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Actin-membrane linkers: Insights from synthetic reconstituted systems

Feng-Ching Tsai^{a,*}, Gwendal Guérin^a, Julien Pernier^b, Patricia Bassereau^{a,*}

^a Institut Curie, Université PSL, Sorbonne Université, CNRS UMR168, Physics of Cells and Cancer, Paris 75005, France

^b Tumor Cell Dynamics Unit, Inserm U1279, Gustave Roussy Institute, Université Paris-Saclay, Villejuif 94800, France

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ABSTRACT

At the cell surface, the actin cytoskeleton and the plasma membrane interact reciprocally in a variety of processes related to the remodeling of the cell surface. The actin cytoskeleton has been known to modulate membrane organization and reshape the membrane. To this end, actin-membrane linking molecules play a major role in regulating actin assembly and spatially direct the interaction between the actin cytoskeleton and the membrane. While studies in cells have provided a wealth of knowledge on the molecular composition and interactions of the actin-membrane interface, the complex molecular interactions make it challenging to elucidate the precise actions of the actin-membrane linkers at the interface. Synthetic reconstituted systems, consisting of model membranes and purified proteins, have been a powerful approach to elucidate how actin-membrane linkers direct actin assembly to drive membrane shape changes. In this review, we will focus only on several actin-membrane linkers that have been studied by using reconstitution systems. We will discuss the design principles of these reconstitution systems and how they have contributed to the understanding of the cellular functions of actin-membrane linkers. Finally, we will provide a perspective on future research directions in understanding the intricate actin-membrane interaction.

1. Introduction

The surface of eukaryotic cells comprises the plasma membrane with integrated and attached proteins (Kusumi et al., 2012; Jacobson et al., 2019; Nicolson and Ferreira de Mattos, 2023). The plasma membrane is physically supported by the actin cortex, an approximately 200 nm thick layer of cross-linked actin filaments (Salbreux et al., 2012; Chugh and Paluch, 2018; Svitkina, 2020). The actin cortex has a significant influence on the organization of the plasma membrane. It acts as corrals, restricting the movements of lipids and membrane-associated proteins (Kusumi et al., 2012; Jacobson et al., 2019). The contractile forces generated by myosin II motors in cortical actin drive some membrane-associated proteins into clusters (Rao and Mayor, 2014; Köster et al., 2016), and regulate the surface tension and its propagation on the cell surface (Sens and Plastino, 2015; Sitarska and Diz-Muñoz, 2020; De Belly et al., 2023). For a plethora of membrane remodeling processes in cells, the principle driving forces are the polymerization of actin filaments, their subsequent assembly into different structures, and the contractile forces generated by myosin II motors (Mogilner and Oster, 1996; Murrell et al., 2015). Actin-dependent membrane remodeling includes local membrane deformation for endocytosis and

exocytosis, the generation of membrane protrusions like lamellipodia, filopodia, microvilli and tunnelling nanotubes, large-scale phagocytosis and macropinocytosis, as well as global cell migration and division (Blanchoin et al., 2014; Goode et al., 2015; Kessels and Qualmann, 2021; Jaumouillé and Waterman, 2020; Mylvaganam et al., 2021; Ljubojevic et al., 2021; Blake and Gallop, 2023; Sauvanet et al., 2015; Morales et al., 2023; Rottner and Schaks, 2019; Faix, 2006; Faix and Rottner, 2022; Yang and Svitkina, 2011; Mattila and Lappalainen, 2008).

To assist the actin cytoskeleton in reorganizing and reshaping the plasma membrane, proteins that connect the actin cytoskeleton to the membrane are crucial players (Bezanilla et al., 2015; Diz-Muñoz et al., 2010; Janmey et al., 2018; Saarikangas et al., 2010; Sheetz, 2001; Sitarska and Diz-Muñoz, 2020; Suetsugu et al., 2014). A prevalent molecular aspect of actin-membrane linkers is that they possess a domain that interacts with the membrane by binding to lipids or to trans-membrane proteins, and another domain that interacts with actin filaments or with actin regulatory proteins. In addition, actin-membrane linkers possess specific molecular properties associated with how they are regulated to function as actin-membrane linkers at specific times and membrane locations, which we have used to classify them into four categories: phosphorylation-regulated linkers, membrane

* Corresponding authors.

E-mail addresses: feng-ching.tsai@curie.fr (F.-C. Tsai), patricia.bassereau@curie.fr (P. Bassereau).

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curvature-sensitive linkers, force-sensitive linkers, and linkers with ATPase activity. This classification is intended to aid in the identification of the various functions of these linkers.

This review focuses on actin-membrane linkers that interact directly with the membrane through binding to lipids. It occurs via electrostatic interaction with negatively charged lipids such as phosphatidylserine (PS) or phosphoinositides lipids (PIs), including phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) and phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂) (Janmey et al., 2018; Senju and Lappalainen, 2019). These PIs are locally enriched or synthesized in the plasma membrane, depending on cellular processes (Posor et al., 2022; Saarikangas et al., 2010; Senju and Lappalainen, 2019; Suetsugu et al., 2014; van Meer et al., 2008). The local enrichment of PIs assists cells in regulating the spatial-temporal recruitment and activation of actin-membrane linkers on the membrane. Conversely, some actin-membrane linkers that are equipped with specific lipid interaction domains, such as PH and FERM domains, have demonstrated an ability to affect the distribution of PIP₂ on membranes (Yamamoto et al., 2020; Ehret et al., 2023; Brown, 2015; Wen et al., 2021). The locally enriched PIP₂ by the actin-membrane linkers can regulate actin assembly (Bezanilla et al., 2015; Janmey et al., 2018; Suetsugu et al., 2014; Suetsugu and Gautreau, 2012).

Given the intricate and reciprocal interactions between actin and the membrane, it is widely acknowledged that to attain a comprehensive mechanistic understanding of the actin-membrane interplay and its function in membrane remodeling, the membrane and the actin cytoskeleton have to be investigated jointly as a composite system (Sitariska and Diz-Muñoz, 2020; Doherty and McMahon, 2008; Köster and Mayor, 2016; Harris et al., 2018; Liu and Fletcher, 2009). Over recent decades, synthetic reconstituted systems have been widely used as effective platforms for elucidating the precise functions of the actin-membrane linkers at the actin-membrane interfaces (Ganzinger and Schwille, 2019; Jia and Schwille, 2019; Lopes dos Santos and Campillo, 2022; Baldauf et al., 2022). This review will discuss the discoveries made so far with such systems and some of the four types of actin-membrane linkers. Our focus will be on physiologically relevant actin-membrane linkers only, and synthetic linkers such as biotin-streptavidin will not be discussed. For *in vitro* studies using non-physiological actin-membrane linkers or actin nucleators such as WASP or its VCA domain, we refer the readers to a list of reviews and recent articles (Lopes dos Santos and Campillo, 2022; Lemièrre et al., 2016; Suzuki et al., 2015; Muresan et al., 2022; Sakamoto et al., 2023; Hsu et al., 2022; Sciortino and Bausch, 2021; Baldauf et al., 2023). Finally, future perspectives will be proposed to conclude this review.

2. Phosphorylation-regulated actin-membrane linkers: ERM proteins

To date, one of the most well-studied actin-membrane linkers are the proteins from the Ezrin/radixin/moesin (ERM) family (Fehon et al., 2010; Fiévet et al., 2007; Michie et al., 2019; Senju and Tsai, 2022; McClatchey, 2014). At the N-terminus, ERM proteins contain a FERM (Four-point-one, Ezrin, Radixin, Moesin) domain responsible for membrane association by interacting with PI(4,5)P₂ (Ehret et al., 2023). Their C-terminus contains an actin-binding domain (C-ERMAD) for their direct interaction with actin filaments. ERM proteins exhibit auto-inhibition through the intramolecular association of the N- and C-ERMAD, which masks the F-actin binding site. Using bioluminescence resonance energy transfer (BRET)-based conformational biosensors, a pool of inactive but membrane-associated ezrin was found in cells (Leguay et al., 2021). The disinhibition of ERM proteins i.e., the dissociation of the C-ERMAD from the FERM domain, relies on several regulatory pathways, including binding to PI(4,5)P₂ and notably post-translational modifications such as phosphorylation by ROCK kinase (Bosk et al., 2011; Jayasundar et al., 2012; Lubart et al., 2018; Pelaseyed et al., 2017; Ramalho et al., 2020; Senju and Tsai, 2022; Shabardina et al., 2016). Activated ERM proteins can link the actin

cytoskeleton to the plasma membrane (Senju and Tsai, 2022; McClatchey, 2014).

ERM proteins have been well-recognized to play crucial roles in regulating and stabilizing a variety of actin-dependent cellular processes. For instance, they contribute to the structural integrity and stability of microvilli, thin (about 100 nm wide), finger-like membrane protrusions on the apical surface of epithelial cells by establishing stable connections between the membrane and the actin bundles. At the cell surface, ERM proteins connect the plasma membrane and the actin cortex composed of cross-linked actin filaments forming a meshwork (Salbreux et al., 2012; Chugh and Paluch, 2018; Svitkina, 2020; Fehon et al., 2010; Bretscher et al., 2002). To retract outward membrane deformations such as blebs or the rear of cells during migration via actomyosin-II contractility, cells have to recruit ezrin to these regions to establish actin-membrane anchorage (Charras et al., 2006; Tsujita et al., 2021). Conversely, to initiate cell protrusions such as filopodia or to facilitate directed cell migration, the amount of actin-membrane linkers, such as ezrin, is reduced at specific membrane regions (Welf et al., 2020; Bisaria et al., 2020). In addition, the ability to modulate the adhesion between the plasma membrane and the actin cortex allows ERM proteins to control the tension of the cell surface, which is essential for processes such as migration and mitosis (Sitariska and Diz-Muñoz, 2020; Diz-Muñoz et al., 2010; Tsujita et al., 2021; Larson et al., 2010; Kunda et al., 2012; Liu et al., 2012; Rouven Brückner et al., 2015; Paraschiv et al., 2021).

Extensive studies using model membranes along with purified ERM proteins, particularly ezrin, have provided a detailed understanding of how phosphorylation of ERM proteins regulates their interactions with membranes and actin. Using PI(4,5)P₂-containing liposomes of various sizes - giant unilamellar vesicles (GUVs, typical diameters larger than a few μm), multilamellar vesicles (MLVs, typical diameters larger than 500 nm), large unilamellar vesicles (LUVs, typical diameters of 100 – 1000 nm), and small unilamellar vesicles (SUVs, typical diameters smaller than 100 nm) - combined with co-sedimentation assays or fluorescence correlation spectroscopy (FCS), the apparent dissociation constant (K_d) was found to be around 5 μM for ezrin and moesin (Blin et al., 2008; Maniti et al., 2013; Senju et al., 2017). The binding of ERM proteins to PI(4,5)P₂ has been shown to be cooperative for the lipids. On the one hand, by combining computer simulations and supported lipid bilayers (SLBs), the multivalent binding of the FERM domain of ezrin and PI(4,5)P₂ was shown, with an average stoichiometry of 1:4 (Ehret et al., 2023). In the case of moesin, the phosphomimetic moesin was shown to be able to bind two PI(4,5)P₂ molecules but not the wild type moesin (Lubart et al., 2018). On the other hand, by performing quartz crystal microbalance (QCM) and scanning force microscopy (SFM) to study the binding process of ezrin on PI(4,5)P₂-containing SLBs, it was proposed that ezrin exhibits lateral protein-protein interactions, resulting in a cooperative adsorption of an ezrin monolayer on the SLBs (Herrig et al., 2006).

Upon PIP₂ binding, ezrin undergoes a conformational change (Jayasundar et al., 2012; Shabardina et al., 2016). Phosphorylation of ezrin at T567 enhances its conformational change towards an active state upon binding to PIP₂, enabling its interaction with actin filaments (Fig. 1A) (Bosk et al., 2011; Shabardina et al., 2016; Tsai et al., 2018). In the presence of PI(4,5)P₂, a larger number of bonds between ezrin and actin filaments can be formed (Braunger et al., 2014). In a more recent study using SLBs, it was found that the addition of actin cross-linkers, fascin and α-actinin, rearrange the organization of ezrin-mediated actin networks on membranes (Schön et al., 2019). This suggests that ezrin-anchored filaments on the membrane possess a certain degree of freedom, allowing actin cross-linkers to reorganize them. Besides, a recent study of contractile actin-myosin II networks coupled to SLBs by ezrin demonstrated that the presence of negatively charged PS lipids together with PI(4,5)P₂ influence the connectivity and contractility of the actin filaments in the network (Fig. 1B) (Liebe et al., 2023). Notably, another study using contractile actomyosin networks anchored to SLBs

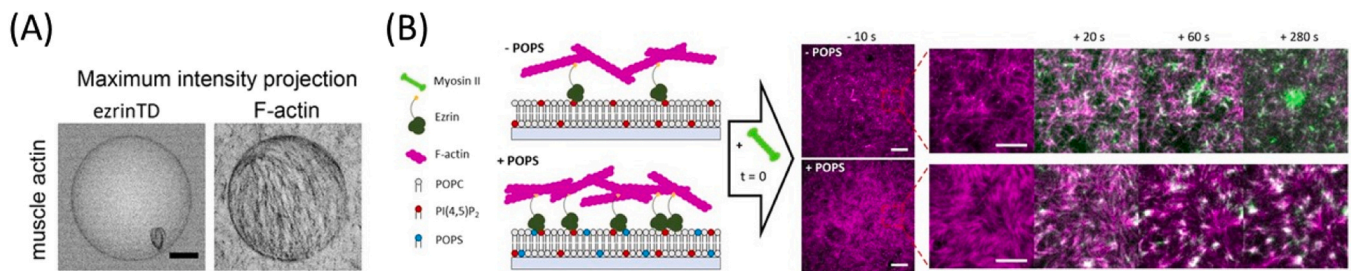


Fig. 1. ERM protein ezrin recruits actin filaments to flat membranes containing PI(4,5)P₂. (A) Phosphorylation of ezrin at T567 (ezrinTD) anchors actin filaments on the surface of giant unilamellar vesicles. Scale bar, 5 μm. Figure adapted from (Tsai et al., 2018) (B) Re-organization of the actin-myosin II contractile networks anchored on supported lipid bilayers by ezrin, in the absence (Top) and presence (Bottom) of POPS in the bilayers. Scale bars, 5 μm (Left) and 10 μm (Right). Figure adapted from (Liebe et al., 2023).

through a truncated ezrin (its C-terminal domain) demonstrated that the contractile forces can be transmitted to the membrane, thereby driving the re-organization of ezrin on the membrane (Köster et al., 2016). In summary, using reconstituted systems, we have gained some molecular understanding of how ERM proteins are regulated by phosphorylation to link the actin cytoskeleton to membranes, which relates to cellular structures such as the actin cortex. In the future, it would be insightful to elucidate how the different types of actin nucleation and polymerizing factors, including formin and Arp2/3 complex, and actin cross-linkers such as α-actinin found in the actin cortex, work together with myosin II contractile forces to modulate the spatial distribution of actin-membrane linkers (Rao and Mayor, 2014; Köster et al., 2016; Köster and Mayor, 2016; Muresan et al., 2022; Sakamoto et al., 2023; Fritzsche et al., 2014, 2013).

Using a single-molecule optical trap assay, a recent study demonstrated that ezrin forms complexes that can slide along an actin filament over micrometer distances when subjected to a pulling force perpendicular to the filament (Korkmazhan and Dunn, 2022). This observation provides mechanistic insights into how ezrin provides a stable link between the membrane and the actin cytoskeleton, acting as a force mediator so that the contractile forces are not transmitted directly to either the actin filaments or the membrane but instead mediating their relative sliding with each other. This force-mediating effect of ezrin may help to protect the structural integrity of the actin cortex from disruption by myosin II forces, as demonstrated in *in vitro* reconstitution systems (Murrell and Gardel, 2012). It would be enlightening to investigate how the presence of the membrane affects the sliding of ezrin along actin filaments and how this in turn affects the modulation of membrane tension (De Belly et al., 2023; Sakamoto et al., 2023).

3. Membrane curvature-sensitive actin-membrane linkers: BAR-domain proteins

Besides being able to interact with both the actin cytoskeleton and the plasma membrane, two key molecular features of the Bin/Amphiphysin/Rvs (BAR) domain proteins are their ability to sense and generate curved membranes as well as to regulate actin assembly (Suetsugu and Gautreau, 2012; Qualmann et al., 2011; Nishimura et al., 2018; Simunovic et al., 2019). BAR domains form intrinsically curved, anisotropic antiparallel homodimers (Carman and Dominguez, 2018; Snider et al., 2021). Based on their crystal structures, BAR domains can be divided into three main subfamilies: classical BAR, longer and less curved Fes/CIP4 homology-BAR (F-BAR) and inverse-BAR (I-BAR) domains. Some classical BAR domains with an N-terminal amphipathic helix are called N-BAR domains. BAR domains themselves are known to interact with membranes via binding to negatively charged lipids, including PS lipids, and more specifically to PI(4,5)P₂ (Mattila et al., 2007; McMahon and Gallop, 2005; Suetsugu et al., 2014). Some BAR proteins are equipped with extra lipid-binding domains including the PH and PX domains (Carman and Dominguez, 2018). The interaction of

BAR proteins and actin filaments can be mediated by the electrostatic interaction between the BAR domains and the filaments (Disanza et al., 2006; Rocca et al., 2008; Kostan et al., 2014; Dräger et al., 2017). Notably, some BAR domain proteins are equipped with a SRC Homology 3 (SH3) domain, allowing them to interact with many actin regulator proteins, including notably the WASP-WAVE family proteins, which regulates the Arp2/3 complex for the assembly of branched actin networks (Takenawa and Suetsugu, 2007).

The activity of BAR proteins is known to be modulated so that they are in an auto-inhibited state in the cytoplasm, limiting remodeling of the plasma membrane and actin cytoskeleton (Nishimura et al., 2018; Carman and Dominguez, 2018). The release of BAR proteins from auto-inhibition requires their interaction with several effectors (Nishimura et al., 2018; Carman and Dominguez, 2018). For example, binding of IRSp53 to Cdc42, PIP₂, and several actin regulatory proteins, including Eps8, VASP and N-WASP, is known to activate IRSp53, leading to the formation of membrane ruffles and filopodia-like protrusions (Disanza et al., 2006, 2013). To maintain IRSp53 in the auto-inhibition state, it relies on the binding of 14–3–3 to phosphorylated IRSp53, which inhibits IRSp53-membrane binding and counteracts the activation of phosphorylated IRSp53 by Cdc42, Eps8 and VASP, thereby impairing filopodia formation and dynamics as well as directed cell migration (Kast et al., 2014; Kast and Dominguez, 2019a,b).

BAR proteins are commonly found associated with curved regions on the plasma membrane to facilitate membrane deformation that can be primarily driven by actin assembly. A prominent well-studied example is endocytosis where various BAR proteins are involved, including F-BAR protein FCHSD2, N-BAR proteins endophilin and amphiphysin, BAR protein SNX9, and I-BAR protein IRSp53 (Kaksonen and Roux, 2018; Thottacherry et al., 2019). Moreover, BAR proteins, including IRSp53 and MIM/ABBA, contribute to the formation of flat membrane protrusions such as lamellipodia as well as finger-like protrusions such as filopodia, microvilli, and tunnelling nanotubes (Ljubojevic et al., 2021; Disanza et al., 2013; Lim et al., 2008; Kast and Dominguez, 2019b; Postema et al., 2018; Bisi et al., 2020; Henderson et al., 2022). Moreover, BAR proteins such as SNX9 and GAS7 have also been implicated in membrane ruffling, a precursor to macropinocytosis and phagocytosis (Miki et al., 2000; Yazar et al., 2007; Hanawa-Suetsugu et al., 2019).

Studies using model membranes and purified BAR proteins have shown that at low surface density, BAR domains can “sense” membrane curvature while at a high surface density, they can generate curved membranes (Nishimura et al., 2018; Simunovic et al., 2019, 2018; Snider et al., 2021; Saarikangas et al., 2009; Baumgart et al., 2011; Kozlov and Taraska, 2023; Suetsugu, 2016). The classical BAR proteins associate preferentially with membranes exhibiting positive curvature (Heinrich et al., 2010; Sorre et al., 2012; Zhu et al., 2012; Hsieh et al., 2012), whereas I-BAR proteins preferentially associate with membranes with a negative curvature (Prevost et al., 2015). In addition, BAR proteins have been recognized for their significant role in controlling actin assembly (Suetsugu and Gautreau, 2012; Nishimura et al., 2018;

Carman and Dominguez, 2018). The BAR-mediated actin assembly is primarily attributed to the direct interaction between the SH3 domain of BAR proteins and the actin nucleation-promoting factor N-WASP (Suetsugu and Gautreau, 2012). Taken together, since the recruitment of BAR proteins to membrane sites can be regulated by membrane curvature, we thus classify BAR proteins as membrane curvature-sensitive actin-membrane linkers.

While there is a wealth of information regarding BAR protein-membrane interactions, the understanding of how BAR proteins regulate actin assembly on membranes remains largely unexplored. Several studies have used reconstituted systems to address this question in the context of endocytosis. Using PI(4,5)P₂-containing LUVs, it was demonstrated that SNX9 synergize with PI(4,5)P₂ to activate N-WASP, leading to the formation of Arp2/3 complex-mediated branched actin network (Yarar et al., 2007, 2008). The addition of PI(3)P in PI(4,5)P₂-containing membranes facilitates BAR protein-associated actin assembly (Gallop et al., 2013). Notably, membrane curvature and the presence of PI(4,5)P₂ and PI(3)P synergistically allow SNX9 to mediate N-WASP-Arp2/3 complex-mediated actin assembly on curved membranes (Fig. 2A and B) (Daste et al., 2017). These studies clearly demonstrate the critical and synergistic roles of PIs and membrane curvature in BAR protein-mediated actin assembly, providing a molecular understanding of how BAR-mediated actin assembly occurs in endocytosis.

Aiming to understand how filopodia are initiated at specific membrane locations in cells, a recent study using a reconstituted system consisting of GUVs as model membranes demonstrated that IRSp53 can form clusters on the membrane, due to its intrinsic curvature and membrane deformation ability (Jarín et al., 2021). These IRSp53 clusters recruit the actin polymerase VASP to the membrane, which triggers localized actin assembly, ultimately leading to the formation of

filopodia-like membrane protrusions (Fig. 2C and D) (Tsai et al., 2022). Overall, this minimal reconstituted system provides a physical basis for the mechanism of filopodia initiation and formation proposed in (Blake and Gallop, 2023; Dianza et al., 2013). A potential future direction for in vitro reconstitution is to investigate how two types of BAR proteins, with different preferred membrane curvatures, can collaboratively regulate actin assembly and influence membrane morphology. This idea is inspired by a recent study conducted in live neuron cells that demonstrated the cooperative action of the I-BAR protein IRSp53 and the N-BAR protein ArhGAP44 in inducing and inhibiting actin assembly at specific membrane regions, ultimately facilitating the formation of membrane protrusions (Mancinelli et al., 2021). Besides, it would be insightful to use reconstituted systems to investigate how BAR proteins mediate different actin structures i.e., Arp2/3-mediated branched actin networks or actin bundles, to generate different types of membrane deformations, such as finger-like filopodia, sheet-like lamellipodia and less well-defined geometries in phagocytosis and macropinocytosis.

Of note, some BAR proteins, specifically the I-BAR IRSp53, the F-BAR pacsin2, the N-BARs PICK1 and Bin1 are able to bind to actin filaments via their BAR domains, resulting in the generation of actin bundles (Dianza et al., 2006; Rocca et al., 2008; Kostan et al., 2014; Dräger et al., 2017). These observations highlight the molecular complexity of the interactions between BAR proteins and membranes, as well as BAR proteins and actin, and call for detailed analysis on how physiologically relevant biochemical and biophysical conditions modulate BAR protein-membrane and BAR protein-actin interactions.

4. Mechano-sensitive linkers: the focal adhesion integrin complex

Focal adhesions contain many proteins, including transmembrane

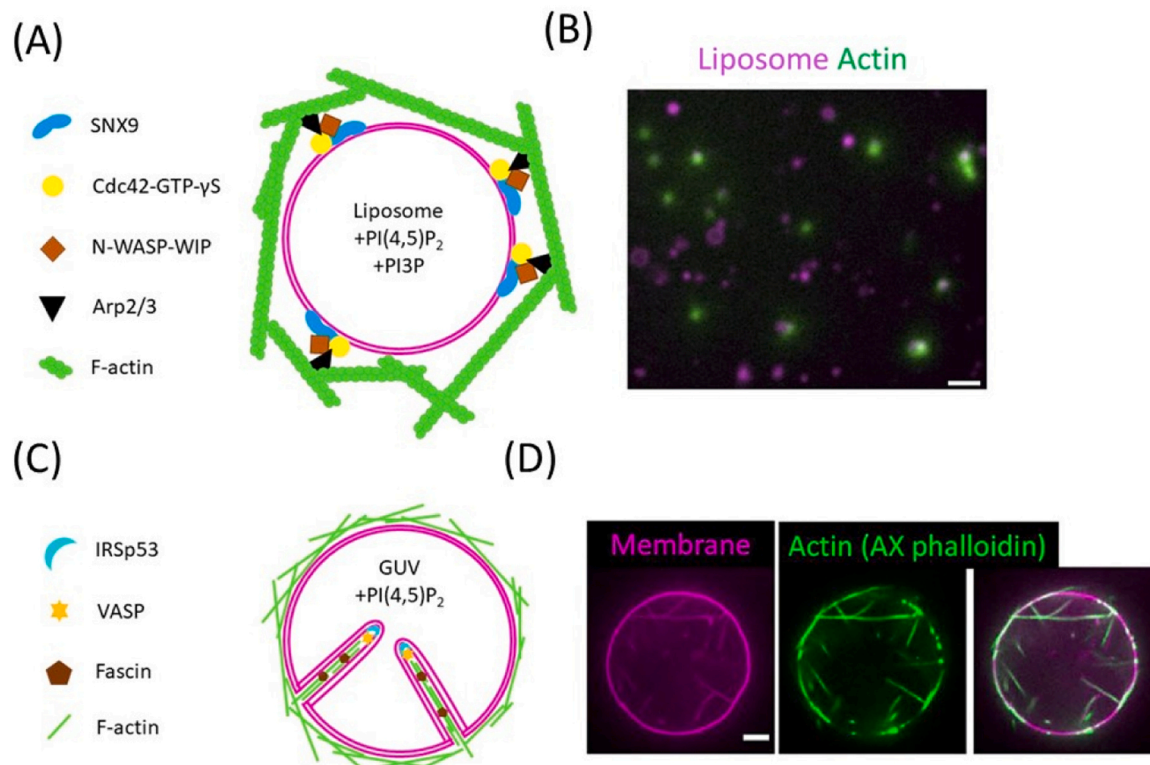


Fig. 2. Actin assembly on membranes in the presence of curvature sensing BAR proteins, SNX9 (A and B) and IRSp53 (C and D). (A and B) Dense accumulation of actin (in green) on liposomes (a mixture of SUVs, LUVs and GUVs) containing PI(4,5)P₂ and PI(3)P (magenta) in the presence of the BAR protein SNX9, the Arp2/3 complex, Cdc42-GTP-γS, N-WASP-WIP complex, and actin. Branched actin networks form at the surface of highly curved liposomes. Scale bar, 6 μm. Figure adapted from (Daste et al., 2017) (C and D) Filopodia-like membrane protrusions formed on Giant Unilamellar Vesicles (GUVs) (magenta) in the presence of the I-BAR protein IRSp53, VASP, capping protein, profilin, AX488 phalloidin, fascin, and actin (green). Scale bar, 5 μm. Figure adapted from (Tsai et al., 2022).

integrins as the main elements, that link the extracellular environment to the actin cytoskeleton inside the cell through cytoplasmic proteins. The integrin complex acts as a mechano-sensitive linker by establishing a mechanical reciprocity between the rigidity of the extracellular matrix and pulling forces exerted by the cytoskeleton (Sun et al., 2016). Integrins have 18 different α chains and 8 types of β subunits in humans, which can assemble into 24 different integrin heterodimeric members (Kechagia et al., 2019). Integrin heterodimers share a similar domain organization. The extracellular domains bind to ligands, the transmembrane domains and the cytoplasmic tails interact with the actin cytoskeleton via cytoplasmic adaptor proteins.

The main function of integrins is in cell adhesion and they are consequently involved in a wide range of biological processes including cell migration, proliferation, differentiation, hemostasis and thrombosis (Takada et al., 2007). These functions depend on the activation and clustering of integrins. Integrin activation is regulated by outside-in and inside-out signaling pathways. Outside-in signaling mediates cellular responses induced by extracellular ligand binding to integrins. Inside-in signaling activates the ligand binding. Among cytoplasmic adaptor proteins, talin and kindlin are indispensable for integrin activation and function cooperatively (Lu et al., 2022; Calderwood et al., 2013).

Talin is a large protein, composed of a N-terminal head domain, containing a 4.1-ezrin-radixin-moesin (FERM) domain (F0 to F3), followed by a linker and a rod domain (R1 to R13), and a C-terminal dimerization domain (Goult et al., 2018). The FERM domain includes a PIP₂-binding interface. The F3 domain interacts with the membrane proximal NPxY motif of the β integrin cytoplasmic tail to permit integrin conformational changes leading to an increased affinity for extracellular ligands (Wehrle-Haller, 2012). Talin contains also three actin-binding sites (Hemmings et al., 1996).

Kindlin also contains a FERM domain (F0 to F3), that binds to another membrane distal NxxY motif in the β integrin cytoplasmic tail. Unlike talin, kindlin F2 domain has an inserted pleckstrin homology (PH) domain that interacts with phosphoinositides with higher affinity for PIP₃ than for PI(4,5)P₂ (Liu et al., 2011). The FERM domain is required for integrin activation (Bouaouina et al., 2012).

Different biomimetic *in vitro* systems containing platelet transmembrane α IIb β 3 integrin have been developed to understand how they are activated and clustered during adhesion. Although challenging, the purified inactive integrins were reconstituted into SUVs and giant GUVs as well as nanodiscs (Frohnmayr et al., 2015; Streicher et al., 2009; Souissi et al., 2021; Gingras et al., 2013; Dransart et al., 2022). These reconstitution assays allow the organization of integrins to be observed and their level of activation to be measured. This is reflected either by the binding of the PAC1 antibody specific for the active form, or by adhesion to extracellular ligands and spreading of GUVs containing integrins on ligand-coated surfaces, or by interaction with cytoplasmic regulators. Using GUVs, it has recently been shown that, in addition to their individual ability to cluster integrins, talin and kindlin act synergistically to induce the formation of larger integrin clusters that are key for integrin activation (Pernier et al., 2023). An important role of phosphoinositides for their recruitment to the membrane was demonstrated, as also shown with an *in vitro* study for talin and vinculin (Kelley et al., 2020). Moreover, kindlin increases the surface density of talin and integrin. Finally, kindlin significantly enhances the segregation of talin-integrin clusters, which conversely reinforces the pulling force induced by actomyosin contractility, leading to the formation of tubules on the GUV (Fig. 3) (Pernier et al., 2023). These studies now open the possibility to develop new *in vitro* systems to determine how extracellular ligands influence the organization of clusters induced by

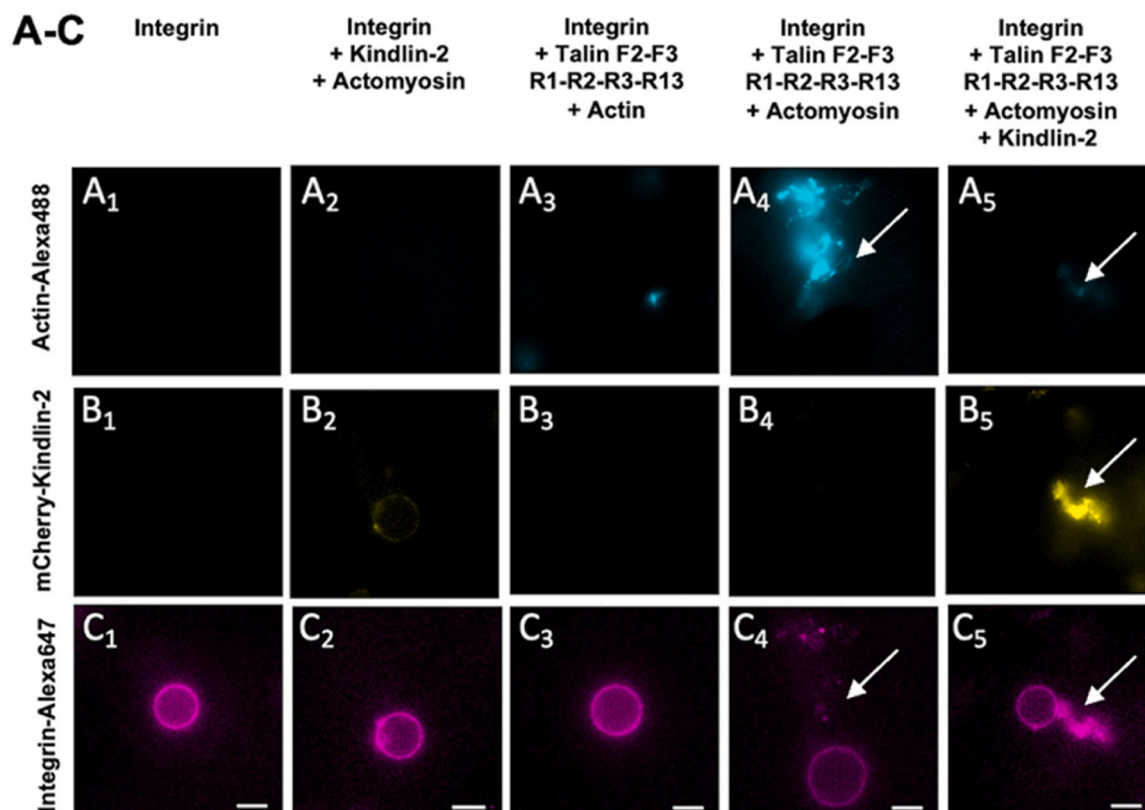


Fig. 3. Kindlin enhances the actomyosin-dependent segregation of integrins. (A–C) Representative epifluorescence images of integrin (magenta) containing GUVs without and with actin (2 μ M, 2% Alexa Fluor 488 labeled, cyan) and myosin II (50 nM), supplemented with talin F2-F3-R1-R2-R3-R13 (200 nM), mCherry-kindlin-2 (200 nM, yellow), and both talin and kindlin. White arrows indicate membrane deformations (tubules) mediated by actomyosin recruitment on area enriched in integrins in the presence of kindlin. The mean background was subtracted for each image. Scale bars: 10 μ m.

Figure adapted from (Pernier et al., 2023).

cytoplasmic proteins and by forces produced by actomyosin contractility, and conversely. This perspective is in line with the emergence of new developments in synthetic cell biology. New promising approaches involving microfluidics and pico-injection into droplet-stabilized giant unilamellar vesicles (dsGUVs) have allowed to reconstitute integrins in an efficient manner (Weiss et al., 2018). Active contractility systems and integrin regulators, including vinculin, might be further encapsulated in these artificial cells, potentially allowing for the reconstitution of functional mechanosensitive focal adhesions in artificial cells in the future (Kelley et al., 2020). Altogether, these more advanced systems should help in the future to progress in our understanding of focal adhesions.

5. Linkers with ATPase activity: type I myosin motors

Many unconventional myosin motors have the capability to simultaneously interact with the actin cytoskeleton and the plasma membrane (Batters and Veigel, 2016; Hartman et al., 2011; Fili and Toseland, 2020; Houdusse and Titus, 2021; Woolner and Bement, 2009). In this section, we will focus on Class I myosins (Myosin-1) (McConnell and Tyska, 2010; McIntosh and Ostap, 2016).

Myosin-1 motors are part of the myosin super-family. In vertebrates, there are 8 types of myosin-1 named as Myo1a to Myo1h (McConnell and Tyska, 2010). The N-terminal end of myosin-1 contains a motor domain that includes a nucleotide pocket for ATP hydrolysis to perform motor activity while interacting with actin filaments. The tail domain at the C-terminal end of myosin-1 contains a Tail Homology 1 (TH1) domain which includes a Pleckstrin Homology (PH) domain capable of binding to anionic lipids, specifically to PI(4,5)P₂ (Feeser et al., 2010a; Hokanson et al., 2006a; Pyrpasopoulos et al., 2010a; McKenna and Ostap, 2009; Hokanson and Ostap, 2006). The interaction with specific lipids is crucial for the recruitment of myosin-1 to specific membrane regions in the plasma membrane and intracellular organelles. Notably, the tail domains of Myo1e and Myo1f include an additional SH3 domain that can interact for instance with WASP, which allows these motors to directly regulate actin assembly (Evangelista et al., 2000). The head and tail of the myosin-1 motors are linked by a neck domain of the protein, formed of several IQ domains (1–6) binding to Calmodulin (CaM). This neck domain functions as a mechanical lever arm for myosin-1 motors (Greenberg et al., 2016; Greenberg and Ostap, 2013).

The ability of myosin-1 to dynamically couple the actin cytoskeleton and the membrane is involved in various cellular activities, as exemplified in the following (McIntosh and Ostap, 2016; Pernier and Schauer, 2022). A prominent example is that myosin-1 motors modulate the tension of the cell surface by bridging the plasma membrane to the underlying actin structures (Diz-Muñoz et al., 2010; Dai et al., 1999; Nambiar et al., 2009a). Operating at the interface of actin bundles and plasma membrane, Myo1a facilitates vesicle shedding at the tip of microvilli (Tyska and Nambiar, 2010; McConnell and Tyska, 2007). Myo1c controls exocytosis by facilitating the anchoring of actin structures at the exocytosis sites, which is required for the compression of the vesicles fused on the plasma membrane (Kittelberger et al., 2016). Phagocytosis requires Myo1g and Myo1e to spatially target and reorganize actin structures at phagocytotic cups (Barger et al., 2022, 2019; Dart et al., 2012). Moreover, Myo1e regulates the efficiency of endocytosis by regulating actin assembly at the endocytic sites (Cheng et al., 2012). Finally, Myo1b regulates the propagation of actin structures along axons to facilitate the formation of membrane protrusions for neuronal development (Iuliano et al., 2018). However, despite the well-recognized contributions of myosin-1 motors in many cellular activities, their precise mechanistic roles on the actin cytoskeleton, on the plasma membrane, or both remain elusive.

Extensive studies using optical tweezers combined with reconstituted assays, in which myosin-1 motors are anchored on solid substrates, have revealed the ATP hydrolysis cycles of myosin-1 motors in great molecular detail. In particular, it has been reported that Myo1b

and Myo1c are mechanosensitive, acting as catch-bonds (Greenberg et al., 2016), i.e. the binding lifetime to actin filaments increases when the motors are exposed to loading forces in the direction opposite to their movement (Laakso et al., 2008, 2010; Greenberg et al., 2012). In addition, the motor activity of myosin-1 can be characterized using actin motility assays in which the motors are anchored on flat substrates while interacting with single actin filaments suspended in solution. Using the forces generated by their power strokes, myosin-1 motors collectively propel the actin filaments, thereby causing them to glide laterally. Using these motility assays, the actin gliding velocities driven by several myosin-1 motors, such as Myo1b, Myo1c and Myo1d have been reported ranging from 5 to 150 nm/sec (Greenberg et al., 2016; O'Connell et al., 2007).

The interaction of myosin-1 motors with specific lipids have been studied using model membranes, notably LUVs. Myo1b, Myo1c and Myo1e have been shown to have a strong preference to negatively charge lipids like PS or PIs through their PH domain (Hokanson and Ostap, 2006; Feeser et al., 2010b; Pyrpasopoulos et al., 2010b; Dawicki McKenna and Ostap, 2009; Hokanson et al., 2006b; Komaba and Coluccio, 2010).

To elucidate how myosin-1 motors interact with actin filaments at the actin-membrane interface, several studies have used in vitro systems. On substrate-supported membranes, Myo1c has been demonstrated to generate forces of about 1 pN on actin filaments, parallel to the membrane plane (Pyrpasopoulos et al., 2016). Moreover, membrane-bound Myo1b was shown to facilitate the depolymerization of actin filaments at the barbed end, thanks to its catch-bond capability (Pernier et al., 2019). This Myo1b-mediated actin depolymerization is thought to impact actin organization by reducing the length of actin filaments, thereby the connectivity of the actin cortex and consequently the cortical contractility (Pernier et al., 2019; Ennomani et al., 2016; Alvarado et al., 2017). In addition, the Myo1b motors can collectively deform the membrane of GUVs by pulling membrane nanotubes along actin bundles immobilized on substrates (Fig. 4) (Yamada et al., 2014), recapitulating the formation of membrane tubules from the Golgi or endosomes reported in cellulo (Salas-Cortes et al., 2005; Almeida et al., 2011).

Using in vitro assays, it has been reported that both membrane-bound mouse Myo1c and *Drosophila* Myo1d drive actin filament gliding in a curved path (counterclockwise), with the later been implicated to contribute to the Left-Right symmetry breaking during *Drosophila* development (Serapion Pyrpasopoulos et al., 2012; Spéder and Noselli, 2007; Báez-Cruz and Ostap, 2023; Lebreton et al., 2018). This asymmetric, circular actin gliding originates from the motor domain and not from the tail domain of the myosin. Notably, this curved actin gliding is not universal, as the actin gliding of human Myo1a, mouse Myo1b and *Drosophila* Myo1c does not show this turning bias.

So far, while much is known about the motor activity of myosin-1, our understanding of how these molecular motors modulate the organization and the architecture of the actin cytoskeleton at the actin-membrane interface remains incomplete (McIntosh and Ostap, 2016; Pernier and Schauer, 2022). A recent study showed that when anchored on glass substrates, Myo1b breaks apart branched actin structures assembled by the Arp2/3 complex (Pernier et al., 2020), providing a mechanism for its effect on the morphology of growth cones (Iuliano et al., 2018). It remains to be investigated if and how membrane-bound myosin-I motors influence the assembly and organization of different actin structures given that myosin-I motors are known to interact with branched actin networks, for instance during endocytosis and phagocytosis, as well as actin bundles as in filopodia and microvilli (McIntosh and Ostap, 2016).

6. Perspectives

Here, we propose three aspects involving actin-membrane linkers that could be addressed by using in vitro reconstitution assays.

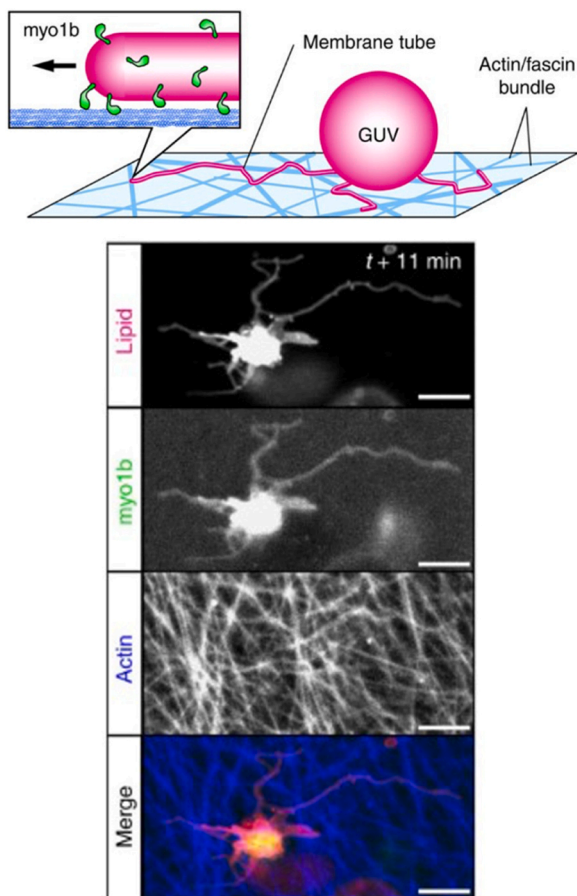


Fig. 4. Membrane tubulation driven by Myo1b. Myo1b motors, bound to the surface of a Giant Unilamellar Vesicle containing PI(4,5)P₂, collectively pull membrane nanotubes by moving on actin bundles immobilized on a glass surface. Scale bars, 5 μ m. Figure adapted from (Yamada et al., 2014).

To elucidate the precise molecular mechanisms of actin assembly taking place on membranes, regulated by the interplay between actin-membrane linkers and membrane-associated actin regulatory proteins or nucleation promoting factors. Many actin regulatory proteins, including profilin, formin, and actin nucleation promoting factors (NPFs), such as WAVE and WASP, have been shown to interact with the plasma membrane, either by interacting with small GTPase at the plasma membrane (including RhoA, Cdc42 and Rac1) or by binding to PIs, in particular PI (4,5)P₂ (Bezanilla et al., 2015; Doherty and McMahon, 2008; Janmey et al., 2018; Senju and Lappalainen, 2019). The assembly of actin structures thus occurs in close proximity to membranes, for example through the recruitment and activation of NPFs at specific membrane locations enriched in PI(4,5)P₂ (Senju and Lappalainen, 2019; Suetsugu et al., 2014; Posor et al., 2022; Mullins et al., 2018; Papayannopoulos et al., 2005; Liu and Fletcher, 2006). Furthermore, it has been shown that the residence time of N-WASP and the Arp2/3 complex can be extended through liquid-liquid phase separation of the Nephren-Nck-N-WASP signaling pathway on membranes (Case et al., 2019). To date, only a handful of studies have provided molecular insights into how actin-membrane linkers synergize with membrane-associated NPFs to trigger localized actin assembly. For example, SNX9 has been shown to facilitate N-WASP-mediated actin assembly at curved membranes, for instance during endocytic vesicle formation (Yarar et al., 2007, 2008; Gallop et al., 2013; Daste et al., 2017). While it has been recognized that a synergistic BAR-NPF interaction facilitates actin-driven membrane reshaping in several cellular processes, such as lamellipodia and filopodia formation and intracellular trafficking, we are still in the early stages of unravelling how actin

assembly on membranes is spatially regulated by the interplay between actin-membrane linkers and membrane-associated NPFs (Suetsugu and Gautreau, 2012; Nishimura et al., 2018; Mancinelli et al., 2021; Sitarska et al., 2023). In addition, we know little about how membrane mechanical properties, such as viscosity, stiffness, and tension, in turn modulate the linker-NPF interplay and thereby affect actin assembly processes on the membrane.

To measure how actin-membrane linkers mediate the transmission of mechanical forces from the actin cytoskeleton to membranes. The transmission of forces between an actin polymerization machinery and a membrane remains a key challenge that needs to be addressed (Doherty and McMahon, 2008). Currently, we lack suitable tools for accurately measuring the distribution and magnitude of forces exerted by actin assemblies to a membrane at the molecular level. One promising approach to measure molecular forces sensitive at the pN range is Förster resonance energy transfer (FRET)-based tension sensors that have been used to measure forces transmitted through e.g. focal adhesion proteins vinculin and talin in cells (Guo et al., 2014; Ringer et al., 2017; Grashoff et al., 2010). Notably, FRET tension sensors have also been used to measure forces transmitted from the assembly of actin networks to the plasma membrane through Sla2-Ent1 linkers that bridge actin to the plasma membrane in budding yeast endocytosis (Abella et al., 2021). Sla2 was modified to incorporate a mechanosensitive peptide connected by two fluorophores undergoing FRET, which allows the reporting of pN ranged forces (Abella et al., 2021). Notably, a recent study introduced a force sensor based on protein condensation (Ren et al., 2023). In this study conducted in fission yeast, the actin-membrane linker End4p was engineered to carry coiled-coil force sensors, enabling the visualization of spatial force distribution around the endocytic pit. In the future, it would be valuable to incorporate similar force sensors into *in vitro* reconstituted systems. This would offer fresh insights not only into the mechanisms of force transmission at actin-membrane interfaces but also into how membrane properties, such as membrane tension and curvature, can influence actin force generation and the mechanical resistance (Harris et al., 2018; Jarsch et al., 2016; McMahon and Gallop, 2005; Romet-Lemonne and Jégou, 2013; Sens and Plastino, 2015; Simon et al., 2019; Sitarska and Diz-Muñoz, 2020). Moreover, integrating reconstitution systems with fluorescent polarization super resolution microscopy, which provides detailed information on actin structural organization, would allow for a comprehensive understanding of both forces distribution and actin's structural dynamics at the actin-membrane interface (Rimoli et al., 2022; Cruz et al., 2016).

To elucidate the mechanistic contributions of actin-membrane linkers in membrane tension propagation. Both ERM protein ezrin and myosin-I motors are known to regulate the tension of the cell surface, a measure of the sum of membrane tension and the energy per unit area required to detach the membrane and the cortex (Dai et al., 1999; Diz-Muñoz et al., 2010; Nambiar et al., 2009b; Sens and Plastino, 2015; Sitarska and Diz-Muñoz, 2020). Recent biophysical studies have investigated the rate of membrane tension propagation across the plasma membrane by pulling membrane tethers from the plasma membrane of living cells (De Belly et al., 2023; Shi et al., 2018, 2022; Gomis Perez et al., 2022; Barnoy et al., 2023). While several mechanisms have been proposed to explain the wide range of tension propagation rates observed in cells, including frictional drag arising from actin-membrane linkers, direct validations of these proposed mechanisms are still lacking (De Belly et al., 2023; Shi et al., 2018, 2022; Gomis Perez et al., 2022; Barnoy et al., 2023). *In vitro* reconstituted systems provide an ideal platform to directly test these proposed mechanisms. For example, by pulling membrane tethers from GUVs encapsulating Arp2/3 complex-mediated actin networks assembled by actin-nucleation-promoting factors anchored to GUV membranes via biotin-streptavidin linkers, it was shown that the presence of the actin shell restricts lipid mobility and thereby membrane flow, similar to the corral-like effect of the actin cortex in cells (Guevorkian et al., 2015). Notably, thanks to the recent advances in the cDICE method, based on

the use of water-in-oil-based emulsion droplets in microfluidic devices, we can generate cell-sized GUVs that encapsulate well-controlled molecular compositions, including actin networks anchored to the inner surface of the GUVs by biologically relevant actin-membrane linkers at a controlled density (Baldauf et al., 2023; Litschel et al., 2021; Loiseau et al., 2016; Van de Cauter et al., 2021; Abkarian et al., 2011; Bashirzadeh et al., 2021). Therefore, by performing tether pulling experiments as in cells, but using actin-filled GUVs, one can address how actin-membrane linkers contribute to the propagation of membrane tension in the presence of tension gradients.

CRedit authorship contribution statement

Gwendal Guérin: Writing – original draft. **Feng-Ching Tsai:** Writing – review & editing, Writing – original draft, Conceptualization. **Patricia Bassereau:** Writing – review & editing, Conceptualization. **Julien Pernier:** Writing – review & editing, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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