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An Updated Look at Actin Dynamics in Filopodia

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Cells dynamically interact with and probe their environment by growing finger-like structures named filopodia. The dynamics of filopodia are mainly caused by the actin rich core or shaft which sits inside the filopodial membrane and continuously undergoes changes like growth, shrinking, bending, and rotation. Recent experiments combining advanced imaging and manipulation tools have provided detailed quantitative data on the correlation between mechanical properties of filopodia, their molecular composition, and the dynamic architecture of the actin structure. These experiments have revealed how retrograde flow and twisting of the actin shaft within filopodia can generate traction on external substrates. Previously, the mechanism behind filopodial pulling was mainly attributed to retrograde flow of actin, but recent experiments have shown that rotational dynamics can also contribute to the traction force. Although force measurements have indicated a step-like behavior in filopodial pulling, no direct evidence has been provided to link this behavior to a molecular motor like myosin. Therefore, the underlying biochemical and mechanical mechanisms behind filopodial force generation still remain to be resolved. © 2015 Wiley Periodicals, Inc.

Key Words: filopodial dynamics; membrane-cytoskeleton interactions; retrograde flow; helical buckling; filopodial rotation; embryonic compaction; mesenchymal cells; HEK293 cells

Introduction

The boundary of a cell continually undergoes rapid remodeling of the membrane and cytoskeleton to facilitate dynamic interactions with the extracellular matrix and other cells. During migration the lamellipodium propagates forward and transient finger-like spikes, named filopodia, protrude from the lamellipodium. Filopodia enable cells to interact with and probe their surroundings and provide them with information about their physical and chemical environment. Several important functions seem to rely on the formation of filopodia, for instance neuronal guidance [Lowery and Van Vactor, 2009]. and wound healing [Vasioukhin et al., 2000]. Filopodia have also been found to assist in the uptake of bacteria in macrophages [Moller et al., 2013] and to facilitate efficient viral infection between cells [Sherer et al., 2007] Recently, new types of filopodia have been discovered in embryonic cells which are critical for the correct coordination of embryonic development [Sanders et al., 2013] (see Fig. 1). Importantly, certain cancer cells contain large numbers of filopodia putatively due to the importance of filopodia in efficient cellular migration and adhesion [Arjonen et al., 2011]. We note that the length of visible filopodia in cells range from ~1 μm to ~100 μm and that several subclasses have been defined. Very short filopodia are often called microspikes while longer filopodia forming long distance intercellular bridges are called cytonemes [Sanders et al., 2013] or tunneling nanotubes (TNTs) [Davis and Sowinski, 2008] which can be regarded as a subclass of canonical filopodia. Other protrusive structures like invadopodia and podosomes, which are structures that facilitate enzymatic degradation of the extracellular matrix during invasion [Murphy and Courtneidge, 2011], are more distantly related to filopodia. Invadopodia can be distinguished from filopodia by their long-lived projections into the surrounding tissue and by the enzymatic secretion at their tip used for degradation of extracellular tissue. This review focuses mainly on how the dynamic behavior of filopodia is linked to the internal actin structure with emphasis on the most recent findings. For comprehensive and broader reviews on similar topics we refer the reader to previous reviews [Mattila and Lappalainen, 2008a; Lowery and Van Vactor, 2009; Bornschlogl, 2013].

Abbreviation used: TEM, transmission electron microscopy; TNT, tunneling nanotube.

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Structure and Composition of Filopodia

Here we review some of the most prominent components found within filopodia and their role in filopodial dynamics

such as growth and retraction. For further information regarding linear growth and retraction of filopodia we refer to a recent review [Bornschlogl, 2013] in which retrograde flow and filopodial growth and retraction velocities are carefully reviewed. The main component in filopodia is filamentous actin which is bundled into parallel arrays by, e.g., fascin cross linkers with the barbed end of actin pointing towards the tip, as sketched in Fig. 2A [Small and Celis, 1978; Small et al., 1978; Small, 1981; Khurana and George, 2011]. In other cells, like epithelial cells, villin, fimbrin or espin cross-linkers are responsible for bundling the actin [Khurana and George, 2011]. Unlike fascin, villin cross-linkers have the ability to link the actin to the plasma membrane as well [Kumar et al., 2004; Khurana and George, 2011]. Transmission electron microscopy (TEM) images have revealed detailed information regarding the actin arrangement, its continuous structure, and the number of actin filaments within a filopodium (typically around 10–30 filaments) [Svitkina et al., 2003]. In slow growing filopodia the actin filaments can be continuous all the way from the lamellipodium to the filopodial tip [Svitkina et al., 2003]. However, recent Cryo-TEM experiments have shown that the actin inside dynamic filopodia from *Dictyostelium discoideum* cells can be discontinuous in regions along the axis, thus suggesting that some biochemical interactions can trigger a change in the actin structure [Medalia et al., 2007; Ben-Harush et al., 2010; Breitsprecher et al., 2011]. Actin severing proteins like gelsolin and cofilin are known to trigger actin depolymerization inside filopodia; neuronal cells devoid of gelsolin exhibit delayed retraction, thus indicating the role of gelsolin in the disassembly and retraction of filopodia [Lu et al., 1997]. Cofilin, which has recently been detected in the filopodia of several cell types [Breitsprecher et al., 2011; Sanders et al., 2013], is involved in disassembly of retracting filopodia, as shown in Figs. 2C–2E [Breitsprecher et al., 2011]. Although the exact role of cofilin in filopodia is not yet entirely understood this protein could be a key player in the mechanical regulation of filopodia through severing of actin filaments. These results suggest that substantial reorganization and regulating mechanisms occur in the filopodial actin shaft during the protrusion-retraction cycle.

Other molecular players important in filopodial dynamics and structure, some of which are depicted in Fig. 2A, include the membrane shaping proteins containing an IBAR domain [Zhao et al., 2011; Linkner et al., 2014], myosin-X [Zhang et al., 2004], as well as β3-integrins [Hu et al., 2014], and E-cadherins [Fierro-Gonzalez et al., 2013] which can function as linkers between the actin and the external substrate. Factors that promote filament elongation are ENA/VASP proteins which are found at the filopodial tip. They exhibit an anti-capping activity and prevent the branching of actin [Han et al., 2002; Krause et al., 2003; Kwiatkowski et al., 2007]. Another protein which is critical for filopodia formation and elongation is

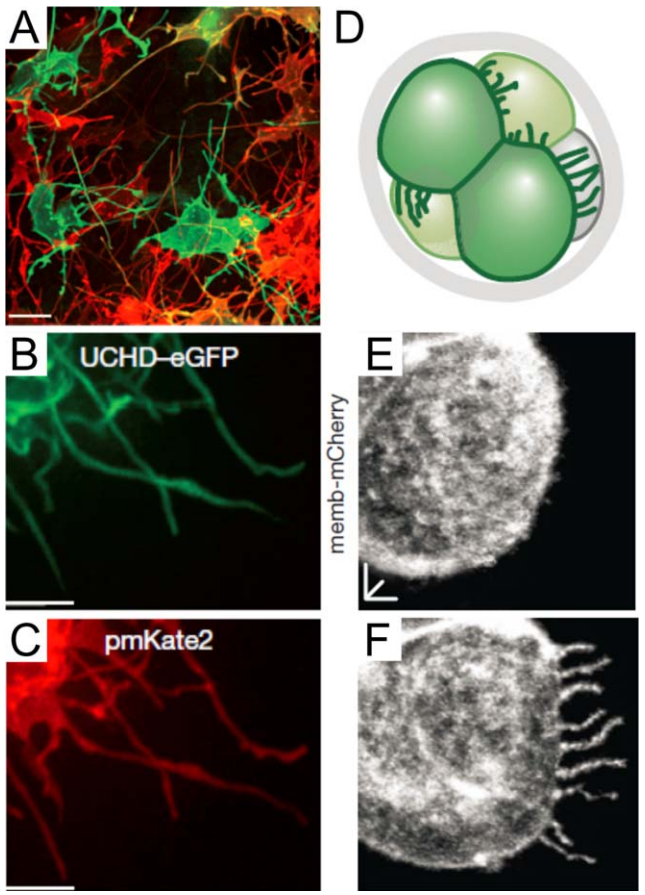

Fig. 1. Newly discovered biological functions of filopodia in mesenchymal cells of the developing mouse limb bud and in mouse embryonic cells. (**A**) Mesenchymal cells display long cytoplasmic projections which are a special type of filopodia that can span up to several cell diameters. The green and red colors represent pmeGFP and pmKate2 which are palmitoylated fluorescent proteins targeted to the membrane and used to label distinct cells with green and red color, respectively. (**B**,**C**) Visualization of the F-actin with UCHD-eGFP (Utrophin Calponin Homology Domain fused to enhanced Green Fluorescent Protein) (B) and the membrane with pmKate2 (C) shows that actin filaments extend all the way to the tip of the filopodium. Scale bar, 3 μm. Reproduced with permission from [Sanders et al., 2013]. (**D**) E-cadherin dependent filopodia control compaction in the mouse embryo. At the 8-cell stage the embryo undergoes a necessary compaction step which depends critically on the growth of E-cadherin dependent filopodia into neighboring cells. (**E**,**F**) Fluorescent images of the cell membrane of a single cell from a mouse embryo (as sketched in D) show that these filopodia are slowly growing. (E) is taken at $t = 0$ min just prior to any filopodia formation (scale bar, 5 μm) and (**F**) is after $t = 220$ min. Figure reproduced with permission from [Fierro-Gonzalez et al., 2013].

the formin Dia2 (diaphanous-related formin-2) which promotes filament elongation [Schirenbeck et al., 2005; Mattila and Lappalainen, 2008b] and has been found to synergistically enhance filopodia elongation at the tip together with VASP in *Dictyostelium discoideum* cells [Schirenbeck et al., 2006]. Other types of the formin protein family have been found at the filopodial tip like FMNL2

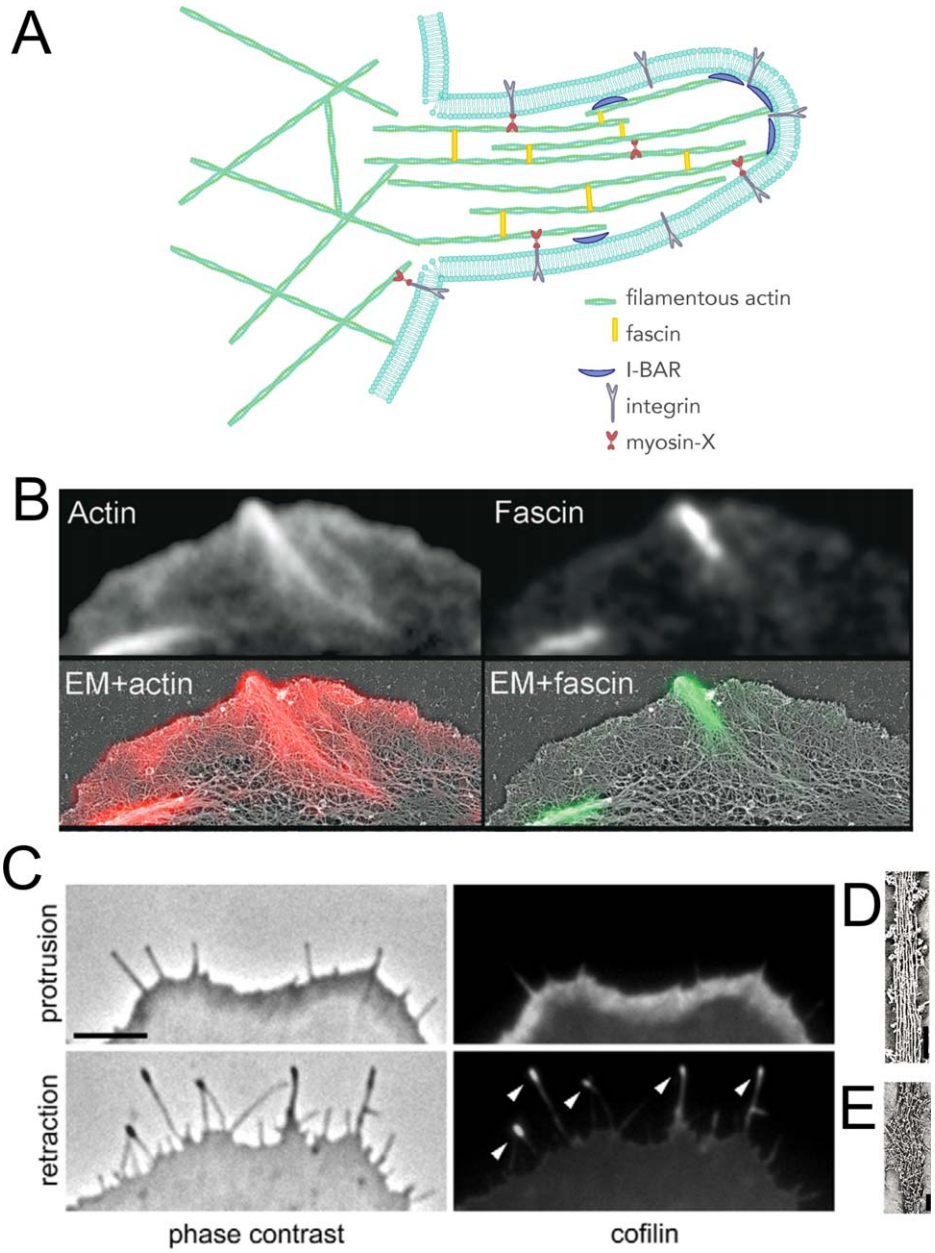

Fig. 2. Internal composition and structure of filopodia. (**A**) Schematic depiction of several important proteins found in filopodia. A bundle consisting of ~10–30 actin filaments is cross-linked by fascin into a stiff structure. Membrane shaping proteins like IBARs bind to the membrane and are associated with initiation of filopodia formation. Transmembrane proteins such as integrins link the actin to extracellular substrates to facilitate force transduction onto substrates. Myosin-X transports membrane components, like integrins, to the distal filopodial tip, but is also suggested to be involved in initiation of filopodia formation by collecting several actin filaments to a single spot (converging filament mechanism). Other important proteins not shown here include the tip complexes mDia2, ENA/VASP as well as cofilin. (**B**) Fluorescence and electron micrographs of filopodia from B16F1 melanoma cells. Top row shows fluorescent images of actin filaments (left) and fascin (right) in the protruding filopodium. Lower row shows an overlay of EM and fluorescence images of actin and fascin. The images reveal high concentrations of fascin in the actin bundle. Reproduced with permission from [Svitkina et al., 2003]. (**C**) Top row shows protruding filopodia and the bottom row shows retracting filopodia. Cofilin clearly localizes to the distal end of retracting filopodia whereas protruding filopodia do not contain significant densities of cofilin. Scale bar, 5 μm. (**D**,**E**) Corresponding EM images reveal that protruding filopodia (D) contain an actin bundle of continuous actin filaments whereas retracting filopodia (E) contain more disorganized and discontinuous actin filaments. Scale bars, 100 nm. Reproduced with permission from [Breitsprecher et al., 2011]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

which also has a function in elongating actin filaments in cooperation with profilin [Block et al., 2012]. For further information on the molecular composition of filopodia we refer to other reviews focusing on the composition of filopodia [Mattila and Lappalainen, 2008b; Faix et al., 2009; Khurana and George, 2011].

Force Generation in Filopodia Is Actin Mediated

Several studies have confirmed the dynamic behavior of actin in filopodia which involves polymerization at the tip [Mallavarapu and Mitchison, 1999], retrograde flow [Aratyn et al., 2007; Bornschlogl et al., 2013], rotation [Tamada et al., 2010; Zidovska and Sackmann, 2011; Leijnse et al., 2015), and bending [Nemethova et al., 2008]. Disruption of actin efficiently suppresses filopodial dynamics as reported in several studies [Kress et al., 2007; Khatibzadeh et al., 2012; Fierro-Gonzalez et al., 2013; Leijnse et al., 2015]. Since the actin bundle exists within a tubular membrane a necessary condition for force generation is that the actin is linked to the external substrate by transmembrane linkers like integrins or E-cadherins. Cellular filopodia on continuous 2D substrates from embryonic chick forebrain neurons generate substantial traction which was quantified by using deformable substrates with embedded tracer particles [Chan and Odde, 2008]. Traction was observed as transient events due to molecular 'clutches' linking the actin to the substrate across the filopodial membrane.

The traction force generated by filopodia has been shown to depend on the number of linkages with the external substrate. Optical trapping of integrin coated beads at the tip of filopodia revealed that epithelial cancer cells pull with higher force on beads with higher density of integrin [Romero et al., 2012] which clearly demonstrates that filopodia dynamically sense and respond to changes in the density of extracellular ligands.

The range of forces exerted by filopodia has been measured to span from pico-Newtons into the nano-Newton range which likely reflects their combined sensory and mechanical function. In Kress et al. (2007), filopodial traction was found to slow down with increased load in macrophages where filopodia were tracked in 3D with a trapped bead at the tip. Interestingly, the function of filopodia apparently includes interactions with pathogens. Surface attached bacteria were found to be shoveled off surfaces and pulled towards the cell body by macrophage filopodia [Moller et al., 2013]. Also, filopodia have been shown to interact with artificial nano-topographies and to align and bend silicon nanowires by pulling with nano-Newton forces [Albuschies and Vogel, 2013].

Optical trapping studies have provided important information regarding the mechanism of force generation. In several studies, membrane tethers have been pulled and held or further extended using an optically trapped bead bound to the membrane [Raucher et al., 2000; Pontes et al., 2011; Khatibzadeh et al., 2012; Farrell et al., 2013; Pontes et al., 2013]. Actin has been observed in membrane tethers in some studies [Pontes et al., 2011, 2013] by fixation and subsequent staining using phalloidin. No actin was observed in, e.g., membrane tethers pulled from NIH-3T3 fibroblasts [Raucher et al., 2000]. It is not clear from the present literature whether membrane tethers artificially pulled from cells contain actin or not. Also, in a typical experiment, it is very hard to distinguish whether one might be pulling a pre-existing very short and bent filopodium or whether one is truly pulling from a flat part of the membrane. In two recent works existing filopodia were pulled by attaching them to optically trapped beads and the filopodia were further elongated while the actin dynamics was imaged using proteins tagged with GFP [Bornschlogl et al., 2013; Leijnse et al., 2015]. The force extension curves in all such pulling experiments show an initial energy barrier due to the adhesion of the actin to the cytoskeleton followed by a stationary level determined by the membrane tension [Khatibzadeh et al., 2012; Pontes et al., 2013]. The forces exerted by extended filopodia remain more or less constant for up to 100 s after which transient load-and-fail behavior occurs, see Figs. 3B and 3C. The pulling on the trapped bead by the filopodium appears to be correlated with the presence of actin near the tip, indicating the important role of the actin shaft in force transduction [Bornschlogl et al., 2013; Farrell et al., 2013; Leijnse et al., 2015].

In Bornschlogl et al., (2013), the dynamics of actin in a filopodium was imaged using confocal fluorescent microscopy while the force was measured simultaneously with an optically trapped bead at the tip, see Fig. 3A. These experiments revealed that the force on the bead depended on the difference in the rate of actin polymerization at the tip and retrograde flow. Retrograde flow was imaged by bleaching the actin near the tip as well as at the cell cortex from which correlative movement of the bleached regions was inferred. Other studies have also documented the presence of retrograde flow in filopodia [Aratyn et al., 2007]. The driving force behind the retrograde flow could be polymerization at the tip region and a pulling mechanism within the lamellipodium like the suggested frictional coupling mechanism [Bornschlogl et al., 2013]. Notably, several studies have revealed step-like behavior in the force curves of filopodia [Kress et al., 2007; Farrell et al., 2013; Leijnse et al., 2015], however, in no study has this behavior been linked to a specific motor. Interestingly, inhibition of myosin-II, using the small molecule myosin-II inhibitor blebbistatin, did not suppress the step-like behavior [Kress et al., 2007].

The forces that were measured for filopodia mostly involve transient load-and-fail behavior superimposed on the background holding force needed to counteract the membrane tension. Membrane tension has been suggested to be a master regulator of several membrane associated processes like endo- and exocytosis [Diz-Munoz et al., 2013], but could also significantly influence filopodial activity. Localized laser ablation of filopodia revealed that filopodia sustained interfacial tension in the apical membrane within the mouse embryo which facilitated cell flattening during compaction of the embryo [Fierro-Gonzalez et al., 2013].

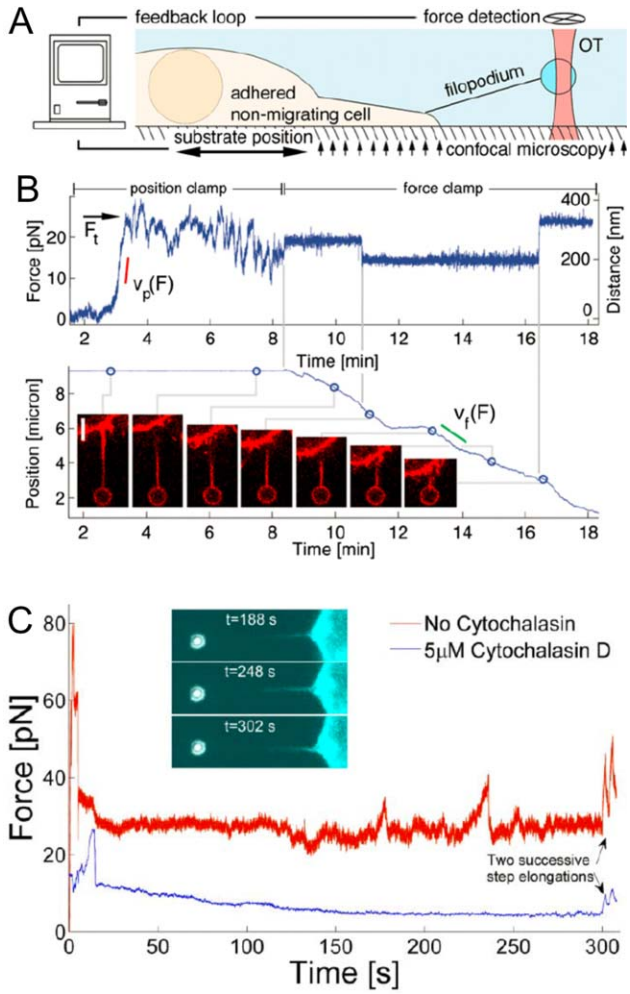

Fig. 3. Force dynamics in filopodia held by an optically trapped bead. (**A**) Monitoring the position of an optically trapped particle at the tip of a filopodium allows the force to be measured [Bornschlogl et al., 2013]. (**B**) Simultaneous force measurement (top panel) and imaging (lower panel) of the filopodial membrane using the lipophilic dye FM4-64. The optical trap can be operated in either force clamp (constant force) or position clamp (constant position) mode. Figure reproduced with permission from [Bornschlogl et al., 2013]. (**C**) Force curves for two filopodia that were extended to a length of 10 μm. The force was monitored for 300 s after the extension. The red curve is the force when the actin was not disrupted, whereas the blue curve represents the force when the actin was disrupted using 5 μM cytochalasin D. At $t = 300$ s the filopodia were rapidly further elongated with 1 μm/s twice resulting in an increase in the dynamic force. Inset images show that the dynamics of the actin (GFP-utrophin) does not change even after >300 s when disrupted using cytochalasin D. Figure reproduced with permission from [Leijnse et al., 2015]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

During formation of filopodia a significant energy barrier needs to be overcome to bend and protrude the plasma membrane which is under tension. Initiation of the protrusion has been suggested to occur via two main mechanisms: (i) a cooperative pushing by converging actin filaments (Lambda precursor mechanism) [Zhao et al., 2011], or (ii) a de novo formation mechanism [Mattila and Lappalainen, 2008b]. Other actin independent mechanisms for tube extensions have been identified such as membrane shaping due to binding of IBAR domain containing proteins [Zhao et al., 2011; Linkner et al., 2014], and membrane tubulation via the water transporting membrane protein, aquaporin, that may have a critical role in the formation of filopodia-like tubular extensions [Karlsson et al., 2013]. However, it is not straightforward to identify a single protein responsible for initiating filopodia formation in cells since filopodia-like structures can form in model membrane systems in the presence of, e.g., only actin or IBAR [Liu et al., 2008; Zhao et al., 2011; Linkner et al., 2014]. Although the role of IBAR domain proteins and aquaporin is poorly understood in the context of filopodia formation it is possible that these proteins function synergistically with actin and other actin associated proteins to form cellular filopodia.

Buckling of Filopodia

Filopodia are known to undergo buckling which has been monitored using fluorescence microscopy [Forscher and Smith, 1988; Schaefer et al., 2002; Nemethova et al., 2008]. Buckling behavior has been modeled by considering the actin as a rigid bundle of N_{fil} filaments within a membrane protrusion which exerts an axial load on the actin bundle [Pronk et al., 2008; Daniels and Turner, 2013]. These models predict a stable helical buckling conformation of $N_{\mathrm{fil}} = 6$ not cross-linked filaments inside a filopodial tube with a radius of $R \sim 100$ nm [Pronk et al., 2008] as shown in Fig. 4C.

The membrane tension is most likely an important factor when filopodia buckle during retraction, however, the helical conformation, as sketched in Fig. 4, has not been experimentally verified yet. Although axial compression by the membrane tension would tend to buckle the actin, it is interesting to note that a recent experiment showed that this effect was not required: A helical conformation of the actin shaft was experimentally measured in filopodia extended from HEK293 cells using an optical trap as shown in Figs. 5D and 5E [Leijnse et al., 2015]. When filopodia are held steady by an optical trap no buckling should be caused by axial load from the membrane tension, nevertheless, buckling was observed, thus suggesting a different mechanism contributing to the buckling of actin in filopodia. Therefore, a mechanism that combines torsional twist of the actin shaft with traction was put forward to explain the observed simultaneous buckling and traction [Leijnse et al., 2015]. The proposed mechanism is similar to the pulling and bending that arises when twisting a rubber band at one end and keeping the other end fixed.

The frequent kinks observed in filopodia indicate that the actin structure is weakened somewhere along the shaft which makes it more prone to buckling at certain locations.

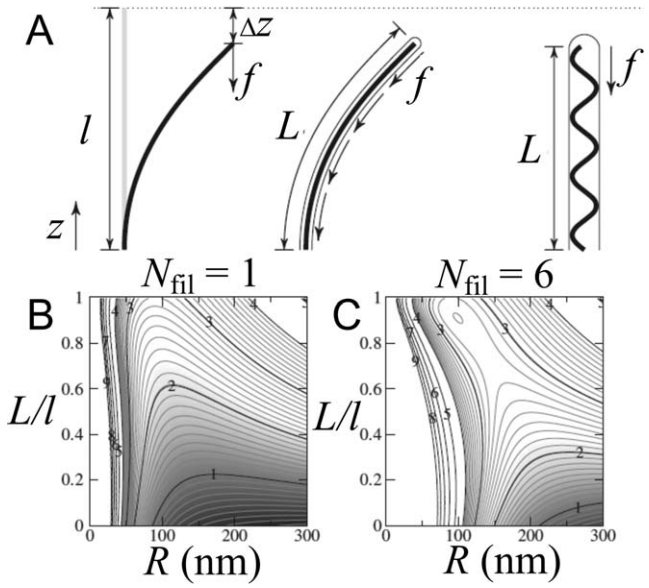

Fig. 4. Theoretical prediction of helical buckling of actin inside filopodia. (**A**) Depiction of a single actin filament buckling under an axial compressive force, *f*, inside a membrane tube of length *L*. The contour length of the actin filament is *l*. (**B**) Energy diagram (unit is K_BT/nm) for a single actin filament plotted for different relative shortenings (L/l) and different radii of the membrane tube. Darker colors represent lower energies. (**C**) Same as (B) but for six parallel actin filaments. An energy minimum exists for $R \sim 100$ nm and $L/l \sim 0.95$. Reproduced with permission from [Pronk et al., 2008].

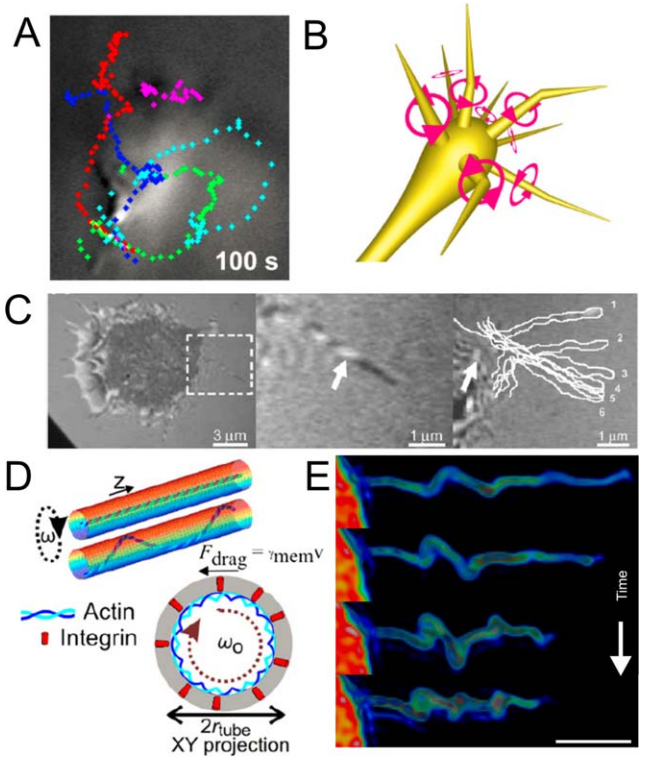

Fig. 5. Rotational dynamics in filopodia. (**A**) Filopodia in neurites rotate in a right-screw direction. The tip of the filopodium is tracked in neuronal growth cones embedded in a collagen gel. The angular velocity was found to be ~0.1 rad/s. Figure reproduced from [Tamada et al., 2010]. (**B**) Schematic drawing of the rotational behavior of filopodia in (A). (**C**) Macrophages also have rotating filopodia which rotate with 1.2 rad/s. The images are acquired using reflection interference contrast microscopy which exploits the change in contrast with height. Figure reproduced with permission from [Zidovska and Sackmann, 2011]. (**D**) Model of actin rotating within filopodia of HEK293 cells. Attachment of the actin to the filopodial membrane generates friction, consequently torsional twist accumulates in the actin shaft and this can induce buckling of the shaft. (**E**) Three dimensional image of helical buckling of the actin shaft within a filopodium in a HEK293 cell. The image is acquired using actin labeled with GFP-utrophin and reconstructed as a 3D image from a stack of confocal images. Scale bar, 3 µm. Figure is reproduced with permission from [Leijnse et al., 2015].

As shown in Fig. 2D,E [Breitsprecher et al., 2011] the actin filaments within filopodia can indeed be discontinuous, hence, the structure can be weaker in some regions. Also, this study showed that cofilin could induce discontinuities in the actin structure and cause rapid integration of retracting filopodia into the lamellipodium.

Rotation of Filopodia

Filopodia exhibit rotational motion which is most clearly observed in live cell microscopy when rotation occurs simultaneously with bending, see Fig. 5 [Tamada et al., 2010; Zidovska and Sackmann, 2011; Leijnse et al., 2015]. The mechanism behind the rotational motion has been ascribed to the spiral interaction between myosin-V and actin [Ali et al., 2002] occurring at the base of the filopodium [Tamada et al., 2010]. The frequency of rotation has been measured to be 1.2 rad/s for macrophages in normal cell culture [Zidovska and Sackmann, 2011] and a somewhat slower angular speed of ~0.1 rad/s was measured for growth cone filopodia in a collagen gel [Tamada et al., 2010]. Interestingly, the right-screw direction in growth cone filopodia was found to drive the turning of neurites [Tamada et al., 2010].

Two different modes of rotational behavior can be distinguished in filopodia, (i) a sweeping motion in which the tip performs a circling motion with the filopodium fixed at the base [Zidovska and Sackmann, 2011; Bornschlogl, 2013], and (ii) rotational motion around the axis of the filopodium [Tamada et al., 2010; Leijnse et al., 2015]. These two different modes can be difficult to discern and might also be superimposed in a filopodium performing a sweeping motion while simultaneously rotating around its own axis. Protruding filopodia typically appear more straight and a possible rotation is therefore more difficult to detect than in retracting filopodia which typically disassemble or fold back during integration into the lamellipodium.

Recently, it was shown that the rotation of an actin bundle within a membrane tube contributes to buckling of the actin shaft (as shown in Figs. 5D and 5E) [Leijnse et al., 2015].

The rotation of the actin shaft within the membrane tube generates friction, hence, torsional twist is accumulated within the actin shaft [Wada, 2011]. The frictional coupling between the membrane and the actin originates from transmembrane integrins [Hu et al., 2014], but also other proteins could be linking the actin to the membrane in different cell types, e.g., myosin-I [Conrad et al., 1993], myosin-X [Zhang et al., 2004], VASP [Breitsprecher et al., 2008] or E-cadherin [Fierro-Gonzalez et al., 2013]. The biological role of actin bending and rotational/sweeping dynamics within filopodia seems to involve steering of neuronal growth cones, traction generation, and enabling cells to explore a larger space during their mechanical and chemotactic sensing of the environment.

Conclusion and Future Directions

Recently, much information has been gained regarding the composition as well as the active dynamics of filopodia and the pivotal role of actin in force transduction is now well established. However, the molecular mechanisms behind rotation and traction still remain to be elucidated. Technological improvements in microscopy combined with genetic manipulations of cells will open up exciting new possibilities to achieve a better understanding of filopodial function. Superresolution techniques can image biological material with a resolution down to ~50 nm and will therefore offer a way to explore the nanostructure of the actin during elongation, retraction, and bending. Also, it will be exciting to explore the dynamic localization of fascin and integrin inside filopodia in parallel with imaging the actin structure. The origin of the observed discrete pulling force still remains to be resolved and we envisage that a comprehensive screening with myosin knock-out experiments could reveal if the pulling mechanism and the discrete steps observed can be ascribed to any myosin motor. Also, more work needs to be done to understand mechanotransduction and signaling in filopodia, this could be achieved, e.g., by coating optically trapped beads with extracellular components, which trigger signaling within the filopodia and activate a cytoskeletal response, while measuring the resulting force on the bead. Proteins like formins which control actin polymerization at the tip [Zhuravlev and Papoian, 2009] have been found to sense and generate forces in vitro [Jegou et al., 2013] and need to be further studied with regard to mechanosensing in filopodia. Finally, we note that discoveries of filopodia in cells like mesenchymal cells expand our knowledge of the diverse biological functions of filopodia and future experiments will most likely reveal other types of specialized filopodia which remain to be explored for their function and composition.

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