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REVIEW

In vitro reconstitution of small GTPase regulation

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Small GTPases play essential roles in the organization of eukaryotic cells. In recent years, it has become clear that their intracellular functions result from intricate biochemical networks of the GTPase and their regulators that dynamically bind to a membrane surface. Due to the inherent complexities of their interactions, however, revealing the underlying mechanisms of action is often difficult to achieve from *in vivo* studies. This review summarizes *in vitro* reconstitution approaches developed to obtain a better mechanistic understanding of how small GTPase activities are regulated in space and time.

Keywords: biochemical networks; *in vitro* reconstitution; lipid-modified proteins; small GTPases

What can we learn from *in vitro* reconstitution?

The living cell is a highly complicated self-organized system where millions of molecules dynamically interact. It contains membrane-bound and membrane-less compartments, which constantly emerge, disappear and change shape and their biochemical identities [1–4]. Small GTPases play an essential role in this intercellular organization as they control gene expression, cell differentiation, cell motility, membrane traffic and compartmentalization [1,5–9]. These small proteins have a highly conserved fold and alternate between inactive GDP-bound and active GTP-bound conformations [10].

Depending on the bound nucleotide, small GTPases engage in interactions with different binding partners in the cell. Upon GTP binding, these proteins often bind to the membrane, where they interact with effector proteins. Membrane binding of small GTPases can be facilitated by lipid anchors. For example, proteins of the Arf subfamily undergo a large conformational change to expose an N-terminal amphipathic helix that inserts into membranes [11,12]. In the case of Arf GTPases, this helix is further modified by a myristate anchor. Rab and Rho subfamilies are prenylated at the end of a hypervariable C-terminal region, which allows for membrane interaction. In the GDP-bound state, Rabs and Rhos engage in a complex with a guanosine nucleotide dissociation inhibitor (GDI) keeping the lipidated protein soluble.

Abbreviations
BSA, bovine serum albumin; GAPs, GTPase-activating proteins; GDI, guanosine nucleotide dissociation inhibitor; GEFs, guanine nucleotide exchange factors; GGTase-II, geranylgeranyltransferase type II; GUV, giant unilamellar vesicles; HDX-MS, hydrogen deuterium exchange mass spectrometry; NMT-1, N-myristoyl-transferase; RBD, Ras-binding proteins; REP, Rab Escort protein; Sf9, *Spodoptera frugiperda*; SOS, Son of sevenless; TIRF, total internal reflection fluorescence; UBD, Ubiquitin binding domain; WRC, Wave Regulatory Complex.

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Specific enzymes, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Fig. 1A), catalyse the transition between the two different nucleotide states and are subject to complex regulation *via* protein–protein and membrane interactions. Together, these components are the basis for more complex biochemical networks that control a diverse array of cellular processes. The specific players, as well as the molecular details of their interactions, have often been identified [5,13–16] (see also references [17,18] for excellent summaries). In addition, we have obtained structural information about proteins and their complexes [19–21] and can now use machine-learning methods to predict hypothetical structures [22,23]. However, this wealth of information is often insufficient to explain where and when small GTPases are activated in the cell, how their biochemical signals are recognized and transmitted along signalling pathways and how they are efficiently switched off.

In contrast to the living cell, biochemical studies using purified components can be highly reductionist. For example, many experiments are performed using GTPases that lack their lipid anchors, without GDIs, in the absence of phospholipid membranes and in chemical equilibrium. These experiments were instrumental in identifying the biochemical specificities of GTPase regulators and characterizing the catalytic activities with their cognate GTPases [24–26]. They have also helped to pave the way to understanding how pathogens rewire signalling pathways during infection [27–30]. However, as these assays did not reflect the inherent biochemical complexity of the living cell, it is not straightforward to use their results to explain the dynamic activity patterns of small GTPases found *in vivo*. Therefore, a mechanistic understanding of how they function and operate in the living cell often seems out of reach.

Rebuilding individual cellular functions one by one using a manageable subset of components in *in vitro* assays offers the chance for a more mechanistic understanding of biochemical modules [31]. Starting from a minimal system of only a single protein species, it is possible to systematically increase the complexity of the reconstituted system by including additional biochemical players or by introducing additional experimental boundary conditions such as geometric constraints [31–33]. Furthermore, *in vitro* reconstitution experiments can help to identify the minimal number of components required for specific cellular functions, to elucidate the role of an individual molecular player and to learn more about additional unknown functions or interaction partners. Using a sufficiently complex set of components, this bottom-up synthetic biology approach can also recreate emergent, out-of-equilibrium properties [34–37]. In addition, as *in vitro* reconstitution studies are often more suitable for meticulous quantitative analyses and mathematical modelling, they can provide a detailed mechanistic understanding that is usually challenging to obtain using other approaches.

Recreating physiological conditions to study small GTPase regulation *in vitro* was often hindered by the experimental difficulties associated with the nature of small GTPase signaling. First, the post-translational modifications with lipid anchors often make proteins insoluble in aqueous buffers lacking detergents and complicate the purification of their native forms. Second, GTPase regulators are large, multidomain proteins that frequently contain non-structured regions and are, therefore, difficult to obtain from bacterial expression systems. Third, one has to use appropriate biomimetic membrane systems that mimic the properties of different cellular membranes, including membrane curvature, lipid packing and composition, without sacrificing the ability for a quantitative biochemical and biophysical analysis. With advances in recombinant protein purification [38,39], the possibility to prepare different classes of biomimetic lipid membranes [40], new microscopy technologies [41–43] and computational analyses [22], it is now possible to reconstitute and study more and more complicated biochemical systems *in vitro*.

In this review, we will summarize and discuss *in vitro* studies on the functions and regulation of small GTPases. We will describe the experimental challenges in preparing lipidated Arf, Rab, Ras and Rho GTPases, how to mimic their interactions with a membrane surface and how fluorescence microscopy offers a means to study the out-of-equilibrium properties of small GTPases signalling systems.

Why and how to prepare lipid-modified proteins

The small GTPase superfamily is divided into five main families: Ras, Rho, Ran, Rab and Arf GTPases. Apart from Ran, which organizes nucleocytoplasmic transport during interphase and mitotic spindle assembly in mitosis, small GTPases exert their function on the surface of phospholipid membranes. The membrane surface plays a critical role in biochemical signalling reactions [44]. It concentrates and pre-orients interaction partners bound to the membrane surface and actively participates in the regulation of small GTPases by influencing the activity of GEFs and GAPs. In addition, it provides a template for the large-scale organization of GTPase effectors, such as

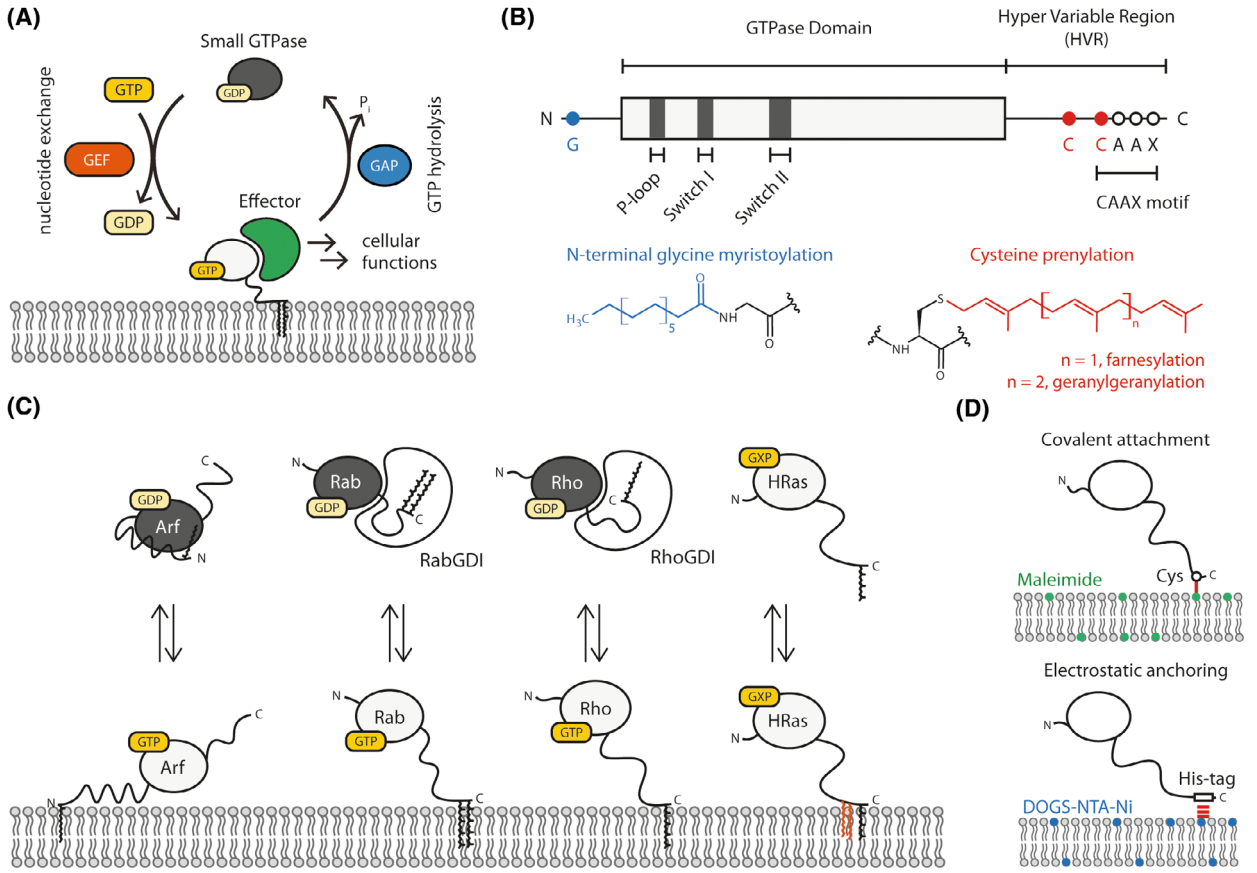

Fig. 1. (A) Small GTPases are molecular switches that typically transition between a membrane-bound GTP-bound ON state and a soluble GDP-bound OFF state. GEFs and GAPs catalyse nucleotide exchange and hydrolysis, respectively. The active, GTP-bound GTPase recruits effector proteins to the membrane to perform cellular functions. (B) GTPases are characterized by a central GTPase domain containing conserved structural motifs required for their functions (P-loop, switch I and switch II). Small GTPases differ in their lipid modifications: Arf and Arf-like GTPases have an N-terminal myristoyl anchor attached to an N-terminal amphipathic helix. Rab, rho and Ras have prenyl anchors at C-terminal cysteines. (C) GTPases differ in how their membrane binding is regulated. Arf GTPases do not require a GDI and also weakly interact with the membrane in their GDP-bound state. Rho and Rab GTPases rely on a soluble GDI that shields the hydrophobic lipid moieties. Fully lipid-modified Ras GTPases are permanently attached to the membrane, while de- and repalmitoylation cycles driven by transferases and thioesterases allow for their redistribution in the cell. (D) Membrane binding of soluble proteins can be mimicked by either attaching the protein to maleimide-lipids *via* a terminal Cystein-residue or a his-tag and membranes with Ni^{2+}-NTA lipids.

the oligomerization of coat proteins that deform the membrane to generate vesicles [45] or the assembly of a dynamic actin network pushing the membrane forward [46]. For the *in vitro* reconstitution of small GTPase systems one therefore not only needs an appropriate membrane substrate, but also small GTPases able to bind the membrane.

With Sar1 being the exception, membrane-binding small GTPases undergo post-translational protein lipidation facilitating their interaction with membranes (Fig. 1B). Ras and Rho proteins possess a C-terminal CAAX motif, which is post-translationally prenylated with a farnesyl group [47]. Ras GTPases are further modified with one or two adjacent S-palmitoyl groups, which determine their intracellular localization [48]. Rab GTPases usually undergo double geranylgeranylation within C-terminal CC or CXC motifs [49]. Finally, Arf and Arf-like (Arl) GTPases are modified with a myristoyl fatty acid attached to the N-terminal glycine of their N-terminal amphipathic helix [50] (Fig. 1C). Preparing and working with lipid-modified proteins has remained challenging and a major bottleneck for *in vitro* assays. Early biochemical studies, therefore, often used non-modified, soluble small GTPases in combination with the catalytic domains of their regulators to perform experiments in solution. Soluble proteins have also been beneficial for performing systematic characterizations of GEF families [24]

and particularly for structural studies on complexes of the GTPase, its effectors and regulators [19,25,26,51]. However, we now know that the membrane surface provides more than only a two-dimensional platform for the interaction of small GTPases with their regulators. As effector proteins can also change the biochemical and biophysical properties of phospholipids, the membrane plays an important, active role in biochemical networks with self-organized emergent properties [44,52–55]. For a more complete understanding of small GTPase regulation, it is therefore essential to perform experimental assays that include native, lipid-modified GTPases, full-length GEFs and GAPs and a close mimic of the biological membrane.

Originally, lipid-modified proteins were prepared directly from mammalian tissues [56,57]. While this approach commonly yields 100% lipid-modified proteins, these protocols are often labour-intensive and do not necessarily exclude the presence of other GTPases [58]. Since then, powerful new methods have been developed to obtain recombinant proteins from heterologous expression systems or by post-translational modifications performed using purified proteins *in vitro*. The optimal purification strategy depends on the protein of interest.

Myristoylated Arf1 and Arf6 have been more extensively studied than other Arf-GTPases. The myristoylated protein can be obtained from bacterial expression systems by co-expressing the protein of interest with human or yeast N-myristoyl-transferase (NMT-1) either from an additional plasmid [58,59] or using a single bicistronic expression vector [60]. To solubilize the highly hydrophobic fatty acid, the myristate is often first allowed to bind to fatty acid-free bovine serum albumin (BSA) before a highly concentrated solution of this complex is added to the growth media [61]. Other protocols directly add the solid myristate salt [62,63]. After cell lysis, the protein is present in the clarified bacterial lysate as a mixture of the modified and non-modified forms. Myristoylated Arf can be isolated *via* ammonium sulfate precipitations and ion exchange chromatography [58,59,63]. Alternatively, the protein can be purified from the soluble fraction using hydrophobic interaction chromatography, taking advantage of the greater hydrophobicity of the myristoylated protein [62]. While this protocol can provide highly pure myristoylated Arfs, the yield from this rather complex purification protocol is relatively low [64]. This might be due to the poor solubility of myristate in aqueous solutions, plasmids loss during protein expression and the loss of the modified protein during cell fractionization and the NH_4SO_4 precipitation steps.

Myristoylation of proteins can also be achieved using the purified non-modified small GTPase, NMT-1 and myristate-CoA *in vitro* [65]. As demonstrated by Padovani *et al.*, *in vitro* myristoylation can yield high amounts of lipid-modified protein [64]. In this important study, Arf6 was purified with a C-terminal His-tag to keep the N-terminal glycine available for the myristate anchor. In case the C-terminal modification is found to alter protein interactions [66], native, non-modified Arf6 can be obtained using an N-terminal 6x His-SUMO tag, which is entirely removed by the protease Ulp1 [67]. If necessary, myristoylated Arf6 can be further enriched by ammonium sulfate precipitation. *In vitro* myristoylation has also been applied to obtain modified Arl3 [68] and Arl5B [69], as well as the myristoylated SRC family kinases [70].

Only non-modified, soluble Rho and Rab GTPases can be obtained from bacterial expression systems. While these proteins can still bind and hydrolyse GTP, only their prenylated forms can bind to membranes and form a complex with RabGDI. Post-translationally modified Rab and Rho GTPases have been obtained *via* expression in *Spodoptera frugiperda* (Sf9) insect cells [71–75]. While Hi5 cells from *Trichoplusia ni* offer higher protein yield, we found that prenylation efficiency was low. Yeast [76,77] and human cell culture [78] have been used to prepare modified Rac and Rho GTPases. From all these expression systems, prenylated proteins are obtained from the membrane fraction of the lysed cells *via* detergent-mediated membrane extraction. A complex of the GTPase with its purified GDI (expressed either in insect cells or in bacteria) can be formed during dialysis into a detergent-free buffer, the complex isolated after size exclusion chromatography and stored at −70 to −80 °C. Recently, this protocol was modified and the GTPase and its GDI were produced by co-infection and isolated directly as a GTPase-GDI complex *via* affinity purification and size exclusion chromatography [79].

Lipid-modified Rho and Rab GTPases can also be obtained *via in vitro* prenylation using the respective enzymes and substrates. Here, mammalian or yeast geranylgeranyltransferase type II (GGTase-II) and Rab Escort protein (REP) are used to catalyse the transfer of two geranylgeranyl groups from geranylgeranyl pyrophosphate onto the C-terminal cysteine residues of a Rab GTPase, which yields double prenylated Rab in complex with REP [80]. In case the reaction is performed in the presence of stoichiometric amounts of a GDI, the GDI:GTPase complex can be isolated *via* size exclusion chromatography [81]. Similarly,

monoprenylated Rab and Rho GTPases can be obtained using geranylgeranyltransferase type I [82,83].

Strategies to mimic membrane binding of lipidated proteins

The preparation and handling of lipidated GTPases represent a bottleneck for many *in vitro* reconstitution experiments. Two different experimental strategies have been demonstrated to attach GTPases to membranes that circumvent lipid-modified proteins (Fig. 1D): First, proteins can be covalently attached to lipid membranes using thiol-maleimide crosslinking [52,84,85]. In this strategy, the small GTPase Ras containing a C-terminal cysteine was permanently coupled to membranes containing defined amounts of lipids with a maleimide headgroup. By tethering Ras-GDP to small unilamellar vesicles, it was found that the activity of the catalytic unit of the GEF Son of sevenless (SOS) on membrane-bound Ras was about 500 times faster than in solution, emphasizing the ability of the membrane surface to enhance biochemical reactions [52].

In an alternative and more popular approach, polyhistidine tagged proteins are attached to membrane surfaces using lipids with Ni^{2+}-chelating headgroups. For example, His-tagged RhoA and vesicles containing Ni^{2+}-NTA-lipids have been used to study the nucleotide exchange activity of RhoGEFs that coordinate the activity of Rho GTPases with heterotrimeric G-proteins. These studies revealed that active RhoA-His_6-GTPγS preexisting on the membrane could enhance the activity of different RhoGEFs with PRG DH-PH domains towards RhoA- His_6-GDP [86]. Similarly, it was found that Rab5-His_{10}-GTP attached to Ni^{2+}-NTA-membranes can increase the activity of Rabex5:Rabaptin5 towards the prenylated Rab5-GDP in complex with its GDI [73]. Other Rab GEFs that were characterized using His-tagged Rab GTPases on Ni^{2+}-NTA-membranes are Mon1:Ccz1 with its substrate Ypt7-His_6 [87] and TRAPPII with Ypt1-His_7 and Ypt32-His_7 [81]. Coyle *et al.* used His-tagged Ras immobilized to solid Ni^{2+}-NTA surfaces to study the out-of-equilibrium properties of protein systems composed of the GTPase, its GEF SOS and different GAPs [88]. More recently, His-tagged Arf1-His_8-GTP and Rac-His_8-GTP on supported membranes containing Ni^{2+}-lipids were used to demonstrate their ability to cooperatively activate the actin nucleation ability of the Wave Regulatory Complex (WRC) [89]. In 2017, Peurois *et al.* systematically characterized the activation of membrane-bound His-tagged Arf, Rab, Rac and Rho GTPases by their respective GEFs [90]. Comparing the nucleotide exchange activity of the GEF Arno towards myristoylated Arf1 and Arf6 and their His-tagged versions showed that the kinetic efficiencies were in a similar range. At the same time, Arno showed much lower activity on the soluble versions of these proteins. Similar observations were made for RhoG-His_6 and Rac1-His_6 with the GEF Trio, while, interestingly, DrrA-catalysed nucleotide exchange on Rab1-His_6 and Rab35-His_6 was not enhanced by protein binding. It was suggested that the bacterial GEF DrrA binds too strongly to PI4P present in the membrane, such that it can activate only a small pool of the membrane-localized GTPases.

Together, these studies showed that the artificial recruitment of small GTPases is a powerful strategy to mimic membrane binding of lipidated proteins. This approach is particularly advantageous for precisely defining the density of membrane-bound proteins. As Ni^{2+}-chelating and maleimide headgroups are chemically orthogonal, it is, in principle, also possible to attach different combinations of proteins at different density ratios to the membrane. This strategy can allow exploring the ability of protein systems to integrate different combinations of signalling inputs present on the membrane and to study the corresponding output response.

However, it is also important to be aware of this approach's limitations. First, the orientation and distance from the membrane might be different for a protein using a polyhistidine tag rather than their native membrane anchor. This issue is particularly relevant for Arf GTPases, which sit relatively close to the membrane surface and perform a significant conformational change during nucleotide exchange and hydrolysis. A specific distance between the protein interaction site and membrane surface might affect not only their intrinsic GTPase activity but also their affinity towards their regulators and effectors [91]. Second, permanent attachment of the protein is incompatible with a continuous exchange or GDI-facilitated removal of the protein from the membrane. The absence of constant turnover might affect protein–protein interactions on the membrane surface and therefore affect the emergent spatiotemporal activity patterns of small GTPases [73,82].

GEFs and GAPs have auxiliary domains controlling their activities

Small GTPases need other proteins to accelerate nucleotide exchange and hydrolysis. While their GEFs and GAPs are often identified by their conserved catalytic cores, different regulatory enzymes vary greatly

in their auxiliary domains (Fig. 2) [51,92,93]. In the absence of upstream signalling inputs, GEFs are often autoinhibited, preventing promiscuous activation of their cognate GTPases in the cell [18,94,95]. Intra- and intermolecular interactions of the regulatory domains control the catalytic activity and define the

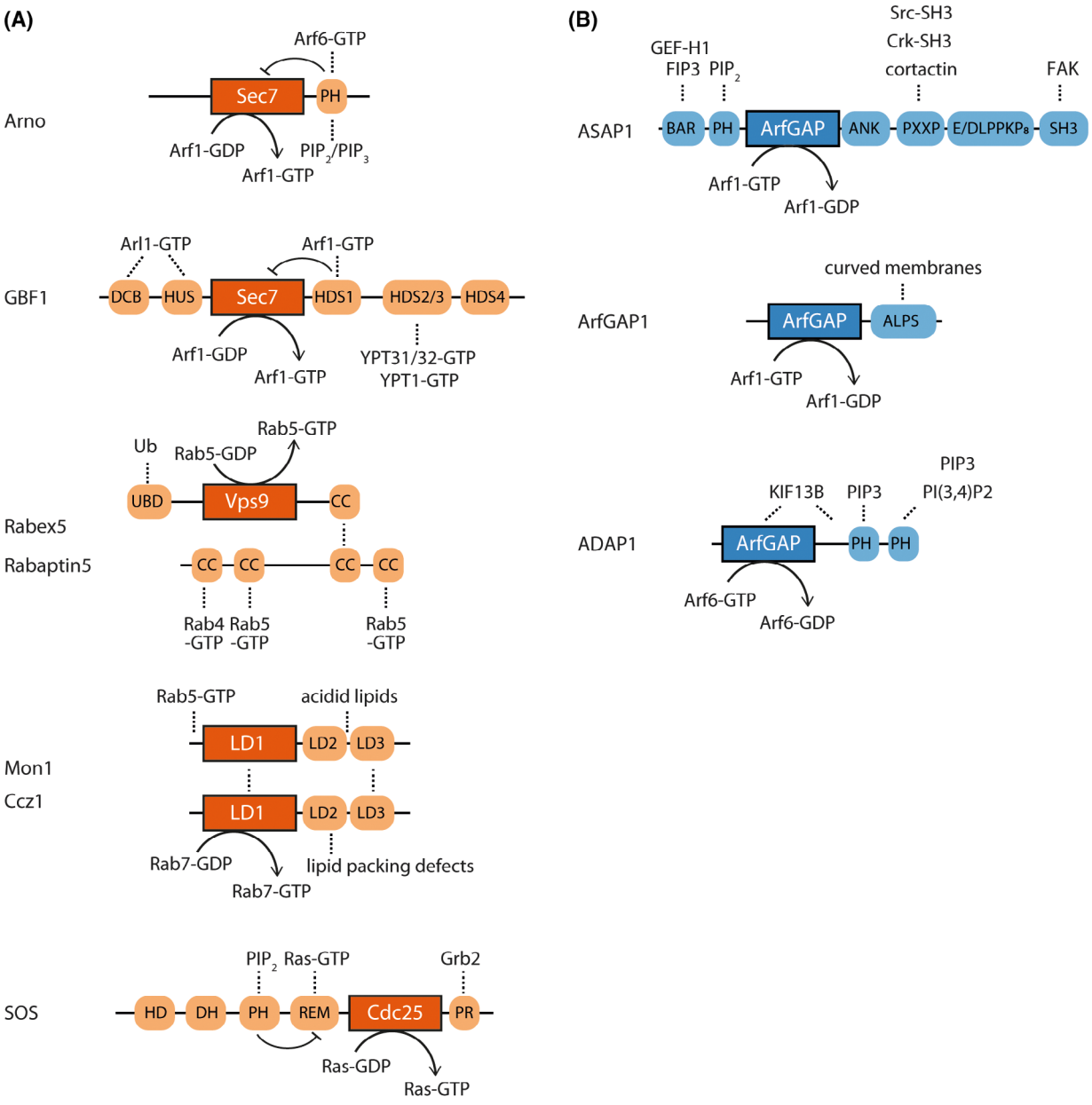

Fig. 2. Examples of how auxiliary domains of small GTPase regulators allow for multiple layers of regulation, which control their location in the cell and as a consequence the spatiotemporal activity patterns of GTPases. (A) for example, the Arf GEFs Arno and Sec7/GBF1 exist in an autoinhibited state due to intramolecular interactions between their catalytic Sec7 and regulatory domains. Similarly, the catalytic core of Rabex5 is highly active in isolation. Autoinhibition by the C-terminal coiled-coil of Rabex5 (CC) is partially relieved in a complex with Rabaptin5. The localization and activity of Mon1:Ccz1 is in part controlled by the interaction with Rab5-GTP and acidic lipids including phosphoinositides, in addition, an amphipathic helix can detect lipid packing defects. The Ras GEF SOS is regulated on multiple levels to respond to upstream signals detected by EGF receptors, phosphoinositides and active Ras-GTP. (B) the activities of Arf GAP domains are controlled *via* regulatory domains that bind to different phosphoinositides or proteins, thereby coupling the catalytic activity of GAPs to regulatory inputs in the cell. Due to their PH-domains, ASAP1 and ADAP1 respond to the presence of phosphoinositides in the membrane, while ArfGAP1 has an amphipathic lipid packing sensor that responds to high membrane curvature.

intracellular location of the GEF complex. Several layers of autoregulation can efficiently control where and when a small GTPase is activated in the cell [52,96]. The hierarchy of these interactions and possible cooperative or combinatorial effects often still need to be discovered.

In vitro experiments were often instrumental in understanding the molecular mechanisms of these interactions. For example, like all Arf GEFs, Arno has a catalytic Sec7 domain, which is autoinhibited in solution *via* an interaction with an amphipathic helix at the C terminus of its PH domain [97]. This inhibition is released *via* two independent interactions: first, the binding of the PH domain to phosphoinositides and second, its interaction with Arf6-GTP [98,99] and to a lesser degree Arf1-GTP on the membrane [100]. As Arf1-GTP and Arf6-GTP are the products of Arno's catalytic activity, this coupling of the catalytic domain with a regulatory domain may result in a positive feedback of GTPase activation and their functional coupling of GTPases in cascades [101–104]. The combination of autoinhibition and positive feedback due to product binding has also been found to control the activity of the Sec7-domain containing GEFs of the BIG/GBF family [105,106].

Several different layers of autoregulation have been identified for the Ras GEF SOS. Next to its catalytic CDC25 domain, SOS contains an allosteric site to bind to Ras-GTP, a PH domain to interact with PIP_2 and a proline-rich domain for interacting with the adaptor protein Grb2. An internal interaction between its N-terminal DH-PH and REM domains seems responsible for the autoinhibition of SOS, which is relieved upon binding to PIP_2 and Ras-GTP [107]. Autoregulatory mechanisms are also involved in the Rab5-specific GEF Rabex5:Rabaptin5. Here, the catalytic core of Rabex5 is a helical bundle-VPS9 domain tandem. Rabex5's GEF activity is autoinhibited by its C-terminal coiled-coil domain and activated by forming a contact with Rabaptin5 [92,108]. It also has two N-terminal ubiquitin-binding domains (Zinc finger ubiquitin binding domain, ZnF UBD, and a motif interacting with ubiquitin, MIU), which direct the GEF complex to the plasma membrane or early endosomes. Recent hydrogen deuterium exchange mass spectrometry (HDX-MS) experiments in combination with biochemical reconstitution assays supported an additional autoregulatory interaction between the N-terminal ubiquitin binding domain (UBD) and the Vps9 domain, which resulted in a mild increase in activity in the presence of Ubiquitin-chains [109]. As Rabaptin5 also has several different binding partners, it will be interesting to see how they affect the activity of the entire GEF complex.

Similarly, the activity of GAPs can be controlled by auxiliary domains. ASAP1, which regulates cell migration and the formation of invadopodia and podosomes, is particularly structurally complex [110]. Its catalytic core is comprised of an Arf-GAP domain, N-terminal BAR and PH domains as well as an Ankyrin repeat immediately C-terminal of the catalytic GAP domain. A proline-rich and SH3 domain mediates the targeting of the protein to focal adhesion sites on the plasma membrane [111]. The BAR-PH domains mediate binding to $PI(4,5)P_2$, which increases the GAP activity of ASAP1 about 10 000-fold [112]. Detailed biochemical and structural studies on the mechanism of ASAP1 regulation revealed that the PH domain controls the enzymatic activity of the Arf GAP domain *via* two different mechanisms [112–114]. First, it has two binding sites for $PI(4,5)P_2$ that result in cooperative binding to negatively charged phospholipids and potentially allow for a switch-like membrane-binding and activation of ASAP1 [115]. Second, the PH domain was found to interact with the Arf GAP domain, blocking its lipid binding domain [110]. Membrane attachment *via* the PH domain is therefore thought to lead to a significant reorientation of the PH and catalytic domain, increasing the catalytic activity of ASAP1 towards Arf1 [115]. Cooperative membrane binding also controls the activity of the Arf6-GAP ADAP1, which has two PH domains binding to PIP_3 [67]. Other Arf GAPs do not appear to be regulated by the density of negatively charged phospholipids, but by membrane topology. The best studied example for such a mechanism is likely ArfGAP-1, which senses membrane curvature through lipid packing defects *via* a C-terminal amphipathic lipid motif. As a result, membrane binding as well as the activity of catalytic GAP domain of Arf GAP1 increases with membrane curvature [116–118]. This property could allow Arf1-GTP to accumulate to flat membranes to initiate COPI-coat assembly, while ArfGAP1 would gradually remove Arf1-GTP from the membrane as it becomes more curved [119,120].

These examples show that *in vitro* reconstitution studies are able to suggest mechanisms to explain the spatiotemporal activity patterns of small GTPases found *in vivo* [121,122]. They also demonstrate that GEFs and GAPs usually do not act as sole agents. Instead, their activity results from a web of interactions with themselves and many other molecular players, proteins as well as phospholipids. To identify and understand the different layers of regulation and their hierarchy, it is therefore often required to include full-length native proteins in *in vitro* reconstitution experiments.

In vitro experiments to study small GTPase regulation

To determine the influence of regulators, effectors and the membrane surface on the activity state of small GTPase, it is necessary to follow the nucleotide state of the protein, ideally in real-time (Fig. 3).

In the case of Arf GTPases, nucleotide exchange and hydrolysis and the associated conformational changes can be continuously followed by monitoring the fluorescence intensities of their native tryptophan residues. The signal change is likely due to conserved tryptophan residues in the switch II region (Trp66 and Trp78 in Arf1, Trp62 and Trp74 in Arf6) [123]. Their fluorescence intensities (excitation at 290 nm and emission at 350 nm) are increased up to twofold [123] upon GTP binding as they move into a more solvent-protected environment [124]. This property has been routinely exploited to study the activity of GEFs and GAPs on Arf GTPases [62,67,100,125,126]. One disadvantage of this assay is that the range of GTPase concentrations that can be used is limited. In the case of Arf1, the fluorescence signal already saturates at 2–3 μM [126]. In addition, changes in tryptophan fluorescence might not be limited to the GTPase in question, but be associated with conformational changes or interaction with other proteins present in the assay [127]. This can make the characterization of more complex protein systems highly challenging.

Intrinsic fluorescence intensity has also been used to measure Rab GTPase activity [128]. However, GEF-catalysed nucleotide exchange is more commonly measured using small GTPases loaded with fluorescent guanine nucleotide analogs such as N-Methyl-3′-O-anthranoyl (mant)- or bodipy-GDP [129,130], where fluorophores are attached to the ribose subunit of the nucleotides. Mant is smaller than most other fluorophores and therefore expected to minimally interfere with its binding to the protein. The fluorescence of mant-labelled nucleotides is significantly higher when bound to the protein than in solution. GTP exchange can therefore be followed by preparing the mant-GDP loaded GTPase and recording the fluorescence decrease after the addition of a GEF, either directly by exciting the fluorophore at 333–365 nm or *via* tryptophan-FRET by exciting at 290–295 nm and recording at 440–488 nm [73,131–133]. Fluorescent nucleotides have been used to study non-modified Rab GTPases in solution, but also prenylated Rabs [73,81] and myristoylated Arfs in the presence of membranes [100]. While this approach is extremely useful, it might not always be generally applicable. For example, modified guanine nucleotides do not always fully activate small GTPases, limiting their use for *in vitro* studies [134,135]. GAP-dependent GTP hydrolysis is usually monitored by quantifying the phosphate release in a coupled colorimetric assay [136,137]. In the case of Rac1, a fluorescent version of GTP (tamra-GTP) could be used as a fluorescent reporter as its intensity is sensitive towards conformational changes associated with GTP hydrolysis [138,139].

Discrete sampling to measure nucleotide exchange and hydrolysis at specific time points can be more sensitive and accurate. For example, radiolabelled [γ-^{32}P] GTP can be used to measure GTPase activity [140,141] by quantifying the release of the ^{32}P. Nucleotide exchange can be followed by measuring the uptake of [γ-^{35} S] GTPγS [61] or [γ-^{32}P] GTPγS [92]. It is also possible to extract the protein-bound nucleotide from the isolated GTPase and identify the GTP/GDP ratio

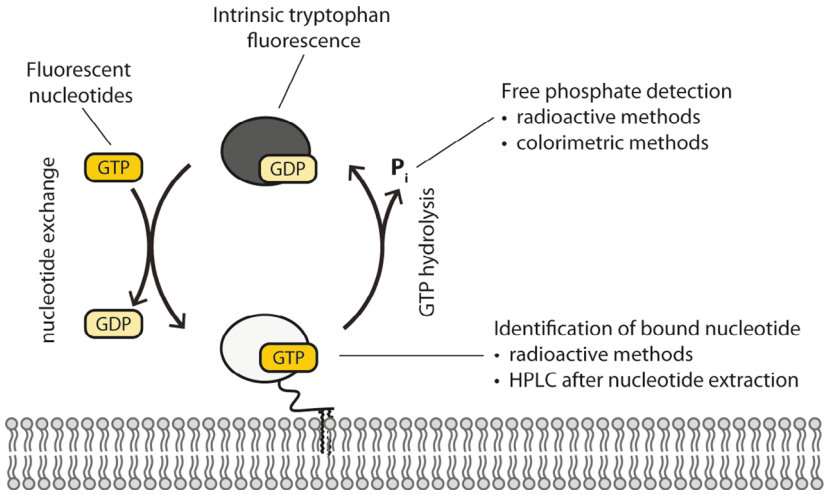

Fig. 3. Experimental assays to follow the nucleotide state of the GTPase. Nucleotide exchange can be monitored with the help of the intrinsic fluorescence of tryptophan residues. Alternatively, fluorescent nucleotides like mant-GDP can be used, whose fluorescence is significantly enhanced when in complex with the protein. The bound nucleotide can also be identified using radioactive nucleotides or *via* high-pressure liquid chromatography after extracting the nucleotide from the protein. To quantify GAP activities, the generation of free phosphate after GTP can be analysed using either radioactive or colorimetric methods.

in high-performance liquid chromatography-based assays [51,67,139,142].

Fluorescence microscopy assays to study small GTPase networks

The nucleotide state of the small GTPase is the result of many molecular steps that precede the actual exchange or hydrolysis. Even a minimal signalling circuit composed of only the GTPase and its GEF can be surprisingly complicated: Starting from the soluble GTPase in its GDP-bound state, its activation is initiated from its transient interaction with the membrane surface. Here, it meets its cognate GEF, which previously existed in an autoinhibited conformation in the cytoplasm. The presence and lifetime of the membrane-bound active state depend on the interaction of auxiliary domains with their interaction partners on the membrane surface. In the case of Arno and Rabex5:Rabaptin5, a signalling circuit of only GEF and GTPase is sufficient to generate an ultrasensitive or even bistable behaviour [73,79,84]. Next, the accumulation of active GTPase initiates the recruitment of effector proteins, which could be either passive passengers of the GTPase or actively participate in the biochemical network. Their most immediate impact could be direct competition with the GEF, inhibiting their recruitment and breaking the positive feedback [100]. In addition, as effector proteins can be lipid kinases or phosphates, their enzymatic activity can change the biochemical composition of the membrane to trigger protein binding and tune their biochemical activities [55,143,144].

While the activity of the effector depends on its recruitment to the membrane by an active GTPase, their respective membrane residence times might significantly differ. As demonstrated by the recovery of fluorescence after photobleaching, the GTPases can continuously cycle on and off the membrane, while the steady-state density is constant during the duration of the biochemical signal [140,145]. At the same time, effectors could remain bound due to rapid rebinding or avidity effects [146]. In this case, it is required that all GTPase molecules switch off and leave the membrane surface collectively to terminate the biochemical signal. The biochemical networks that would trigger such a cooperative behaviour still need to be better understood.

To quantitatively characterize the molecular steps as well as the emergent properties of small GTPase networks, more complex biochemical reconstitutions and experimental assays are required. Using fluorescence microscopy in combination with biomimetic membranes, it is possible to visualize and quantify the spatiotemporal dynamics of minimal protein systems in great detail. Total internal reflection (TIRF) microscopy and supported lipid bilayers even allow studying the behaviour of fluorescently labelled proteins on a single molecule level, making it possible to obtain quantitative information often missed in ensemble spectrophotometric measurements (Fig. 4). In pioneering studies on the Ras-SOS system, Iversen *et al.* [84] developed an experimental assay that allowed observing the GEF activity of individual SOS molecules on membrane-bound Ras-GDP. This study used micropatterned surfaces that confined lipids and membrane-bound proteins on individual membrane patches. SOS was then added at a concentration where every membrane patch only contained a single GEF acting on a membrane-bound layer of Ras-GDP. To follow the activity of this single enzyme, the GTPase was previously loaded with Atto488-labelled GDP. The exchange of this fluorescent nucleotide with non-labelled GTP caused a continuous decline in fluorescence intensity on individual membrane patches, producing kinetic traces corresponding to the activities of individual GEFs. This study revealed that the final output of a biochemical system, that is if an individual membrane compartment is switched on, can be dominated by highly active, individual GEFs on this compartment, rather than by the average of reaction rates. In a later study, a similar approach was used to image fluorescent SOS directly and to quantify the time difference between its arrival on the membrane and effector recruitment due to Ras activation [85]. The observed activation delay likely corresponds to the time required for the allosteric activation of SOS and the release of its autoinhibition. SOS was also found to sometimes leave the membrane before activating Ras. In combination with a theoretical analysis, it was suggested that Ras activation by SOS follows a kinetic proofreading mechanism, where long residence times increase the likelihood of Ras activation [85].

These experiments focused on membrane recruitment and detachment of the GEF, while the GTPase was permanently attached to the membrane surface. To understand the reversible binding dynamics of Arf, Rab and Rho GTPases, functional, lipid-modified and fluorescent versions of the proteins need to be prepared. While fluorescent fusion proteins are commonly used for studying small GTPases *in vivo*, the presence of this fluorescent label can perturb the function of the protein. Instead, small organic dyes should be preferentially used, as they provide a brighter fluorescent signal while having little effect on enzyme activity. Fluorophores can be specifically attached to the N or

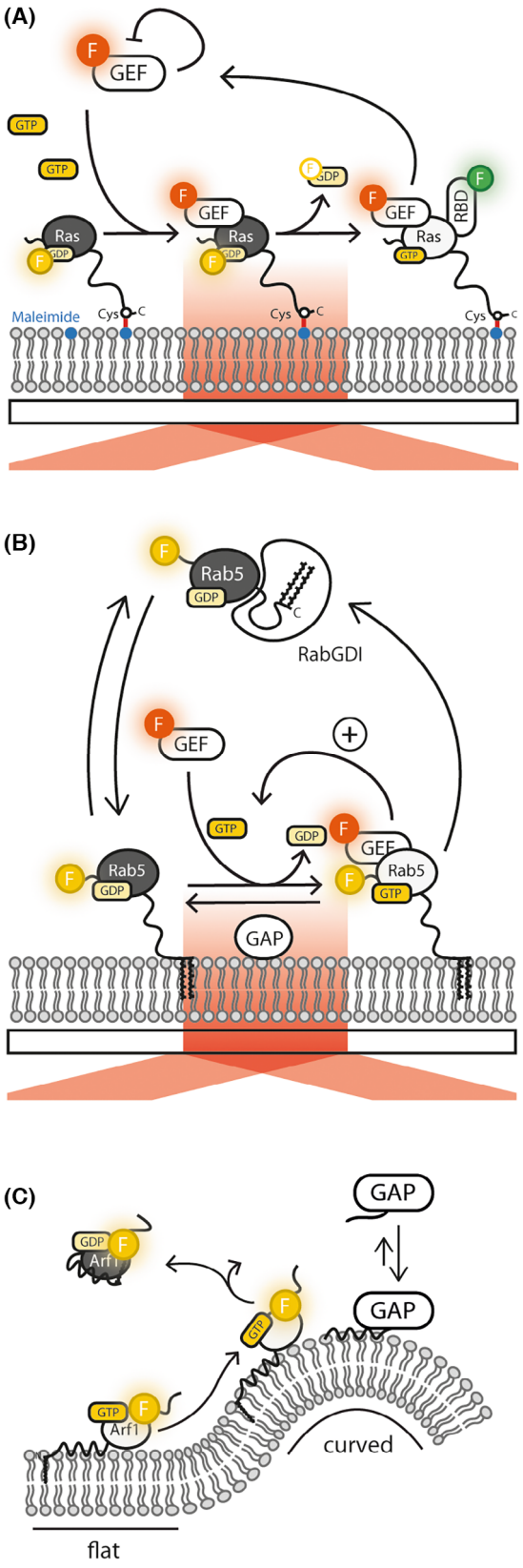

Fig. 4. Fluorescent microscopy assays can reveal the molecular steps underlying GTPase activation. (A) For example, using TIRF microscopy and fluorescent versions of Ras GEF SOS, Ras-binding proteins (RBD) as well as GDP, it was possible to quantify the lifetime of SOS on membrane-bound Ras-GDP and the rate of Ras activation. (B) A minimal regulatory network controlling the activity of Rab5 could be analysed using TIRF and fluorescently labelled GTPase and GEF. These experiments made it possible to quantify the lifetime of Rab5-GDP on the membrane prior to activation and to demonstrate ultrasensitive activation by Rabex5:Rabaptin5. (C) Using fluorescently labelled Arf1 and GUVs, curvature-dependent detachment of the protein due to a lipid-packing sensor in ArfGAP1 could be directly visualized.

C terminus of the protein *via* Sortagging [73,147,148], to endogenous or extra cysteines *via* maleimide labeling [118], flexible loops using Ybbr labeling [149] or genetically encoded artificial amino acids in combination with click-chemistry [150,151].

Using N-terminally sortagged, prenylated Rab5, it was possible to obtain experimental evidence for an ultrasensitive, switch-like activation of the GTPase by Rabex5:Rabaptin5 [73] due to positive feedback encoded in the regulatory network. This study could also identify a rapid equilibrium between Rab5-GDP in complex with its GDI and bound to the membrane prior to nucleotide exchange. Specifically, it was found that Rab-GDP spends around 0.5 s on the membrane, while an individual Rab-GTP can be found on the membrane for more than 10 s, likely limited by its intrinsic GTPase hydrolysis activity. In the additional presence of RabGAP-5 and increased GTP hydrolysis, the protein system even shows characteristics of bistability, that is the ability of a biochemical system to have two stable steady states [152]. In the case of the Rab5 regulatory network, this allowed for the coexistence of the collective ON- and OFF-states in the same experiment and the presence of waves of Rab5 activation traveling across the membrane surface [73]. Despite the significant difference in spatial scales, these spatiotemporal patterns might originate from similar mechanisms as the spatiotemporal activity distributions of Rab GTPases found *in vivo* [153].

Supported lipid bilayers in combination with TIRF microscopy were also successfully used to quantify the RhoGDI-catalysed membrane extraction rate of fluorescently labelled Rho in different nucleotide states [82]. Using this approach, it was possible to directly demonstrate that the RhoGDI extracts both the inactive and the active GTPase from the membrane Rho [154,155]. The result that the GDI can also extract active Rho GTPases is in stark contrast to the

behaviour of Rab GTPases that are stably attached to the membrane in their GTP-bound state [83], suggesting that different GTPases use distinct mechanisms to achieve their specific dynamic spatiotemporal distribution in the cell.

Finally, *in vitro* reconstitution of protein systems also allows us to directly visualize the effect of membrane curvature on the spatial distribution of GTPases. For example, the Arf1 GAP ArfGAP1 has an N-terminal lipid packing sensor domain, which makes membrane binding of the protein hypersensitive to curvature. Using optical tweezers and micropipette aspiration, Ambroggio *et al*. pulled membrane tubes from giant unilamellar vesicles (GUV) generating continuous membranes with effectively flat and highly curved surfaces. When myrArf1-GTP was allowed to bind to these vesicles, it was found on both the curved and flat areas. The activity of ArfGAP1, however, was predominantly localized to the high curvature of the membrane tube. This differential distribution of the GAP generated a gradient of Arf1, originating from the GUVs reaching towards the tip of the membrane tube [118].

Conclusions and perspectives

In vitro reconstitution of protein systems offers opportunities to obtain a mechanistic understanding of cellular functions that are not available from experiments in the living cell. This review showcased *in vitro* reconstitution studies to understand how small GTPases are regulated in time and space. In recent years, new protocols have been developed to prepare fully functional fluorescent proteins with post-translational modifications and large protein complexes of native proteins. We have also obtained new ways to prepare biomimetic membranes that closely mimic the *in vivo* situation in experimental assays, while allowing complete experimental control and quantitative characterization. Thanks to novel microscopy techniques that offer a higher spatiotemporal resolution, we can now directly visualize molecular behaviour in the nm range and on a millisecond time scale. All these techniques are invaluable for understanding the emergent properties of protein systems. The limiting factor for all *in vitro* reconstitution experiments is to have included the molecular players required for a specific cellular function. At the same time, every new additional player exponentially increases the complexity of the experimental system. Therefore, multi-laboratory collaborations will likely be necessary to succeed in the reconstitution of more and more complex cellular behaviour.

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