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REVIEW

Molecular principles of bidirectional signalling between membranes and small GTPases

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Most small GTPases actuate their functions on subcellular membranes, which are increasingly seen as integral components of small GTPase signalling. In this review, we used the highly studied regulation of Arf GTPases by their GEFs to categorize the molecular principles of membrane contributions to small GTPase signalling, which have been highlighted by integrated structural biology combining *in vitro* reconstitutions in artificial membranes and high-resolution structures. As an illustration of how this framework can be harnessed to better understand the cooperation between small GTPases, their regulators and membranes, we applied it to the activation of the small GTPase Rac1 by DOCK-ELMO, identifying novel contributions of membranes to Rac1 activation. We propose that these structure-based principles should be considered when interrogating the mechanisms whereby small GTPase systems ensure spatial and temporal control of cellular signalling on membranes.

Keywords: Arf; ArfGEF; DOCK-ELMO; GTPases; integrated structural biology; Rac1; subcellular membranes

It is well established that most small GTPases, GEFs, GAPs, and effectors carry out their functions by assembling complexes at the surface of intracellular membranes (reviewed in ref. [1]). Membranes are thus taking centre stage as *bona fide* components of small GTPase signalling, with pivotal roles in defining specificities, regulating efficiencies or determining the shape and duration of signals. During the last decade, integration of structural, biophysical, and imaging methods using purified proteins and artificial membranes (Integrated Structural Biology, Box 1) has generated unprecedented insights into the molecular determinants of small GTPase systems on membranes that were not attainable otherwise. These approaches highlighted

that there are many ways that the membrane can modulate small GTPase signalling. This knowledge now constitutes a robust framework to assist in the interpretation of experimental observations made in cells or in diseases.

Arf GTPases coordinate most aspects of membrane traffic along the secretory and the endocytic pathways, such as the regulation of vesicles biogenesis, membrane contact sites assembly lipid metabolism, and in the coordination of membrane traffic and cytoskeleton remodelling (reviewed in refs [2,3]). As a consequence of their pivotal functions in cells, they are also involved in severe pathologies, such as cancer, diabetes, or developmental diseases and diverted by

Abbreviations

Arf, ADP-ribosylation factor; ArfGAP, Arf GTPase-activating protein; ArfGEF, Arf guanine nucleotide exchange factor; COPI, coat protein complex I; cryo-EM, cryo-electron microscopy; GBF1, Golgi brefeldin A-resistant guanine nucleotide exchange factor; GDP, guanosine diphosphate; GTP, guanosine 5'-triphosphate; HDX-MS, hydrogen deuterium exchange mass spectrometry; PDB, protein data bank; PH, pleckstrin homology; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-bisphosphate; PS, Phosphatidylserine; Rac1, Ras-related C3 botulinum toxin substrate 1; TRAPP, transport protein particle.

Box 1. Integrated Structural Biology

Integrated structural biology refers to an ensemble of structural, biophysical, and imaging methods using purified proteins, which, together, allow to general multiscale insight into protein structures and functions. The method of choice for determining the structure of protein assemblies in atomic details, X-ray crystallography, is restricted to essentially rigid structures and informs poorly on flexible structures, which in addition often hamper crystallization. It is increasingly challenged by single-particle cryo-electron microscopy (cryo-EM), which allows to visualize much larger assemblies that can have multiple conformations, and is now nearing atomic resolution. In complement, small-angle X-ray scattering (SAXS) provides information on the overall shape of soluble particles at low resolution, somehow similar to envelopes obtained by negative staining microscopy, and in case the proteins have flexible regions, on conformational ensembles. Currently, these methods have not yet been widely used to study protein structures and protein/lipid interactions at the protein–membrane interface. Alternatively, several sophisticated low-to-medium resolution structural methods were successfully used with proteins reconstituted in artificial membranes, such as vesicles or lipid nanodiscs, including information on the protein/membrane interface by nuclear magnetic resonance (NMR), hydrogen/deuterium exchange coupled to mass spectrometry (HDX-MS) and structures of protein assemblies on liposomes by cryo-electron tomography. However, important efforts are still required for such methods to become widely accessible to the structural biology community, notably cryo-electron tomography. Other approaches include protein folding prediction methods, notably the AlphaFold Database. Finally, our understanding of protein–membrane interactions at the atomic level still relies mostly on predictions from molecular dynamics (MD) simulations.

Besides structural approaches, quantitative kinetics measurements inform on the modulation of small GTPase activities by membranes. For instance, activation of small GTPases by their GEFs can be monitored by fluorescence spectroscopy using intrinsic protein fluorescence or fluorescent nucleotides, using proteins reconstituted in liposomes of defined curvature and lipid composition. In such reconstituted systems, the effect of additional protein components or of variations in the membrane composition can readily be assessed. Finally, small GTPase systems reconstituted in lipid supported bilayers can be observed by total internal reflection fluorescence (TIRF) microscopy, documenting the kinetics of protein recruitment to the membrane and the shape and expanse of the activation zone.

pathogens in infections. As they function on most endomembranes, they are representative of the remarkable diversity of regulatory modalities supported by membranes that have been uncovered by integrated structural biology. There are five human Arf GTPases (reviewed in ref. [4]) which are activated by 15 guanine nucleotide exchange factors (ArfGEFs), recognizable by their conserved catalytic domain of about 200 amino acids, named Sec7 domain with reference to its homology with yeast Sec7p [5]. ArfGEFs are classified in different subfamilies, each with a different set of domains flanking the Sec7 domain (Fig. 1A, reviewed in refs [6–8]).

In this review, we used the highly studied regulation of Arf GTPases by their GEFs to categorize the molecular principles of membrane contributions to small GTPase signalling. We then applied these principles to analyse the activation of the Rac1 (Ras-related C3 botulinum toxin substrate 1) GTPase by its GEFs of the DOCK family in the context of membranes, illustrating how this framework can be harnessed to understand how small GTPases function on membranes. The PDB ID's of all major structures used in this review are listed in Table 1.

Interactions of Arf and ArfGEFs with membranes

A first step to understand how membranes modulate the function of small GTPase systems is to delineate the structural elements that are involved in their binding to membranes and how these elements recognize specific lipids and/or physicochemical characteristics of the membrane.

On the small GTPase side, with only few exceptions, an essential feature is their reversible association to membranes through N- or C-terminal tails, which extend from the GTPase core and are post-translationally modified by one or several lipids (membrane anchors hereafter). Association of the lipidated tails to membranes involves both the lipid and proteins residues, which together establish various interactions with the lipid bilayer, which still remains poorly understood at the atomic level. In Arf GTPases, the membrane-binding region is an N-terminal amphipathic α -helix carrying one [9,10] or two [11] myristate lipids, and is currently one of the best characterized. The myristate is masked by intramolecular interactions with the GTPase core in the cytosol, which requires that the helix toggles to insert into the membrane to

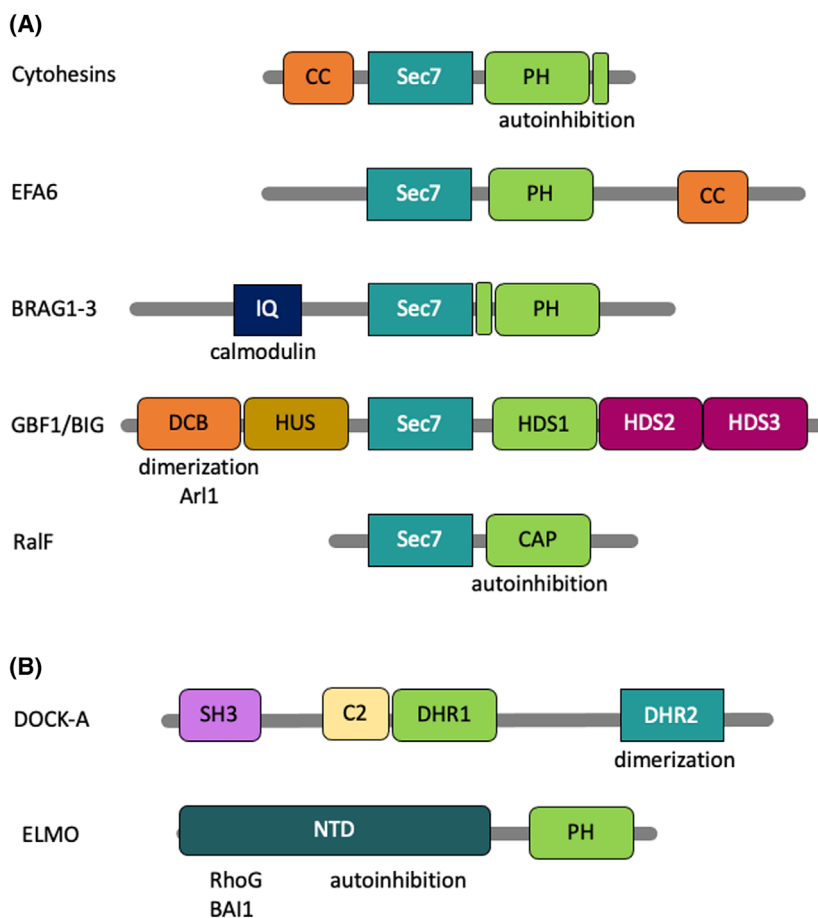


Fig. 1. Domain organization of GEFs described in this review. (A) ArfGEFs (UNIPROT IDs: IQSEC1, Q6DN90; cytohesin2, Q99418; EFA6, A5PKW4; BIG, Q9Y6D6; RalF, Q8RT31). (B) DOCK-A/ELMO (UNIPROT IDs: DOCK1, Q14185; ELMO1, Q8BPU7). GEF domains are coloured in emerald green and membrane-interacting domains in lime green. CAP, capping domain; CC, coiled-coil; DCB, dimerization and cyclophilin binding; DHR, DOCK homology region; HDS, homology downstream of Sec7; IQ, IQ calmodulin-binding motif; NTD, N-terminal domain; PH, pleckstrin homology; SH3, Src homology 3.

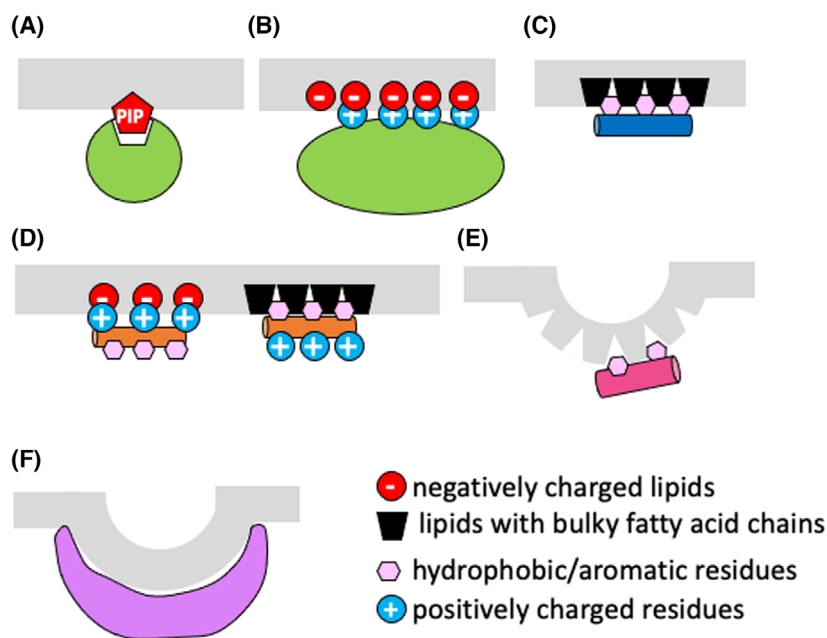
Table 1. List of structures used in this review.

PDB ID	Structure
1R8S	Arf1-GDP/Sec7 domain complex with interswitch toggled [31]
4C0A	Arf1-GDP in complex with BRAG2 ^{Sec7-PH} [23]
6FNE	Bragsin inhibitor bound to the PH domain of BRAG2 ^{Sec7-PH} [34]
1 U29	PIP3 lipid headgroup bound to the PH domain of Cytohesin2 [20]
1XSZ	Autoinhibited <i>legionella</i> RalF [28]
4KAX	Arf6-GTP bound to the PH domain of cytohesin1 [38]
7URO ^a	Arf1 bound to full-length yeast GEA2 [27]
5EE5	Arl1-GTP bound to the DCB domain of BIG1 [44]
6TGC ^a	DOCK2-ELMO1-Rac1 ternary complex [62]
7DPA ^a	DOCK5-ELMO1-Rac1 ternary complex [63]
6TGB ^a	Autoinhibited DOCK2-ELMO1 complex [62]
2VSZ	ELMO1 PH domain [70]
6IDX	BAI1 peptide bound to the NTD of ELMO2 [71]
6UKA	RHO-GTP bound to the NTD of ELMO1 ^{NTD} [62]
1HH4	Lipidated full-length Rac1 bound to RhoGDI [67]
5A1U	Structure of the COP1 coat triad [76]
8D9V	AP-1, Arf1 and Nef lattice on membrane tubes [15]

^aStructure determined by cryo-EM.

allow GDP-to-GTP exchange [10,12]. Importantly, the myristoylated helix is connected to the GTPase core by a short linker that functions as hinge [12,13], implying that the GTPase core of Arf GTPases is close to the membrane. Of note, Arf1-GTP functions on endo-membranes from the early Golgi to the plasma membrane (reviewed in refs [2,3]), indicating that the sequence of its amphipathic N-terminal helix is permissive to a broad range of membrane compositions and shapes. Importantly, membrane-bound Arf GTPases can form multimers that associate more specifically with curved membranes. Notably, Arf GTPases at high concentration form dimers that induce membrane tubulation, which were predicted from HDX-MS experiments to involve the nucleotide-sensor switch two region [14]. Likewise, cryo-electron tomography revealed that Arf dimers bridge AP-1 complexes in a liposomal system to induce a tubular lattice, in which the dimer interface is located outside the nucleotide-sensor switch regions [15]. In the asymmetric unit (also called leaf) of the COP1 coat triad, two Arf1 molecules are found in two different environments, and one

Fig. 2. Modalities of the interaction of Arf regulators and effectors with membranes. (A) Interaction with specific phosphoinositide headgroups. (B) Multiple electrostatic interactions between negatively charged lipids and positively charged amino acids. (C) Insertion of amphipathic helix into packing defects resulting from lipids with unsaturated fatty acid chains. (D) Dual specificity interactions, in which positively charged residues recognize anionic lipids, and hydrophobic residues recognize loosely packed membranes. (E) Amphipathic helix inserting hydrophobic residues in lipid packing defects resulting from positive curvature. (F) Complementarity of shape with positively or negatively curved membranes.



of them forms a loose trimer in the model of the triad [16].

In contrast, a diversity of membrane recognition features has been identified in ArfGEFs, ranging from specific interactions with individual phosphoinositides to multiple interactions recognizing physicochemical characteristics of membranes (Fig. 2A–D). The enrichment in low-abundance phosphoinositides is often considered a signature of specific endomembranes, for instance, the signalling lipids PI(4,5)P₂ (PIP2) and PI(3,4,5)P₃ (PIP3) at the plasma membrane (reviewed in refs [17,18]). Specific recognition of individual PIP2 and/or PIP3 lipids has been observed so far only in cytohesins [19]. The lipid headgroup binds deeply into a positively charged pocket of their PH domain, where it is held by multiple hydrogen bonds [20] (Fig. 2A). Besides, the presence of such rare signalling lipids, intracellular membranes can be divided into two broad territories based on their general physicochemical characteristics: electrostatics at the cell periphery and loose lipid packing in the cell interior (reviewed in ref. [21]). The plasma membrane, for example, has a high content in negatively charged lipids, which attract proteins that carry positively charged lysine (K) and arginine (R) residues. Conversely, ER and Golgi membranes are less negatively charged and are enriched in lipids with bulky fatty acid chains that create packing defects, which will favour the binding of proteins with aliphatic or aromatic residues. Both cytohesins and BRAG/IQSEC establish non-specific electrostatic interactions with anionic membranes. Cytohesins use a polybasic helix located after its PH domain, which

reinforces the specific interaction of its PH domain with PIP2 and PIP3 [20,22]. In contrast, strong binding of BRAG2/IQSEC1 with anionic membranes requires mostly non-specific electrostatic interactions [23–25]. The membrane-binding region of BRAG2/IQSEC1 comprises flexible polybasic loops of the PH domain, which otherwise lacks a canonical phosphoinositide-binding pocket, and the adjacent linker connecting the Sec7 and PH domains [23] (Fig. 2B). The geometry of the Sec7-PH tandem brings isolated positively charged residues in the Sec7 domain close to the membrane, where they add to the interaction [25].

Amphipathic α -helices are a recurrent lipid-binding motif able to recognize almost any type of endomembranes (reviewed in ref. [26]), which are used by ArfGEFs in different membrane contexts. GEA2, the yeast orthologue of human GBF1, activates Arf GTPases in Golgi-ER traffic, where it is predicted to interact mostly with the loosely packed membrane territory. A recent cryo-EM study predicted that GEA2 exposes a membrane-binding amphipathic helix that is well-suited for shallow insertion into lipid packing defects [27] (Figs 2C and 4C). Highlighting the importance of the structural biology approach in understanding how small GTPase interacting proteins bind to membranes, this structure showed that previously proposed membrane-binding sites were in fact buried in the protein interior. Legionella RalF activates Arf1 at the surface of the Legionella-containing vacuole (LCV) during infection [28]. It is recruited to the LCV by two twin helices with an unusual combination of

positively charged and aromatic residues, encoding a dual specificity for both negatively charged membranes, such as found in the immature LCV, and membranes with packing defects, such as those of the mature LCV [29] (Fig. 2D).

The roles of membranes in the function of ArfGEFs

Membranes cooperate with Sec7 domains to activate Arf

A remarkable feature of Arf GTPases is that membrane-binding and activation by GTP are allosterically coupled through a structural element located in the G protein core (called the interswitch), which functions as a structural toggle switch (reviewed in ref. [30]). The interswitch can only toggle after the myristoylated N-terminal helix has been displaced by membranes, explaining why Arf GTPases are resistant to activation by their GEFs in solution [5]. How the Sec7 domains harness this requirement for membranes has been elucidated by crystallographic studies that trapped intermediates of the exchange reaction. Remarkably, the first action of Sec7 domains is to promote the toggle of the interswitch to the ON position while Arf is still bound to GDP, indicating that Arf-GDP must be associated with the membrane before the GDP/GTP exchange reaction initiates [31]. Thus, membranes can be considered as mandatory co-factors that cooperate with ArfGEFs to activate Arf GTPases. Interestingly, the recent cryo-EM structure of yeast GEA2, which is also the first structure of a full-length ArfGEF, revealed that its Sec7 domain adopts several positions, one of which could be sterically compatible with the binding of soluble, autoinhibited Arf-GDP [27] (see structure of GEA2 in Fig. 4C). Accordingly, GEA2 was proposed to capture Arf-GDP in the cytosol and escort it to the membrane to facilitate its activation [27]. However, soluble autoinhibited Arf-GDP binds to Sec7 domains with an extremely low affinity [32], suggesting that productive Arf-GDP/GEA2 interactions are unlikely in the cytosol. Alternatively, the positional flexibility of the Sec7 domain could support its sequential rotations with respect to membrane-anchored Arf, as was observed in structural intermediates of the exchange reaction [31,33].

Membranes potentiate the GEF efficiency through 3D coincidence detection

Simple geometry considerations imply that for two peripheral membrane proteins to interact productively

at the surface of a membrane, their protein–membrane and protein–protein interfaces must align in three dimensions. We refer to this as 3D coincidence detection. For instance, the nucleotide-binding site of membrane-anchored GTPases and the active site of GEFs must be properly aligned to yield productive interactions. The importance of geometry was demonstrated by the structural, biochemical, and chemical biology study of the Sec7-PH domains BRAG2/IQSEC1. The GEF activity of BRAG2 is strongly potentiated by anionic liposomes, despite it is not autoinhibited in solution [23,24]. The crystal structure of BRAG2/Arf1 together with MD simulations highlighted that the complex adopts a close-packed organization that is precisely apposed to the membranes, suggesting that the increase in activity is due to optimization of its geometry by the membrane [23,25]. Accordingly, perturbation of this geometry by Bragsin, a small molecule that binds to the PH domain/membrane interface without dissociating BRAG2/IQSEC1 from the membrane, results in a dramatic decrease of its GEF activity [34] (Fig. 3A). Incidentally, this study also provided proof-of-concept for a novel drug discovery strategy through targeting protein–membrane interfaces. In a related manner, the geometry imposed by membranes on GTPases and their regulators can act as a specificity determinant. While this has not been identified in the Arf field, it has been shown for Rab GTPases, which bind to membranes by lipidated C-terminal peptides of variable lengths, hence present their nucleotide-binding site at different distances from the membrane. This distance was shown to be “measured” by RabGEFs of the TRAPP subfamily to distinguish between related membrane-anchored Rab GTPases [35], reviewed in ref. [36].

Coupling membrane association to autoinhibition release

At least two families of ArfGEFs are strongly autoinhibited in solution and must undergo large conformational changes to reach their active conformation: cytohesins and bacterial RalF. Remarkably, in both cases, autoinhibition involves membrane-binding elements that are masked by intramolecular interactions.

In cytohesins, access to the Arf-binding site is blocked by the C-terminal membrane-binding polybasic helix, which sequesters the PH and Sec7 domains by binding at their interface [20] (Fig. 3B). The structure of an active, Arf-bound full-length cytohesin is currently missing, however, steric considerations indicate that the PH domain must undergo a large displacement to make the Sec7 active site available.

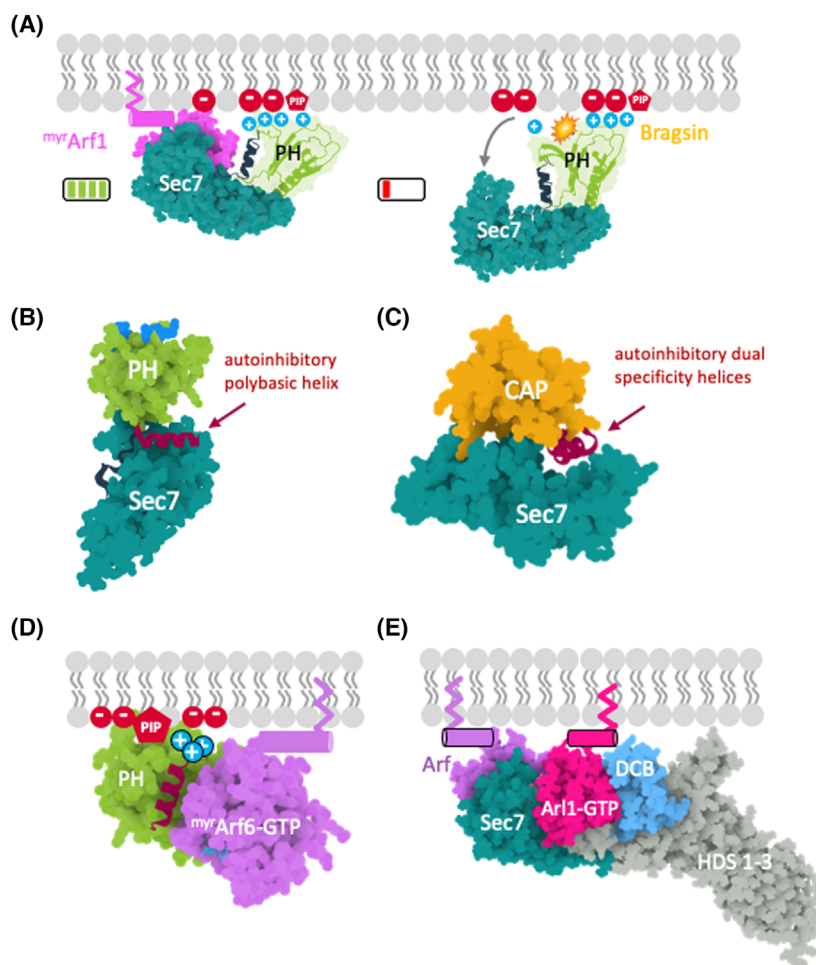


Fig. 3. Molecular aspects of the role of membranes in the function of ArfGEFs. (A) Close-packed electrostatic interactions of BRAG2/IQSEC1 with membranes. Left: BRAG2/IQSEC1 establishes multiple electrostatic interactions with anionic lipids, which optimize the geometry of the Arf/ArfGEF complex to yield maximal activity (PDB ID 4C0A [23]); Right: the small molecule Bragsin binds at the interface between the PH domain and the membrane, which disturbs the geometry of the complex with respect to the membrane, resulting in inhibition (PDB ID 6FNE [34]). (B) Cytohesins are autoinhibited in solution by their membrane-binding C-terminal polybasic helix (in burgundy, arrow), which interlocks the Sec7 and PH domains. Of note, the PIP2-binding pocket of the PH domain (in blue) is constitutively accessible (PDB ID 1U29 [20]). (C) The membrane-binding helices of bacterial RalF (in burgundy, arrow) are blocked by autoinhibitory intramolecular interactions with the Sec7 domain (PDB ID 4D7R [28]). (D) The positive feedback loop in membrane-attached cytohesins is mediated by membrane-anchored Arf-GTP (PDB ID 4KAX [38]). (E) Model of recruitment of monomeric BIG1 to membranes by membrane-anchored Arl1-GTP (PDB IDs: 7URO [27] and 5EE5 [44]). The myristoylated helix of Arf and Arl GTPases is indicated. See text for details.

Surprisingly, the lipid-binding pocket of the PH domain is readily available in autoinhibited cytohesins, suggesting they can bind to signalling PIP2 and PIP3 lipids independently to their activation state. The ensuing proximity to a negatively charged membrane may then facilitate the subsequent extraction of the polybasic helix from the PH/Sec7 interface, eventually setting the PH domain free to move away from the Sec7 domain to complete autoinhibition release. *Legionella* RalF is strongly autoinhibited in solution by a capping domain that masks the Arf-binding site on the Sec7

domain [28]. Similar to cytohesins, autoinhibition also involves membrane-binding elements that must be displaced through a large conformation change, with the difference that the membrane-binding region of autoinhibited RalF is entirely masked by the Sec7 domain [29] (Fig. 3C). In both ArfGEFs, autoinhibition thus provides a means to strongly couple the GEF activity to the recruitment to a specific membrane. It is interesting to note that the loss of intramolecular interactions upon conversion from their close, autoinhibited, to their open, active conformation is energetically

balanced by alternative protein–membrane interactions. This compensation effect is lacking in solution, which may compromise the GEF activity entirely as in RalF proteins.

Feedback loops by activated Arf GTPases

In several ArfGEFs, activated GTPases, the product of the GDP/GTP exchange reaction, have been shown to rebind outside the GEF active site – in other words, to recognize GEFs as effectors rather than as regulators. This has been best characterized for cytohesins, in which Arf-GTP implements a positive feedback loop. Arf6-GTP binds directly to the PH domain of cytohesins [37], in a manner that is not compatible with the autoinhibited conformation [20,38] (Fig. 3D). Accordingly, cytohesins activity is amplified by membrane-attached Arf-GTP [39]. In the structure of Arf-GTP bound to the PH domain of cytohesin, all membrane-binding elements in Arf-GTP, in the PH domain and in the C-terminal helix are nicely aligned to bind simultaneously to membranes [38], which are likely to stabilize an otherwise labile conformation of the GEF, eventually resulting in the positive feedback effect. A positive feedback effect mediated by binding of Arf1-GTP outside the Sec7 domain has also been proposed for the yeast Golgi ArfGEF SEC7 (the orthologue of the mammalian large ArfGEF BIG) [40]. The precise mechanism is currently not known, notably why this effect was not observed in full-length SEC7 [40]. Opposite to these positive effects, binding of Arf-GTP to a region located downstream of the Sec7 domain in EFA6, another PH domain-containing ArfGEF, resulted in a negative feedback effect, the structural basis for which is not known [41]. While membrane-attached EFA6 could activate both Arf1 and Arf6 (incidentally contradicting its original name “exchange factor for Arf6”), only Arf6-GTP was able to implement this effect. This suggested that EFA6 may function upstream of cytohesins to implement an ArfGEF cascade with mixed negative/positive feedback effects [41].

Additional levels of regulation by membrane-associated regulators

The basic properties of ArfGEFs on membranes, such as their conformations or their protein–membrane interactions, can in principle be harnessed by membrane-proximal or transmembrane proteins. We mention here, in a non-exhaustive manner, two classes of proteins that have been reported to interact with ArfGEFs: activated small GTPases (other than in feedback loops) and various transmembrane receptors.

Several GTP-bound, membrane-anchored small GTPases have been reported to target ArfGEFs to specific endomembranes. For example, Rab1-GTP interacts with the N-terminus of the large ArfGEF GBF1 to modulate its recruitment at ER exit sites [42], and with the C-terminal domains of GEA1 and GEA2, the homologs of GBF1 in yeast, to recruit them at the Golgi [43]. Similarly, Arl1-GTP binds to the N-terminal DCB domains of mammalian BIG1 and of yeast SEC7 to recruit them at the Trans Golgi Network (TGN) [44]. Unlike GBF1/GEA, BIG1/SEC7 does not form dimers through its DCB domain [44]. Hence, this subfamily of ArfGEF does not duplicate its membrane-binding elements, which may be compensated by membrane-attached Arl1-GTP. Consistently, overlaying the crystal structures of Arl1-GTP/DCB^{BIG1} or Arl1-GTP/DCB^{Sec7} [44] onto the cryo-EM structure of the GEA2/Arf complex [27] suggests that Arl1-GTP and the Arf1-GDP substrate would bind to BIG1 in a manner that their lipidated tails can interact with the membrane simultaneously (Fig. 3E). A related small GTPase, Arl4, has been proposed to recruit cytohesins to the plasma membrane by binding to its PH domain [45,46]. Although the modalities of this interaction are currently not known, a possibility is that it contributes to autoinhibition release, in a manner similar to Arf6-GTP [38].

Various studies report functional links between ArfGEFs and transmembrane receptors, which in some cases have been proposed to occur through direct interactions between ArfGEFs and membrane-proximal receptor elements. For example, the epidermal growth factor receptor (EGFR) has been proposed to interact with the PH domain of BRAG2/IQSEC1 by its cytoplasmic tail [47] and with the Sec7 domain of cytohesin1 through its juxtamembrane domain [48]. Likewise, BRAG/IQSEC subfamily members were reported to interact with the AMPA [49] and NMDA [50] receptors, and the V-ATPase complex was proposed to recruit cytohesin2/ARNO to early endosomes [51], possibly leading to inhibition of its Sec7 domain [52]. The molecular basis of these interactions remains to be elucidated in the context of membranes, and how they function is currently speculative. One possibility is that they couple receptor signalling to Arf pathways by concentrating Arf-GTP and its effectors in the vicinity of activated receptors, thus establishing “receptor nanodomains”. Again, such accessory interactions must be sterically compatible with the geometrical constraints imposed by the membrane, and therefore, care must be taken when analysing results established with peptides or carried out in solution, where the protein and/or membrane environments are lacking.

Defining Arf signalling zones in time and space

The presence of specific signalling lipids together with physicochemical characteristics of the membrane, such as electrostatics, packing defects resulting from unsaturated lipids, or flatness/curvature, constitute signatures of subcellular membranes and organelles (reviewed in refs [17,21]). Thus, by combining recognition of specific lipid headgroups to non-specific recognition of such characteristics, membrane-binding elements in ArfGEFs can restrict the localization of ArfGEFs to subcellular organelles and territories. Given that Arf1 lacks overall membrane specificity, the subcellular localization of Arf activity is likely to be almost entirely defined by the membrane determinants carried by ArfGEFs. Of note, there are no known examples, to our knowledge, of ArfGEFs that recognize curved membranes. However, positive curvature is directly recognized by certain Arf effectors such as arfaptin (reviewed in ref. [53], Fig. 2E), and by ArfGAP1, which uses an atypical amphipathic helix, called the ALPS motif, to detect the curvature of budding vesicles [54] (Fig. 2F). Whether negative curvature is also relevant to Arf1 signalling is currently unknown, although the functional role of Arf1 in endocytic vesicle fission leaves the question open [55].

How ArfGEFs interact with lipids and membranes may also contribute to define the size, shape and persistence of Arf signalling zones. For example, ArfGEFs forming strong interactions with PIP2 or PIP3 lipids may reside a long time within a restricted area of the membrane. This would result in a small-sized, persistent activation zone with a high density of Arf-GTP, possibly at the expense of the total number of activated Arf GTPases (Fig. 4A). Conversely, ArfGEF forming weaker non-specific interactions may drift more easily on the membrane and/or dissociate/rebind, resulting in activation of Arf on a broader area and at a lesser density (Fig. 4B). These supramolecular aspects of small GTPase signalling are only beginning to be explored and they remain speculative in the Arf field.

Dimeric ArfGEFs: a role in shaping Arf signalling zones on membranes?

Dimerization is a common feature in small GTPase regulators and effectors, which duplicates their active sites and their membrane-binding elements (see for example, Arf effectors, reviewed in ref. [53]). However, whether dimerization fulfils specific functions remains unclear. Structural analysis of ArfGEF dimers provided some insights into this question. Yeast GEA2 is representative of a rigid, essentially symmetrical dimer, such that the predicted membrane-binding helices are located at fixed

positions where they can bind simultaneously to the membrane [27] (Fig. 4C). In that case, dimerization may increase both the number of active sites and the residence time of the ArfGEF on the membrane, hence, the probability that it encounters an Arf GTPase substrate over a large area. In contrast, cytohesins dimerize through a central coiled-coil to which the Sec7 and PH domains are loosely connected, leading to a flexible and asymmetrical dimer observed by SAXS in solution [56]. Reconstitution of cytohesins and myristoylated Arf in liposomes showed that cytohesins are also asymmetrical on membranes, where they bind one monomer at a time, hence, activate one Arf GTPase at a time [56] (Fig. 4D). It is thus possible that membrane-bound cytohesins dimers have one monomer in the active conformation at the membrane and the other in the autoinhibited conformation in solution (Fig. 4D). The dimeric organization of cytohesins may play a role in the way they “navigate” on the membrane in search of Arf substrates, possibly through “drifting” or “walking” rather than by dissociation/rebinding. Future studies are now needed to further investigate the modalities of symmetrical or asymmetrical binding of dimeric GEFs to membranes, and the extent to which they contribute to defining the size and Arf-GTP density of the Arf signalling zone.

Organizing signalling nanodomains through bidirectional signalling: lipid segregation and protein nanoclustering

Bidirectional signalling between lipids and proteins at the periphery of membranes is currently attracting huge interest in small GTPase biology. Understanding its molecular and structural inner workings experimentally has remained difficult and still relies largely on MD simulation-based predictions. With that caveat in mind, two major concepts are currently emerging: proteins bound at the surface of membranes can signal to the membrane through lipid segregation (reviewed in ref. [57]) or membrane deformation (reviewed in ref. [58]), and conversely, specific lipids can drive protein nanoclustering, as highlighted for K-Ras (reviewed in ref. [59]) and Rac1 [59,60]. We refer to such domains undergoing bidirectional signalling as “signalling nanodomains”. In the case of the ArfGEFs BRAG2, MD simulations predicted that multiple, short-lived interactions with PIP2 lipids would result in PIP2 clustering near BRAG2 and Arf through repeated dissociation and recapture [25]. It can be speculated that PIP2 clustering could then fuel a positive feedback effect by recruiting more ArfGEFs to generate more Arf-GTP molecules. Likewise, it could implement a feedforward effect by attracting downstream effectors and regulators with avidity for PIP2, such as

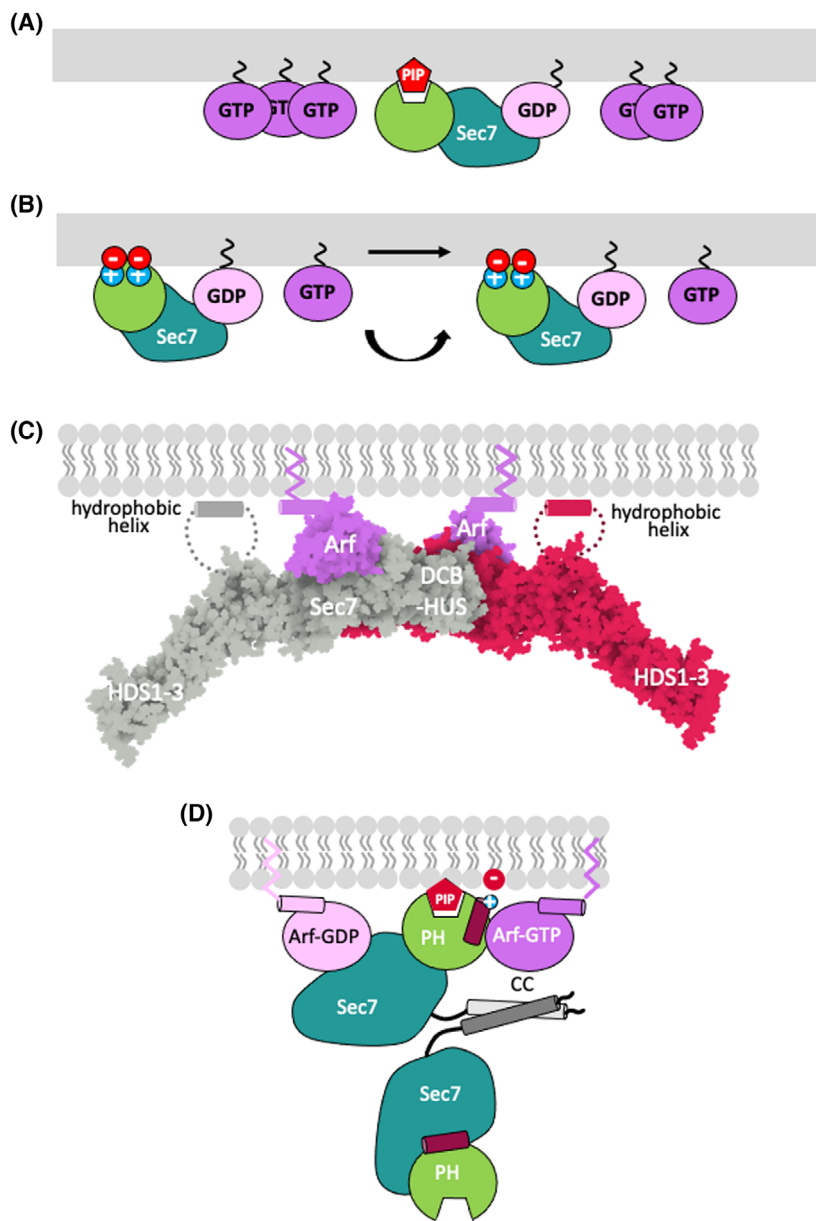


Fig. 4. Supramolecular organization of GEFs on the membrane. (A) Strong interaction of a GEF with a membrane, for instance through specific recognition of individual phosphoinositides (PIP), increases its residence time, resulting in a high density of activated GTPases. The membrane-binding domain of the GEF is in green, the substrate, GDP-bound GTPases in light pink, the product, GTP-bound GTPases in dark pink. (B) Weak interactions, for instance through non-specific electrostatic interactions, allow the GEF to diffuse on the membrane (horizontal arrow), or to bind/dissociate from the membrane (curved arrow) to activate GTPases on a broader surface at a lower density. Colour-coding is as in Fig. 4A. (C) Symmetrical binding of the GEA2 dimer to membranes allows simultaneous activation of two Arf molecules. Cryo-EM structure of the GEA2/Arf1 complex is from PDB ID 7URO [27]. (D) Cytohesin dimers bind asymmetrically to the membrane, with one GEF-competent monomer at a time. Colour-coding is similar to Fig. 4A.

lipid-modifying enzymes. In this manner, signalling nanodomains could assemble where the density of relevant components is increased, whether by direct protein–protein interactions or by proximity effect through lipids (reviewed in ref. [61]), which constitute exciting challenges for future experimental studies.

A framework to analyse the regulation of small GTPase regulators with membranes: the example of DOCK RacGEFs

The above overview provides a framework to dissect how membranes shape the structure, regulation and

activity of regulators of small GTPases (Box 2). As an example, we used this framework to examine the contribution of membranes to the activation of Rac GTPases by their GEFs of the DOCK (dedicator of cytokinesis) family, using landmark cryo-EM structures of unbound and Rac-bound DOCK that have been published recently ([62,63], reviewed in ref. [64]) (Fig. 1B). The small GTPase Rac1 controls the remodelling of the actin cytoskeleton at the plasma membrane during a variety of cellular events, including cell migration. It is activated by multiple GEFs, of which DOCK family members are involved in physiological processes, such as development, autoimmunity, and bone homeostasis (reviewed in refs [65,66]). DOCK-A members (DOCK1/

Box 2. Questions to consider when analysing the regulation of a protein of interest by membranes*Binding*

- What are the membrane-binding elements?
- Do they have features to recognize specific phospholipids, anionic membranes, membranes with lipid packing defects, flat or curved membranes?
- What are the kinetics of membrane recruitment and dissociation?

Regulation

- Are membrane-binding sites available or are they inhibited by intramolecular interactions?
- If the protein has more than one membrane-binding elements, are they aligned for simultaneous interaction with the membrane?
- If yes, are there additional domains apposed to the membrane, which could assist in membrane-binding?
- Are there hinge regions or flexible elements to support conformational changes that could bring additional domains near the membrane?

Activity

- Does the interaction with membranes modulate the protein activity: autoinhibition release, feedback loops?
- Does it modulate its specificity by 3D coincidence detection?
- Do membranes affect the symmetry of dimeric or multimeric proteins and does this affect their activity?
- Do regulators function by modulating the protein–membrane interface, for example by establishing additional membrane anchors?

Supramolecular effects

- Does the protein cluster in membrane nanodomains?
- Does it cluster specific lipids?

180, DOCK2 and DOCK5) form heterotetramers with ELMO (Engulfment and Cell Motility) proteins, which regulate its activity. Both Rac1 and DOCK interact directly with negatively charged membranes: Rac1 through its polybasic C-terminus and its attached geranylgeranyl lipid [67], and DOCK proteins by binding PIP3 through its DHR1 domain [68].

To examine the interaction of DOCK-A proteins with membranes and the role of membranes in their regulation, we used a wealth of structural information that has been garnered over the last decade, including cryo-EM structures of full-length autoinhibited DOCK2-ELMO, of Rac1-bound DOCK2-ELMO [62] and DOCK5-ELMO [63]. In addition, we adopted crystal structures of smaller constructs or complexes: full-length lipidated Rac1 in complex with RhoGDI [67], the catalytic (DHR2) domain of DOCK2 bound to Rac1 [69], the phosphoinositide-binding domain (DHR1) of DOCK1 [68], the PH domain of ELMO1 [70], and the N-terminus of ELMO bound to RhoG-GTP [62] and to an intracellular peptide from BAI1 [71]. The PDB IDs of the structures used in this analysis are listed in Table 1.

Active ELMO/DOCK/Rac1 complexes have six potential membrane-targeting elements

The ELMO-DOCK2 and ELMO-DOCK5 complexes bound to Rac1 have a similar organization ([62,63], reviewed in ref. [64]) (Fig. 5A). Both forms elongated symmetrical heterotetramers, in which the catalytic/dimerization DHR2 domains are located in the centre and the membrane-binding DHR1 domains in the middle. One ELMO protein interacts with the DHR1 and DHR2 domains in each monomer, extending the structure on both ends. The dimeric Rac1/DOCK/ELMO complex presents two lipid-binding elements on each half of the complex, all located on the same side of the complex: the phosphoinositide-binding pocket of the DHR1 domain [68] and the prenylated, polybasic C-terminus of Rac1 (Rac1^{Ct}, ¹⁷⁸CPPPVKRKRKCLLL¹⁹²), which has been described in the structure of the Rac1/RhoGDI complex [67]. Thus, an approximate orientation of the complex with respect to the membrane can be deduced, which can be used to search for extra membrane-binding elements. Interestingly, ELMO

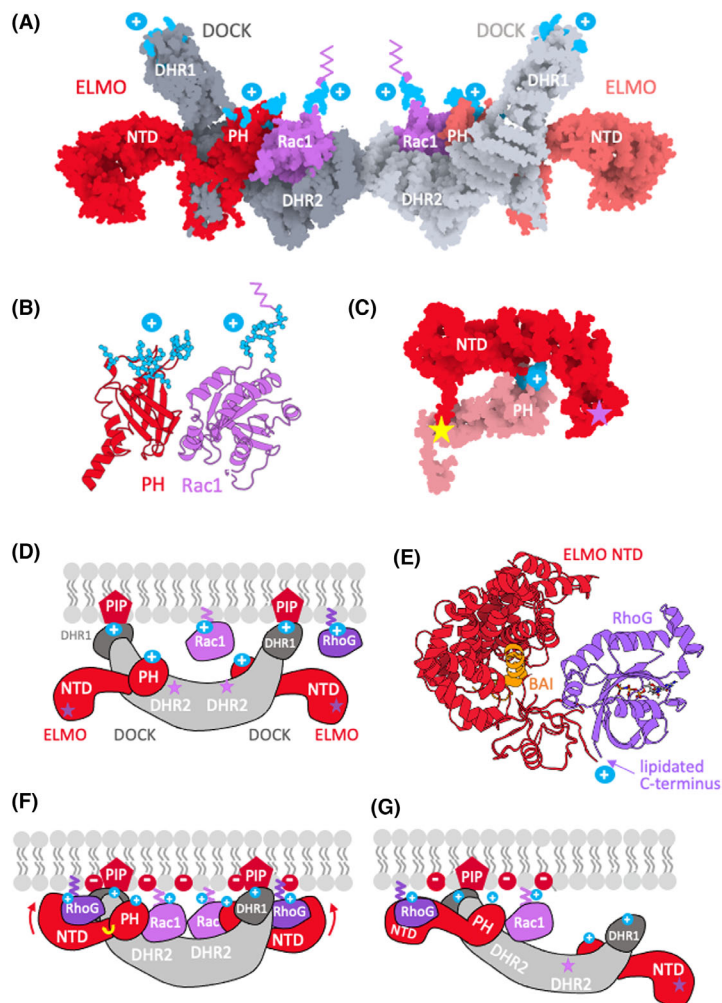


Fig. 5. DOCK/ELMO on membranes: a structural perspective. (A) The active ELMO/DOCK heterotetramer bound to two Rac1 GTPases has six potential membrane-binding elements in each heterodimer: the DHR1 domain of DOCK, the PH domain of ELMO and the lipidated C-terminus of Rac1. Positively charged residues are in blue. The structure is from PDB ID [6TGC](#) [62]. (B) Polybasic loops in the PH domain of ELMO and the lipidated polybasic C-terminus of Rac1 are close to each other in the structure of the DOCK/ELMO/RAC1 complex, indicating that they can associate simultaneously to the membrane. Positively charged residues are shown in blue. PDB IDs: ELMO PH: [2VSZ](#) [70], lipidated full-length Rac1 from the Rac1/RhoGDI complex: [1HH4](#) [67]. (C) ELMO inhibitory conformation observed in the closed DOCK/ELMO complex. The NTD domain (in bright red) blocks access to the Rac1-binding site in the DHR2 domain of the DOCK subunit (purple star). In this conformation, the NTD also blocks the proposed membrane-binding site (in cyan) of its own PH domain located in its C-terminus domain (in salmon). The position of the hinge between the NTD and PH domain-containing C-terminal domain is shown by a yellow star. PDB ID: [6TGB](#) [62]. (D) Association of the open conformation of DOCK/ELMO complex to phosphoinositides by its two DHR1 domains does not allow the DHR2 GEF domains (pink star) to reach membrane-anchored Rac1 and nor the NTD of ELMO (violet star) to reach membrane-anchored RhoG. (E) RhoG-GTP and the BAI1 cytosolic peptide have different binding sites in the NTD of ELMO. The orientation of the NTD of ELMO is as in Fig. 5A. The cartoon is a superposition of RhoG-GTP/ELMO^{SH3} and BAI1/ELMO^{NTD} onto the NTD domain of ELMO in the cryo-EM structure of active DOCK/ELMO/Rac1. RhoG is in pink, BAI1 in orange, ELMO^{NTD} in red. The location of the C-terminus of RhoG is indicated. PDB IDs: Rho-GTP/ELMO: [6UKA](#) [62]; BAI1/ELMO: [6IDX](#) [71]; DOCK/ELMO/Rac1: [6TGC](#) [62]. (F) A model for the conversion of DOCK/ELMO into a GEF-competent conformation by membrane-anchored RhoG-GTP. In this model, the NTD of ELMO captures membrane-anchored RhoG through rotation around the NTD/PH hinge (yellow arrow), in a manner that it pushes the C2-DHR1 domains to align with the other membrane-binding elements. This brings the DHR2 domain close to membrane-anchored Rac1, allowing GDP/GTP exchange. The fully activated complex is symmetrical, with one RhoG-GTP bound to each ELMO subunit. (G) Model for asymmetrical activation of DOCK/ELMO by a single RhoG-GTP. Alternatively, the DOCK/ELMO dimer binds a single RhoG-GTP, asymmetrically aligning only half its membrane-binding elements with minimal conformational changes. The DHR2 active site of the second DOCK monomer (pink star) and the RhoG-binding site of the second ELMO molecule (Purple star) remain unbound.

displays a PH domain (ELMO^{PH}), but this domain lacks the canonical phosphoinositide-binding pocket and a role as a membrane-binding domain was originally ruled out [70]. However, in the DOCK/ELMO/Rac1 complexes, ELMO^{PH} is located immediately next to Rac1^{Ct}, where it exposes multiple positively charged residues that are typical of non-specific electrostatic interactions with anionic membranes (R563, K564, R568, R569, R570, K573, K628, K635, K659) (Fig. 5B). Accordingly, it can be predicted with confidence that in the context of the active ELMO/DOCK/Rac1 structures, ELMO^{PH} is a third membrane-binding element, and that ELMO^{PH} and Rac1^{Ct} can interact with anionic membranes simultaneously (Fig. 5B). Because of their dimeric organizations, the DOCK/ELMO/Rac1 complexes thus feature six potential membrane-targeting elements, all located on the same face so that they should contribute concurrently to the interaction with the membrane (Fig. 5A).

Inhibition of the DHR2 active site by ELMO blocks the lipid-binding surface of its own PH domain

Unbound and Rac1-bound DOCK2/ELMO complexes differ essentially in the conformation of the ELMO subunit [62]. In unbound DOCK2/ELMO, the entire N-terminus of ELMO (NTD) swings into the active site of the DHR2 domain to block access to Rac1 (Fig. 5C). Remarkably, in this conformation, the NTD also masks the predicted lipid-binding surface of the PH domain of ELMO (Fig. 5C). Thus, the structures suggest that the PH domain has a dual contribution to the regulation of DOCK by ELMO: it contributes to inhibition by stabilizing the inhibitory conformation of the NTD, which also results in autoinhibition of its membrane-binding surface; and it participates in activation through its membrane-binding capability. Of note, the lipid-binding pocket of the DHR1 domains is readily available in both inhibited and active configurations, suggesting that unlike ELMO^{PH}, its binding to membranes does not depend on the activation state of the DOCK/ELMO complex and may thus be constitutive.

How to resolve the misalignment of membrane-binding elements in the DOCK/ELMO/Rac1 complexes?

A surprising discovery from the cryo-EM structures of Rac-bound DOCK/ELMO complexes is that the DHR1 domains and Rac1^{Ct} dimers are located at different distances from the membrane ([62,63], reviewed in ref. [64]), which we extend here to include ELMO^{PH}. The DHR1 domains tower the DHR2

domains by about 40 Å, such that symmetrical binding of the two DHR1 domains to phospholipids would position the DHR2 active sites away from the membrane by the same distance. Importantly, Rac1 must be delivered to negatively charged lipids through its polybasic C-terminus prior to its activation by its GEFs [72]. The membrane anchors of K-Ras and Rac1 were recently shown to form multiple electrostatic interactions between their positively charged lysine and arginine residues and specific negatively charged lipids, leading to lipid and protein nanoclustering [59,73]. Through these interactions, the polybasic C-terminus of Rac1 is thus expected to position the GTPase core close to the membrane, such that the DHR2 domain would be unable to reach it (Fig. 5D). Hence, symmetrical binding of the ELMO/DOCK complexes through their DHR1 domains alone results, *de facto*, in inhibition of the GEF activity.

The question thus arises of the conformational changes in the DOCK/ELMO complexes that would be needed to align all membrane-binding elements in a GEF-competent configuration. One possibility could be that the DHR1 domains, together with their associated C2 domain, adopt another position with respect to the DHR2 domain and ELMO. Alternatively, ELMO/DOCK could bind asymmetrically to membranes without major conformational change, using a single ELMO^{PH}, Rac1^{Ct} and DHR1 domain. In this configuration, one DHR2 domain would be brought close to the membrane where it could activate Rac1, while the other DHR2 domain would be displaced further away. Whether such asymmetrical activation of DOCK is compatible with the observation that abrogating dimerization through DHR2 reduces activation of Rac1 [74] remains to be investigated. A third possibility could be that the complex has a preference for positively curved membranes, hence allowing all membrane-binding elements to be reached without conformational change. We discuss below why this seems unlikely in the context of the activation of ELMO by membrane-attached RhoG.

Membrane-proximal regulators may contribute to align membrane-binding elements to activate ELMO/DOCK

The small GTPase RhoG, whose GTP-bound form is anchored to the membrane by its lipidated C-terminus similarly to Rac1, and the transmembrane adhesion G Protein-Coupled Receptor BAI1, have been proposed to modulate the GEF activity of ELMO/DOCK by binding to ELMO, through mechanisms that have remained poorly understood. A plausible mechanism,

in the light of the cryo-EM structures, is that they contribute to alleviate the constraints imposed by membranes on the activity of the DOCK/ELMO complexes. Crystal structures of RhoG-GTP bound to the N-terminal domain of ELMO1 [62], and of a BAI1 peptide bound to the armadillo repeats of ELMO2 [71] show that the binding sites of both regulators are available only in the active, Rac1-bound ELMO/DOCK configuration (reviewed in ref. [64]) (Fig. 5E).

RhoG, as Rac1, has a polybasic membrane anchor that is predicted to interact with negatively charged lipids, hence to position the GTPase core close to the membrane. In a configuration where ELMO/DOCK is bound symmetrically to the membrane through both DHR1 domains, overlay of the RhoG-GTP/ELMO structure predicts that RhoG would be located at about the same distance from the membrane as Rac1, thus not in a position to interact with the membrane. In other words, membrane-anchored RhoG and Rac1 cannot bind to the complex in that configuration (Fig. 5D). This also makes it unlikely that the active DOCK/ELMO complex binds to positively curved membranes, as the membrane would thus need to be doubly curved near both Rac1 and RhoG to accommodate them. Moreover, the position of the membrane anchor of RhoG would be pointing opposite to the membrane (Fig. 5E), confirming that it cannot bind to the DOCK/ELMO complex in that configuration. Thus, to capture membrane-anchored RhoG-GTP, ELMO should have a conformation different from that seen in the cryo-EM structures. ELMO has a flexible hinge connecting its N-terminus to its PH domain, which supports its large conformational change [62]. This hinge would be well-suited to allow its N-terminal domain to move closer enough to the membrane to bind to membrane-anchored RhoG-GTP. RhoG-GTP-bound ELMO may then exert a “push and pull” effect to relocate the C2-DHR1 domains at the same distance from the membrane as the other membrane-binding elements (Fig. 5F). This would probably require that two RhoG-GTP molecules are simultaneously bound to the ELMO/DOCK complex, as both DHR1 must be displaced to “flatten” the structure of the complex.

Alternatively, binding of a single membrane-anchored RhoG-GTP molecule to ELMO would make the complex asymmetrical, thus enabling its asymmetrical association to the membrane through simultaneous binding of a single RhoG^{Ct}, Rac1^{Ct}, ELMO^{PH}, and DOCK^{DHR1} (Fig. 5G). In this configuration, RhoG-GTP would stimulate the GEF activity by securing an orientation of ELMO/DOCK with respect to the membrane that optimizes the position of one DHR2 domain to activate one membrane-anchored

Rac1. RhoG-GTP/ELMO/DOCK would thus hold one GEF-competent DHR2 domain at a time, possibly leaving the second ELMO/DOCK monomer in the autoinhibited conformation.

The intracellular BAI1 peptide also binds to the N-terminus of ELMO, although at a different location that is also available only in active ELMO [71] (Fig. 5E). It is currently unknown if the distance between the intracellular BAI1 peptide and the membrane would match the distance between ELMO and the membrane as seen in the cryo-EM structures, or if ELMO must undergo a conformational change to bind the peptide, but the possibility of a mechanism similar to that proposed for RhoG can be envisioned. Another variation of such mechanism could be used by phosphorylated receptor-associated Crk, an adaptor protein that binds to the C-terminus of DOCK and activates it (reviewed in ref. [75]). Whether for RhoG, BAI1 or Crk, these mechanisms are of course speculative at this stage, and future efforts are needed to test them and resolve these mysteries.

Concluding remarks: “Think membrane”!

Integrated structural biology has been instrumental in uncovering how membranes modulate small GTPase systems at the molecular level, a knowledge that would not have been attainable otherwise. It revealed that membranes play essential roles in determining the recruitment, the efficiency, the specificity and the supra-molecular organization in time and space of small GTPases systems. Membranes should, in fact, be considered in the same terms as any other protein component of small GTPase signalling networks. General principles governing bidirectional signalling between membranes and small GTPase systems can now be established, some firmly and others still in need of experimental investigations (Box 2). With the example of DOCK/ELMO/Rac1, we illustrated how such principles can be harnessed, generating testable hypothesis about the fully activated configuration of DOCK/ELMO on membranes. We propose that these structure-based principles should be considered when interrogating the mechanisms whereby small GTPase systems ensure spatial and temporal control of cellular signalling on membranes. We anticipate that they will also be valuable for a better understanding of small GTPase failures in diseases, and to assist in drug discovery. They underline, however, the need for the development of, and broader access to, integrated structural biology approaches able to deliver high-resolution information on protein–membrane interfaces.

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