The making of filopodia

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Summary

Filopodia are rod-like cell surface projections filled with bundles of parallel actin filaments. They are found on a variety of cell types and have been ascribed sensory or exploratory functions. Filopodia formation is frequently associated with protrusion of sheet-like actin filament arrays called lamellipodia or membrane ruffles, but as compared to the latter, the molecular details of initiation and maintenance of filopodia are just beginning to emerge. Here we summarize recent advances in our understanding of the molecular requirements for filopodia protrusion, compare them with lamellipodia, and discuss prominent models on the inter-relationships between these two 'sub-compartments' of the protrusive actin cytoskeleton.

Introduction and terminology

The exploration of new space during cell migration is mediated by protrusion of actin-rich organelles at the cell front, followed by their adhesion to extra-cellular matrices or cells and by cell body translocation. The best-characterized protrusive structure is the lamellipodium, comparable to the "pseudopod" in *Dictyostelium*, which is built of a dense meshwork of branched or crosslinked actin filaments [1,2]. Continuous lamellipodia protrusion and ruffling is frequently accompanied by the formation of bundles of parallel actin filaments, most frequently termed filopodia [3], which are particularly prominent in individual cells such as fibroblasts or axonal growth cones, but also at the free front of migratory tissue sheets. Well-known examples for the latter are found during developmental stages in *Drosophila* and *C. elegans*, called dorsal closure and ventral enclosure, respectively, in which filopodia from opposing tissue layers approach each other and contribute to the zippering of these epithelial layers [4]. B16-F1 melanoma cell or fibroblast lamellipodia frequently contain actin filament bundles remaining embedded in these structures during continuous protrusion, which we refer to as microspikes [3] and which can develop into filopodia by protruding beyond the lamellipodium edge [●●5 and Figure 1b]. According to this definition, microspikes never bend to protrude beyond the xy-plane of the lamellipodium, while filopodia frequently do. Anchorage of filopodial shafts in substrate contacts called focal complexes [6] may support the latter behaviour, while microspikes have been implicated in the initiation of these nascent contact sites below the lamellipodium [2]. Since the orientation of the large majority of microspike bundles is not perpendicular to the lamellipodium front, their protrusion, which is exclusively driven by actin polymerization at their tips [7], is accompanied by significant lateral movement with respect to the substrate and the lamellipodium, with rapid changes in direction, thereby causing frequent collision and fusion of individual microspikes or filopodial bundles [3]. However, the molecular hardware driving this complex dynamic behaviour is largely elusive. Interestingly, non-protrusive peripheral actin filament bundles known as retraction fibres and frequently associated with the sides or rear of a migrating cell can develop into filopodia when protrusion resumes [●●5]. Other cellular processes that were proposed to be related to filopodia include myopodia on differentiating muscles cells [8], lymphocyte and brush border microvilli or stereocilia on cochlear cells [9,10].

Signalling to filopodia formation

Similar to other prominent cellular actin-based structures such as stress fibres or membrane ruffles, several signalling pathways have been proposed to drive the protrusion of filopodia.

Not surprisingly, most of them involve activation and subsequent effector binding of Rho-family GTPases [11]. The paradigm of such a signalling pathway in vertebrates certainly constitutes Cdc42-mediated filopodia formation [6], which was most frequently proposed to be driven by direct interaction with proteins of the WASP subfamily (haematopoietic WASP and ubiquitous N-WASP), well-established activators of Arp2/3-complex-catalysed actin filament assembly [12]. Initial studies have shown that Cdc42 activation, stimulated for instance by bradykinin treatment, causes microspike/filopodia formation [13], which in many cell types is amplified by simultaneous suppression of Rac activity [6]. Two lines of evidence suggested a direct link of Cdc42-activation to N-WASP-mediated actin polymerisation in or at the tips of filopodia. Firstly, co-expression of both proteins in COS cells generated exceptionally long filopodia [14], and secondly, *in vitro* experiments demonstrated the capability of Cdc42, in synergy e.g. with the phospholipid PIP2 [15], to unlock the auto-inhibited conformation of N-WASP leading to nucleation of Arp2/3-dependent actin filaments [reviewed in 12]. However, the initial expectation that filopodia are nucleated via a complex of Cdc42-WASP-Arp2/3 is not supported by more recent studies utilizing time-lapse microscopy and gene inactivation. As opposed to lamellipodia, Arp2/3-complex appears absent from microspike bundles in fibroblast and B16F1 lamellipodia [●●5,16]. Moreover, deletion of the N-WASP gene in murine cells did not abolish Cdc42-induced filopodia protrusion [17,18]. Notably, in line with observations on growth cone filopodia [●19], attempts to detect any specific enrichment of this protein at the tips of filopodia by employing both GFP-tagged N-WASP or antibodies specific for the endogenous protein, were so far unsuccessful (unpublished). In addition, WASP knockdown in *Drosophila* S2 cells by RNA interference did also not affect cell morphology, in particular lamellipodia and filopodia protrusion [●20]. Finally, careful analysis of cell surface alterations in T-cells lacking the haematopoietic WASP isoform revealed no observable defects in microvilli formation [9]. Instead, an increasing number of observations suggests that WASP/N-WASP rather function in trafficking processes and endocytosis, as exemplified e.g. by abrogation of T-cell receptor internalisation in $WASP^{-/-}$ T-cells [21] and reduced EGF receptor internalisation in $N\text{-}WASP^{-/-}$ cells [22]. Nevertheless, neither of these observations exclude a contribution of WASP-Arp2/3-mediated actin assembly to signalling processes eventually culminating in filopodia formation, but they preclude an essential contribution to actin polymerisation at filopodia tips (see also below).

More recently, a number of additional Cdc42 effectors have been implicated in signalling to actin assembly, and may therefore constitute promising candidates driving filopodia protrusion downstream of Cdc42. Of particular interest in this respect is the diaphanous related formin Drf3, called mDia2 in the mouse [23] (see also below), which was reported to bind to Cdc42, but also other Rho-family GTPases [24,●25]. Another link to filopodia formation may arise from a more detailed characterization of the Cdc42 effector insulin receptor substrate p53 (IRSp53), also known as IRS-58 [26], which through its interaction with Ena/VASP family members may contribute to efficient filopodia formation [27]. IRSp53 is now grouped into a protein family with MIM/ABBA subfamily proteins [●28]. All these proteins share an intriguing actin filament bundling activity at their N-termini mediated by the so called IMD-domain, whose over-expression can reportedly induce filopodia formation [●28,●29]. Finally, similar to group A p21-activated kinases (e.g. PAK1), group B PAKs like PAK4 and PAK5, which preferentially bind to Cdc42, have also been implicated in filopodia formation, although the molecular mechanism linking these kinases to actin assembly remained elusive [30].

Besides Cdc42, activation of several of the 22 mammalian Rho-GTPases has recently also been described to induce filopodia formation [31], such as the Cdc42 subclass GTPase Wrch-1 or RhoD and Rif, the latter of which can interact with mDia2 proposed to drive filopodia formation induced by this GTPase [●25]. In agreement with these observations, genetic deletion of Cdc42 does not abolish filopodia formation [●●32]. Thus, Cdc42 loss of function in signalling to filopodia protrusion can either be compensated for by expression of other Rho GTPases or by GTPase-independent pathways. For instance, increased c-Abl tyrosine kinase activity has recently been correlated with increased filopodial persistence and neurite branching [33], which was unaffected by over-expression of dominant-negative Cdc42. More recent data employing murine knockout cell lines demonstrated significantly reduced filopodia numbers during cell spreading in the absence of the Abl tyrosine kinases Abl and Arg (Abl related gene), the Abl substrate p62 docking protein (Dok1) and its interaction partners Nck1 and Nck2 [●34]. Hence, Abl-mediated Dok1 phosphorylation may trigger its association to SH2-domains of Nck adaptors contributing to filopodia protrusion. However, the molecular link of this signalling pathway to filopodial actin assembly remained uncertain. A very recent study suggests an alternative signalling pathway to filopodia protrusion in *Xenopus* neuronal growth cones, also involving tyrosine phosphorylations, but this time including both Cdc42 signalling and phosphorylation of Src-family kinase substrates such as

PAK1 [●19]. Again, a direct link to actin assembly at filopodia tips remains to be established, but as opposed to earlier studies, the induction of tyrosine phosphorylation at filopodia tips seemed to directly correlate with protrusion, and hence the onset of actin assembly [35] and coincided with the accumulation of Src, Cdc42 and PAK in the filopodial tip complex [●19].

Together, an ever growing list of GTPases, kinases and adaptor proteins appear to associate with signalling pathways leading to the formation of these finger-like protrusions. However, the exact sequence of events - including the establishment of essential *versus* redundant factors – leading to engagement of a core actin polymerisation unit constituting the filopodial tip complex - is far from being unravelled. Such a challenging task should include comparison of key signalling pathways conserved in vertebrates and other, genetically more easily tractable model organisms such as *Drosophila* and *Dictyostelium*. Three Rac proteins (Rac1A, B and C) were previously shown to induce filopodia formation in *Dictyostelium* [36]. Interestingly, one of the *Dictyostelium* diaphanous related formins, dDia2 was shown recently to recruit to the tips of protruding filopodia (Figure 1 and see below) and to be essential for efficient filopodia protrusion, presumably acting downstream of Rac1A [●●37]. These data point towards a similar function for a formin such as mDia2 in filopodia protrusion in vertebrates [23,●25, see also Figure 2], although the relevance of this protein e.g. by gene deletion or RNAi experiments remains to be directly addressed.

The molecular hardware of filopodia protrusion

Our current understanding of the molecular mechanism of filopodia formation is still fragmentary. It appears that in many of the studied systems filopodia emerge from lamellipodia suggesting that the lamellipodium serves as a precursor structure [●●5,●20,38]. The dendritic nucleation model of lamellipodia protrusion suggests that continuous actin assembly occurs by branching of new filaments off the sides or tips of pre-existing filaments mediated by Arp2/3-complex and its regulators [39]. The filaments push the membrane forward as they elongate by polymerization, until growth is blocked by barbed end capping proteins. According to this model, these activities lead to a densely packed network of short, stiff and branched filaments exerting the force required for protrusion and motility.

The necessity of capping proteins for motility was also demonstrated in reconstituted systems using purified components. Arp2/3-dependent *in vitro* motility of *Listeria* and *Shigella* was dependent on capping proteins, which maintained a high G-actin pool required for unidirectional growth of actin filaments at the surface of the bacteria [40]. In this scenario

high Arp2/3 and capping activities promote the protrusion of lamellipodia, although excess capping activity for instance effected by depletion of the capping protein inhibitor mCARMIL blocked lamellipodia protrusion entirely [●●41].

Which activities then promote filopodium formation? In contrast to lamellipodia, filopodia lack Arp2/3-complex and contain bundles of parallel and linear actin filaments that grow at their tips [16,35]. This notion implies that lamellipodia and filopodia are formed by different molecular mechanisms triggered by separate pathways. Interestingly, formins which constitute molecular machines generating linear actin filaments have recently been localized to filopodia tips in mammalian and *Dictyostelium* cells [23,●25,●●37]. Moreover, formins were shown to reside at the barbed ends of individual actin filaments thereby acting as processive motors that can generate forces of ~1.3 pN/filament [●●42,●●43]. Finally, knock-out and overexpression studies in *Dictyostelium* revealed that filopodia formation directly correlates with expression of the Diaphanous-related formin dDia2 [●●37]. Since formins are capable of *de novo* nucleation and elongation of pre-existing actin filaments, it is conceivable that this class of proteins can generate cellular actin structures in the absence of other nucleators like the Arp2/3-complex. These data corroborate the view of independent formation of lamellipodia and filopodia, although formal proof for Arp2/3-complex independent filopodia formation is still missing.

Previous data however suggested an alternative mechanism of filopodia formation. Detailed studies employing electron microscopy and live cell imaging with GFP-fusion proteins led to the proposal of the convergent elongation model of filopodium formation [●●5]. It proposes coalescence and selective elongation of pre-existing lamellipodial filaments into bundles to form a filopodium. Notably, the existence of such a mechanism was demonstrated by Arp2/3-complex dependent *Listeria* motility, which could be shifted *in vitro* into Arp2/3-independent, solely elongation-based movement simply by Arp2/3-complex sequestration [●●44]. Filament growth in filopodia is initiated by assembly of a tip complex harbouring molecular activities promoting filopodia protrusion, such as uncapping and/or elongation. Proteins that could mediate uncapping include formins such as dDia2, which was shown to remove barbed end capping proteins *in vitro* [●●37]. The significance of low capping activity for filopodia protrusion was clearly demonstrated by massive induction of these structures upon capping protein depletion in both *Dictyostelium* and mammalian cells [45,●●46]. Moreover, several experiments suggested that promotion of actin filament assembly at the plasma membrane e.g.

by Ena/VASP proteins [47,48] stimulated filopodia protrusion [●49]. Hence, at first glance low capping activity and high rate of filament elongation both favour the conversion of peripheral actin structures into filopodia. However, the fact that capping protein depletion in Mena/VASP$^{-/-}$ cells did not simply reverse the capping protein knock-down phenotype, but stimulated membrane ruffling, points towards additional functions of Ena/VASP proteins in filopodia formation. Interestingly, Ena/VASP proteins were also shown previously not only to bundle actin filaments *in vitro* but also to be crucial for filopodia formation *in vivo* [50,●51]. Thus, bundling activity may be a key feature of filopodia formation, as also evidenced by the implication in this process of proteins such as IRSp53/MIM or fascin [52, see also Figure 2], although the relative contribution of all these proteins to filament bundling in filopodia remains to be established.

In addition to proteins required for nucleation, elongation and cross-linking of actin, there is increasing evidence for involvement of unconventional myosins in filopodium formation. Mammalian myosin-X accumulates at the tips of growing filopodia and its over-expression induces formation of these structures [●53]. Myosin-X interacts with ß-integrins through its FERM domain and downregulation of myosin-X by RNA interference affects integrin-mediated cell adhesion, which led to the proposal that myosin-X may productively link the actin cytoskeleton to integrins during cell adhesion or filopodia formation [54]. Although *Dictostelium* cells lack a true myosin-X orthologue, they express the related myosin-VII. Similar to myosin-X, myosin-VII is strongly enriched in filopodia tips, and *Dictyostelium* mutants lacking this protein display impaired adhesion, a decreased rate of phagocytosis and an almost complete lack of filopodia [●55]. Moreover, a recent search for myosin-VII binding proteins led to identification of the adhesion-associated talinA [56], which had been implicated in filopodia formation previously. Finally, interaction of myosin-X with Mena implicated a role in Ena/VASP delivery to filopodia tips [57]. Together, these results suggest that specific unconventional myosins function in actin-based transport of cell-adhesion molecules and possibly other cargo during adhesion and/or filopodia extension. Additional evidence for the role of unconventional myosins in cargo delivery to the tips of actin-filled protrusions has also been obtained from the analysis of stereocilia on hair cells in the inner ear. A number of myosins including myosin Ic, VI, VIIa and XVa are essential for proper stereocilia formation , and mutations in these genes lead to hearing loss and balance disorders in vertebrates and *Drosophila* [58]. Of these, only myosin-XVa is exclusively localized to the apices of stereocilia and was shown to target the PDZ domain–containing protein whirlin to

stereocilia tips - and also to the tips of filopodia upon expression of both proteins in COS cells. Most significantly, myosin-XVa-mediated whirlin recruitment required the respective interaction surfaces for each partner protein and a functional motor domain of the myosin [●●59]. Although the precise functions of most of these motor proteins still remain to be established, these findings strongly suggest that a plus-end directed motor activity is required for accumulation of constituents at the tips of stereocilia and filopodia.

Concluding remarks

In recent years we have witnessed significant progress in our understanding of the molecular regulation of protrusive cell-membrane structures like lamellipodia and filopodia. Several models for the mechanism of filopodia protrusion have been introduced. A key issue in the field is whether filopodia arise from lamellipodia or can form independently of the latter. As yet, several key modulators of actin filament dynamics have been implicated in affecting filopodia formation, e.g. capping and bundling proteins as well as filament nucleators such as formins. Key questions for future studies include assessment of the precise molecular mechanism of filopodia initiation (nucleation versus elongation) and/or the relevance of Arp2/3 complex-mediated actin filament assembly for filopodia formation. A full understanding may only come from establishment of the complete molecular inventory of filopodia and of the specific functions of the involved components. Finally, it will be exciting to determine the similarities and differences in the molecular mechanisms of filopodia, microvilli and stereocilia formation.

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Figure legends

Figure 1: Formins from different species are strongly enriched in filopodia tips. (**a**) A *Dictyostelium* cell expressing GFP-tagged full length dDia2 was fixed and labelled with anti GFP antibodies to visualize dDia2 (green) and with phalloidin to visualize F-actin (red). The merged image shows strongest dDia2 accumulation at the ends of filopodial actin bundles. Bar, 5 μm. (**b**) Phase contrast (left) and fluorescence (right) images of a mouse melanoma cell moving on laminin and expressing full-length human Drf3/mDia2 fused to EGFP. Note that filopodia, but not lamellipodia protrusion coincides with significant Drf3 accumulation. Representative frames from a time-lapse series. Time is in seconds and bar equals 2μm.

Figure 2: Schematic model depicting the molecular activities promoting the formation, protrusion and retraction of filopodia. Grey bars represent actin filaments. During the initiation phase, a filopodium tip complex (FTP) is assembled, presumably at or very close to the plasma membrane. Note that some FTP proteins (such as formins) accumulate exclusively at these sites, while others like Ena/VASP or IRSp53 family proteins are also found at the tips of lamellipodia (see Table 1). In addition, the FTP may contain proteins required for both actin filament nucleation and for reorganization of pre-existing filaments. The latter step is thought to include the removal of capping proteins from actin filament barbed ends and subsequent filament elongation by actin monomer addition (blue squares). Notably, *de novo* nucleation by factors other than Arp2/3-complex would have to be accompanied by rapid association to or anchorage into pre-existing cortical filaments. Force generation is mediated by actin polymerization at the tip, presumably driven by the action of formins, and by cross-linking of parallel nascent filopodial actin filaments by actin-bundling proteins (green bars). During later stages of filopodium protrusion, actin-bundling proteins such as fascin stabilize elongating actin filaments also in the shaft of the filopodium (red bars). Filopodium protrusion is positively and negatively regulated by filament elongation and actin rearward flow, respectively. Given that the rate of rearward flow in filopodia is more or less constant as observed in growth cone filopodia [35], net filopodium retraction occurs upon reduction or abolishment of tip elongation. For the sake of simplicity, the potential function of myosins is not depicted.

Table 1: Localizations and biochemical properties of selected actin binding proteins implicated in filopodia formation

Protein	Lamellipodium	Filopodium	Biochemical Activities	Potential Functions	Refs
Arp2/3-complex	tip + meshwork	-	- generates branched filament arrays	- proposed to generate filaments for filopodia elongation	[••5,16,•20, 39]
SCAR/WAVE 1 SCAR/WAVE 2/3	tip tip	- tip	- activate Arp2/3-complex - associate in multi-protein complexes *in vivo*	- proposed to generate lamellipodial filaments for filopodia protrusion - function at filopodia tips unknown	[3,12,•20,62]
Formins (mDia2/dDia2)	-	tip	- nucleate and elongate unbranched filaments - compete with capping proteins for barbed filament ends	- dDia2 essential for efficient filopodia formation	[•25,••37, ••42,••43]
Capping proteins	tip + meshwork	-	- block growth of filament barbed ends	- counteract (excess) filopodia formation	[••5,39,••41, 45,••46]
Ena/VASP family	tip	tip	- enhance actin polymerization and motility - reported to exert anti-capping, nucleation and bundling activity - reported to accelerate Arp2/3 dissociation from activator	- *Dictyostelium* VASP required for efficient filopodia formation - mammalian family members implicated in initiation and elongation of filopodia	[••5,47,48, •51,61]
Fascin	meshwork (weak)	tip and shaft	- bundles actin filaments	- implicated in stabilization of filopodial actin bundles	[••5,52]
IRSp53	tip	tip	- N-terminal IMD domain bundles actin filaments	- proposed to drive filopodia formation by bundling	[•28,•29,60]
Myosins (myosin-X, VII)	-	tip	- barbed end directed motor proteins	- proposed to transport cargo to filopodia tips	[•53,54,•55, 56]

Figure 1:

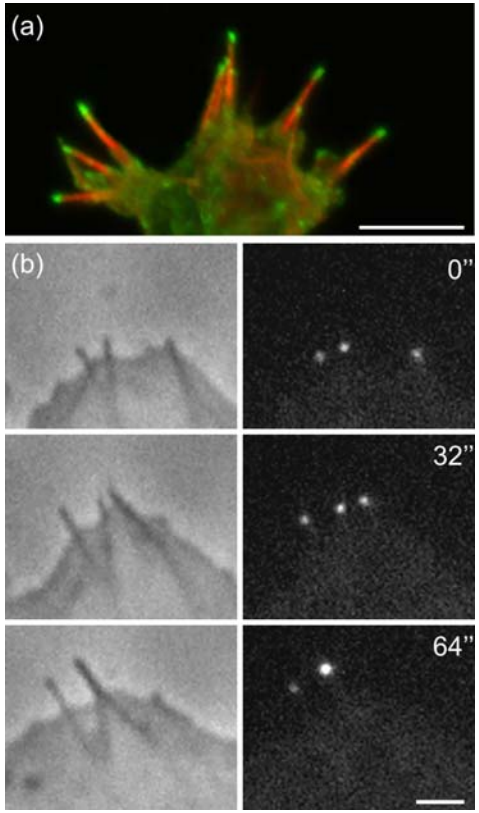
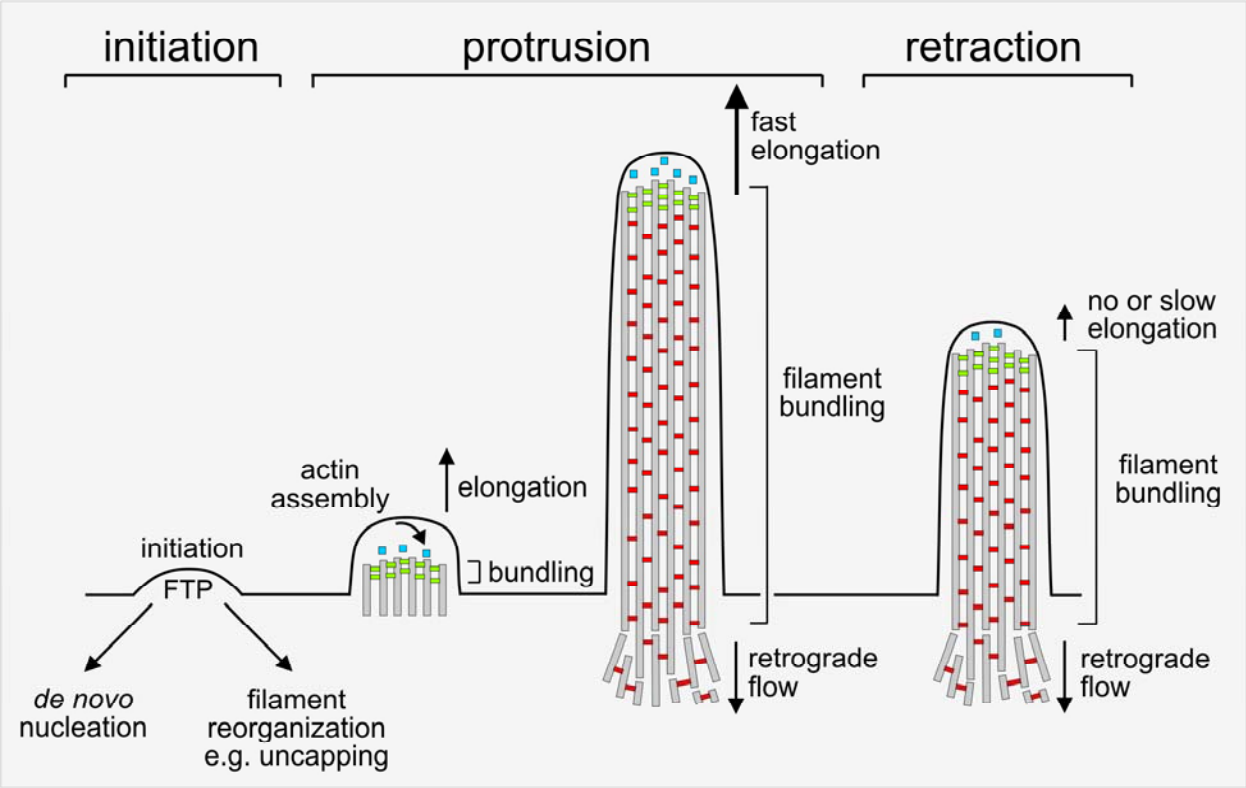


Figure 2:


initiation
protrusion
retraction
fast
elongation
filament
bundling
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flow
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elongation
filament
bundling
retrograde
flow
actin
assembly
elongation
bundling
initiation
FTP
de novo
nucleation
filament
reorganization
e.g. uncapping