ANNUAL REVIEWS

Annual Review of Cell and Developmental Biology

Filopodia In Vitro and In Vivo

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Annu. Rev. Cell Dev. Biol. 2023. 39:307–29

First published as a Review in Advance on July 5, 2023

The *Annual Review of Cell and Developmental Biology* is online at cellbio.annualreviews.org

https://doi.org/10.1146/annurev-cellbio-020223-025210

Keywords

formin, myosin, actin, Ena/VASP, protrusion, fascin

Abstract

Filopodia are dynamic cell surface protrusions used for cell motility, pathogen infection, and tissue development. The molecular mechanisms determining how and where filopodia grow and retract need to integrate mechanical forces and membrane curvature with extracellular signaling and the broader state of the cytoskeleton. The involved actin regulatory machinery nucleates, elongates, and bundles actin filaments separately from the underlying actin cortex. The refined membrane and actin geometry of filopodia, importance of tissue context, high spatiotemporal resolution required, and high degree of redundancy all limit current models. New technologies are improving opportunities for functional insight, with reconstitution of filopodia in vitro from purified components, endogenous genetic modification, inducible perturbation systems, and the study of filopodia in multicellular environments. In this review, we explore recent advances in conceptual models of how filopodia form, the molecules involved in this process, and our latest understanding of filopodia in vitro and in vivo.

Contents

INTRODUCTION

Filopodia, narrow finger-like protrusions from cells, are formed of parallel bundles of actin filaments surrounded by the plasma membrane and found throughout multicellular eukaryotes. Filopodia grow and shrink, mechanically probing the environment, forming adhesions, and transducing guidance cues and other signals to the rest of the cell. Filopodia contribute to tissue development and neuronal connectivity, and increases in filopodia are associated with cancer metastasis. Filopodia are important for the subversion of host systems by pathogens as they are induced by viral replication and used as routes of cell entry and for propagation between cells, recently becoming a putative therapeutic target for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Many regulatory components of filopodia have been identified (**Figure 1**), although it is not well understood how they combine to drive filopodial protrusion in different conditions. Key questions include how lipid signals, membrane curvature, and mechanical forces are integrated with actin regulation and how the dynamic behavior of individual filopodia is translated to cellular-scale decision-making. The roles of individual components during filopodial initiation have been debated, with differences attributed to cell-type specificity, while, more recently, heterogeneity between filopodia of the same cell has become apparent (Dobramysl et al. 2021, Pokrant et al. 2023, Urbančič et al. 2017). As an ancient and robust system of proteins that needs to respond to diverse extracellular signals, the molecular machinery's extensive redundancy is not surprising. We have come to think of filopodia as resulting from a system of mechanistic activities provided in combination across time and multiple players with molecular components tuned by layers of regulation. Such regulation can be provided by binding interactions, conformational change, protein oligomerization, and higher-order clustering, amongst other biochemical processes. Thus, like many other aspects of biology, filopodia emerge from a complex heterogeneous network of interactions.

The filopodia that we consider in this review are associated with cell migration, including microspikes that can be largely embedded in the lamellipodium. Other reviews can be explored for specialized types of filopodia with distinguishable subsets of functions, such as cytonemes, stereocilia, and dendritic filopodia, the precursors to spines, as their molecular bases are distinct (Daly et al. 2022, Houdusse & Titus 2021, McGrath et al. 2017, Wit & Hiesinger 2022).

FILOPODIA USE CORE ACTIN REGULATORY PRINCIPLES

Regulatory mechanisms used throughout the actin cytoskeleton apply in filopodia. Actin monomers are added to the growing filament barbed ends by processive elongators such as Ena/VASP and formins clustered at the filopodial tip (**Figure 1**), with depolymerization at the base leading to treadmilling (Mallavarapu & Mitchison 1999). Similar to the formation of other actin superstructures, filopodial formation is activated by upstream Rho GTPase signaling, while early studies of filopodia made it clear that they are distinct from lamellipodia in their Rho GTPase signaling and rates of extension (Nobes & Hall 1995). Filopodia have exponentially distributed rates of extension and shrinkage, with a mean growth rate of approximately 2 μm/min and reaching 25 μm/min (Dobramysl et al. 2021, Richier et al. 2018). Filopodial actin bundles are formed of 10–30 filaments tightly cross-linked by fascin (**Figure 1**), reaching lengths of 5–35 μm and diameters of 60–400 nm (Jacinto & Wolpert 2001). The enveloping plasma membrane is likely an active participant in filopodial formation, through both signaling to the actin machinery and mechanical effects (Mattila et al. 2007, Prévost et al. 2015, Saarikangas et al. 2015). Filopodia are responsive to the wider actin and membrane conditions through sharing the actin monomer pool with the wider network and responding to forces on the membrane, for example, from shaped transmembrane proteins (Ma et al. 2017, Rotty et al. 2015, Skruber et al. 2018).

USING MECHANISTIC MODELS AS LENSES TO UNDERSTAND FILOPODIAL FORMATION

We regard the various models of how filopodia form as useful lenses to view different aspects of the process at the molecular level (**Figure 2**). Our view is that the extent to which each model comes to the fore depends on which mechanisms are the most critical in any particular circumstance, for example, depending on membrane tension, protein expression level, or the dynamics or lifetime of the filopodium demanded by the biological context.

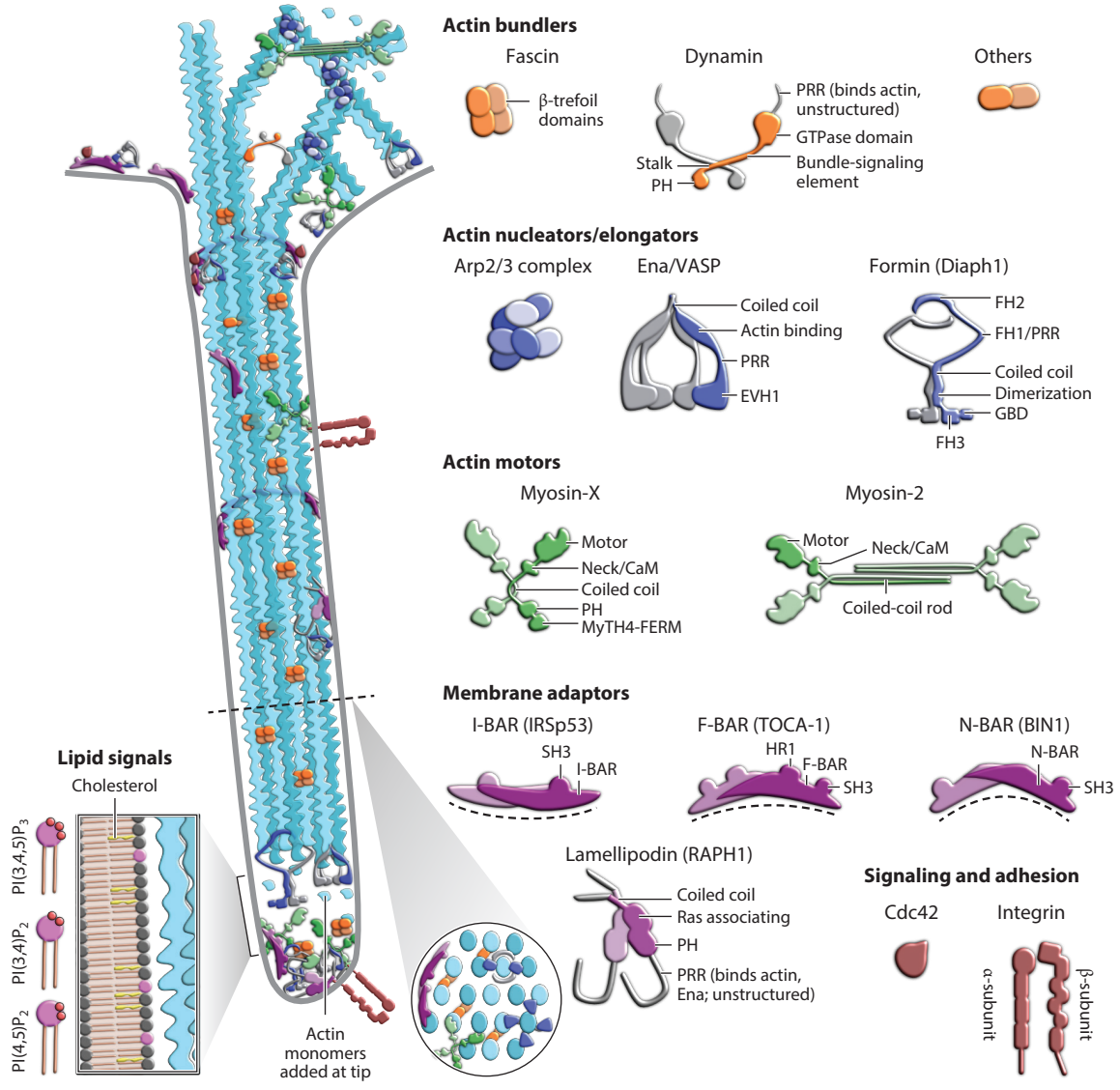
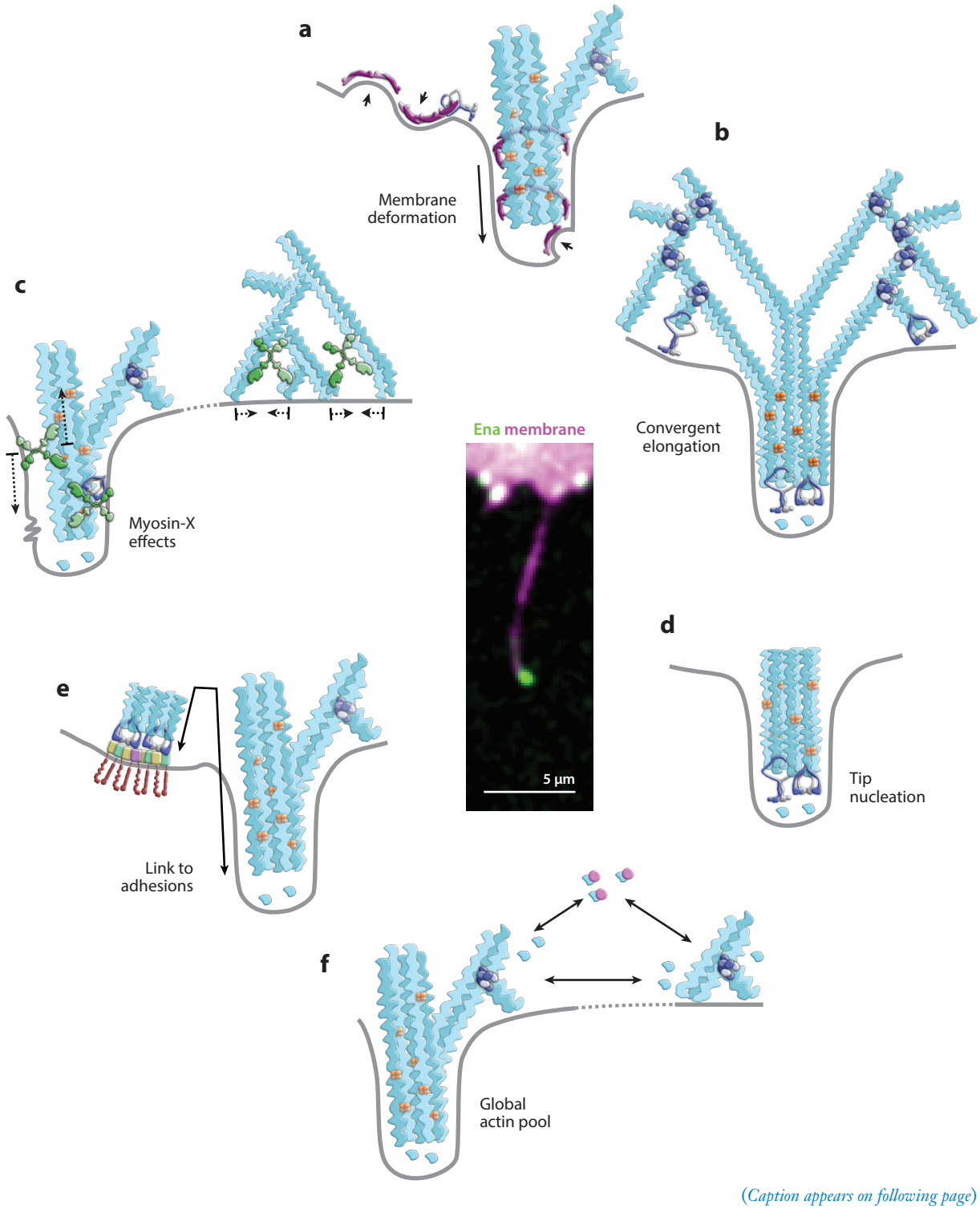


Figure 1

The molecular architecture of filopodia. Bundling proteins (*orange*) are responsible for the tight apposition and hexagonal packing of actin filaments (*cyan*) in filopodia. Actin nucleators and elongators (*blue*) are involved mainly at the tip and at the base of an emerging filopodium, including Ena/VASP proteins, which bind focal adhesion components and lamellipodin using the Ena/VASP homology 1 (EVH1) domain, and formins. Formins are released from autoinhibition by Rho GTPases binding the GTPase-binding domain (GBD), and then the formin homology 2 (FH2) domain adds profilin–actin monomers to the barbed end after they are recruited by the FH1 domain. Myosin-X is enriched at the filopodial tip and traffics through the shaft, with calmodulin (CaM) bound to the myosin lever arm, while myosin-2 has roles in the actin cortex at the base (*green*). Filopodia have distinctive membrane curvature and lipid microenvironments, highlighted in cross section (phosphoinositide lipids depicted in *pink* and cholesterol in *yellow*), and are stabilized by membrane adaptor proteins (*purple*), which interact with membranes using BAR domains or pleckstrin homology (PH) domains. Various protein–protein interactions are mediated by transient interactions between Src homology 3 (SH3) domains and proline-rich regions (PRRs). Domain shapes were derived in part from AlphaFold predictions (Jumper et al. 2021, Varadi et al. 2021).



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Different mechanistic models contribute to understanding how filopodia form. The molecules and mechanisms involved in making filopodia can be viewed in different ways depending on the primacy given to different elements of the regulatory machinery. It is likely that all these mechanisms contribute. The filopodium shown is a live total internal reflection fluorescence micrograph of a *Xenopus* retinal ganglion cell expressing mEos3.2-Ena with GAP43-RFP to mark the membrane. Convergent elongation and tip nucleation models were informed by Yang & Svitkina (2011).

Membrane Deformation Models Actively Consider the Membrane

As long narrow tubes, filopodia are areas of very high negative membrane curvature. The lipid distribution between the inner and outer leaflets therefore needs to change in concert with actin protrusion (**Figure 2*a***). In endocytic processes where there is positive membrane curvature, the role of clathrin in stabilizing curvature rather than actively bending the membrane led to the identification of BAR domain proteins that bend and stabilize high membrane curvature (Peter et al. 2004). Proteins with similar sequence identity and the opposite curvature have been found to bend and stabilize the negative curvature in filopodia: the I-BAR proteins and a subset of the F-BAR proteins (Guerrier et al. 2009, Mattila et al. 2007). Regulated IRSp53 interactions with the membrane stabilize curvature and recruit actin filament–elongating proteins such as VASP (Disanza et al. 2013, Tsai et al. 2022). Optogenetic control of IRSp53 recruitment demonstrates that it can dynamically induce and elongate filopodia (Jones et al. 2020). Whether these protrusions can fulfill the functions of filopodia is not yet clear, but new optogenetic tools give the opportunity to test filopodia generated via membrane curvature within native settings.

Surprisingly, BAR proteins associated with positive curvature, including ARHGAP44, FCHSD2, and TOCA-1, can also generate filopodia and recruit actin filament–elongating proteins (Blake et al. 2023, Bu et al. 2009, Galic et al. 2014, Zhai et al. 2022). BAR proteins binding to a positively curved membrane could stabilize stochastic membrane fluctuations, working with I-BAR proteins, reducing the energetic barrier to the highly curved membrane, and ensuring robust membrane responsiveness to actin bundles (Mancinelli et al. 2021). More straightforwardly, there are side-binding modes of F-BAR proteins adapted for neutral curvature, allowing F-BAR proteins to act as oligomeric membrane scaffolds for other regulatory proteins (Frost et al. 2008). Alternatively, convoluted or positively curved membranes could persist in filopodia after their initial formation (Galic et al. 2014), although this has not been widely observed in recent cryo-electron tomography studies of hippocampal neuron filopodia (Atherton et al. 2022, Hylton et al. 2022, Jasnin et al. 2013).

The Convergent Elongation Model Emphasizes the Reorganization of Existing Actin Networks

The convergent elongation model (**Figure 2*b***) is based on correlative light and electron microscopy studies of filopodia and sites of filopodial emergence in B16F1 melanoma cells (Svitkina et al. 2003). It is an influential model because of support from numerous in vitro studies that demonstrate bundling of actin networks induced by fascin (Vignjevic et al. 2006) and by membranes in purified systems with Arp2/3 complex–generated actin (Liu & Fletcher 2006). In studies with cell extracts and $PI(4,5)P_2$-supported lipid bilayers, filopodia-like structures also rely on initial Arp2/3 complex–generated actin to allow further elongation using other proteins such as Ena/VASP or formins (Lee et al. 2010). Light and electron microscopy in neurons provides evidence that actin patches precede the outgrowth of filopodia downstream of PI3K activation (Spillane et al. 2011).

Myosin-X Models Integrate Mechanical Force, Actin Bundling, and Cargo Recruitment

Myosin motors have diverse roles in producing force, controlling and organizing the actin network, and interacting with cargo proteins such as receptors or actin regulators (**Figure 2*c***). Myosin-X-driven rearrangements of the cortical actin network are proposed to lead to filopodial emergence (Pokrant et al. 2023, Tokuo et al. 2007). Using artificial dimers of myosin-X lacking the cargo-transporting tail domain, a model was proposed where myosin-X is activated by dimerization at the leading edge, driving convergence and bundling of actin filaments and leading to filopodial initiation (Tokuo et al. 2007). Artificially targeting myosin-X to membranes promotes filopodial formation and growth, likely due to the reorganization of actin filaments in addition to transporting other regulators to the filopodium tip and reducing the mechanical barrier to membrane deformation (Fitz et al. 2022, Zhang et al. 2021).

The Tip Nucleation Model Explains That Filopodia Can Be Separable from Lamellipodia

Filopodia have physical and dynamic properties distinct from any underlying actin network and can emerge independently of it (**Figure 2*d***). Actin filament elongators that can also nucleate actin are implicated in forming filopodia independently of another actin network. The discovery of the actin filament nucleation activity of formins is important, and filopodia form profusely using formin-mediated processes when the Arp2/3 complex is removed from cells (Faix et al. 2009). Because filopodia can be seen to initiate independently of a preexisting network and in the absence of other filament elongators, a simple, formin-driven mechanism can explain filopodial formation (Barzik et al. 2014).

A Link with Adhesions as a Site for Filopodial Growth

Filopodia-driven traction forces are important in many migrating cells. The distinctive components of filopodial integrin adhesions have been mapped and can develop into focal adhesions (Jacquemet et al. 2019). Integrin complexes are capable of triggering actin polymerization in vitro (Butler et al. 2006). Integrin-based adhesion foci are proposed to provide the sites for filopodial initiation (**Figure 2*e***), stabilize and decrease filopodial growth rate, and act as points where filopodia change direction (Gallop 2020, Gardel et al. 2010, He et al. 2017, Lee et al. 2012, Schäfer et al. 2009). Experiments involving the mechanical pulling of filopodia show that their actin polymerization activity responds to external forces, in particular, their connections to the extracellular matrix (ECM) (Alieva et al. 2019).

Global Transcriptional or Competitive Effects from the G-Actin Pool

Experiments depleting cells of actin regulatory proteins provide evidence that there are global effects on how actin architectures adjust to conditions (Kadzik et al. 2020). A major change in cell state—such as from transcription factor activities leading to epithelial–mesenchymal transition or kinase activity, for example, Src activation—could alter filopodial formation in multiple ways, both direct and indirect (**Figure 2*f***). A high level of profilin–actin drives the protrusion of Ena/VASP-generated filopodia, particularly in the absence of the Arp2/3 complex (Rotty et al. 2015). When lamellipodial actin is limited by removing or inhibiting the Arp2/3 complex, the overall balance of substrate for other nucleation and elongation pathways is increased, allowing more bundled parallel actin in microvilli (Faust et al. 2019). Increasing the G-actin pool that is not profilin-bound with, for example, increased cofilin and capping protein activity or decreasing

profilin levels favors Arp2/3 complex–generated branched filament architectures (Shekhar et al. 2015, Skruber et al. 2020).

RECENT PROGRESS IN UNDERSTANDING THE MOLECULES INVOLVED IN FILOPODIA

Actin in Filopodia

The architecture of filopodial actin filaments is being revealed by cryo-electron tomography studies of cultured cells and neurons, with new quantitative analysis tools helping to explore actin-binding proteins and actin reorganization in response to signals (Dimchev et al. 2021a). There are similarities between filopodia and other filament architectures: Hexagonally packed actin filament bundles with 12–13-nm spacing are found in comet tails, stress fibers, and filopodia in *Listeria*-infected PtK2 cells (Jasnin et al. 2013). Filopodia show long-range order of filaments over lengths of 200–300 nm. Cryo-electron tomography of mouse hippocampal growth cones has revealed the detailed architecture of the P-zone, where filopodia emerge from lamellipodia (Atherton et al. 2022). Small vesicles are present in filopodia, consistent with active membrane remodeling and ribosomes being strongly enriched in filopodia, suggesting a large role for de novo translation, as previously proposed (Leung et al. 2006). Individual filopodia contain multiple distinct bundles of actin, leading to filopodia being of different widths, with densely packed actin filaments extending beyond the membrane sheath into the P-zone (Atherton et al. 2022, Hylton et al. 2022). In rat hippocampal growth cones, there is a transition between fascin-bound and cofilin-bound actin (cofilactin), with fascin-bound bundles extending into the cell further than the membrane sheath (Hylton et al. 2022). Cofilactin filaments are stabilized in the P-zone and have a different repeat length, 27 nm instead of 37 nm, due to tighter twisting (Atherton et al. 2022, Hylton et al. 2022). Because cofilin changes the helical pitch, fascin-mediated/hexagonal actin filament packing can no longer occur, so cofilactin filaments segregate within the bundle. Filopodia can have segments of both types of bundles, with cofilactin bundles dominating at the intragrowth cone base. Mixed cofilactin and fascin bundles are linked to filopodial bending: 20% of filopodia undergoing lateral searching movements generated by myosin-2 have kinks and bends associated with cofilactin bundles (Hylton et al. 2022). This approach of combining dynamic light microscopy analysis with high-resolution electron microscopy links structural information to the cellular context.

Actin Bundling

Fascin has long been known as a major bundling protein in filopodia (Vignjevic et al. 2006), with the compact orientation of actin-binding domains leading to the tight packing of actin filaments (Jansen et al. 2011). While other actin bundlers are also implicated in filopodial formation, the specific fascin-bundle geometry contributes to both the mechanical properties of the actin bundle and the interactions with other actin-binding proteins, such as limiting certain myosin motors to either filopodia or microvilli (Fitz et al. 2022, Matoo et al. 2021). In view of its multidomain interaction, fascin is surprisingly dynamic and is exchanged between filopodia on a timescale of 30 s (Pfisterer et al. 2020). The phosphorylation of fascin controls its dynamics as well as localization, while artificially stabilizing filaments with jasplakinolide reduces fascin incorporation. The mobility of slow-moving, bound fascin is reduced in 3D collagen environments, with stiffer environments promoting fascin mobility (Pfisterer et al. 2020).

As well as fascin, plastins/fimbrins closely bundle actin filaments, mechanically supporting larger filopodia, which are needed to bridge gaps between areas of the ECM (Garbett et al. 2020, Li et al. 2020). A talin-related protein, TLNRD1, contributes to filopodial formation triggered by myosin-X overexpression in U2OS cells (Cowell et al. 2021). Many filopodia proteins are found

to have actin-bundling activity (including IRSp53, VASP, formins, and myosin-X). While better known for its function in vesicle scission in endocytosis, dynamin also has roles in bundling actin filaments. Dynamin works with IRSp53 during filopodial initiation (Chou et al. 2014) and stabilizes actin bundles in growth cone filopodia, working alongside the actin-binding protein cortactin (Yamada et al. 2013). During myoblast fusion, dynamin forms an oligomeric helix to bundle up to 16 filaments along its outer rim, unlike proteins that bundle pairs of filaments, and dynamin can simultaneously bind membrane nanotubes, providing a mechanism for coupling between actin filaments and membrane (Zhang et al. 2020).

Actin Filament Nucleation and Elongation

There are ~15 metazoan formins, and many have been implicated in making filopodia (Mellor 2010, Peng et al. 2003). Because they can nucleate actin filaments as well as elongate them, they are key candidates for driving filopodial protrusion when there is no preexisting lamellipodial network (Faix & Rottner 2006, Schirenbeck et al. 2005). Multiple diaphanous-related formins (Drfs) localize to filopodial tips where they polymerize actin monomers in a mechanosensitive manner, increasing elongation activity when pulled but strongly reducing activity when sterically hindered (Cao et al. 2018, Otomo et al. 2005, Suzuki et al. 2020). Knockdown of FMNL2 and FMNL3 in HeLa cells leads to significantly reduced filopodia length and number, whereas DAAM1 depletion (which is more implicated in bundling) increases filopodial lifetime without changing number and length (Jaiswal et al. 2013, Pfisterer et al. 2020). FMNL2 depletion also has significant effects on fascin mobility, unlike FMNL3 and DAAM1 (Pfisterer et al. 2020). In human melanoma cells, FMNL3 localizes to the tips of filopodia, consistent with a traditional role in elongating filament barbed ends, whereas FMNL2 localizes along the shaft and works with I-BAR protein IRTKS, promoting filopodial formation by a distinct role in membrane bending and tethering (Fox et al. 2022). In Arp2/3 complex knockout cells, filopodia are produced, sometimes profusely, due to upregulation of FMNL2 and FMNL3 (Dimchev et al. 2021b).

Ena, VASP, and Evl are a family of barbed end–binding proteins that localize to adhesions and filopodia, where they promote processive elongation of actin filaments after tetramerization and higher-order clustering (Breitsprecher et al. 2011, Hansen & Mullins 2015). Interestingly, when actin filaments are bundled by fascin, the trailing filaments are a better substrate for Ena/VASP elongation activity, suggesting a mechanism for the alignment of filopodia actin filaments (Harker et al. 2019). Ena and VASP coalesce into larger clusters before initiation (Cheng & Mullins 2020, Disanza et al. 2013, Svitkina et al. 2003), with abundance peaking at the moment of filopodial initiation (Urbančič et al. 2017). When they extend filopodia, they work with multiple membrane adaptors, including lamellipodin, IRSp53, and TOCA-1 (Blake et al. 2023, Cheng & Mullins 2020, Disanza et al. 2013). Ena and VASP are required for cortical neurite initiation and are important in axonal growth cone filopodia (Dent et al. 2007) and for 2D migration and microspike formation within melanoma cells (Damiano-Guercio et al. 2020). During the response of axonal growth cones to the guidance cue netrin-1, VASP activity is regulated by cycles of ubiquitination by the related E3 ligases TRIM9 and TRIM67. In the presence of netrin-1, VASP inhibition by TRIM9 ubiquitination, which results in less stable filopodia, is antagonized by TRIM67 binding to VASP, resulting in proper axon turning (Boyer et al. 2020, Menon et al. 2021).

The Arp2/3 complex generally seems dispensable for filopodial formation, although it is likely to have specific roles in some circumstances (Goley & Welch 2006). In fibroblasts, an inducible knockout of Arp3 greatly increased the number of filopodia, likely due to increased FMNL2 and FMNL3 expression, so cells were still mobile in the absence of the Arp2/3 complex (Dimchev et al. 2021b). The Arp2/3 complex was not needed for filopodia-driven durotaxis, and removal

of Arpc2 led to more and longer filopodia at the leading edge (Hakeem et al. 2023). However, the Arp2/3 complex is sometimes involved in filopodial formation, by making relevant actin-rich precursor structures (Spillane et al. 2011), and the Arp2/3 complex accumulates at filopodia adhesion and re-extension points (He et al. 2017). Filopodia can nucleate lamellipodia, which suggests an indirect purpose for the Arp2/3 complex localizing to filopodia shafts (Guillou et al. 2008). Cadherin adhesions were recently observed to be formed of interdigitated actin-rich protrusions, where Arp3 is important as well as the Ena/VASP family member Evl (Li et al. 2021).

Actin Motors in Filopodia

Myosins have a defined, highly conserved motor domain that usually moves toward the barbed end of actin filaments (the filopodial tip), a neck domain that allows myosins to take different-sized steps, and a highly variable tail region that allows them to act as processive or contractile motors or mechanosensitive membrane tethers (Robert-Paganin et al. 2020). Myosin-X dimerizes via an antiparallel coiled coil and has long, flexible lever arms, allowing it to take unusually large steps of >50 nm, well-suited to stepping along and gathering together adjacent filaments to make an actin bundle and to drive initiation of filopodial formation (Ropars et al. 2016, Tokuo et al. 2007). Myosin-X, along with myosin-7 and myosin-15, has MyTH4-FERM tandem domains that organize bundled actin structures (Fitz et al. 2022, Liu et al. 2021). Experiments in brush border epithelial cells carrying both filopodia and microvilli showed that myosin-X has a strong preference for filopodia, based on mode of dimerization, cargo interactions, and motor domain properties (Matoo et al. 2021).

A model of myosin-X as an organizer of actin structure was supported by the use of endogenous genetic knockouts in B16F1 melanoma cells, showing that myosin-X, but not other nonmyosin membrane adaptors, is essential for microspike formation and VASP clustering at the leading edge, forming a potential initiation complex (Pokrant et al. 2023). The myosin-X knockout mouse is semilethal and shows defects in filopodial formation; melanoblast migration; and eye, limb, and brain development (Bachg et al. 2019, Heimsath et al. 2017). Other functions for myosin-X-induced filopodia have been found in myoblast fusion (Hammers et al. 2021) and fibroblast migration in wound healing (Nozaki et al. 2022). Because of its multiple domains, it is unclear which functions of myosin-X (organization of actin filaments, cargo transport, or direct force production) are directly involved in promoting filopodial formation and at which stage it acts (Houdusse & Titus 2021).

Myosin-X interacts with cargo, including binding microtubules, transporting VASP to filopodial tips, supporting talin-mediated activation of integrins at filopodial tips, and tethering lamellipodin (RAPH1) at filopodial tips (Miihkinen et al. 2021, Popović et al. 2023, Tokuo & Ikebe 2004, Weber et al. 2004). Whether myosin-X acts as a more general transporter of cargo or even membrane vesicles [via the pleckstrin homology (PH) domains] remains to be seen. Supply and tethering of filopodia-promoting factors may be important for later stages of filopodia extension when diffusion-based supply is more difficult, where a role for myosin-X at adhesions and filopodia re-extension has been demonstrated (He et al. 2017).

Uncoupling the cargo role from the force role has recently been achieved by artificial myosin tetramers and inducible membrane tethering (Fitz et al. 2022, Zhang et al. 2021). Recruitment of single, isolated motor domains to the membrane elongated filopodia, consistent with a mechanical role at later stages of filopodial protrusion, by either softening the membrane or pushing back the bundle to make space for monomer addition, though this approach could not test the function of dimeric motors (Fitz et al. 2022). Studies on *Dictyostelium* Myo7 (the only MyTH4-FERM myosin present) showed that motor mutants deficient in force production could not promote filopodial formation (Arthur et al. 2019).

Non-MyTH4-FERM myosins also play roles in promoting filopodial formation, including leukocyte myosin-1f (Hensel et al. 2022); myosin-3 (Fitz et al. 2022); myosin-16, which was necessary for netrin-1-induced filopodia at the axonal growth cone of hippocampal neurons (Menon et al. 2021); and myosin-19, which processively moves along filopodia and transports mitochondria (Sato et al. 2022). Together, they point to a broader role for myosins in regulating and utilizing filopodia. This includes myosin-2, which drives retrograde flow at the filopodium base and the broader mechanical properties of the actin cortex, correlates with longer filopodial lifetime, and was required for filopodial adhesion formation (Alieva et al. 2019).

Membrane Adaptors in Filopodia

Specialized scaffolding membrane adaptor proteins recruit and cluster actin regulators, integrating upstream signaling and membrane curvature. I-BAR proteins such as IRSp53 have been heavily implicated in filopodia, being activated by Cdc42, recruiting VASP, and deforming membranes (Disanza et al. 2013, Krugmann et al. 2001, Mattila et al. 2007, Tsai et al. 2022). IRSp53 activation is subject to tight spatial control by the coincidence of inhibitory phosphorylation by 14-3-3 protein, Cdc42 activation, and $PI(4,5)P_2$, accounting for its localization to filopodia that are pulled from only dynamic regions of the cell (Kast & Dominguez 2019a,b; Tsai et al. 2022). This spatial regulation is also found within the filopodium, with super-resolution imaging showing that full-length IRSp53 segregates to just the lateral parts of the filopodial membrane, unlike the isolated I-BAR domain (Sudhaharan et al. 2019). However, in some systems, IRSp53 localizes to the filopodial membrane separately from the actin bundle or VASP clusters (Cheng & Mullins 2020, Sudhaharan et al. 2019), and endogenous genetic deletion of all I-BAR proteins did not affect VASP clusters (Pokrant et al. 2023). These differences are partly explained by significant redundancy between I-BAR proteins and other membrane adaptors and differences in membrane architectures between microspikes and membrane-wrapped filopodia (Damiano-Guercio et al. 2020, Fox et al. 2022, Pokrant et al. 2023).

Lamellipodin (RAPH1), an MRL family protein, is proposed to play a similar role as a membrane scaffold for clusters of VASP, forming dynamic filopodial initiation complexes at the leading edge (Cheng & Mullins 2020, Hansen & Mullins 2015). Unlike I-BAR proteins, its endogenous genetic deletion impairs cell motility and VASP cluster formation at microspike tips in B16F1 melanoma cells (Dimchev et al. 2020, Pokrant et al. 2023). Although these studies mostly focused on B16F1 melanoma cell lines, which are typically studied for microspikes in lamellipodia, though they also produce membrane-wrapped filopodia (Pokrant et al. 2023), lamellipodin has also been found at filopodial tips in other cell types, trafficked or anchored there by myosin-X (Popović et al. 2023). Ezrin, an ERM domain protein, is abundant at the plasma membrane and partitions into filopodia, where it tethers the actin bundle to the membrane, binding to $PI(4,5)P_2$ (Fehon et al. 2010). It is released from autoinhibition by phosphorylation and therefore is a candidate protein for transducing signals to changes in actin architecture (Gandy et al. 2013, Tsai et al. 2018). Depending on the binding partner, ezrin localizes to negatively curved membrane with IRSp53 (Tsai et al. 2018) or positively curved membrane at the base of myoblast filopodia-like structures with N-BAR protein BIN1 (Picas et al. 2022).

Other BAR proteins associated with positively curved membrane are implicated in filopodial formation, due either to positively curved membrane present during filopodial formation or protrusion or alternative binding modes (Frost et al. 2008, Galic et al. 2014, Mancinelli et al. 2021, Shimada et al. 2007). F-BAR protein TOCA-1 localizes to neuronal filopodial tips, where it coincides with Ena during filopodial protrusion (Blake et al. 2023), and the related proteins FBP17 and CIP4 are associated with either endocytic tubules or membrane protrusions in neurons, depending on the isoform expressed (Taylor et al. 2019). PX-BAR protein SNX9 is present

in a subset of filopodia, where it may also be involved in the budding of extracellular vesicles (EVs), similar to what has been reported for the missing in metastasis (MIM) protein (Jarsch et al. 2020, T. Nishimura et al. 2021). Finally, formins have membrane-binding and membrane-bending activity as well as their roles in actin nucleation and elongation (Fox et al. 2022, Kühn et al. 2015).

Lipids and Membrane Environment in Filopodia

As PI(4,5)P_2 is generally enriched at the membrane, it is likely to be involved in filopodial regulation, acting to stimulate actin polymerization via activation of Cdc42, IRSp53, ezrin, and other autoinhibited proteins and sequestering actin-severing proteins (Ma et al. 1998, Senju et al. 2017, Tsai et al. 2018). PI(3,4,5)P_3 localizes to filopodial tips and supports filopodial formation by activation of myosin-X (Plantard et al. 2010), and recent use of newly developed phosphoinositide probes has made it clear that PI(3,4)P_2 has a notable enrichment to filopodial tips, likely localizing lamellipodin and VASP (Hansen & Mullins 2015, Jacquemet et al. 2019, Popović et al. 2023, Yoshinaga et al. 2012). Filopodia are also proposed to be sites of cholesterol-rich membranes and slowed lipid flow (Gaus et al. 2003, Kishimoto et al. 2020).

The high-curvature membrane of filopodia acts as a distinct local environment for transmembrane proteins. One recent notable example is Piezo1, a nonselective mechanosensitive cation channel involved in transducing stretch and shear forces. The distinctive shape of the multitransmembrane protein means that it is excluded from the highly curved filopodial membrane, and activation-induced conformational changes increase Piezo1 sorting into filopodia (Yang et al. 2022). Though Piezo1 is dispensable for calcium signals in filopodia, Piezo1 knockout increased filopodia numbers, suggesting that it may have global effects on the ability of cells to protrude filopodia (Efremov et al. 2022, Yang et al. 2022).

HOW TO BUILD A FILOPODIUM: INSIGHTS FROM BIOCHEMICAL RECONSTITUTION

The actin field has a rich history of quantitative measurement and biochemical reconstitution. When common proteins are used for separable functions across the cell, simplified systems come into their own. Advanced microscopy of cell-free reconstitutions makes it possible to draw parallels and test mechanistic hypotheses about cellular architecture and dynamics using biochemical principles of reduction and quantitative measurement.

Bundled Actin Filament Systems

Early systems of filopodial reconstitution used nucleation promotion factors (NPFs) conjugated to beads [e.g., an N-WASP verprolin homology, central, and acidic (VCA) domain fused with glutathione S-transferase] to act as a focus for Arp2/3 complex–mediated nucleation of actin filaments (Bernheim-Groswasser et al. 2002, Vignjevic et al. 2003). In extracts, at low levels of capping protein, star-like actin bundles emanate from VCA-coated beads in a parallel orientation and are bundled by fascin with similar morphologies (Vignjevic et al. 2003), and this can be recapitulated with the purified Arp2/3 complex and fascin (Vignjevic et al. 2003). Fascin-mediated bundles of actin with 8-nm spacing that arise from NPF-coated beads segregate from filaments bundled with 35-nm spacing by α-actinin, offering a physical explanation for how filopodia separate from other actin networks in the cell (Winkelman et al. 2016). Fascin domain lengths on the actin bundles are exponentially distributed, which is evocative of the exponential length distributions of filopodia seen in vivo. Radial micropatterning of the VCA domain in purified systems leads to regions of branched, elongated antiparallel and parallel bundles of filaments (Reymann et al. 2012). The addition of myosin-6 leads to selective contraction and disassembly of the branched antiparallel

networks, while parallel (filopodia-like) bundles became further elongated, presumably due to the increased monomer availability (Reymann et al. 2012). These results show that the locations and spacing of NPF activation can help determine the resulting actin architecture, together with motor protein availability and the regulation of bundling proteins.

Bundled Actin Filament Systems with Membranes

In reconstitutions using actin-binding proteins alone, the influence of the cell membrane and membrane-bound regulatory factors is not present, showing that many of the activities related to making the actin bundle within filopodia can be found within the wider actin machinery. To capture the full range of filopodial behavior, the holistic regulatory and signaling environment needs to be considered, and there has been much recent progress with both membrane and actin reconstitutions using extracts and purified proteins.

Supported lipid bilayers form spontaneously on glass coverslips when small unilamellar liposomes are applied to clean surfaces at physiological ionic strength. They offer a contiguous area of membrane fixed to the coverslip where fluidity and composition can be controlled, with an open setup making it straightforward to add extracts or proteins and simplifying microscopy. Membrane anchoring of VASP to supported lipid bilayers in the presence of actin and capping protein leads to actin filament bundling (Nast-Kolb et al. 2022). The VASP and membrane lipids need to be mobile, and the actin bundling is dependent on the particular VASP polymerization process rather than bundling alone because Dia (a formin) and α-actinin are not sufficient to create these results, either individually or together (Nast-Kolb et al. 2022).

When exposed to cell extracts, supported lipid bilayers containing $PI(4,5)P_2$ recapitulate the nucleation of a spontaneously self-assembling tip complex resembling that found at tips of filopodia (Lee et al. 2010). The growth and shrinkage dynamics of these filopodia-like structures mirror those of filopodia in vivo (Dobramysl et al. 2021, Richier et al. 2018). There is focal Cdc42 activation, N-WASP recruitment, and Arp2/3 complex activation and also recruitment of filopodial actin regulators Drf, Ena, and VASP, resulting in bundled actin filaments. The order of actin regulator recruitment is highly dependent on actin, starting with membrane adaptor proteins, NPFs, the Arp2/3 complex, and Cdc42 activation followed by Drf3, Ena, and fascin, substantially after actin itself has assembled (Dobramysl et al. 2021, Lee et al. 2010). Surprisingly, rather than a defined tip complex, four-color imaging of the tip complexes alongside the growth dynamics of the resulting actin bundle demonstrated a high level of redundancy and heterogeneity in the molecular mechanisms underpinning FLS growth (Dobramysl et al. 2021). This work suggests that many simple reconstitutions of three or four proteins are capturing different aspects of the wider ways in which biology can make filopodia-like actin bundles.

Giant unilamellar vesicles (GUVs) are cell-like vesicles of 1–200-μm diameter. Typically, they are largely composed of phosphatidylcholine, and actin regulators are incorporated by using biotin-conjugated lipids and streptavidin tags. The deformability and large, flat surface of GUVs have been useful when purified protein reconstitution systems are added on the outside: NPFs and Arp2/3 complex–generated actin filaments can generate inward spike-shaped actin protrusions on GUVs, equivalent to outward filopodia-like protrusions from cells (Gat et al. 2020, Liu & Fletcher 2006, Simon et al. 2019). A recent reconstitution demonstrated that low levels of VCA lead to filopodia-like protrusions into GUVs; however, at high VCA concentrations, no spikes were apparent (Gat et al. 2020). This shows that concentration-dependent effects of sparsely localized proteins on membranes are important for forming discontinuities in actin networks over the surface of GUVs and that these discontinuities prompt the protrusion of spikes into the GUV. In linking actin bundles to the membrane, IRSp53 alone can bind and cluster on the outside of

negatively charged GUVs containing PI(4,5)P_2 or phosphatidylserine, and, in combination with actin, VASP, and fascin, it makes protrusions into the GUV (Tsai et al. 2022).

Progress in encapsulation methods means that generating filopodia-like structures from the inside of the GUV has recently become a viable option, giving similar constraints to cells (Litschel & Schwille 2021). When VCA is anchored to the outer surface of the GUV, an actin cortex is formed (Baldauf et al. 2023, Wubshet et al. 2023). Adding fascin within GUVs without membrane anchoring of VCA leads to actin bundles that protrude from the membrane, showing that membranes of simple compositions will deform to follow the actin template, while addition of α-actinin into the GUV instead leads to less rigid bundles that conform to the spherical membrane (Bashirzadeh et al. 2020). Membrane linkage in the presence of fascin can generate a mixed actin network showing polarity and bundling (Wubshet et al. 2023). Supporting a role for stochasticity and inhomogeneities, reconstitutions with lower concentrations of the Arp2/3 complex and increased concentrations of actin can form a protrusion of bundled actin even without fascin present (Baldauf et al. 2023). In 10% of cases, low Arp2/3 complex concentrations caused clustering of filopodia-like protrusions in bouquets, similar to the profuse filopodia seen in migrating cells (Baldauf et al. 2023). While such synthetic cell systems offer important progress in understanding the biophysical basis of filopodial protrusion, it comes with the caveat that there are no transmembrane proteins present, and there is no extracellular environment and no membrane trafficking, offering several reasons why cells may be different.

FILOPODIA IN VIVO

Mechanical Properties of Filopodia and Adhesions

Filopodial behavior in cells is intimately linked to the external mechanical environment, with some substrates favoring filopodia-based motility in culture, such as soft substrates with fast stress relaxation (Adebowale et al. 2021). Filopodia probe the local environment, typically pulling with 5–25 pN of force, depending on actin retrograde flow and myosin-2 pulling at the filopodium base, and they are important for durotaxis (Alieva et al. 2019, Bornschlögl et al. 2013, Hakeem et al. 2023). Filopodia respond to pulling forces at the tip by calcium influx via L-type channels, potentially mediating guidance decisions, and by increasing adhesion formation via mechanosensitive regulators upstream of integrin activation, including talin and p130Cas (Anthis & Campbell 2011, Jacquemet et al. 2019, Miihkinen et al. 2021). Filopodial adhesions have a composition distinct from other adhesions and act as sites of re-extension and direction change (He et al. 2017, Jacquemet et al. 2019). Focal adhesions can be nucleated at filopodial tips by clustering of paxillin and VASP, before the developing adhesion moves down the filopodium and is eventually overtaken by the lamellipodium, where the focal adhesions mature. Strong interaction with the ECM stabilizes extending filopodia and inhibits the formation of new filopodia (Alieva et al. 2019, Jacquemet et al. 2019).

Effective cell migration depends on reciprocal interactions between filopodia and the ECM, as filopodia apply force to, organize, and are regulated by ECM components such as hyaluronan and fibronectin (Kyykallio et al. 2020, Summerbell et al. 2020). In lung cancer cells, upregulation of myosin-X promotes long-lived filopodia that reorganize fibronectin into efficient tracks for collective 3D migration (Summerbell et al. 2020); however, premalignant breast cancer interactions between filopodia and the ECM support intact basement membranes, inhibiting cancer cell invasion (Peuhu et al. 2022). During development of the *Xenopus* embryo mucociliary epithelium, multi-ciliated cells intercalate into the superficial layer using filopodia to probe the junctions between epithelial cells and preferentially integrate at stiff junctions formed of four or more cells (Ventura et al. 2021).

As well as experiencing linear traction forces, filopodia undergo sweeping, bending, and rotational motion in three dimensions, depending on spiral motions of both myosin-5 and myosin-X around an actin filament and arising from spinning the actin bundle within the filopodium rather than twisting the whole filopodium around the base (Leijnse et al. 2022, Tamada et al. 2010). In a 3D matrix, this rotation encounters friction, leading to buckling and coiling of filopodia, which increase the pulling force and the exploratory range of filopodia in a complex environment (Leijnse et al. 2022).

Endogenous and Hijacked Roles for Vesicle Secretion and Trafficking at Filopodia

Membrane trafficking, both vesicle secretion and endocytosis, is observed at filopodia with roles in cell motility and signaling. Components of the endocytic machinery are enriched at the base of filopodia (Bu et al. 2009, Chou et al. 2014) and vesicles bud and traffic in a retrograde direction along filopodial actin bundles in growth cones (Nozumi et al. 2017). Membrane scission and the secretion of filopodia-derived EVs have recently been observed in HEK293 cells in both 2D and 3D culture, linked with I-BAR protein overexpression and responsive to knockdown of the I-BAR protein MIM (T. Nishimura et al. 2021). Interestingly, filopodia-derived EVs (which are enriched for Nectin-2, IRS4, and Rac1) stimulate migration in recipient cells, showing that filopodia can contribute to motility beyond the cells that they protrude from. Protrusion and retraction of filopodia and lamellipodia are also linked to the release of 30-nm plasma membrane–derived particles enriched in cholesterol from macrophages, especially when focal adhesion disassembly is inhibited, with possible roles in cholesterol transport (Hu et al. 2019).

Mechanisms and roles of vesicle secretion and uptake at filopodia are of interest given their roles as precursors to synapses and in viral infectivity. Filopodia are frequently induced by viral infections, and multiple viruses have been shown to be pulled and grabbed by filopodia as well as surfing along them to enter cells at endocytic hotspots at the filopodial base (Lehmann et al. 2005). Recently, the SARS-CoV-2 virus was also shown to induce filopodia, and global phosphorylation profiling identified casein kinase 2 (CK2) as a possible target for a therapy focused on the host cell, and the action of CK2 is proposed to be mediated via effects on filopodia (Bouhaddou et al. 2020). The filopodia induced by SARS-CoV-2 are mediated by fascin and Cdc42 (Zhang et al. 2022), and viral-like particles move and traffic within the filopodia and tunneling nanotubes, allowing the virus to spread between cells (Pepe et al. 2022).

Multicellular Roles and Impacts of Filopodia in Tissues

Recent work in explanted *Drosophila* tissues has illustrated how filopodial activity mediates tissue-scale forces and organization (Bischoff et al. 2021, Popkova et al. 2020). The reduced redundancy in *Drosophila* compared with vertebrates means that it is a useful system to examine filopodial formation.

For example, filopodia were shown to dominate the migration of myotubes in explants of developing testis, and filopodia are important for linking the tissue together (Bischoff et al. 2021). When the filopodia were disrupted, prominent holes were seen in the muscle sheath, similar to holes formed by inhibition of actin contractility (Bischoff et al. 2021). Inhibition of formins with pan-formin inhibitor SMIFH2 severely impaired cell motility and filopodial morphology, though reducing the levels of single formins by RNA interference did not, pointing to redundancy [off-target effects of SMIFH2 on myosins could confound these results (Y. Nishimura et al. 2021)]. Supporting flexibility in molecular mechanisms within native contexts, the Arp2/3 complex was shown to promote cell motility and formation of branched filopodia in this system (Bischoff et al. 2021).

Another case of filopodia providing mechanical coupling across a tissue comes from experiments with explants from the *Drosophila* ovary, where interdigitating filopodia connect and organize stress fibers across cells (Popkova et al. 2020). Cdc42, N-WASP, and Dia are all involved in making the egg chamber follicle cell filopodia. Photoactivatable Cdc42 and a dominant-negative mutant used across clusters of cells (clones) showed that altered activities in modified cells propagate to adjacent cells. Ena could rescue the effects of dominant-negative Cdc42, indicating that filopodia themselves, rather than the particular molecule, are important (Popkova et al. 2020). Roles of filopodia in cell-to-cell adhesion and in linking supracellular actin cables thus confer tissue-level organization that is important for function.

Understanding how molecular functions result in wider tissue-level changes is important for considering how the manipulation of filopodia could have therapeutic value. For example, myosin-X is associated with poor outcomes in metastatic cancer (Arjonen et al. 2014), but in a model of pre-invasive breast cancer, knockdown of myosin-X makes cells more invasive in mouse xenografts, possibly due to downstream effects on ECM architecture or upregulation of FMNL3 (Peuhu et al. 2022). Heterogeneous roles of core regulators such as myosin-X in vivo may mean that they have less potential as targets than cell-type-specific activities.

FUTURE PERSPECTIVES AND CONCLUSIONS

As advanced microscopy tools become more available, combined with quantitative, deep learning–equipped image analysis and mathematical modeling, the complex and dynamic migratory processes organisms use will become open to precise molecular understanding. Endogenous CRISPR/Cas9 knockouts, knock-ins, and base editing are beginning to link genomic and molecular regulation to cellular morphology and allowing filopodial components to be functionally characterized in combination. Where global regulatory networks mean that perturbations are likely to have nonlinear effects, perturbation-free approaches such as fluctuation analysis (Welf & Danuser 2014) or acute interventions with small molecules or optogenetics are particularly important. Visualizing actin filament architecture alongside actin regulator organization, such as by cryo-electron tomography, molecular fitting, and correlative light microscopy or by use of novel actin and other direct probes with super-resolution microscopy, will provide key molecular detail underlying cellular behavior. Together with explant and 3D culture systems that allow us to consider filopodia in context as dynamic structures dependent on their mechanical and signaling environments, these powerful tools are opening up the study of filopodia so that we can answer key questions: How are their dynamic and physical properties determined at the molecular level? How do their activities intersect with mechanical properties of tissues? Are disease-related filopodia (such as in cancer or infection) viable drug targets? Cells control filopodia for specific ends and needs: Let us learn from the cell what is possible.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We acknowledge Jonathan Gadsby, Julia Mason, and Mary-Pia Jeyarajasingham for helpful comments on the manuscript and the Wellcome Trust for funding a Senior Research Fellowship 219482/Z/19/Z to J.L.G.

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