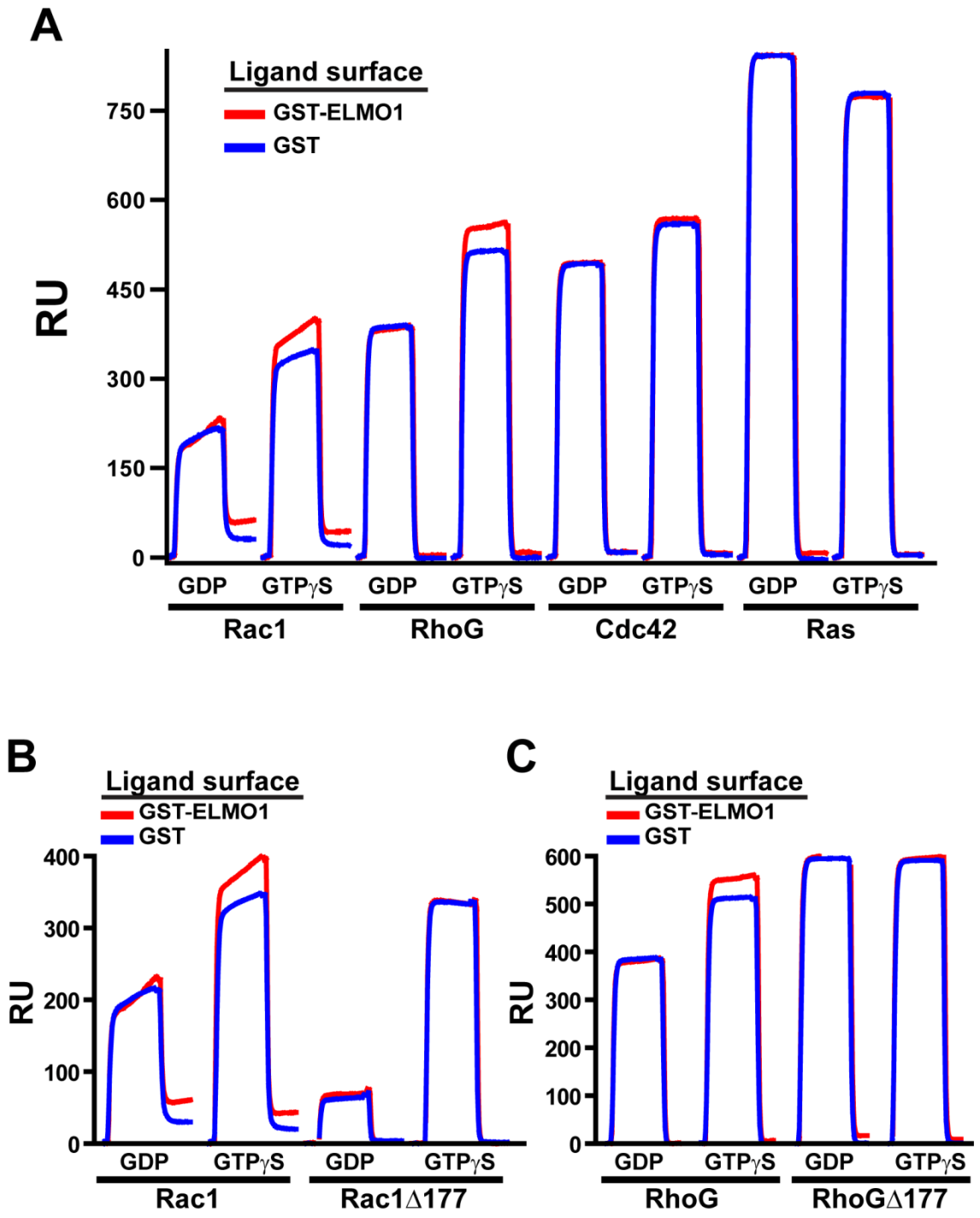


Figure 6: ELMO1 binding to RhoG and Rac1 is nucleotide and PBR dependent.

A-C) Ligand surfaces of GST-ELMO1 or GST were coupled to a CM5 chip modified with covalently-bound anti-GST antibody. Binding of 10 μM GTPases, either GDP- or GTPγS-bound was monitored by the increase in response units (RU) via surface plasmon resonance. Binding to ELMO1 (red) is measured as an increase in RU over that of the GST control (blue).

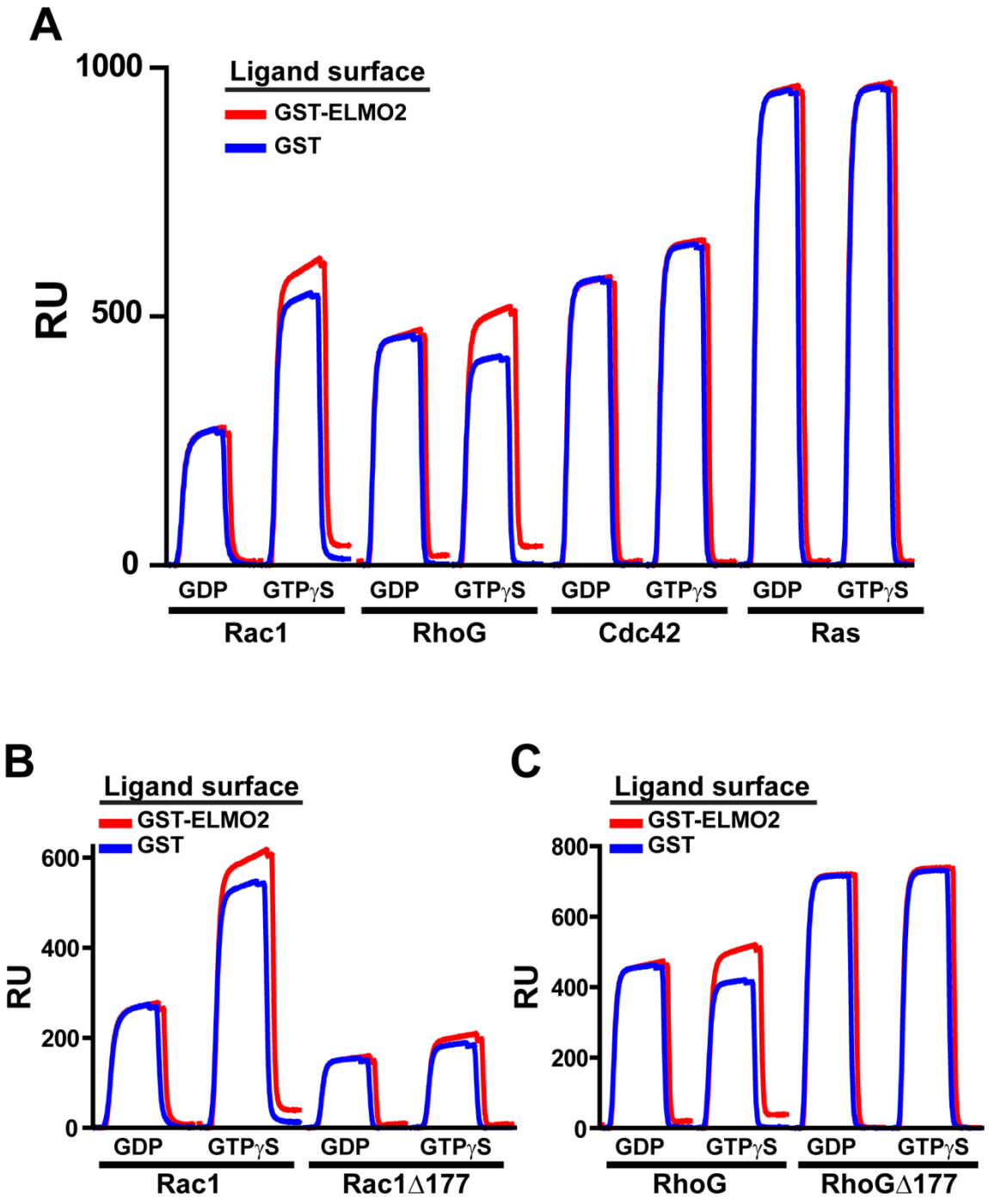


Figure 7: ELMO2 binding to RhoG and Rac1 is nucleotide and PBR dependent.

A-C) Ligand surfaces of GST-ELMO2 or GST were coupled to a CM5 chip modified with covalently-bound anti-GST antibody. Binding of 10 μM GTPases, either GDP- or GTPγS-bound was monitored by the increase in response units (RU) via surface plasmon resonance. Binding to ELMO2 (red) is measured as an increase in RU over that of the GST control (blue).

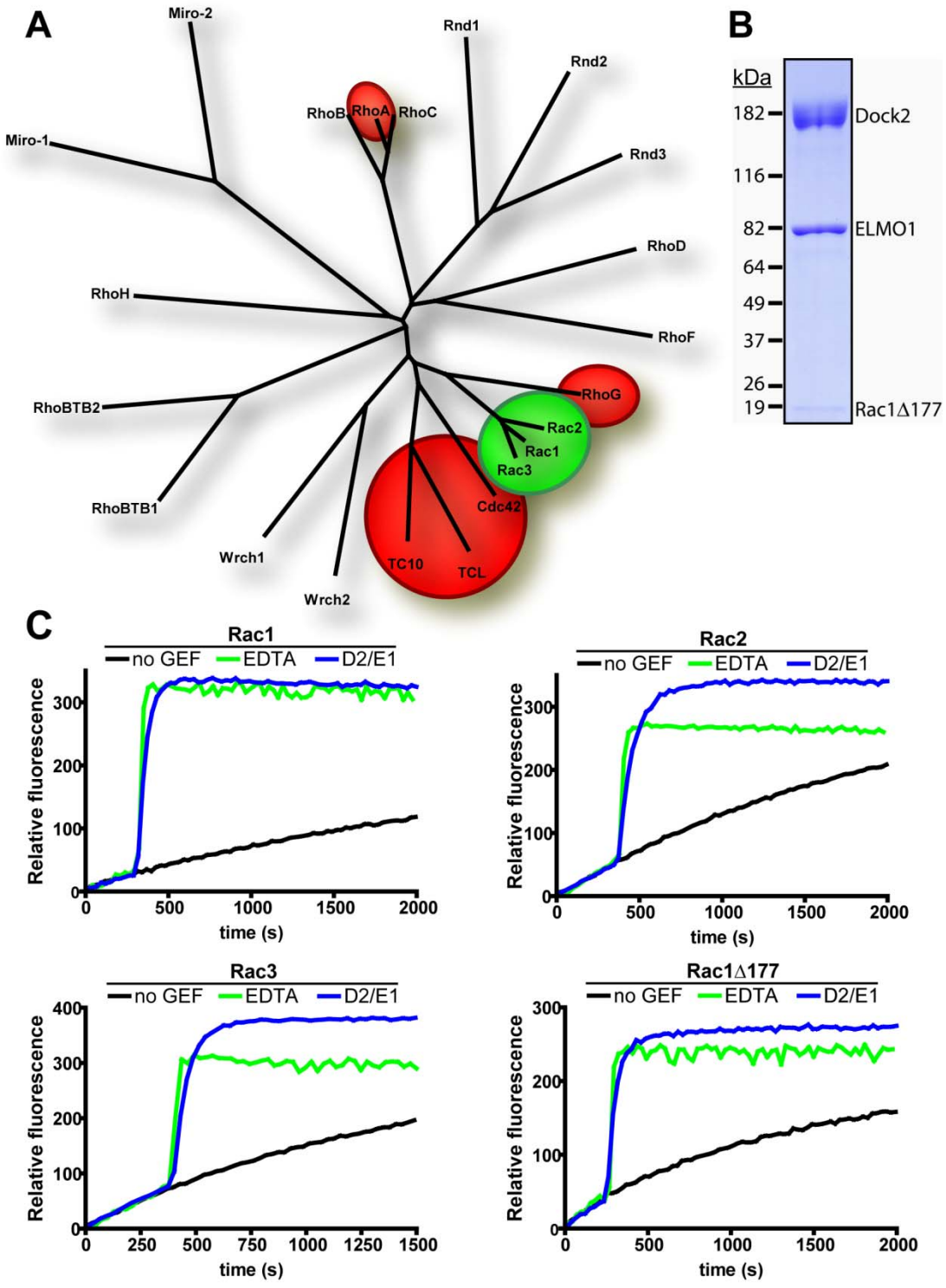


Figure 8: Dock2/ELMO1 is an effective GEF for Rac1, Rac1Δ177, Rac2 and Rac3.

A) Rho-family GTPase tree. Green indicates Dock2/ELMO1 is an effective exchange factor for GTPase. Red indicates lack of exchange activity by Dock2/ELMO1. **B)** Purified Dock2/ELMO1 bound to Rac1. **C)** Fluorescence-based exchange assays monitoring the loading of BODIPY-GDP onto GTPases (2 μM). Dock2/ELMO1 was used at 100 nM and EDTA was used at 20 mM as a control for GTPase activity.

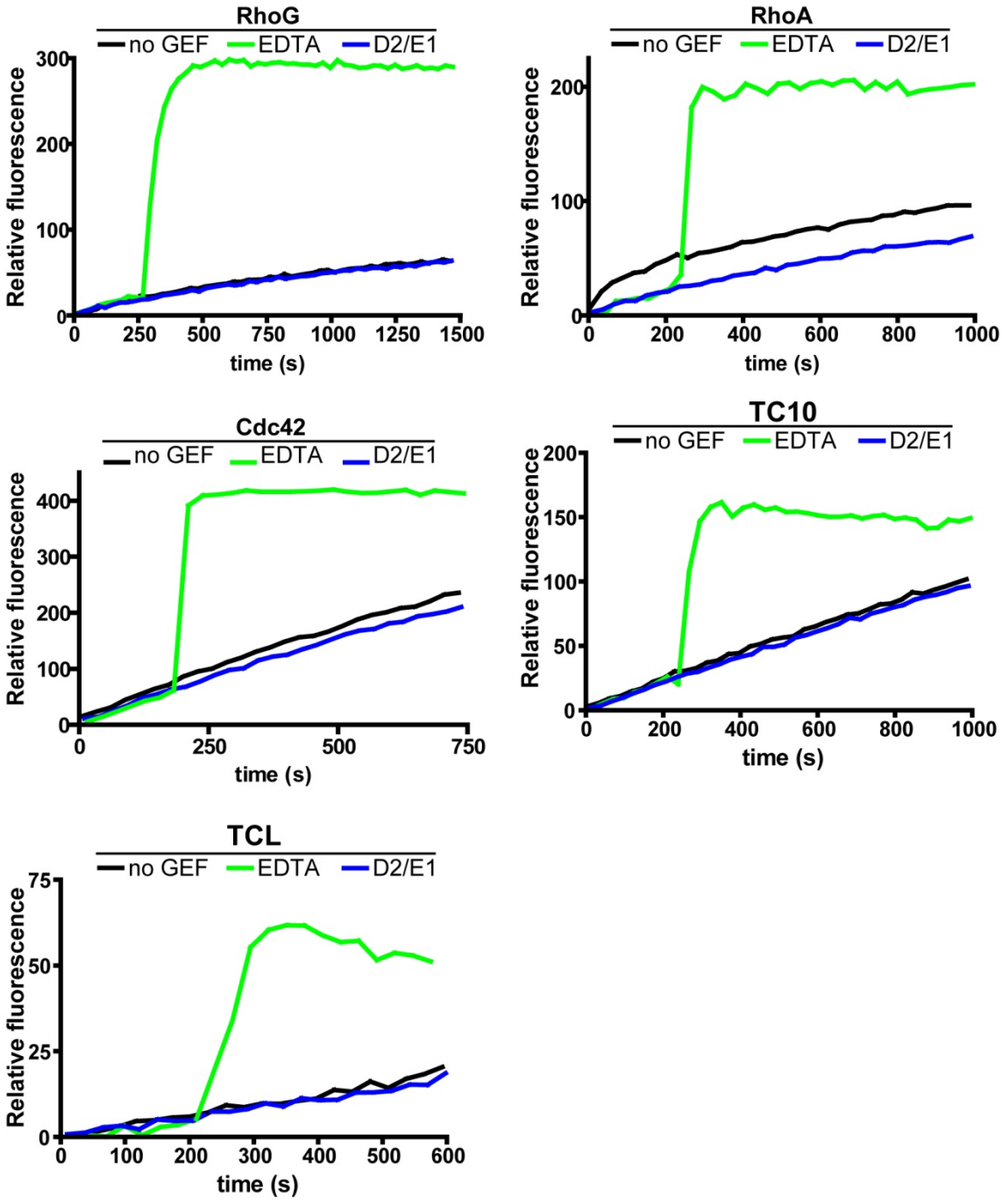


Figure 9: Dock2/ELMO1 does not catalyze exchange on other Rho-family GTPase members.
Fluorescence-based exchange assays monitoring the loading of BODIPY-GDP onto GTPases (2 μM). Dock2/ELMO1 was used at 100 nM and EDTA was used at 20 mM as a control for GTPase activity.

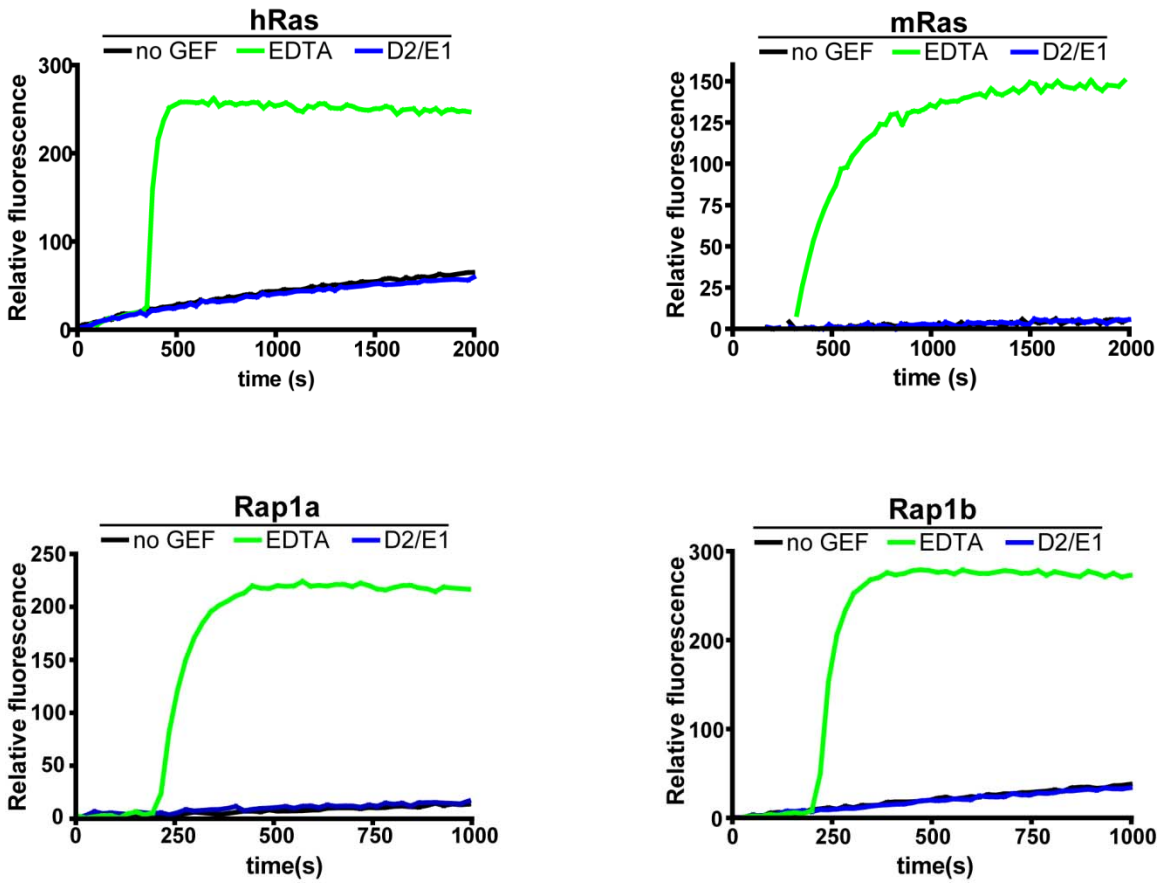
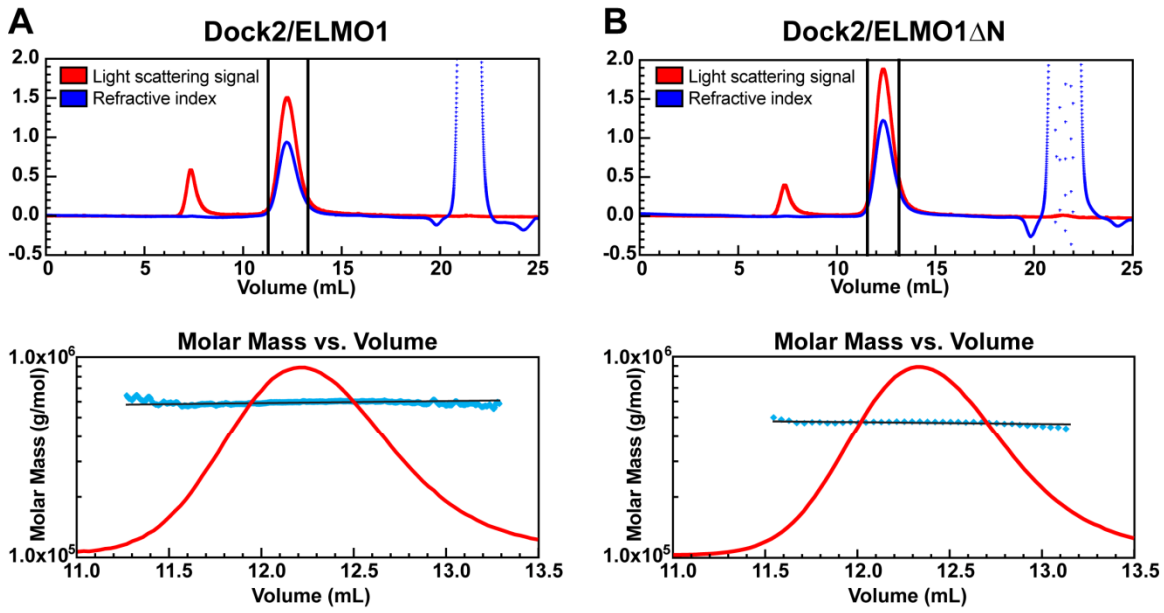


Figure 10: Dock2/ELMO1 does not catalyze exchange on other Ras superfamily GTPase members.
Fluorescence-based exchange assays monitoring the loading of BODIPY-GDP onto GTPases (2 μM). Dock2/ELMO1 was used at 100 nM and EDTA was used at 20 mM as a control for GTPase activity.



C

	Calculated MW: Dock2	Calculated MW: ELMO1	Calculated MW: Heterotetramer	Mean experimental MW*
Dock2/ELMO1 (N=5)	213 kDa	84 kDa	594 kDa	598 ± 23 kDa
Dock2/ELMO1ΔN (N=3)	213 kDa	23 kDa	472 kDa	466 ± 7 kDa

*Data represented as the mean ± 1 standard deviation (σ) of the mean.

Figure 11: Dock2/ELMO1 is a heterotetramer.

A, B) Dock2/ELMO1 and Dock2/ELMO1ΔN samples were subjected to SEC-coupled multi-angle laser light scattering to determine molecular weight. Both complexes eluted as a single, monodispersed peak. Upper panels: An overlay of the light scattering signal (red) and refractive index (blue) of each sample. The areas of peaks used for molecular weight determination are bounded by black lines. The peaks at 7.5 ml contained no detectable protein, and the peaks around 21.5 ml are due to sample buffer components. Lower panels: The molar mass of the selected peak is plotted vs. the elution volume of the peak. The horizontal nature of the lines indicating molar mass indicates the samples are monodispersed. The calculated molar mass is in cyan, the fit of the data to a parabola is in black, and the light scattering signal is in red. **C)** Table showing the predicted and experimentally determined molecular weights (MW) for the Dock2/ELMO1 complexes tested.

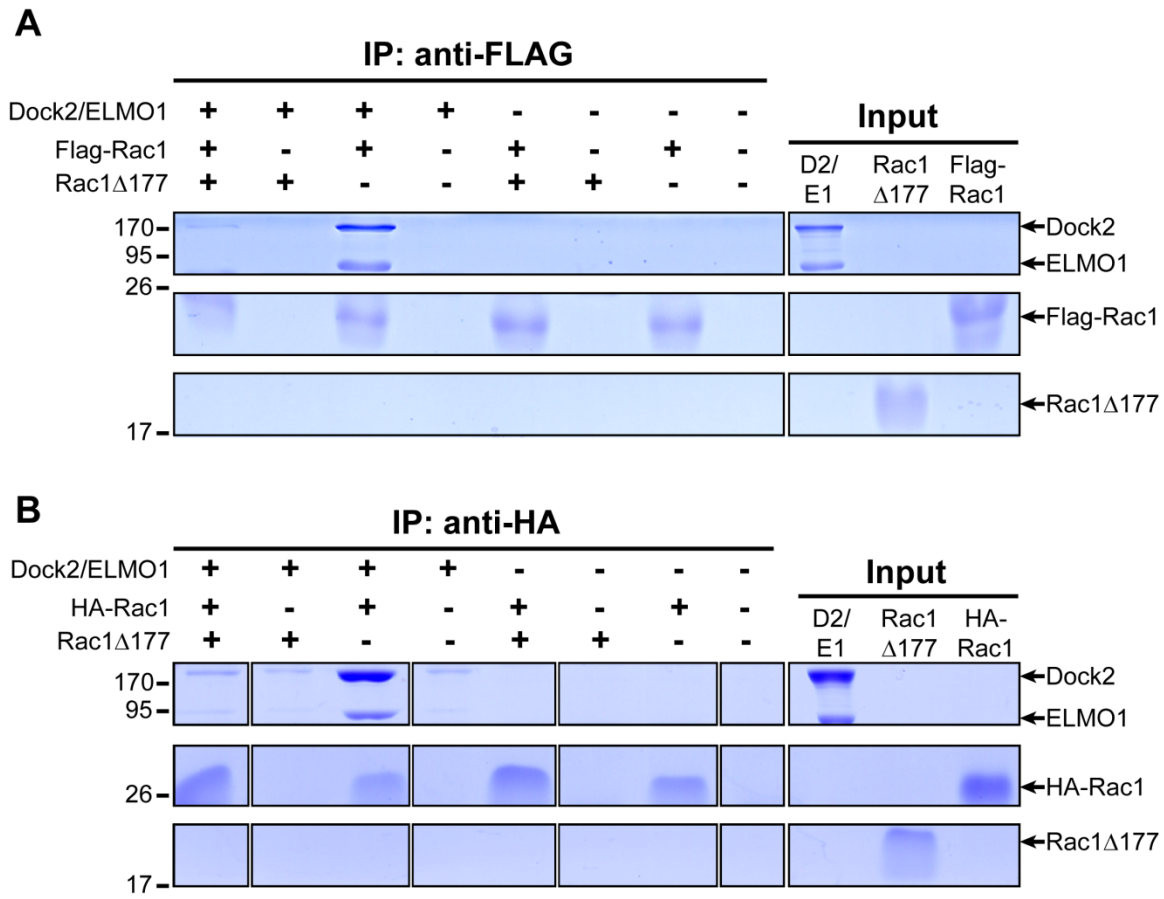


Figure 12: The Dock2/ELMO1 tetramer binds a single Rac1 molecule.

A) Dock2/ELMO1 and bacterially purified Flag-Rac1 and untagged Rac1Δ177, as indicated, were pre-incubated, then precipitated by anti-Flag antibody-coupled beads. Bound proteins were eluted by boiling in SDS-PAGE sample loading buffer and run on an SDS-PAGE gel, stained with Coomassie brilliant blue. Right panel shows purified proteins used. **B)** Experiment was run as in (A), but with HA-tagged Rac1 replacing Flag-Rac1, and anti-HA antibody-coupled beads were used for the immunoprecipitation.

CHAPTER 3 – RHOG DIRECTLY REGULATES THE ACTIVITY OF THE DOCK/ELMO COMPLEX VIA INTERACTION WITH ELMO PROTEINS

Background

The specific, functional role that ELMO proteins play within the context of the Dock/ELMO heterotetramer remains to be defined. Although ELMO1 possesses no independent GEF activity, the PH domain of ELMO1 can interact *in trans* with a complex of Dock1 and nucleotide-free Rac1 independent of other defined Dock/ELMO interactions. This interaction increases the exchange activity of Dock1 by about two-fold *in vitro*. Interestingly, mutation of a tryptophan residue to alanine in the PH domain of ELMO1 can disrupt formation of this trimeric complex, and ELMO1 constructs containing this mutated PH domain are unable to synergize with Dock1 in exchange assays, although the proteins are still capable of interacting through the other defined interactions between Dock1 and ELMO1 [41]. Beyond the PH domain interaction and ELMO1 synergizing with Dock1 to enhance exchange activity [25,71], Makino, *et al.* describe the ability of ELMO1 to inhibit ubiquitylation of Dock1. Potential evidence for this functionality was seen in our own results (Chapter 2), when we were only able to purify significant quantities of Dock2 from insect cells when it was co-expressed with ELMO1.

In addition to whatever direct effects ELMO proteins have on the function of the Dock/ELMO tetramer, RhoG, a member of the Rho-family of GTPases interacts with the

N-terminus of ELMO1 in its active, GTP-bound form. Through this interaction, RhoG is able to translocate the primarily cytoplasmic Dock/ELMO complex to the plasma membrane [47,74]. RhoG activates Rac1 via the Dock/ELMO complex, and this pathway is required for integrin-mediated Rac1 activation and cell spreading, promotion of cell migration, as well as nerve growth factor-induced neurite outgrowth [47,48]. The Dock/ELMO complex is not, however, required for the RhoG-induced suppression of anoikis [125]. The activation of Rac1 by RhoG is usually attributed to the translocation of the Dock/ELMO complex to the plasma membrane.

Here, we present data showing that there is an inhibitory intramolecular interaction between the N- and C-terminal regions of ELMO1. Truncation of the N-terminus of ELMO1 to relieve this interaction results in increased phagocytosis in LR73 cells, as well as increased exchange activity of the Dock/ELMO complex. Activated RhoG can approximate the effect of truncation by increasing the exchange activity of the Dock1/ELMO1 or Dock2/ELMO1 complex. This activation is lost if the form of ELMO protein used is incapable of binding to RhoG. The Dock2/ELMO1ΔN complex is also capable of increased binding to PI(3,4,5)P3 over that of the Dock2/ELMO1 wild-type complex. We propose a model whereby activated RhoG binds the N-terminus of ELMO1 to disrupt the inhibitory intramolecular interaction, thereby causing a conformational change which directly increases the exchange activity of the Dock2/ELMO1 complex.

Experimental Procedures

Plasmids

pEBB-ELMO1-GFP, ELMO1ΔN-Flag, ELMO1ΔC-Flag, ELMO1GL-Flag (previously ELMO1arm2-Flag) have been previously described [40,49] To generate the pGBT10-ELMO1 vector, the Flag-ELMO1 coding sequence was removed from the pEBB-ELMO1-Flag vector with BamHI and NotI. The insert was ligated into the BamHI–NotI site of a modified pGBT10 vector containing an AscI and NotI linker inserted between the BamHI and EcoRI sites with these two restriction sites intact.

ELMO1 constructs for insect cell expression were PCR-amplified and inserted into pFastBac1 using a modified ligation-independent cloning strategy (LIC) [107]. ELMO1 (1-727) and ELMO1ΔN (532-727) were amplified from pGEX4T2-ELMO1 [40], with an N-terminal methionine added to initiate translation for ELMO1ΔN. ELMO1GL was amplified from pGEX4T2-ELMO1arm2 [49].

The pCXN2-Flag-Dock180 construct was from Dr. Michiyuki Matsuda [32,33]. A construct encoding a GST fusion of the PH domain of mouse Grp1 in pGEX2TK (GE Healthcare) was a gift from Dr. Mark Lemmon [126]. Other plasmids were as described in Chapter 2, experimental procedures. All new constructs were verified by automated sequencing.

Protein purification

GST-Grp1-PH was expressed as described [126]. The cells were harvested by centrifugation and lysed in 20 mM Tris pH 7.5, 1 M NaCl, 1% Triton X-100, 2 mM DTT, 0.5 mM PMSF, and 1 tablet protease inhibitor cocktail (Roche) per 25 ml buffer, using an

Emulsiflex-C5 homogenizer (Avestin), and clarified by ultracentrifugation at ~150,000 x *g*. Clarified supernatant was loaded onto a 5 ml GSTrap FF column (GE Healthcare) pre-equilibrated with GST-PH buffer (20 mM Tris pH 7.5, 0.5 M NaCl, 0.1% Triton X-100, 2 mM DTT, 0.5 mM PMSF, 10% glycerol, and 1 tablet protease inhibitor cocktail per ~100 ml), and eluted with GST-PH buffer supplemented with 10 mM reduced glutathione. Fractions containing GST-Grp1-PH were pooled, concentrated using a 10K MWCO Vivaspin 20 ml concentrator, snap frozen in liquid N_2, and stored at -80°C.

Cell culture

Mammalian cells were maintained in a moisturized incubator with 5% CO_2 at 37º C. Human 293T fibroblast cells were maintained in Dulbecco's Modification of Eagle's Medium containing 10% FBS and 1% penicillin/streptomycin/glutamine. The Chinese Hamster Ovary fibroblast cell line LR73 [127] was maintained in Alpha's Modified Eagle's Medium supplemented with 10% FCS and 1% penicillin/streptomycin/glutamine. 293T cells were transiently transfected by the calcium phosphate method and LR73 cells were transiently transfected using Lipofectamine 2000 (GIBCO-BRL) according to the manufacturer's instructions [40]. In all experiments, carrier DNA was added to keep equal plasmid concentration between different samples.

Immunoprecipitation assays

Before lysis, all mammalian cells were washed with ice cold PBS. Cells were then lysed on ice 48 hours after transfection for 10 minutes in lysis buffer containing 50 mM Tris, pH 7.6, 1% (v/v) Triton X-100, 150 mM NaCl, 10 mM Sodium Pyrophosphate, 10 mM Sodium Fluoride, 1 mM orthovanadate, 10 μg/ml AESF, 1 mM DTT, 10 μg/ml

Pepstatin, 10 μg/ml Aprotinin and 10 μg/ml Leupeptin. Lysates were then cleared in a centrifuge for 10 minutes at 4˚C. All beads were washed in lysis buffer prior to incubation with cell lysates or bacterially produced purified protein. Immunoprecipitations were performed as described previously [25,40]. Briefly, for precipitation of Flag tagged proteins, 20 μl of anti-Flag M2 antibody (Sigma) directly coupled to Sepharose was used per lysate from a 100 mm culture dish. Cleared cell lysates were then incubated with the beads for 1-2 hours at 4º C on a Nutator. Beads were then washed four times with 1 ml of washing buffer (20 mM Hepes, pH 7.4, 0.1% (v/v) Triton X-100, 150 mM NaCl, 5 mM Sodium Fluoride, 1 mM Orthovanadate, 10% (v/v) Glycerol, 1mM DTT, 10 μg/ml Pepstatin, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin). Precipitated proteins were assessed by SDS-PAGE and immunoblotting. Antibodies used as indicated were mouse monoclonal anti-GFP (clone B2, used at 0.5 μg/ml, Santa Cruz Biotechnology), Anti-Flag M2 monoclonal antibody (clone M2, used at 1:10,000, Sigma) and purified rabbit polyclonal anti-ELMO1 [25]. HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (both used at 1:10,000) were from GE Healthcare. Immunoblots were developed using enhanced chemiluminescence (Pierce).

Yeast two-hybrid assays

The strain HF7C was employed and growth was monitored on -histidine, -tryptophan, and -leucine selection media. Briefly, constructs cloned into pGBT10 are fused to a DNA binding domain and transformants thrive on tryptophan selection media. Genes cloned into pVP16 are fused to an activation transformants will grow on media lacking leucine. Yeast transformants carrying both plasmids will be able to grow on tryptophan and leucine deficient media. When the proteins of interest interact, those

transformants will also thrive in/on histidine-deficient media. 3-amino-1,2,4-triazole (3-AT) inhibits histidine synthesis, and can be added to media to increase selectivity. Cells were transformed using the Lithium Acetate-based transformation protocol. Genes of interest were cloned into the pVP16 or the pGBT10 vectors as indicated.

Exchange assays

The radioactivity based *in vitro* GEF assay was performed as described previously [25] with minor modifications. 293T cells in 100 mm dish were transfected with either Flag-Dock1 or a Flag-ELMO1 construct + Dock1 and the cell lysates were immunoprecipitated with anti-Flag antibody. Precipitated proteins were then eluted into 120 μl with Flag peptide (Sigma) following manufacturer's recommendation. The eluted proteins were quantitated after Western blotting. Equal amounts of Dock1 were analyzed for GEF activity toward Rac as follows: 5 μg of bacterially expressed and purified Rac was loaded with 50 μCi of [α-^{32}P] GTP (3000 Ci/mmol) in 40 mM MOPS (pH7.1), 1 mM EDTA, 1 mg/ml BSA and 0.3 μM unlabeled GTP, for 20 minutes on ice. $MgCl_2$ was then added to 10 mM and incubated on ice for an additional 10 minutes. 125 ng ^{32}P-GTP loaded Rac was added with 10 μl of eluted proteins (for wild-type Dock1, volume for other samples was adjusted based on the concentration of eluted Dock1) to reaction buffer of 25 mM MOPS, pH7.1, 6.25 mM $MgCl_2$, 0.6mM NaH_2PO_4, 0.5 mg/ml BSA, 1.25 mM GDP (unlabeled) to a final volume of 100 μl. After 15 minutes at 30° C, 50 μl of the exchange reaction was subjected to filter binding to nitrocellulose and quantitated by scintillation counting. The presence of GEF-activity was revealed as loss of radioactivity bound to Rac (i.e. due to the exchange reaction). Relative GEF activity was presented as the percentage of radioactivity loss, with samples from mock-transfected

293T cell set at 0% and samples from wild-type Dock180 alone transfected cells at 100%. Results are representative of at least five independent experiments.

Fluorescence-based exchange assays utilized either Rac1(C189S) or Rac1Δ177 pre-loaded with BODIPY-FL-GDP. To load the GTPase, Rac1 was incubated for 30 minutes at room temperature with 2-5 mM EDTA, and ~3-fold molar excess of BODIPY-FL-GDP. After incubation, 10-20 mM $MgCl_2$ was added and the excess nucleotide was removed using a Hi-Prep desalting column (GE Healthcare) pre-equilibrated with 20 mM HEPES pH 7.5, 150 mM NaCl, 2 mM DTT, 10 mM $MgCl_2$ and 10% glycerol. Aliquots were snap frozen in liquid N_2, and stored at -80°C. RhoG was loaded with GDP and GTPγS in a similar manner, with the exceptions that a 10-fold molar excess of GDP or GTPγS was used. Efficient nucleotide loading for RhoG was confirmed using a radioactive filter binding assay and GTPγ^{35}S. When used in exchange reactions, RhoG was pre-incubated with the indicated GEF for a minimum of 15 minutes at 25°C.

Exchange assays utilizing Rac1 pre-loaded with BODIPY-FL-GDP were carried out in 20 mM HEPES, 200 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 100 μM GDP and 10% glycerol at 10°C on a Perkin-Elmer LS-55 fluorimeter, using excitation and emission wavelengths of 503 and 511 nm. Rac1 was used at 150 nM, Dock2/ELMO1 complexes were added to 15 nM and RhoG was used at 10 μM. Data analysis was carried out in GraphPad Prism software version 4.0.

Flag-Dock1 and Dock1/Flag-ELMO1 complexes were obtained by immunoprecipitation as described for the radioactive GEF assays. Equal amounts of Dock180 were analyzed for GEF activity using 150 nM Rac1-BODIPY-FL-GDP as above.

Phosphoinositide binding assays

PIP-beads precipitation - Purified Dock2/ELMO1 and Dock2/ELMO1ΔN were incubated with PI(3,4,5)P_3 beads (Echelon Biosciences) in varying amounts (5, 25, 50 and 250 pmoles of tetramer per reaction) in buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 0.25% NP-40 and 0.5 mg/ml BSA for 3 hours on a rotator at 4°C, in a 500 µl total volume. 20 µl of bead slurry (washed with buffer) was used per condition. After incubation, beads were collected by centrifugation at 1000 rpm for 3 minutes and the supernatant was aspirated away from the beads. The beads were washed with 1 ml buffer, spun, and the supernatant removed 3 times. Bound proteins were eluted by boiling in 20 µl 2X SDS-PAGE loading dye, and samples were run on SDS-PAGE gels. Dock2/ELMO1 complex binding was measured by the N-terminal 6X-His tag on Dock2 via western blotting by incubating with Penta-His HRP conjugate (Qiagen) antibody, and was detected with enhanced chemiluminescence (ECL, GE Healthcare). To test for specificity, PIP beads conjugated to PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2 and PI(3,4,5)P_3 were utilized in assays as above, with 25 nM pmoles of tetramer per assay.

Fluorescence polarization - ELMO1, ELMO1ΔN or GST-Grp1-PH were titrated into buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 1 mM DTT) containing 50 nM PI(3,4,5)P_3 labeled with BODIPY-FL (Echelon Biosciences). Titrations were performed manually at 25°C, and the change in polarization was measured in triplicate for each concentration assayed [128]. Data were fit to a single-site binding model using non-linear regression with SigmaPlot software to yield parameters for P_{min} (starting polarization), P_{max} (maximum polarization), and K_D (dissociation constant).

Results

Intramolecular interaction within ELMO1

In Chapter 2, size exclusion chromatography and light scattering data show that purified ELMO1 exists as a monomer in solution (Fig. 4). In contrast, immunoprecipitations suggest that full-length Flag-ELMO1 is capable of interacting with full-length GFP ELMO1 when co-transfected into human 293T fibroblasts (Fig. 13). To resolve this conflict, we examined whether the interactions we were seeing in the immuoprecipitations were actually stemming from intramolecular interactions within ELMO1, rather than intermolecular interactions between different ELMO1 molecules.

To test this, we examined the capability of different pieces of ELMO1 (Fig. 13) to interact via immunoprecipitation assays. Full-length Flag-ELMO1 was capable of precipitating full-length GFP-ELMO1, as well as ELMO1ΔN, ELMO1ΔC and ELMO1$_{GL}$, a mutant incapable of interaction with active RhoG. Flag-ELMO1ΔC, encompassing residues 1-550, precipitated full-length ELMO1, but not ELMO1ΔN, while ELMO1ΔN, encompassing residues 532-727, precipitated both full-length ELMO1, and ELMO1ΔC. It is unclear why ELMO1ΔC failed to precipitate ELMO1ΔN, when the precipitation worked in the reverse direction, but this is likely caused by interference due to the nature of antibody binding to the tagged ELMO1 fragments. These immunoprecipitations suggest that there is an interaction within or between ELMO1 molecules that involves both the N- and C-terminal portions of ELMO1

Yeast-two hybrid assays were employed to further examine these interactions. Plasmid constructs encoding ELMO1, ELMO1ΔN and ELMO1ΔC in the appropriate fusions with the DNA binding domain or activation domain were co-transformed in

various combinations in yeast and growth of the transformants were monitored on selective media (Fig. 14A). Of the combinations tested, the interaction between ELMO1ΔC and ELMO1ΔN was the strongest, showing yeast growth comparable to that of the known interaction between ELMO1ΔC and constitutively active RhoG (Q61L). These data, combined, suggest that the detected ELMO1-ELMO1 interactions originate from an intramolecular interaction between the N- and C-terminal portions of ELMO1.

To test the existence of an intramolecular association between the N- and C-terminal portions of ELMO1, we used a yeast-two hybrid assay to compare the interactions between ELMO1, ELMO1ΔC or ELMO1ΔN with $RhoG^{Q61L}$ (Fig. 14B). ELMO1 and ELMO1ΔC both interacted with $RhoG^{Q61L}$ and ELMO1ΔN, which has the region necessary for $RhoG^{Q61L}$ binding removed, did not interact. The strength of the interaction between $RhoG^{Q61L}$ and ELMO1ΔC was greater than the interaction with ELMO1, supporting the hypothesis that an intramolecular interaction between the N- and C-terminal portions of ELMO1 exists. Abolishing this interaction by removal of the C-terminus of ELMO1 increases the ability of the N-terminus of ELMO1 to interact with $RhoG^{Q61L}$.

Relief of the intramolecular interaction within ELMO1 hyper-activates the Dock2/ELMO1 complex and increases phagocytosis

To examine the effect of the ELMO1 intramolecular interaction within the context of the Dock/ELMO tetramer, we tested various ELMO1 constructs using engulfment and exchange assays. Phagocytic LR73 fibroblasts transfected with GFP-ELMO1ΔN and Flag-Dock1 showed an increase in engulfment about 5.5-fold above basal levels – measured by the percent of GFP positive cells containing engulfed beads (Fig. 15A).

Dock1 and full-length ELMO1, on the other hand, only promoted engulfment about 2.5-fold above basal levels, while a Dock1 + ELMO1ΔC (which does not interact with Dock1) only increased phagocytosis by 1.5-fold. ELMO1 constructs transfected without Dock1 did not promote phagocytosis. This increase in activity upon deletion of the N-terminal 531 residues of ELMO1 may reflect an inhibitory effect of the intramolecular interaction between the N- and C-terminal portions of ELMO1. Equivalent protein expression was determined by western blotting (Data not shown).

The increase in phagocytosis caused by the ELMO1ΔN fragment could be caused by an increase in the ability of the Dock/ELMO complex to catalyze exchange. To test the effect of ELMO1ΔN on the exchange activity of the complex, we tested the ability of the ELMO1 constructs to synergize with Dock1 in radioactive exchange assays. We transfected 293T cells with Flag-tagged ELMO1, ELMO1ΔN and ELMO1ΔC, with and without Dock1, and immunoprecipitated the proteins using anti-Flag antibody. After elution with the Flag peptide, we measured the amounts of protein via western blotting, and equalized the quantities of proteins used in a radioactive GEF assay (Fig. 15B). We arbitrarily set the relative level of activity for Dock1 at 100%. The ELMO1 fragments alone showed no ability to promote exchange on Rac1, neither did Dock1/ELMO1ΔC. Dock1/ELMO1 was capable of increased exchange activity at about 175%, however, Dock1/ELMO1ΔN showed an increase in exchange activity above the level of wild-type at about 275%. Combined, the increase in phagocytosis and the increase in exchange activity suggest that the ELMO1 intramolecular interaction may serve as an inhibitory mechanism for Rac1 exchange.

The exchange activity of purified Dock2/ELMO1ΔN and Dock2/ELMO1 complexes was tested using fluorescent-based guanine nucleotide exchange assays (see Fig. 16B for a gel of purified proteins showing equal concentrations of the exchange factors used). Briefly, Rac1Δ177 was pre-loaded with BODIPY-FL-GDP, and the exchange of fluorescent nucleotide for GDP from the buffer was monitored over time as a decrease in fluorescence. The data from a minimum of three assays per condition were fitted to a single exponential decay curve, which provided relative rates of exchange. Dock2/ELMO1 and Dock2/ELMO1ΔN were both efficient exchange factors for Rac1 (Fig. 16A), and Dock2/ELMO1ΔN showed an approximately two-fold increase in the rate of exchange over Dock2/ELMO1 (Fig. 16C).

RhoG-GTPγS enhances exchange activity of immunoprecipitated Dock1/ELMO1

We suggested above that the intramolecular interaction between the N- and C-terminal portions of ELMO1 may be an inhibitory mechanism since removal of the N-terminus resulted in increased exchange activity and phagocytosis. Cells must be able to release this inhibition for this to be a biologically-relevant mechanism. Since RhoG is known to bind to the N-terminus of ELMO1 [47], activated RhoG might be the key for unlocking this intramolecular interaction.

We used purified full-length, non-prenylated RhoG (C188S) as an additive in fluorescence-based exchange assays to test whether RhoG can affect exchange activity of Dock2/ELMO1 complex. RhoG was pre-loaded with either GDP or GTPγS, a non-hydrolysable GTP analogue, and fluorescence-based exchange assays were carried out as above, with the exception that RhoG was pre-incubated with Dock/ELMO where indicated.

Dock1/ELMO1 complexes were immunoprecipitated from 293T cells essentially as done for the radioactive GEF assays. Flag-ELMO1 constructs were co-transfected with Dock1, or Flag-Dock1 was transfected alone. Dock1, by itself, is an active exchange factor in vitro for Rac1, but the Dock1/ELMO1 complex is significantly more active than Dock1 alone (Fig. 17). When RhoG-GDP is incubated with Dock1/ELMO1, there is no detectable difference in the exchange activity of the complex. When RhoG-GTPγS is incubated with Dock1/ELMO1, however, there is a definite increase in the exchange activity of the complex (Fig. 17B). Neither Dock1 alone, nor Dock1 complexes containing ELMO1ΔN or ELMO1GL (G72A, L77S), were activated by either form of RhoG (Fig. 17A and Fig. 18). Both ELMO1ΔN and ELMO1GL are incapable of interacting with RhoG due to an N-terminal truncation and N-terminal point mutations, respectively, although interaction with Dock1 is apparently unaltered.

RhoG-GTPγS enhances the exchange activity of purified Dock2/ELMO1

We tested the purified Dock2/ELMO1 complex in an exchange assay with and without RhoG (Fig. 19A). RhoG-GDP does not increase the rate of exchange for Dock2/ELMO1, but with RhoG-GTPγS, the rate is significantly increased over that of the complex alone. When RhoG, in either form, is added to the Dock2/ELMO1ΔN complex, there is no change in the exchange activity of the complex (Fig. 19B). The purified Dock2/ELMO1GL complex also showed no activation by either form of RhoG (Fig. 19C). While the increase in the rate of exchange seen by Dock2/ELMO1ΔN over wild-type complex is larger, the increase of the relative rate of exchange of the wild-type Dock2/ELMO1 tetramer induced by RhoG-GTPγS is statistically significant ($P<0.01$) as measured by a student's unpaired t-test (Fig. 19E). The purified proteins were run on a

Coomassie brilliant blue-stained SDS-PAGE gel, showing equivalent protein concentrations for the Dock2/ELMO1 complexes (Fig. 19D).

These data suggest that the activation of the Dock2/ELMO1 complex by active RhoG could be a mechanism by which cells release the inhibition caused by the intramolecular interaction between the N- and C-terminal regions of ELMO1. The binding of RhoG is known to require the N-terminus of ELMO1, and to be GTP-dependent. In these assays, we were able to approximate the activation of the complex seen with truncated ELMO1 by adding RhoG-GTPγS to wild-type Dock2/ELMO1. When RhoG binding was eliminated, RhoG was incapable of hyper-activating the complex.

Dock2/ELMO1 phosphoinositide association

The DHR-1 region of Dock2 is known to bind to phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$), likely through the predicted C2 domain [129]. We tested the lipid-binding capabilities of purified Dock2/ELMO1 complexes by incubating with phosphoinositide-coated beads and precipitating the bound proteins. Consistent with published results, Dock2/ELMO1 bound to $PI(3,4,5)P_3$, and not to other phosphoinositides (Fig. 20A). Of interest, when we compared the binding of Dock2/ELMO1 and Dock2/ELMO1ΔN to $PI(3,4,5)P_3$, we found that noticeably more Dock2/ELMO1ΔN bound to the $PI(3,4,5)P_3$ beads than the wild-type tetramer (Fig. 20B).

The contribution of the ELMO1 and ELMO1ΔN molecules to $PI(3,4,5)P_3$ binding by the Dock2/ELMO1 complexes was examined by assaying the ability of bacterially-purified ELMO1 and ELMO1ΔN to interact with $PI(3,4,5)P_3$. Increasing concentrations of purified ELMO1 proteins were titrated into a cuvette containing fluorescently-labeled

$PI(3,4,5)P_3$ and the change in fluorescence polarization was monitored as a direct measure of binding to the phosphoinositide. Neither ELMO1 nor ELMO1ΔN showed appreciable binding when compared to a control protein, a GST-tagged PH domain from Grp1 (Fig. 20C). This suggests that the increased binding to $PI(3,4,5)P_3$ by the Dock2/ELMO1ΔN tetramer is not a result of ELMO1ΔN interaction with $PI(3,4,5)P_3$. This does not discount, however, the possibility that deletion of the N-terminus of ELMO1 artificially exposes a region of Dock2 which can bind phosphoinositides. Potentially, though, the removal of the intramolecular interaction within ELMO1 by deletion of the N-terminus may be responsible for a change in the conformation of the tetramer, which is then capable of an increased interaction with $PI(3,4,5)P_3$ through the C2 domains within the Dock2 molecules.

Discussion

Figure 19 presents a model of Dock2/ELMO1 regulation by RhoG. In this model, the cytoplasmic form of the Dock2/ELMO1 heterotetramer is in an inhibited state. Dock2 and ELMO1 are bound via approximately two interfaces within the C-terminal region of ELMO1 and the N-terminal region of Dock2. The two Dock2 molecules are tightly associated via a dimerization interface located within the DHR-2 regions of the two proteins, potentially via the putative coiled-coil region (Fig. 1). Each ELMO1 molecule is in the inhibitory state, where the N-terminal region is bound to the C-terminal region. As a result of this series of interactions, the PH domains of the ELMO1 molecules are unable to stabilize nucleotide-free Rac in the binding pocket provided by the two Dock2 molecules.

Upon activation of RhoG, the N-terminal regions of the ELMO1 molecules bind to the activated GTPase, translocating the Dock2/ELMO1 complex to the plasma membrane where the substrate Rac molecules are located. The binding of activated RhoG to the N-terminus of ELMO1 releases the inhibited conformation and allows the PH domains to move into a position where they can stabilize the nucleotide-free transition state of the Rac molecule. This rearrangement of ELMO1 spurs a change in the conformation of the Dock2 molecules as well, allowing the C2 domains to contact $PI(3,4,5)P_3$ at the membrane, stabilizing the complex in this position.

This model compiles a body of preexisting literature with the results that we have presented in both chapters 2 and 3. Some of the ideas presented in that model need testing to allow us to confirm or adapt the model. A stepwise analysis of the model and the relevant results that the model is based on follows:

1) Pre-activation conformation of the model (Fig. 21A)
    a. The interactions between Dock2 and ELMO1 have been presented in the literature [41,42,47,48,71,74]. The overall composition of the molecule as a 2:2 heterotetramer comes from the light scattering analysis of the Dock2/ELMO1 complex (Fig. 11).
    b. The suggested dimerization interface between the two Dock2 molecules is predicted based on evidence in the literature [44,118] and our own analysis of a potential coiled-coil region within the DHR-2 domain (Fig. 1).

c. The inhibited conformation of ELMO1 was analyzed in our results using SEC, light scattering, immunoprecipitations and yeast two-hybrid assays (Fig. 4, 13-14).

d. The positioning of the PH domains as inaccessible to stabilize Rac in the binding pocket is based on our observation that deletion of the N-terminus of ELMO1 enhances exchange activity (Fig. 15), and preexisting knowledge that the PH domain of ELMO1 can bind to a Dock/Rac complex and can increase the exchange rate of the complex [49]. Combining those observations, the best-fit model to our data would place the PH domain in a position where it is not accessible to enhance exchange activity while the ELMO1 inhibitory intramolecular interaction is in place.

2) Active conformation (Fig 19B)

a. Localization of the Dock/ELMO complex at the membrane via RhoG binding to ELMO proteins has been described in the literature [47,74].

b. We have no direct evidence for the stoichiometry of the RhoG:tetramer interaction, but have drawn the model with two molecules of RhoG per tetramer based on the based on evidence that purified, monomeric ELMO proteins can interact with activated RhoG (Fig. 5-7). This suggests that each molecule of ELMO1 in the tetramer would have the capability to bind RhoG.

c. Interruption of the ELMO1 intramolecular interaction by RhoG is based on our data that an intramolecular interaction exists (Fig. 4, 13-14), as well

as our exchange assays showing that active RhoG is capable of activating the Dock2/ELMO1 complex similarly to the activation seen by truncating the N-terminus of ELMO1 (Fig. 19). This activation of Dock2/ELMO1 by RhoG is dependent upon its ability to interact with the N-terminus of ELMO1 (Fig. 19).

d. Exposure of the PH domains to allow stabilization of Rac in the binding pocket is based on our data showing the increase in activity upon deletion of the N-terminal region of ELMO1 (Fig. 15-17). This fragment is primarily composed of the PH domain and the C-terminal Dock-interacting residues (Fig 1). The explanation for the increase in activity could be from a change in the overall conformation of the Dock2/ELMO1 tetramer, allowing increased activity – perhaps through changes in the binding pocket. However, evidence for the contribution of the PH domains to the activity of the complex has been published [49], and this seems to be the best explanation with the data available. Experiments to test this concept have been described in chapter 4.

e. Movement of the C2 domains to allow binding to PI(3,4,5)P_3 at the membrane is based upon our data that more Dock2/ELMO1ΔN tetramer binds to PI(3,4,5)P_3 beads than the wild-type tetramer (Fig 18B). At least two explanations can explain this data. Deletion of the N-terminus of ELMO1 may simply cause an area of Dock2 to be exposed that normally would not be exposed in the tetramer, and the increase in lipid-binding could simply be a non-specific effect. The specificity of binding shown

by both mutant and wild-type tetramer (Fig. 20A) would suggest that this is not the case, but we cannot discount the possibility. We would suggest that the deletion of the N-terminus of ELMO1 (or, as in our model, the binding of RhoG to the N-terminus), instead, causes a conformational change within the tetramer that moves the C2 domain to a configuration that allows easier access to lipids for binding. This would be consistent with the RhoG localization of the tetramer to the membrane, as the C2 domain has been shown to be instrumental in membrane localization as well [38].

The model provides an overview for how RhoG directly regulates the exchange capacity of the Dock/ELMO tetramer through binding to ELMO1. The results presented give us a clearer understanding of what contributions ELMO proteins make to the exchange activity of the Dock/ELMO bipartite GEF.

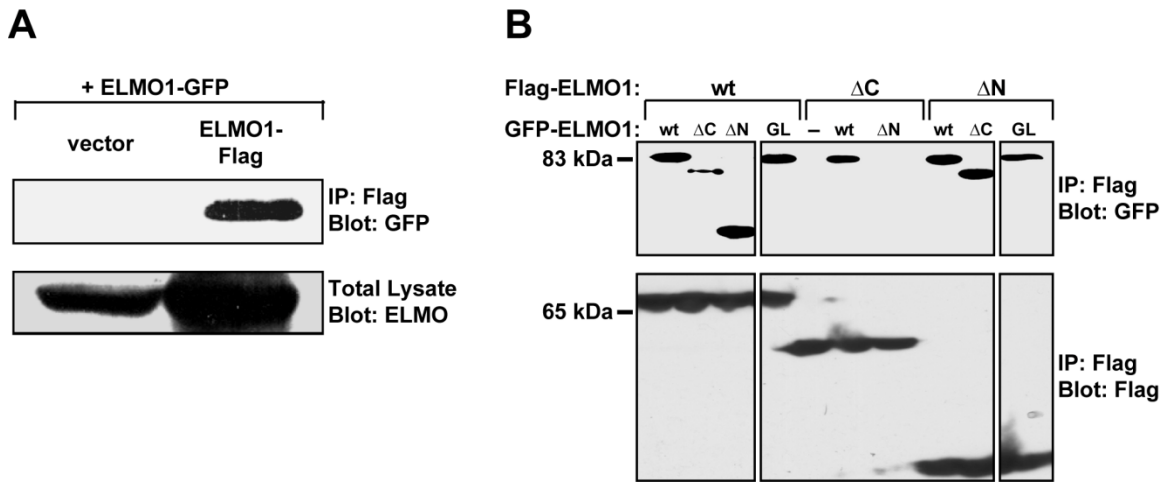


Figure 13: ELMO1 can interact with itself in cells.

A,B) The indicated constructs of Flag and GFP-tagged ELMO1 were co-transfected into 293T cells. Lysates were precipitated with anti-Flag antibody and co-precipitating GFP-tagged proteins were assessed by immunoblotting. The failure of Flag-ELMO1ΔN to precipitate GFP-tagged ELMO1ΔC in panel B is unclear, as the interaction is obvious when the precipitation was done in the reverse direction. *These experiments were performed by Colin deBakker, Ravichandran Lab, University of Virginia.

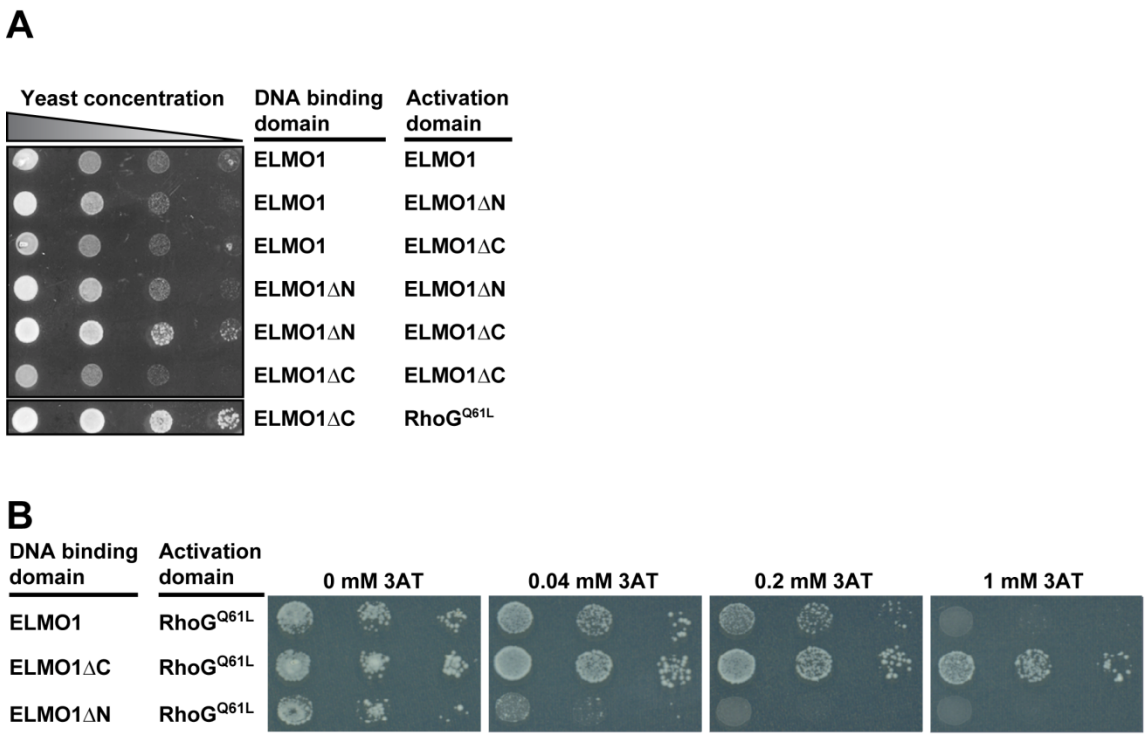


Figure 14: The N-and C-terminal regions of ELMO1 interact

A) Yeast two-hybrid assay. The N- and C-terminal portions of ELMO1 preferentially interact relative to other combinations. The interaction is comparable to the interaction between ELMO1ΔC and RhoG. The HF7C yeast strain was transformed with the indicated constructs using a lithium acetate-based transformation protocol. Colonies were plated in decreasing concentrations on selection media lacking histidine, tryptophan and leucine. **B)** Removing the C-terminus of ELMO1 increases the interaction between ELMO1 and RhoG. The HF7C yeast strain was transformed with the indicated constructs and plated on increasingly selective media. *These experiments were performed by Colin deBakker, Ravichandran Lab, University of Virginia.

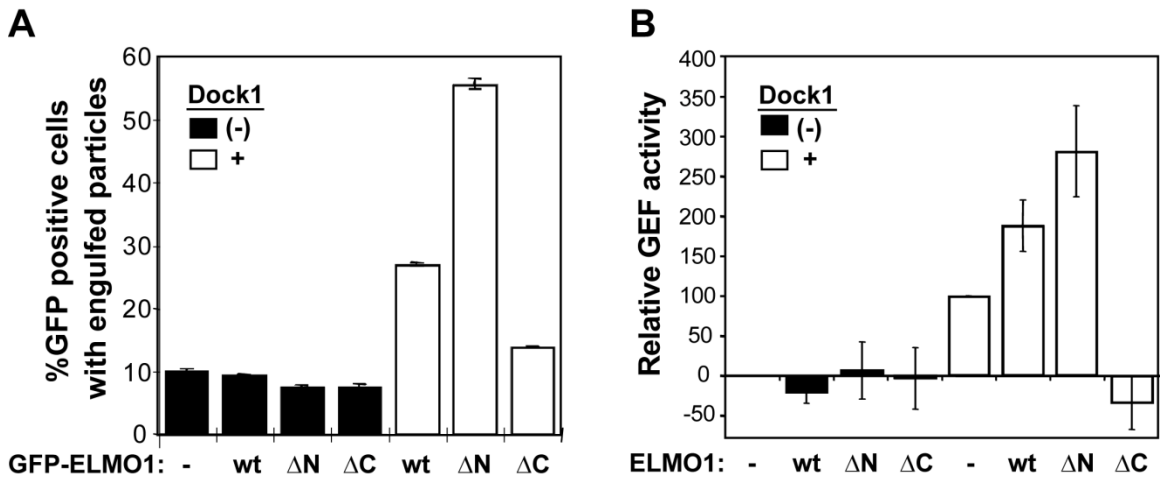


Figure 15: Deletion of the N-terminus of ELMO1 hyper-activates the Dock1/ELMO1 complex.

A) LR73 cells were transiently transfected with the indicated ELMO1 constructs (1.0 μg/well) with or without Flag-Dock1 (1.2 μg/well) in triplicate. Empty Flag vector was added if necessary to ensure equal total plasmid concentrations were used for transfection in each condition. The phagocytosis, as measured as the fraction of GFP positive cells with engulfed particles, is shown. GFP mean fluorescence intensity (MFI) was matched between the different constructs and cells with comparable MFI were analyzed throughout the experiment. Data are representative of at least 5 independent experiments.

B) 293T cells were transiently transfected with the indicated plasmids encoding various constructs of ELMO1, either alone or co-transfected with Dock1. After 48 hours, Flag immunoprecipitations were performed and the samples were tested in an *in vitro* GEF assay using bacterially produced Rac1 pre-loaded with ^{32}P-GTP as a substrate. The activity with Dock1 alone was arbitrarily set at 100%. Comparable expression of the transfected proteins was confirmed by immunoblotting (not shown). *These experiments were performed by Colin deBakker, Ravichandran Lab, University of Virginia.

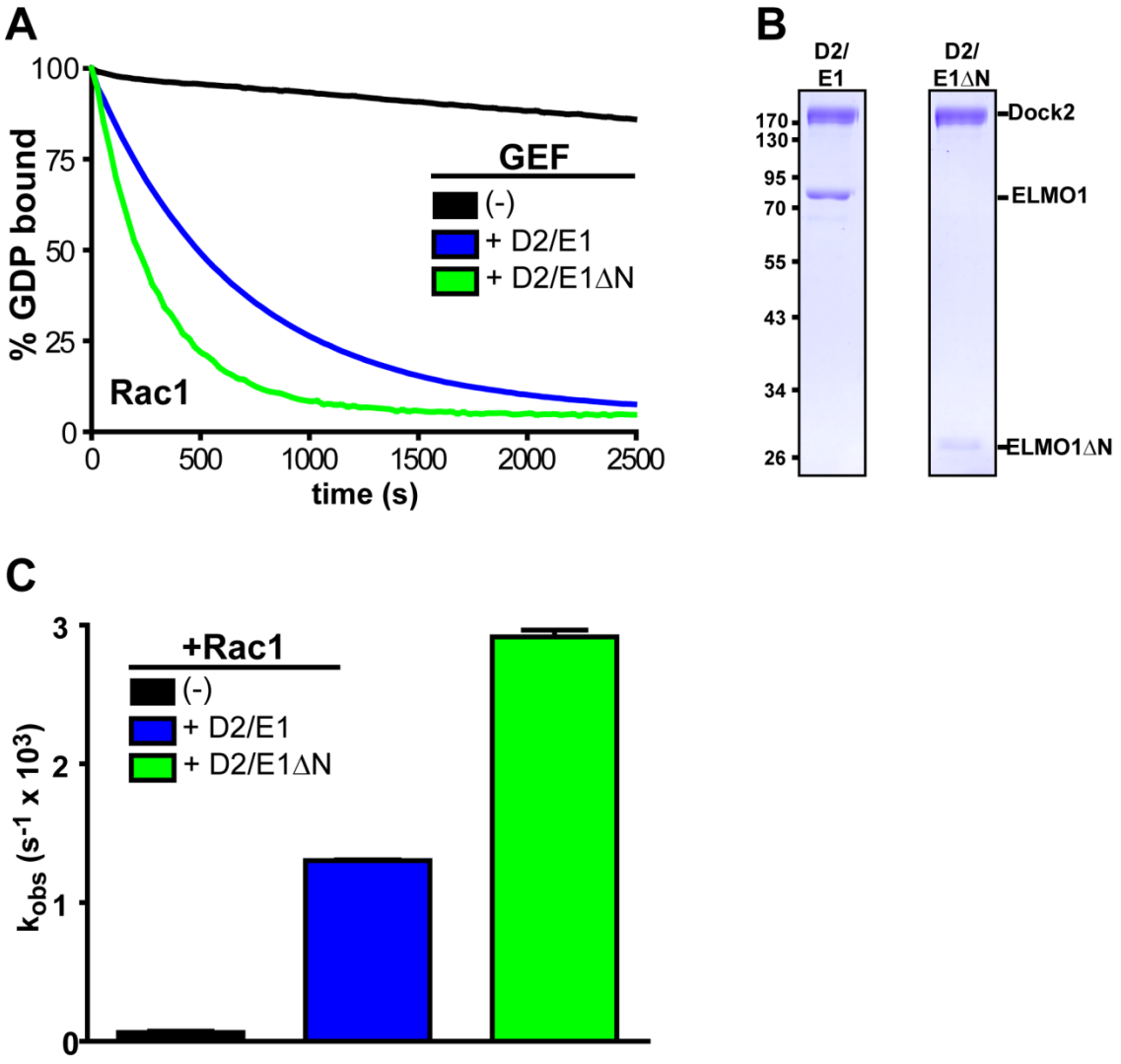


Figure 16: Deletion of the N-terminus of ELMO1 hyper-activates the Dock2/ELMO1 complex.

A) Purified Dock2/ELMO1 (D2/E1) and Dock2/ELMO1ΔN (D2/E1ΔN) complexes (15 nM) were tested for exchange activity using a fluorescence-based assay. Bacterially purified Rac1Δ177 (150 nM) was pre-loaded with BODIPY-FL-GDP. As the BODIPY-FL-GDP is exchanged for GDP the loss of fluorescence is measured. Individual curves shown are representative of 3-4 independent experiments. **B)** Equal amounts of Dock2/ELMO1 and Dock2/ELMO1ΔN were run on an SDS-PAGE gel and visualized with Coomassie brilliant blue staining to show purity and equivalent concentration. **C)** Relative exchange rates determined from fluorescence-based assays as in (A) with a minimum of three independent experiments per condition.

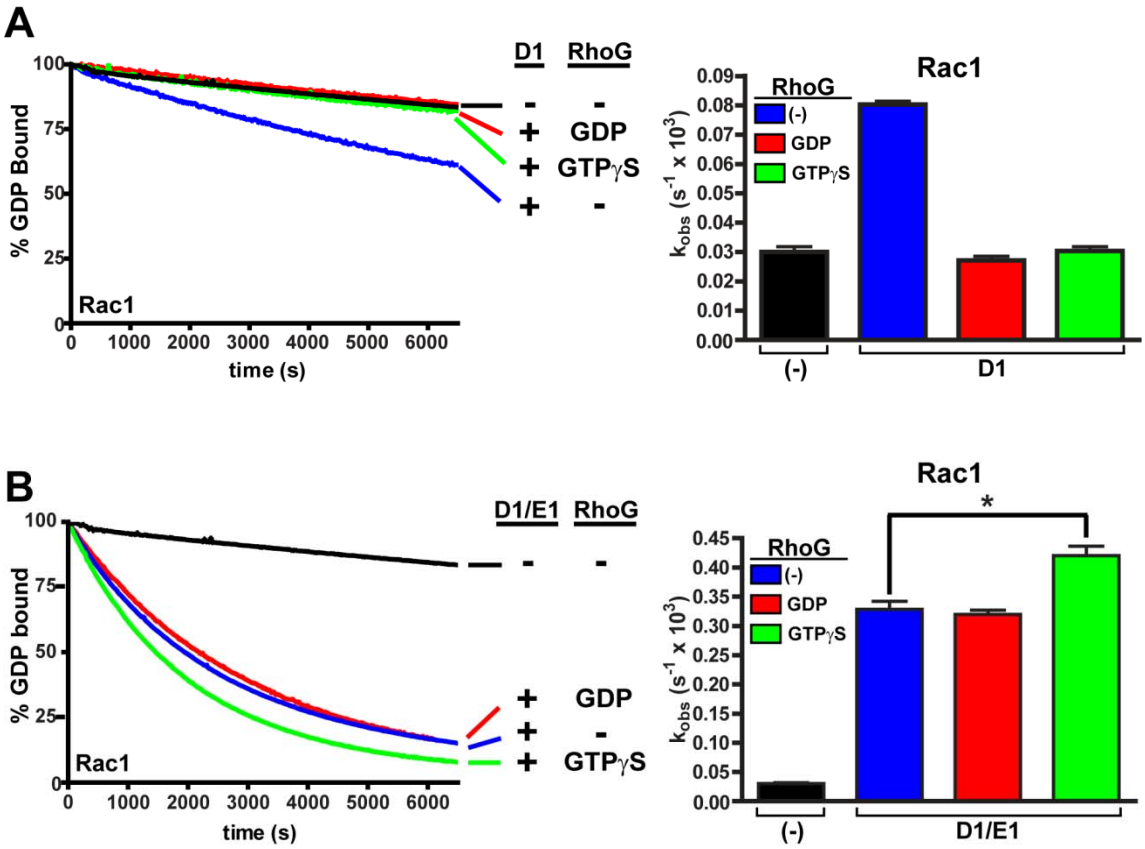


Figure 17: Active RhoG enhances the exchange activity of the Dock1/ELMO1 complex.

A) Active RhoG does not enhance the exchange activity of Dock1. Flag-Dock1 was expressed in 293T cells. Lysates were precipitated with anti-Flag antibody-conjugated beads and proteins were eluted using Flag peptide. Proteins were quantitated by immunoblotting and equivalent amounts were used in each assay. Exchange assays were performed as in Figure 14 with Rac1(C189S) pre-loaded with BODIPY-FL-GDP, with a minimum of three independent experiments per condition. Where indicated, RhoG (final concentration: 10 μM), pre-loaded with GDP or GTPγS was pre-incubated with Dock1. Left panel shows representative curves, right panel shows relative rates of exchange. **B)** Active RhoG enhances the exchange activity of Dock1/ELMO1. Flag-tagged ELMO1 was co-expressed with Dock1 in 293T cells and was immunoprecipitated as above. Exchange activity was measured as above. Left panel shows representative curves, right panel shows relative rates of exchange. * denotes the difference in the means is statistically significant ($P < 0.05$) as determined by a student's unpaired t-test assuming equal variances.

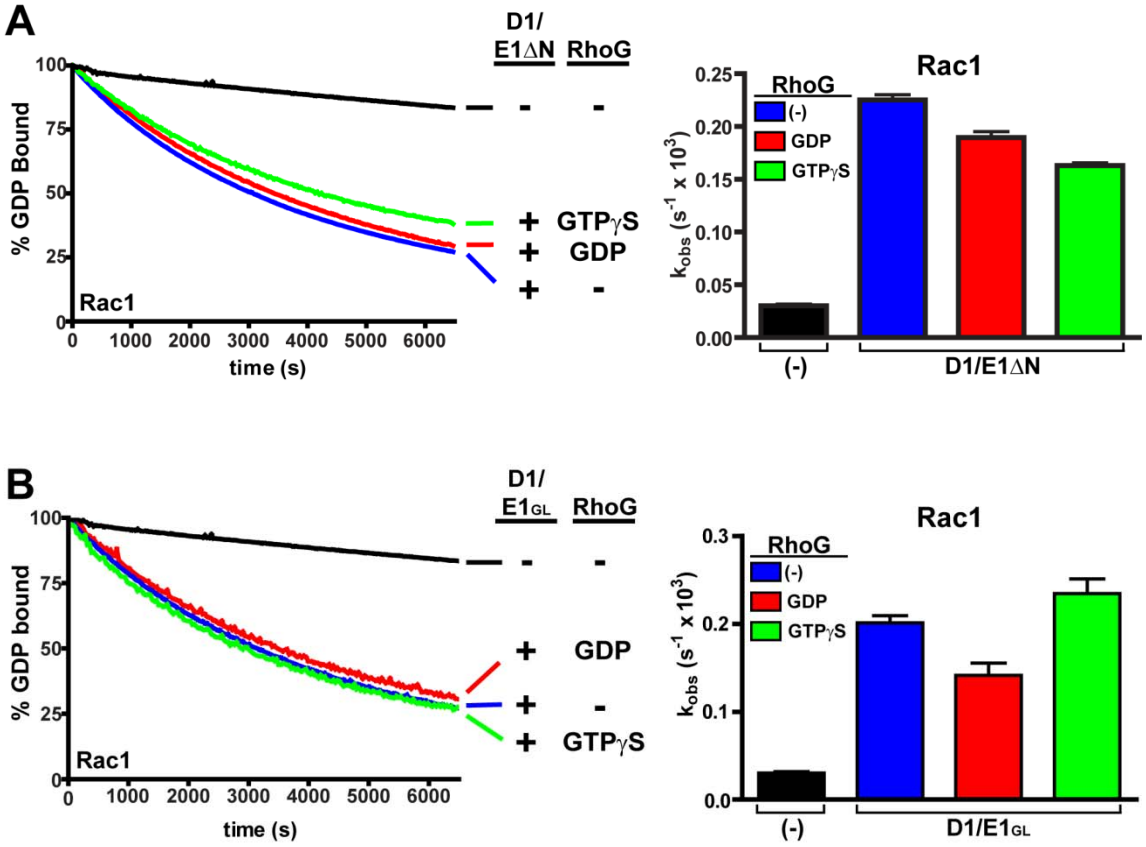


Figure 18: Active RhoG requires the N-terminus of ELMO1 to enhance the exchange activity of the Dock1/ELMO1 complex.

A, B) Indicated proteins were immunoprecipitated from 293T cells as described in Figure 15. Exchange assays were carried out as indicated in Figure 15. Left panels show representative curves and the right panels show relative rates of exchange.

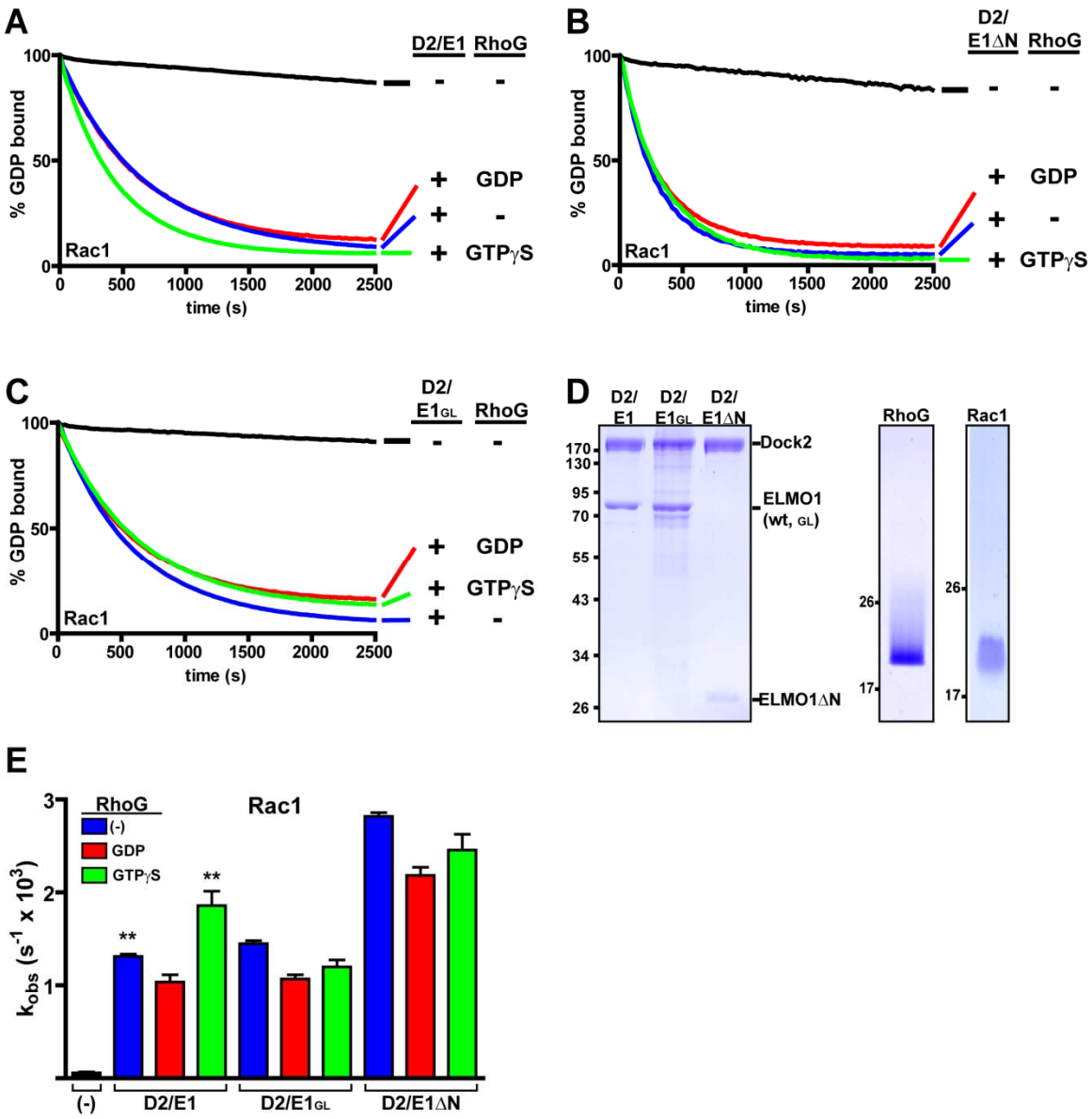


Figure 19: B-D. Active RhoG requires the N-terminus of ELMO1 to enhance the exchange activity of the Dock2/ELMO1 complex.

A-C) Active RhoG enhances exchange activity of Dock2/ELMO1, but does not activate Dock2/ELMO1$_{ARM}$ or Dock2/ELMO1ΔN. Recombinant Dock2/ELMO1 complexes were purified from insect cells, and used at a concentration of 15 nM. Rac1Δ177 (150 nM) and RhoG (10 μM) were purified from bacteria and preloaded as in figures 14 and 15. Curves shown are representative of 3-5 independent experiments. **D)** Coomassie brilliant blue-stained SDS-PAGE gels of purified protein used in (A-C). Left panel shows equal amounts of different Dock2/ELMO1 complexes. Right two panels show purified RhoG and Rac1. **E)** Relative rates of exchange for various Dock2/ELMO1 complexes +/- RhoG. Each rate was calculated from 3-5 independent experiments. ** denotes $p < 0.01$ when comparing the rates between Dock2/ELMO1 and Dock2/ELMO1 + RhoG-GTPγS, as determined by an unpaired t-test assuming equal variances.

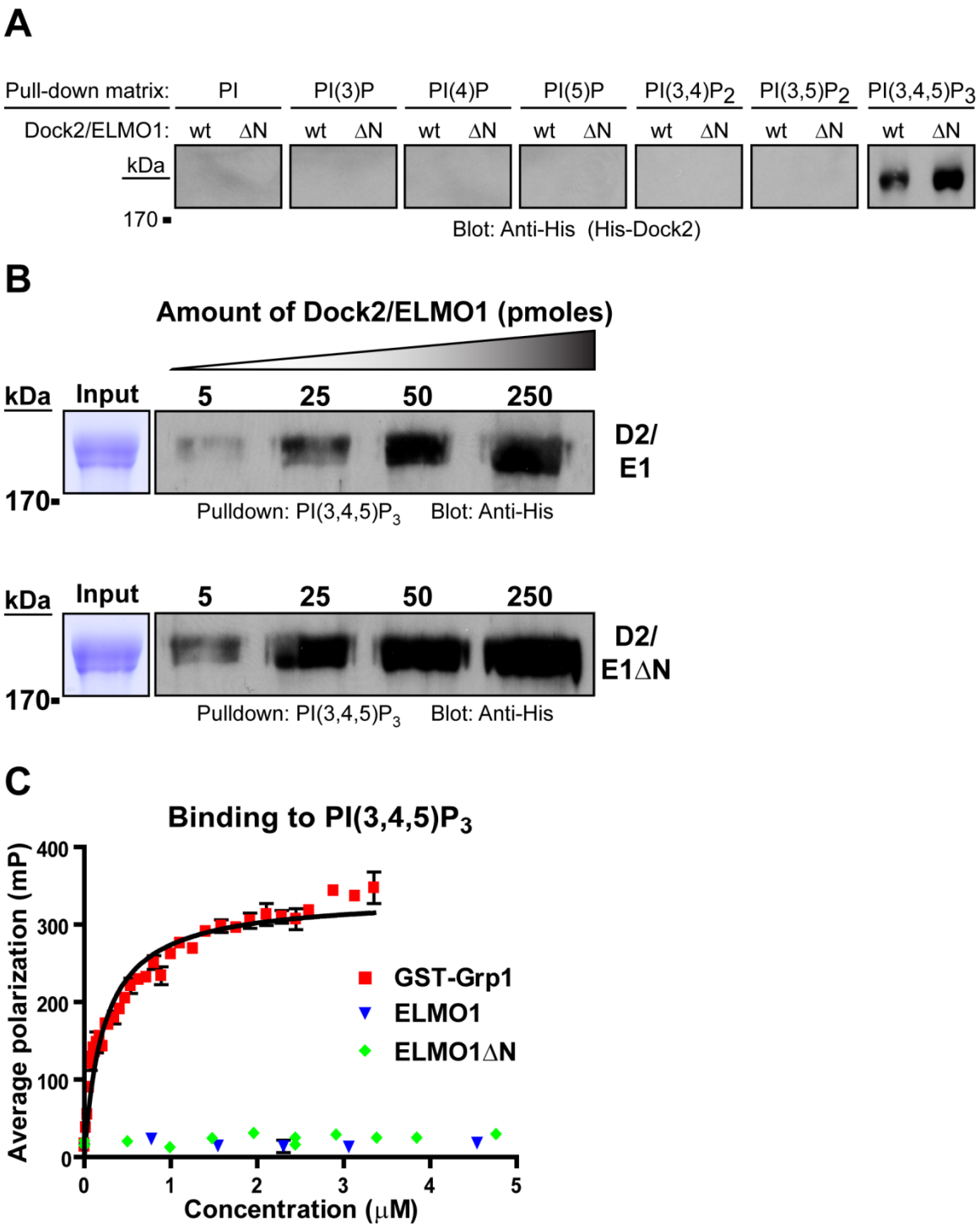


Figure 20: Dock2/ELMO1ΔN specifically binds more PI(3,4,5)P_3 than Dock2/ELMO1.
A) Beads modified with indicated phospholipids were incubated with either Dock2/ELMO1 (wt) or Dock2/ELMO1ΔN (ΔN) as indicated. Bound proteins were analyzed by SDS-PAGE and immunoblotting for His-Dock2. **B)** Varying amounts of total D2/E1 or D2/E1ΔN were incubated with PI(3,4,5)P_3 beads. Bound proteins were analyzed as in (A). **C)** Binding of ELMO1 and ELMO1ΔN to BODIPY-FL labeled PI(3,4,5)P3 was monitored by change in polarization. The GST-tagged PH domain of Grp1 was used as a control for binding. No binding curve could be accurately fitted to ELMO1 and ELMO1ΔN data.

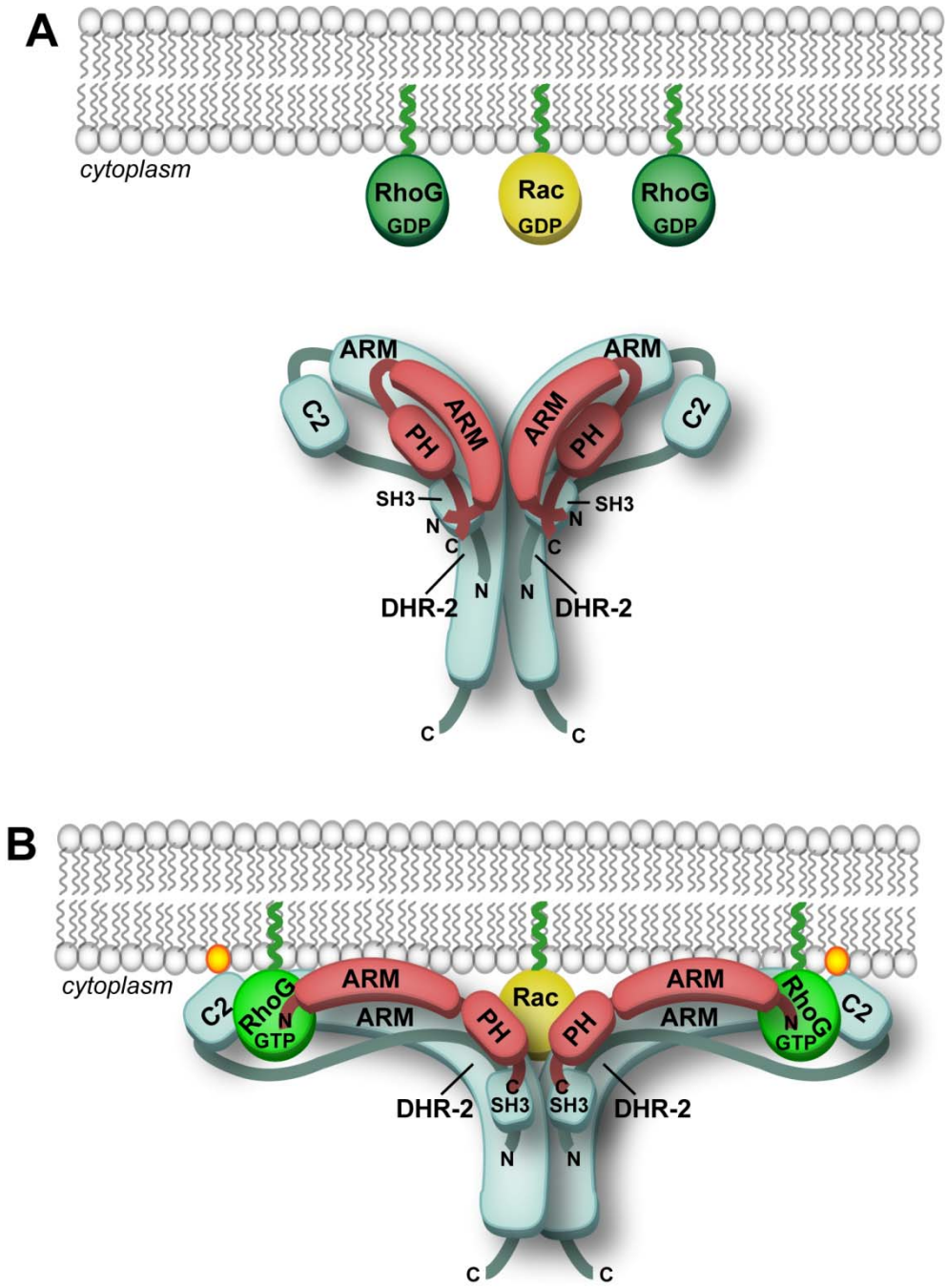


Figure 21: Model of direct activation of the Dock/ELMO tetramer by RhoG.

A) Cytoplasmic Dock/ELMO tetramer. Dock protein in blue, ELMO protein in red, Rac1 in yellow, RhoG in green. The Dock protein SH3 domain and N-terminal residues bind the C-terminal region of the ELMO protein. The N- and C-terminal regions of ELMO1 interact to inhibit the Dock/ELMO complex. RhoG-GDP does not activate Dock/ELMO. **B)** Membrane-associated Dock/ELMO tetramer. RhoG-GTP binds the N-terminus of the ELMO protein to release inhibitory conformation, and localizes the Dock/ELMO complex to the membrane, allowing exchange activity on membrane-bound Rac. The PH domain of the ELMO protein stabilizes nucleotide-free Rac in the binding pocket of the Dock/ELMO tetramer, enhancing exchange activity. The C2 domain of the Dock protein can bind to $PI(3,4,5)P_3$ (yellow) at the membrane.

CHAPTER 4 – CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The Dock family of proteins is rapidly growing in recognized importance within a variety of cellular signaling pathways. Understanding the mechanisms of function and regulation of these relatively new, non-Dbl-family GEFs will lead to insight on potential therapies for diseases in which these proteins play a key role.

We have demonstrated that Dock1 and Dock2 associate with ELMO proteins in a seemingly obligatory complex. The Dock/ELMO complex functions as a 2:2 heterotetramer, binding to and activating one molecule of Rac1, Rac2 or Rac3 at a time. The size of this complex and the existence of the functional unit as a heterotetramer leads us to question how these proteins function together to promote exchange on Rho-family GTPases.

Examination of the effect that ELMO1 had upon the activity of the complex led to data showing that ELMO1 had a direct effect on the activity of the Dock/ELMO complex. Upon further examination, we found that RhoG was capable of modulating this direct effect upon the exchange activity of the Dock/ELMO complex, providing a new mode of regulation of Dock/ELMO complexes by RhoG.

A model, combining research presented here and other published data, provides an overview for the regulation of the Dock/ELMO complex by RhoG. As presented, the Dock/ELMO complex exists in the cytoplasm in a closed state, with the two ELMO1

molecules "closed" through the interaction between the N- and C-terminal regions of the protein. Upon activation of RhoG, the ELMO1 molecules are now able to interact with RhoG and the Dock/ELMO complex is localized to the membrane by this interaction. RhoG binds to the N-terminus of ELMO1, interrupting the N- and C-terminal interaction within ELMO1, allowing the Dock/ELMO complex to shift conformation. The PH domains of the ELMO1 molecules are now able to shift into position to stabilize Rac1 in the nucleotide-free transition state. The entire Dock/ELMO complex may also shift conformation in two ways: 1) the active site may become more accessible upon the release of the inhibitory interaction within ELMO1, and 2) the C2 domains of the Dock proteins may shift in relation to the tetramer to allow association with the membrane through binding to $PI(3,4,5)P_3$ (Fig. 21).

Interestingly, a model for the regulation of Dock11, which does not bind ELMO proteins, shows some striking similarities to our model with the binding of an activated GTPase to increase the exchange activity of Dock11. Briefly, Dock11 is capable of binding activated Cdc42, as well as being an exchange factor for Cdc42. Lin, *et al.* proposed that the N-terminus of Dock11 interacts with the DHR-1 region, and Dock11 has limited GEF activity in this conformation. Upon activation of Cdc42, the active GTPase binds to the N-terminus of Dock11 and this increases the exchange activity [88]. It will be interesting to see if the common threads in the two models might reflect a common mechanism of activation and even translocation for the Dock proteins. The idea that the PH domain in the N-terminus of the Dock D subfamily may be acting similarly to the PH domain provided by ELMO proteins for the Dock A and Dock B subfamilies has been suggested previously [41]. Although no significant sequence homology would

suggest it, is it possible that the N-terminal areas of the Dock D and perhaps even Dock C proteins have evolved to replace ELMO proteins in the mechanism of activation, or vice versa?

Future work that will help refine this model of activation of Dock/ELMO by the interaction of RhoG with ELMO proteins is presented in the following sections.

Future Directions

Testing of PH-domain enhancement of Dock2/ELMO1 Rac1 activation

One assumption made in the model presented (Fig. 21) is that the previously-reported enhancement of exchange activity by the PH domain is responsible for at least some of the activation seen when RhoG binds to ELMO1. We assumed that 1) the PH domain is not available to stabilize the nucleotide-free transition state of Rac1 while the N- and C-terminal regions of ELMO1 are bound in the inhibitory interaction and 2) the relief of this inhibition allows enhancement of exchange activity by allowing the PH domain access to the binding site to stabilize the nucleotide-free transition state.

One way to test this idea would be to make mutant forms of ELMO1 to purify in a complex with Dock2 in insect cells. There are three known mutations within ELMO1 which abrogate the interaction between the isolated PH domain and a Dock/Rac complex [41]. W665A, G559A and FRK➔AAA (662-664) mutations within the PH domain are all capable of interfering with this interaction. If these mutations were introduced into both ELMO1 (wt) and ELMO1ΔN, the resulting Dock2/ELMO1 complexes could be purified and tested for both the activation of Dock2/ELMO1 via truncation of the N-terminus and activation via binding of RhoG. In either case, if the contribution of the PH

domain stabilizing the nucleotide-free transition state is what is responsible for the activation of the Dock/ELMO exchange activity, these mutants should have no more activity that wild-type protein.

Test the membrane contribution of the activation of Dock2/ELMO1 by RhoG

In the exchange assays presented in chapters 2 and 3, the contribution of the membrane to the interactions and activation of the Dock/ELMO complex was not tested. In our assays, the wild-type Dock2/ELMO1 was an efficient exchange factor without any activated RhoG present, and the relatively modest enhancement to the rate upon addition of active RhoG may only be indicative of the partial effect that RhoG has on the Dock/ELMO complex. It is possible that the increase in exchange activity upon binding of RhoG may be much greater than that seen in assays without a membrane present.

To address this question, exchange assays using lipidated Rac1 and lipidated RhoG incorporated into lipid vesicles would provide a way to determine the contribution of the membrane. Lipidated forms of GTPases can be made using baculovirus expression systems, and can be incorporated into reconstituted lipid vesicles after purification. The assays would examine the effects of various combinations of lipidated and non-lipidated Rac1 and/or RhoG. The specific contributions to activity could be clearly defined using the Rac1 and RhoG combinations as well as the Dock2/ELMO1 mutants which are incapable of interacting with RhoG (ELMO1ΔN and ELMO1GL).

Crystallization of Dock2/ELMO1 complexes

The ability to purify a stable Dock2/ELMO1 complex to near homogeneity ant to a relatively high concentration (up to ~40 mg/ml) provides the opportunity to attempt

crystallization of the complex for X-ray crystallographic structure determination. A variety of commercially-available screens have been used to set up crystallization trials for the Dock2/ELMO1 (at 12 mg/ml) and a Dock2/ELMO1/Rac1Δ177 (17 mg/ml) trimeric complex, with no potential crystal conditions yet. Obviously, a great many more crystallization conditions can be tried, at much higher concentrations than used before.

One problem encountered while trying to crystallize this complex will be the relative concentration of the protein. Even at a concentration of 40 mg/ml, the tetramer molar concentration is only approximately 67 μM. This could present difficulties in obtaining crystals. Complexes that would be of interest to crystallize would include Dock2/ELMO1 (wt), Dock2/ELMO1/Rac1, Dock2/ELMO1ΔN, Dock2/ELMO1ΔN/Rac1 and even a structure of ELMO1 without a Dock protein could provide useful information, with or without RhoG.

ELMO1 may show homology to the formin protein GTPase binding domain and the Diaphanous autoregulatory domain.

After several rounds of psi-BLAST [100] analysis of the mouse ELMO1 sequence, the N-terminus of ELMO1 is picked out as homologous to several different formin homology 2 (FH2)-domain containing proteins, including FHOS, a formin protein that is enriched in the spleen [120]. Formins contain multiple domains and govern dynamic remodeling of the cytoskeleton [130]. Interestingly, formins are known to have an autoinhibitory interaction between the N- and C-terminal regions of the proteins, mediated by a C-terminally located diaphanous autoregulatory domain (DAD) and an N-terminally located GTPase binding domain (GBD). The homology between the N-terminus of ELMO1 and FHOS detected by psi-BLAST resides within the N-terminal

region of FHOS, which is where the predicted GBD for that protein lies [130]. FHOS has been shown to interact with Rac1, dependent upon the PBR of Rac1. The predicted homology of ELMO1 to the formin proteins suggests, especially since the homology lies within the GBD of the formins, that the mechanism of the ELMO1 N- and C-terminal interaction may be similar to that of the formin autoinhibitory interactions. Accordingly, there is a region within the C-terminus of ELMO1 which loosely corresponds to the DAD motif of the formins.

Structures are available showing the binding of RhoC to the formin protein, mDia1, as well as showing the DAD bound to mDia1. With an in-depth sequence analysis between ELMO1 and formin proteins within the regions of homology, it should be possible to design mutants to test if this homology between ELMO1 and the formins carries over to the GBD-DAD inhibitory interaction. By designing mutants in the DAD-like region of ELMO1 and/or in the GBD-like region, we may be able to interfere with the inhibitory interaction within ELMO1 as well as with the RhoG binding capabilities. A point mutant which abrogates the ELMO1 intramolecular interaction would be very useful in studying how the ELMO1 molecule affects the exchange activity of the Dock/ELMO complex, and would allow us to refine our model further.

Analysis of Rac1 binding to ELMO1

In chapter 2, we identified an interaction between GTPγS-bound Rac1(C189S) and ELMO1 in addition to the commonly reported RhoG-GTPγS interaction. In a few different publications, the interaction of ELMO1 with RhoG has been reported, but interactions between ELMO1 and Rac1 have been tested for and not detected [40,47]. To understand the interaction between ELMO1 and Rac1, a few different experiments are

possible. First, we need to determine if the RhoG and Rac1 binding sites are the same. Through use of truncated and mutant ELMO1 constructs, we should be able to determine the general region to which Rac1 is binding. ELMO1ΔN and ELMO1 (wt) can both be purified from bacteria, and ELMO1ΔC the ELMO1GL mutant should be able to be purified as well. Between these constructs we should be able to identify if Rac1 is binding to the N- or C-terminus of ELMO1. If Rac1 is binding to the N-terminus of ELMO1, we can use the ELMO1GL mutant to determine if Rac1 is binding in the same region as RhoG. If Rac1 is binding to the same site on ELMO1 as RhoG, it is likely that the similarities between the two proteins are simply allowing Rac1 to bind in an artificial, *in vitro* setting. If, however, Rac1 binds to the C-terminus of ELMO1, this would suggest a potentially biologically-relevant association. As mentioned in chapter 2, the activation of Rac1 could allow Rac1-GTP to bind to ELMO1, perhaps stabilizing the Dock/ELMO complex in its enhanced-activity conformation in a positive feed-forward loop, similar to the reported cases for Ras-Sos interaction and Cdc42-Dock11 [88,123,124].

Summary

The model of how RhoG regulates the activity of the Dock/ELMO heterotetramer through interaction with ELMO proteins is a scaffold on which to build a more thorough understanding of how the Dock-family of exchange factors catalyze the exchange of nucleotide on Rho GTPases, and of how these proteins are regulated. These studies, designed with the model in mind, will allow us to add detail to the mechanisms behind the Dock-family GEFs so that we can further define its role in cellular signaling and disease.

REFERENCES

1. Wennerberg, K., Rossman, K. L., and Der, C. J. The Ras superfamily at a glance. (2005) *J Cell Sci* **118**(Pt 5), 843-846

2. Etienne-Manneville, S., and Hall, A. Rho GTPases in cell biology. (2002) *Nature* **420**(6916), 629-635

3. Wright, L. P., and Philips, M. R. Thematic review series: lipid posttranslational modifications. CAAX modification and membrane targeting of Ras. (2006) *J Lipid Res* **47**(5), 883-891

4. Williams, C. L. The polybasic region of Ras and Rho family small GTPases: a regulator of protein interactions and membrane association and a site of nuclear localization signal sequences. (2003) *Cell Signal* **15**(12), 1071-1080

5. Peck, J., Douglas, G. t., Wu, C. H., and Burbelo, P. D. Human RhoGAP domain-containing proteins: structure, function and evolutionary relationships. (2002) *FEBS Lett* **528**(1-3), 27-34

6. Mitin, N., Rossman, K. L., and Der, C. J. Signaling interplay in Ras superfamily function. (2005) *Curr Biol* **15**(14), R563-574

7. Cox, A. D., and Der, C. J. Ras family signaling: therapeutic targeting. (2002) *Cancer Biol Ther* **1**(6), 599-606

8. Seabra, M. C., and Wasmeier, C. Controlling the location and activation of Rab GTPases. (2004) *Curr Opin Cell Biol* **16**(4), 451-457

9. Rossman, K. L., Der, C. J., and Sondek, J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. (2005) *Nat Rev Mol Cell Biol* **6**(2), 167-180

10. Erickson, J. W., and Cerione, R. A. Structural elements, mechanism, and evolutionary convergence of Rho protein-guanine nucleotide exchange factor complexes. (2004) *Biochemistry* **43**(4), 837-842

11. Schmidt, A., and Hall, A. Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. (2002) *Genes Dev* **16**(13), 1587-1609

12. Berken, A., Thomas, C., and Wittinghofer, A. A new family of RhoGEFs activates the Rop molecular switch in plants. (2005) *Nature* **436**(7054), 1176-1180

13. Berken, A., and Wittinghofer, A. Structure and function of Rho-type molecular switches in plants. (2007) *Plant Physiol Biochem*

14. Thomas, C., Fricke, I., Scrima, A., Berken, A., and Wittinghofer, A. Structural evidence for a common intermediate in small G protein-GEF reactions. (2007) *Mol Cell* **25**(1), 141-149

15. Gupta, S., Lee, A., Hu, C., Fanzo, J., et al. Molecular cloning of IBP, a SWAP-70 homologous GEF, which is highly expressed in the immune system. (2003) *Hum Immunol* **64**(4), 389-401

16. Mavrakis, K. J., McKinlay, K. J., Jones, P., and Sablitzky, F. DEF6, a novel PH-DH-like domain protein, is an upstream activator of the Rho GTPases Rac1, Cdc42, and RhoA. (2004) *Exp Cell Res* **294**(2), 335-344

17. Shinohara, M., Terada, Y., Iwamatsu, A., Shinohara, A., et al. SWAP-70 is a guanine-nucleotide-exchange factor that mediates signalling of membrane ruffling. (2002) *Nature* **416**(6882), 759-763

18. Rojas, R. J. (2007) Coordinating the activation of RhoA by diverse RhoGEFs: SmgGDS and G-alpha-q-responsive Dbl family guanine nucleotide exchange factors. In. *Department of Pharmacology*, University of North Carolina at Chapel Hill, Chapel Hill

19. Isomura, M., Kaibuchi, K., Yamamoto, T., Kawamura, S., Katayama, M., and Takai, Y. Partial purification and characterization of GDP dissociation stimulator (GDS) for the rho proteins from bovine brain cytosol. (1990) *Biochem Biophys Res Commun* **169**(2), 652-659

20. Lanning, C. C., Ruiz-Velasco, R., and Williams, C. L. Novel mechanism of the co-regulation of nuclear transport of SmgGDS and Rac1. (2003) *J Biol Chem* **278**(14), 12495-12506

21. Quilliam, L. A., Rebhun, J. F., and Castro, A. F. A growing family of guanine nucleotide exchange factors is responsible for activation of Ras-family GTPases. (2002) *Prog Nucleic Acid Res Mol Biol* **71**, 391-444

22. Rebhun, J. F., Castro, A. F., and Quilliam, L. A. Identification of guanine nucleotide exchange factors (GEFs) for the Rap1 GTPase. Regulation of MR-GEF by M-Ras-GTP interaction. (2000) *J Biol Chem* **275**(45), 34901-34908

23. Strassheim, D., Porter, R. A., Phelps, S. H., and Williams, C. L. Unique in vivo associations with SmgGDS and RhoGDI and different guanine nucleotide exchange activities exhibited by RhoA, dominant negative RhoA(Asn-19), and activated RhoA(Val-14). (2000) *J Biol Chem* **275**(10), 6699-6702

24. Vikis, H. G., Stewart, S., and Guan, K. L. SmgGDS displays differential binding and exchange activity towards different Ras isoforms. (2002) *Oncogene* **21**(15), 2425-2432

25. Brugnera, E., Haney, L., Grimsley, C., Lu, M., et al. Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex. (2002) *Nat Cell Biol* **4**(8), 574-582

26. Cote, J. F., and Vuori, K. Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity. (2002) *J Cell Sci* **115**(Pt 24), 4901-4913

27. Cote, J. F., and Vuori, K. GEF what? Dock180 and related proteins help Rac to polarize cells in new ways. (2007) *Trends Cell Biol* **17**(8), 383-393

28. Meller, N., Irani-Tehrani, M., Kiosses, W. B., Del Pozo, M. A., and Schwartz, M. A. Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins. (2002) *Nat Cell Biol* **4**(9), 639-647

29. Meller, N., Merlot, S., and Guda, C. CZH proteins: a new family of Rho-GEFs. (2005) *J Cell Sci* **118**(Pt 21), 4937-4946

30. Hasegawa, H., Kiyokawa, E., Tanaka, S., Nagashima, K., et al. DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane. (1996) *Mol Cell Biol* **16**(4), 1770-1776

31. Wu, Y. C., and Horvitz, H. R. C. elegans phagocytosis and cell-migration protein CED-5 is similar to human DOCK180. (1998) *Nature* **392**(6675), 501-504

32. Kiyokawa, E., Hashimoto, Y., Kurata, T., Sugimura, H., and Matsuda, M. Evidence that DOCK180 up-regulates signals from the CrkII-p130(Cas) complex. (1998) *J Biol Chem* **273**(38), 24479-24484

33. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. Activation of Rac1 by a Crk SH3-binding protein, DOCK180. (1998) *Genes Dev* **12**(21), 3331-3336

34. Erickson, M. R., Galletta, B. J., and Abmayr, S. M. Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization. (1997) *J Cell Biol* **138**(3), 589-603

35. Albert, M. L., Kim, J. I., and Birge, R. B. alphavbeta5 integrin recruits the CrkII-Dock180-rac1 complex for phagocytosis of apoptotic cells. (2000) *Nat Cell Biol* **2**(12), 899-905

36. Akakura, S., Singh, S., Spataro, M., Akakura, R., et al. The opsonin MFG-E8 is a ligand for the alphavbeta5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells. (2004) *Exp Cell Res* **292**(2), 403-416

37. Balagopalan, L., Chen, M. H., Geisbrecht, E. R., and Abmayr, S. M. The CDM superfamily protein MBC directs myoblast fusion through a mechanism that requires phosphatidylinositol 3,4,5-triphosphate binding but is independent of direct interaction with DCrk. (2006) *Mol Cell Biol* **26**(24), 9442-9455

38. Cote, J. F., Motoyama, A. B., Bush, J. A., and Vuori, K. A novel and evolutionarily conserved PtdIns(3,4,5)P3-binding domain is necessary for DOCK180 signalling. (2005) *Nat Cell Biol* **7**(8), 797-807

39. Kobayashi, S., Shirai, T., Kiyokawa, E., Mochizuki, N., Matsuda, M., and Fukui, Y. Membrane recruitment of DOCK180 by binding to PtdIns(3,4,5)P3. (2001) *Biochem J* **354**(Pt 1), 73-78

40. Gumienny, T. L., Brugnera, E., Tosello-Trampont, A. C., Kinchen, J. M., et al. CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. (2001) *Cell* **107**(1), 27-41

41. Lu, M., Kinchen, J. M., Rossman, K. L., Grimsley, C., et al. PH domain of ELMO functions in trans to regulate Rac activation via Dock180. (2004) *Nat Struct Mol Biol* **11**(8), 756-762

42. Lu, M., Kinchen, J. M., Rossman, K. L., Grimsley, C., et al. A Steric-inhibition model for regulation of nucleotide exchange via the Dock180 family of GEFs. (2005) *Curr Biol* **15**(4), 371-377

43. Akakura, S., Kar, B., Singh, S., Cho, L., et al. C-terminal SH3 domain of CrkII regulates the assembly and function of the DOCK180/ELMO Rac-GEF. (2005) *J Cell Physiol* **204**(1), 344-351

44. Yin, J., Haney, L., Walk, S., Zhou, S., Ravichandran, K. S., and Wang, W. Nuclear localization of the DOCK180/ELMO complex. (2004) *Arch Biochem Biophys* **429**(1), 23-29

45. Makino, Y., Tsuda, M., Ichihara, S., Watanabe, T., et al. Elmo1 inhibits ubiquitylation of Dock180. (2006) *J Cell Sci* **119**(Pt 5), 923-932

46. Park, D., Tosello-Trampont, A. C., Elliott, M. R., Lu, M., et al. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. (2007) *Nature* **450**(7168), 430-434

47. Katoh, H., and Negishi, M. RhoG activates Rac1 by direct interaction with the Dock180-binding protein Elmo. (2003) *Nature* **424**(6947), 461-464

48. Katoh, H., Hiramoto, K., and Negishi, M. Activation of Rac1 by RhoG regulates cell migration. (2006) *J Cell Sci* **119**(Pt 1), 56-65

49. deBakker, C. D., Haney, L. B., Kinchen, J. M., Grimsley, C., et al. Phagocytosis of apoptotic cells is regulated by a UNC-73/TRIO-MIG-2/RhoG signaling module and armadillo repeats of CED-12/ELMO. (2004) *Curr Biol* **14**(24), 2208-2216

50. Handa, Y., Suzuki, M., Ohya, K., Iwai, H., et al. Shigella IpgB1 promotes bacterial entry through the ELMO-Dock180 machinery. (2007) *Nat Cell Biol* **9**(1), 121-128

51. Li, X., Gao, X., Liu, G., Xiong, W., Wu, J., and Rao, Y. Netrin signal transduction and the guanine nucleotide exchange factor DOCK180 in attractive signaling. (2008) *Nat Neurosci* **11**(1), 28-35

52. Santy, L. C., Ravichandran, K. S., and Casanova, J. E. The DOCK180/Elmo complex couples ARNO-mediated Arf6 activation to the downstream activation of Rac1. (2005) *Curr Biol* **15**(19), 1749-1754

53. Nishihara, H., Kobayashi, S., Hashimoto, Y., Ohba, F., et al. Non-adherent cell-specific expression of DOCK2, a member of the human CDM-family proteins. (1999) *Biochim Biophys Acta* **1452**(2), 179-187

54. Fukui, Y., Hashimoto, O., Sanui, T., Oono, T., et al. Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. (2001) *Nature* **412**(6849), 826-831

55. Sanui, T., Inayoshi, A., Noda, M., Iwata, E., et al. DOCK2 regulates Rac activation and cytoskeletal reorganization through interaction with ELMO1. (2003) *Blood* **102**(8), 2948-2950

56. Sanui, T., Inayoshi, A., Noda, M., Iwata, E., et al. DOCK2 is essential for antigen-induced translocation of TCR and lipid rafts, but not PKC-theta and LFA-1, in T cells. (2003) *Immunity* **19**(1), 119-129

57. Jiang, H., Pan, F., Erickson, L. M., Jang, M. S., et al. Deletion of DOCK2, a regulator of the actin cytoskeleton in lymphocytes, suppresses cardiac allograft rejection. (2005) *J Exp Med* **202**(8), 1121-1130

58. Garcia-Bernal, D., Sotillo-Mallo, E., Nombela-Arrieta, C., Samaniego, R., et al. DOCK2 is required for chemokine-promoted human T lymphocyte adhesion under shear stress mediated by the integrin alpha4beta1. (2006) *J Immunol* **177**(8), 5215-5225

59. Kunisaki, Y., Tanaka, Y., Sanui, T., Inayoshi, A., et al. DOCK2 is required in T cell precursors for development of Valpha14 NK T cells. (2006) *J Immunol* **176**(8), 4640-4645

60. Tanaka, Y., Hamano, S., Gotoh, K., Murata, Y., et al. T helper type 2 differentiation and intracellular trafficking of the interleukin 4 receptor-alpha

subunit controlled by the Rac activator Dock2. (2007) *Nat Immunol* **8**(10), 1067-1075

61. Nombela-Arrieta, C., Mempel, T. R., Soriano, S. F., Mazo, I., et al. A central role for DOCK2 during interstitial lymphocyte motility and sphingosine-1-phosphate-mediated egress. (2007) *J Exp Med* **204**(3), 497-510

62. Janardhan, A., Swigut, T., Hill, B., Myers, M. P., and Skowronski, J. HIV-1 Nef binds the DOCK2-ELMO1 complex to activate rac and inhibit lymphocyte chemotaxis. (2004) *PLoS Biol* **2**(1), E6

63. Brazier, H., Stephens, S., Ory, S., Fort, P., Morrison, N., and Blangy, A. Expression profile of RhoGTPases and RhoGEFs during RANKL-stimulated osteoclastogenesis: identification of essential genes in osteoclasts. (2006) *J Bone Miner Res* **21**(9), 1387-1398

64. Moore, C. A., Parkin, C. A., Bidet, Y., and Ingham, P. W. A role for the Myoblast city homologues Dock1 and Dock5 and the adaptor proteins Crk and Crk-like in zebrafish myoblast fusion. (2007) *Development* **134**(17), 3145-3153

65. Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D. M., et al. Candidate gene for the chromosome 1 familial Alzheimer's disease locus. (1995) *Science* **269**(5226), 973-977

66. Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., et al. Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. (1995) *Nature* **375**(6534), 754-760

67. Kashiwa, A., Yoshida, H., Lee, S., Paladino, T., et al. Isolation and characterization of novel presenilin binding protein. (2000) *J Neurochem* **75**(1), 109-116

68. Chen, Q., Yoshida, H., Schubert, D., Maher, P., Mallory, M., and Masliah, E. Presenilin binding protein is associated with neurofibrillary alterations in Alzheimer's disease and stimulates tau phosphorylation. (2001) *Am J Pathol* **159**(5), 1597-1602

69. Chen, Q., Chen, T. J., Letourneau, P. C., Costa Lda, F., and Schubert, D. Modifier of cell adhesion regulates N-cadherin-mediated cell-cell adhesion and neurite outgrowth. (2005) *J Neurosci* **25**(2), 281-290

70. de Silva, M. G., Elliott, K., Dahl, H. H., Fitzpatrick, E., et al. Disruption of a novel member of a sodium/hydrogen exchanger family and DOCK3 is associated with an attention deficit hyperactivity disorder-like phenotype. (2003) *J Med Genet* **40**(10), 733-740

71. Grimsley, C. M., Kinchen, J. M., Tosello-Trampont, A. C., Brugnera, E., et al. Dock180 and ELMO1 proteins cooperate to promote evolutionarily conserved Rac-dependent cell migration. (2004) *J Biol Chem* **279**(7), 6087-6097

72. Namekata, K., Enokido, Y., Iwasawa, K., and Kimura, H. MOCA induces membrane spreading by activating Rac1. (2004) *J Biol Chem* **279**(14), 14331-14337

73. Yajnik, V., Paulding, C., Sordella, R., McClatchey, A. I., et al. DOCK4, a GTPase activator, is disrupted during tumorigenesis. (2003) *Cell* **112**(5), 673-684

74. Hiramoto, K., Negishi, M., and Katoh, H. Dock4 is regulated by RhoG and promotes Rac-dependent cell migration. (2006) *Exp Cell Res* **312**(20), 4205-4216

75. Yan, D., Li, F., Hall, M. L., Sage, C., et al. An isoform of GTPase regulator DOCK4 localizes to the stereocilia in the inner ear and binds to harmonin (USH1C). (2006) *J Mol Biol* **357**(3), 755-764

76. Miyamoto, Y., Yamauchi, J., Sanbe, A., and Tanoue, A. Dock6, a Dock-C subfamily guanine nucleotide exchanger, has the dual specificity for Rac1 and Cdc42 and regulates neurite outgrowth. (2007) *Exp Cell Res* **313**(4), 791-804

77. Nellist, M., Burgers, P. C., van den Ouweland, A. M., Halley, D. J., and Luider, T. M. Phosphorylation and binding partner analysis of the TSC1-TSC2 complex. (2005) *Biochem Biophys Res Commun* **333**(3), 818-826

78. Rosner, M., Hanneder, M., Siegel, N., Valli, A., and Hengstschlager, M. The tuberous sclerosis gene products hamartin and tuberin are multifunctional proteins with a wide spectrum of interacting partners. (2008) *Mutat Res*

79. Watabe-Uchida, M., John, K. A., Janas, J. A., Newey, S. E., and Van Aelst, L. The Rac activator DOCK7 regulates neuronal polarity through local phosphorylation of stathmin/Op18. (2006) *Neuron* **51**(6), 727-739

80. Borghese, L., Fletcher, G., Mathieu, J., Atzberger, A., et al. Systematic analysis of the transcriptional switch inducing migration of border cells. (2006) *Dev Cell* **10**(4), 497-508

81. Duchek, P., Somogyi, K., Jekely, G., Beccari, S., and Rorth, P. Guidance of cell migration by the Drosophila PDGF/VEGF receptor. (2001) *Cell* **107**(1), 17-26

82. Ruusala, A., and Aspenstrom, P. Isolation and characterisation of DOCK8, a member of the DOCK180-related regulators of cell morphology. (2004) *FEBS Lett* **572**(1-3), 159-166

83. Takahashi, K., Kohno, T., Ajima, R., Sasaki, H., et al. Homozygous deletion and reduced expression of the DOCK8 gene in human lung cancer. (2006) *Int J Oncol* **28**(2), 321-328

84. Griggs, B. L., Ladd, S., Saul, R. A., DuPont, B. R., and Srivastava, A. K. Dedicator of cytokinesis 8 is disrupted in two patients with mental retardation and developmental disabilities. (2008) *Genomics* **91**(2), 195-202

85. Nishikimi, A., Meller, N., Uekawa, N., Isobe, K., Schwartz, M. A., and Maruyama, M. Zizimin2: a novel, DOCK180-related Cdc42 guanine nucleotide exchange factor expressed predominantly in lymphocytes. (2005) *FEBS Lett* **579**(5), 1039-1046

86. Meller, N., Westbrook, M. J., Shannon, J. D., Guda, C., and Schwartz, M. A. Function of the N-terminus of zizimin1: autoinhibition and membrane targeting. (2008) *Biochem J* **409**(2), 525-533

87. Fluge, O., Bruland, O., Akslen, L. A., Lillehaug, J. R., and Varhaug, J. E. Gene expression in poorly differentiated papillary thyroid carcinomas. (2006) *Thyroid* **16**(2), 161-175

88. Lin, Q., Yang, W., Baird, D., Feng, Q., and Cerione, R. A. Identification of a DOCK180-related guanine nucleotide exchange factor that is capable of mediating a positive feedback activation of Cdc42. (2006) *J Biol Chem* **281**(46), 35253-35262

89. Kwofie, M. A., and Skowronski, J. Specific recognition of Rac2 and Cdc42 by DOCK2 and DOCK9 guanine nucleotide exchange factors. (2008) *J Biol Chem* **283**(6), 3088-3096

90. Li, R., and Zheng, Y. Residues of the Rho family GTPases Rho and Cdc42 that specify sensitivity to Dbl-like guanine nucleotide exchange factors. (1997) *J Biol Chem* **272**(8), 4671-4679

91. Snyder, J. T., Worthylake, D. K., Rossman, K. L., Betts, L., et al. Structural basis for the selective activation of Rho GTPases by Dbl exchange factors. (2002) *Nat Struct Biol* **9**(6), 468-475

92. Karnoub, A. E., Worthylake, D. K., Rossman, K. L., Pruitt, W. M., et al. Molecular basis for Rac1 recognition by guanine nucleotide exchange factors. (2001) *Nat Struct Biol* **8**(12), 1037-1041

93. Gao, Y., Xing, J., Streuli, M., Leto, T. L., and Zheng, Y. Trp(56) of rac1 specifies interaction with a subset of guanine nucleotide exchange factors. (2001) *J Biol Chem* **276**(50), 47530-47541

94. Lu, M., and Ravichandran, K. S. Dock180-ELMO cooperation in Rac activation. (2006) *Methods Enzymol* **406**, 388-402

95. Ishimaru, S., Ueda, R., Hinohara, Y., Ohtani, M., and Hanafusa, H. PVR plays a critical role via JNK activation in thorax closure during Drosophila metamorphosis. (2004) *Embo J* **23**(20), 3984-3994

96. Chen, Q., Kimura, H., and Schubert, D. A novel mechanism for the regulation of amyloid precursor protein metabolism. (2002) *J Cell Biol* **158**(1), 79-89

97. Wu, Y. C., Tsai, M. C., Cheng, L. C., Chou, C. J., and Weng, N. Y. C. elegans CED-12 acts in the conserved crkII/DOCK180/Rac pathway to control cell migration and cell corpse engulfment. (2001) *Dev Cell* **1**(4), 491-502

98. Zhou, Z., Caron, E., Hartwieg, E., Hall, A., and Horvitz, H. R. The C. elegans PH domain protein CED-12 regulates cytoskeletal reorganization via a Rho/Rac GTPase signaling pathway. (2001) *Dev Cell* **1**(4), 477-489

99. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. (1997) *Nucleic Acids Res* **25**(24), 4876-4882

100. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. (1997) *Nucleic Acids Res* **25**(17), 3389-3402

101. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., et al. (2005) Protein Identification and Analysis Tools on the ExPASy Server. In: Walker, J. M. (ed). *The Proteomics Protocols Handbook*, Humana Press

102. Ginalski, K., Elofsson, A., Fischer, D., and Rychlewski, L. 3D-Jury: a simple approach to improve protein structure predictions. (2003) *Bioinformatics* **19**(8), 1015-1018

103. Kelley, L. A., MacCallum, R. M., and Sternberg, M. J. Enhanced genome annotation using structural profiles in the program 3D-PSSM. (2000) *J Mol Biol* **299**(2), 499-520

104. Letunic, I., Copley, R. R., Pils, B., Pinkert, S., Schultz, J., and Bork, P. SMART 5: domains in the context of genomes and networks. (2006) *Nucleic Acids Res* **34**(Database issue), D257-260

105. McGuffin, L. J., and Jones, D. T. Improvement of the GenTHREADER method for genomic fold recognition. (2003) *Bioinformatics* **19**(7), 874-881

106. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. SMART, a simple modular architecture research tool: identification of signaling domains. (1998) *Proc Natl Acad Sci U S A* **95**(11), 5857-5864

107. Stols, L., Gu, M., Dieckman, L., Raffen, R., Collart, F. R., and Donnelly, M. I. A new vector for high-throughput, ligation-independent cloning encoding a tobacco etch virus protease cleavage site. (2002) *Protein Expr Purif* **25**(1), 8-15

108. Chhatriwala, M. K., Betts, L., Worthylake, D. K., and Sondek, J. The DH and PH domains of Trio coordinately engage Rho GTPases for their efficient activation. (2007) *J Mol Biol* **368**(5), 1307-1320

109. Worthylake, D. K., Rossman, K. L., and Sondek, J. Crystal structure of Rac1 in complex with the guanine nucleotide exchange region of Tiam1. (2000) *Nature* **408**(6813), 682-688

110. Studier, F. W. Protein production by auto-induction in high density shaking cultures. (2005) *Protein Expr Purif* **41**(1), 207-234

111. Wen, J., Arakawa, T., and Philo, J. S. Size-exclusion chromatography with on-line light-scattering, absorbance, and refractive index detectors for studying proteins and their interactions. (1996) *Anal Biochem* **240**(2), 155-166

112. Snyder, J. T., Singer, A. U., Wing, M. R., Harden, T. K., and Sondek, J. The pleckstrin homology domain of phospholipase C-beta2 as an effector site for Rac. (2003) *J Biol Chem* **278**(23), 21099-21104

113. Andrade, M. A., Perez-Iratxeta, C., and Ponting, C. P. Protein repeats: structures, functions, and evolution. (2001) *J Struct Biol* **134**(2-3), 117-131

114. Hatzfeld, M. The armadillo family of structural proteins. (1999) *Int Rev Cytol* **186**, 179-224

115. Murzin, A. G., Brenner, S. E., Hubbard, T., and Chothia, C. SCOP: a structural classification of proteins database for the investigation of sequences and structures. (1995) *J Mol Biol* **247**(4), 536-540

116. Nishihara, H., Maeda, M., Oda, A., Tsuda, M., et al. DOCK2 associates with CrkL and regulates Rac1 in human leukemia cell lines. (2002) *Blood* **100**(12), 3968-3974

117. Lupas, A., Van Dyke, M., and Stock, J. Predicting coiled coils from protein sequences. (1991) *Science* **252**(5010), 1162-1164

118. Meller, N., Irani-Tehrani, M., Ratnikov, B. I., Paschal, B. M., and Schwartz, M. A. The novel Cdc42 guanine nucleotide exchange factor, zizimin1, dimerizes via the Cdc42-binding CZH2 domain. (2004) *J Biol Chem* **279**(36), 37470-37476

119. (1981) An introduction to polyacrylamide gel elctrophoresis. In: Hames, B. D., and Rickwood, D. (eds). *Gel ectrophoresis of proteins: A practical approach*, IRL Press Limited, London, Washington DC

120. Westendorf, J. J. The formin/diaphanous-related protein, FHOS, interacts with Rac1 and activates transcription from the serum response element. (2001) *J Biol Chem* **276**(49), 46453-46459

121. Rose, R., Weyand, M., Lammers, M., Ishizaki, T., Ahmadian, M. R., and Wittinghofer, A. Structural and mechanistic insights into the interaction between Rho and mammalian Dia. (2005) *Nature* **435**(7041), 513-518

122. Rose, R., Wittinghofer, A., and Weyand, M. The purification and crystallization of mDia1 in complex with RhoC. (2005) *Acta Crystallogr Sect F Struct Biol Cryst Commun* **61**(Pt 2), 225-227

123. Freedman, T. S., Sondermann, H., Friedland, G. D., Kortemme, T., et al. A Ras-induced conformational switch in the Ras activator Son of sevenless. (2006) *Proc Natl Acad Sci U S A* **103**(45), 16692-16697

124. Margarit, S. M., Sondermann, H., Hall, B. E., Nagar, B., et al. Structural evidence for feedback activation by Ras.GTP of the Ras-specific nucleotide exchange factor SOS. (2003) *Cell* **112**(5), 685-695

125. Yamaki, N., Negishi, M., and Katoh, H. RhoG regulates anoikis through a phosphatidylinositol 3-kinase-dependent mechanism. (2007) *Exp Cell Res* **313**(13), 2821-2832

126. Saxena, A., Morozov, P., Frank, D., Musalo, R., et al. Phosphoinositide binding by the pleckstrin homology domains of Ipl and Tih1. (2002) *J Biol Chem* **277**(51), 49935-49944

127. Pollard, J. W., and Stanners, C. P. Characterization of cell lines showing growth control isolated from both the wild type and a leucyl-tRNA synthetase mutant of Chinese hamster ovary cells. (1979) *J Cell Physiol* **98**(3), 571-585

128. Ceccarelli, D. F., Blasutig, I. M., Goudreault, M., Li, Z., et al. Non-canonical interaction of phosphoinositides with pleckstrin homology domains of Tiam1 and ArhGAP9. (2007) *J Biol Chem* **282**(18), 13864-13874

129. Kunisaki, Y., Nishikimi, A., Tanaka, Y., Takii, R., et al. DOCK2 is a Rac activator that regulates motility and polarity during neutrophil chemotaxis. (2006) *J Cell Biol* **174**(5), 647-652

130. Wallar, B. J., and Alberts, A. S. The formins: active scaffolds that remodel the cytoskeleton. (2003) *Trends Cell Biol* **13**(8), 435-446