The role of actin bundling proteins in the assembly of filopodia in epithelial cells

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Abbreviations: EMT, epithelial-mesenchymal transition; PIP₂, phosphatidylinositol-4,5-bisphosphate

The goal of this review is to highlight how emerging new models of filopodia assembly which include tissue specific actin-bundling proteins could provide more comprehensive representations of filopodia assembly that would describe more adequately and effectively the complexity and plasticity of epithelial cells. This review also describes how the true diversity of actin bundling proteins must be considered to predict the far-reaching significance and versatile functions of filopodia in epithelial cells.

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Filopodia play an important role in epithelial cell-cell contact and cell migration. Efficient bundling of actin filaments within filopodia is essential for filopodia formation both in vitro and in cultured cells. Membrane deformation is energetically unfavorable and single actin filaments which are not adequately stiff to push the membrane buckle under the cell surface. Consequently it follows that tight parallel actin bundles are necessary for membrane protrusion and the mechanical stability of filopodia. Fascin is the most widely examined actin-bundling protein that is enriched within filopodia and localizes to the entire length of the filopodia. However, fascin is not a ubiquitous protein. Particularly, fascin is not expressed in epithelial cells nor is it expressed in all carcinomas. So how are actin filaments bundled and filopodia assembled in cells that do not express fascin? Recent studies have identified tissue specific actin-bundling proteins like espin and villin that can assemble filopodia in the absence of fascin.

The routes of filopodia assembly are likely very complex, highly regulated and a single mechanism and a single actin-bundling protein is unlikely to account for the diverse structures, behaviors and functions of filopodia that are found in a wide range of cell types and organisms. Since numerous cells possess filopodia, which differ in length, thickness, dynamics, localization and origin, it has been suggested that by varying these properties of cell surface structures, the cell generates a range of discrete structures to perform specialized functions. We suggest that one way to generate this diversity would be through the recruitment of specific sets of actin-regulatory proteins which may be unique to the cell type or may be unique to the specialized function that these structures perform. Most current models of filopodia assembly for that reason, seem incomplete and based solely on the identification of a limited number of proteins. In this context, the role of tissue specific actin regulatory proteins and their role in the assembly of filopodia are particularly under-appreciated.

Filopodia are Associated with Unique Functions in Epithelial Cells

Filopodia are especially relevant for epithelial cells, where they are required to establish cell-cell contact between epithelial layers and to guide cell motility. Our basic understanding of the molecular mechanism(s) of filopodia assembly and functions of filopodia in epithelial cells nonetheless, remains quite rudimentary. Actin polymerized in filopodia has been shown to move and cluster integrins to spatially position integrins primed to probe the matrix at the very front of cell protrusion creating so-called “sticky fingers” along the leading edge of cells with sensory or exploratory functions. Accordingly in most cells filopodia are used to establish adhesion to the extracellular matrix and for cellular guidance. Since directional cues guide epithelial cell migration, filopodia play a major role in wound-healing, regeneration and maintenance of the epithelium. An impaired ability of epithelial cells for directional migration is linked to chronic inflammatory diseases which are in turn associated with an increased risk for carcinogenesis. Epithelial cell motility is plastic and complex. Epithelial cells can undergo epithelial-to-mesenchymal transition (EMT) and move as single cells. Alternatively, epithelial cells can move as intact clusters, sheets or tubes in a process called “collective cell migration.” Collective cell migration requires directed, coherent movement of cell groups that maintain constant positions while migrating. Collective cell migration is characterized by the movement of cells that maintain their polarized epithelial phenotype and remain connected by apical cell-cell junctions. Therefore, as opposed to single cell migration, collectively migrating cells do not develop a retracting uropod but exert pulling forces on their neighboring cells thus, keeping their positions within the collective structure while moving as 2-D sheets or 3-D strands.

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Collective cell migration is a conserved morphogenetic event that shapes embryos and tissue in both vertebrate and invertebrate systems. This type of cell migration is observed in the caudal migration of lateral line primordial in zebrafish,26 border cell migration in Drosophila ovaries,27 dorsal closure in Drosophila embryos,28 wound healing in epithelial monolayers29 and in vitro models of mammary glandbranching morphogenesis.30 When epithelial cells migrate as groups of cells or collectively as sheets of cells rather than as single cells they can form intermittent or less stable or repetitive short-lived contacts with other cells in the group; alternatively they can maintain intact and stable cell-cell adhesions and cell-cell communication. Whether cells move as tightly adherent sheets or loosely associated with occasional contact but inherent polarity also determines a different migratory mechanism.31-33 Cell-cell junctions can form de novo and resolve again thus, single and collective migration modes are also interconvertible.24 The current thinking is that there may be a combination of localized (for leader cell) and collective signaling (for the remainder of the cells in the group) modes that work to move the loosely adherent or collective sheet of cells forward.34,35 It is speculated that collective sheets may even have different guidance properties relative to solitary migrating cells.35-37 Since single-cell and collective migration modes serve mutually exclusive functions during morphogenesis, tissue regeneration and, in pathological conditions of the epithelium, the role of filopodia in regulating single and collective migration is expected to be similarly complex and likely distinct from what is described for most mesenchymal cells. How epithelial cells migrate directionally in the context of complex multicellular organisms during development or in disease and how they use filopodia to guide such cell migration however, also remains undetermined.

Epithelial tumors cause more than 85% of all cancer-related deaths. Abundant filopodia are a characteristic of invasive, metastatic cancer cells and the number of filopodia correlates with their invasiveness.38,39 Similar to the developmental processes cancer cell migration can occur as single-cell migration (amoeboid or mesenchymal, solitary or in Indian files) or it can occur as collective cell migration (in cell sheets, strands, tubes or clusters).24,40-44 Furthermore, by altering cell-cell and cell-extracellular matrix adhesions cancer cell invasion modes can be altered from multicellular strands to amoeboid dissemination.45,46 Tumor cells choose different migratory mechanisms and the resulting diversity and plasticity contributes to the difficulty of treating metastasis.41 The role of filopodia in these diverse and complex cell migratory mechanisms and their contribution to cancer metastases however remains unknown.

In addition to their role in cell motility, filopodia in epithelial cells also align cells correctly to adhere to their target partner and to close the gap between epithelial sheets, a process called “adhesion zippering.” In this process filopodia interdigitate allowing E-cadherins to interact at sites of contact which then mature into adherens junctions through recruitment of catenins and alignment of the plasma membranes, thereby ‘zipping’ the two epithelial sheets together.20,47 Adhesion zippering is very well described in epithelial sheet fusion in C. elegans,48,49 during dorsal closure of Drosophila embryos20,47 and in cultured epithelial cells undergoing in vitro wound closure.41,50,51 Contractile filopodia also tether lens and retina to coordinate epithelial invagination highlighting the role of filopodia in similar morphogenetic events in vertebrates.52 It is speculated that in these unique situations, filopodia function as mechanosensitive organelles to control cell-cell communication and cell-cell adhesion.33,34,53,54

Several viruses and bacteria that infect epithelial cells, bind at filopodial tips which participate in their internalization and spread in host cells.55-57 Viruses are often associated with the dense microvilli of polarized epithelial cells but also with the filopodia of both polarized and nonpolarized cells.56,58-61 Transport of viruses along filopodia during the attachment and entry process has been documented for a number of viruses.55,62-64 Filopodia have been shown to act as cellular tentacles that serve to increase the efficiency of uptake of pathogens.65 Recent evidence suggests that viruses can themselves activate filopodia formation to enhance viral uptake.55,63,64 Since a majority of HIV transmissions occur across a mucosal surface such as the anorectal mucosa, vaginal mucosa and less frequently, the oral mucosa, several recent studies have demonstrated how profoundly and preferentially epithelial tissue are targeted during all stages of HIV-1 infections and how microvilli/filopodia-like structures are involved in cell-to-cell transmission of HIV virus in epithelial cells.57 Similarly, epithelial wounding has been shown to facilitate papillomavirus infection; the etiological agent of epithelial tumors and cancers.58 Presumably, cells associated with oral and gastrointestinal tract disease pathogenesis also use filopodia and lamellipodia to adhere and invade epithelial cells.66 Enteropathogenic Escherichia coli, Shigella, Salmonella and Citrobacter rodentium, which colonize the gut, have all been shown to contain a WxxxE motif which promotes filopodia assembly by activating Cdc42 to regulate the adhesion, spread and survival of these enteropathogens in host epithelial cells.57,70-74 Interestingly, a similar process has also been described for the retrograde transport of activated epidermal growth factor (EGF) receptors on adenocarcinoma cells.75

In Epithelial Cells the Same Actin Bundling Protein Could Assemble Microvilli and Filopodia

Parallel, bundled actin filaments found in cellular protrusions such as microvilli and filopodia are ubiquitous in eukaryotes, but are particularly well-developed and functionally significant in epithelial cells.76 Actin-polymerization and actin-crosslinking provide the driving force for the formation of these cell surface structures, which can be rapidly extended and retracted thus, modifying cell morphology and function in response to extracellular stimuli or intracellular signaling.77 The molecular mechanism for the assembly and turnover of microvilli and filopodia in fact share several similarities namely, both contain parallel actin bundles; actin nucleation is essential for the assembly of both these structures; the bundles are formed by actin assembly that begins near an electron-dense complex at the tip; the bundles taper, that is the number of filaments decrease as one approaches the bundle tip; bundles are polarized with the barbed ends located at the bundle tip nearest to the plasma membrane;
bundle production is independent of a specific type of crosslinker (multiple actin bundling proteins associated with microvilli and filopodia); the length of the filaments appears to be independent of filament number; the actin bundles must remain at constant length yet treadmill; the microvilli is embedded in the terminal web, while most filopodia are embedded in a lamellipodium. Since crosslinked bundles of actin filaments in a wide variety of cells from different organisms share common features it has been hypothesized that a common mechanism to construct initial actin bundles exists in all cells. In this context it is worth mentioning that apical filopodia of certain cell types have been proposed to be precursors of more specialized protrusions such as brush border microvilli or the sensory stereocilia of the inner ear hair cell. Villin and fascin are actin bundling proteins that are associated with parallel actin bundles in microvilli and filopodia. It follows then that actin-bundling proteins in epithelial cells could build parallel bundled structures in both the microvilli and filopodia. Furthermore, their physiological function may be determined not by their association with one organelle or the other but by their functional characteristics. Most actin bundling proteins are associated with multiple cellular protrusions which suggests that similar actin bundling events regulated by these proteins may occur in many of these cell surface structures. The epithelial cell specific actin-bundling protein villin is associated with microvilli, lamellipodia, microspikes and filopodia. Espin is associated with microvilli, stereocilia and filopodia. Similarly, the actin-bundling protein fascin is associated with microvilli; stereocilia; filopodia and lamellipodia; and in transformed epithelial cell lines that do express fascin, it is associated with cell-cell adherens junctions. The initial bundles assembled in these structures are also continuously modified. For instance, although intestinal and renal epithelial microvilli maintain constant length after initial assembly, the assembly and turnover of microvilli is dynamic, which implies ongoing assembly and disassembly and utilization of actin bundles within these structures. Perhaps the best-studied examples are the shortening of microvilli during starvation and after treatment with cyclohexamide or lectins and re-elongation of the microvilli following the removal of these stimuli or when G-actin is added with cyclohexamide or lectins and re-elongation of the microvilli is possible over many years. Five actin crosslinking proteins are associated with D. melanogaster morphogenesis: singed (fascin), α-actinin, filamin, forked and quail (villin-like). Deletion or mutations in any one of these five proteins is associated with an abnormal phenotype (Table 1). A polarized distribution of two or more actin bundling proteins during cell motility is likewise documented. It is clear from studies done with Dictyostelium that even simple organisms such as amoeba require a whole spectrum of actin-binding proteins to perform basic cellular functions. Therefore, it is not unlikely that in eukaryotic cells, filopodia could be assembled by multiple actin bundling proteins.

Most models of filopodia identify two actin bundling protein in filopodia namely, fascin and IRSp53. It may be noted that neither of these proteins is expressed in normal epithelial cells. On the other hand, most mammalian cells express additional actin crosslinking proteins such as α-actinin, filamin, T-plastin, espin, fimbrin and villin many of which have been localized to the filopodia. At least two of these actin-bundling proteins are expressed in epithelial cell and have been associated with both the microvilli and filopodia, namely, espin and villin. In the absence of fascin, some of these actin crosslinkers can also rescue filopodia assembly. Therefore, it is still uncertain whether fascin is sufficient for filopodia formation or if multiple actin bundling proteins share this role. It is also noteworthy that fibroblasts from fascin-1 deficient mice still extend filopods, suggesting that fascin is not the sole regulator of filopodia assembly. It seems probable then, that while fascin may be the main actin crosslinking protein in cells that do express fascin, other actin crosslinkers are likely used in specialized cells including epithelial cells that do not express fascin or IRSp53 or in specific conditions, such as the recruitment of filamin to filopodia induced by Wnt5a signaling. It is also possible that different sets of proteins form filopodia by related mechanisms depending on which proteins are present and/or active within a particular cell type.

There are discrete stages for most actin bundle formation in vivo. In almost all instances, two or more actin-bundling proteins localize sequentially and have non-redundant functions that are required to generate maximally cross-linked and maximally rigid actin bundles in vivo (Table 1). In general,
one actin crosslinking protein is required to hold adjacent filaments together during the initial stages of elongation and the second is required to zipper together the filaments in tightly packed, well-ordered bundles. Such two-step actin assembly has been described in the development of microvilli in intestinal epithelial cells.\textsuperscript{93,122,135} In other instances, such as the colon adenocarcinoma cell line, Caco-2 there is a downregulation of fascin and an upregulation of villin and α-actinin as cells assemble well structured and functional microvilli.\textsuperscript{111} Could multiple actin bundling proteins have a similar function in the assembly of filopodia? In a recent study, a non-homogenous distribution of actin bundling protein within the filopodia was described with α-actinin localized to the middle section of the filopodia, coronin 1 associated with the base of the filopodia and T-plastin associated with the entire length of the filopodia except the tip.\textsuperscript{129} Similarly, espin has been shown to localize to the proximal part of the filopodia.\textsuperscript{5} In such models, a temporally regulated, sequential non-overlapping function of each of these proteins during actin filament bundling in filopodia can be envisioned. Interestingly only two actin bundling proteins are described so far that associate with the entire length of the filopodia namely fascin and the epithelial cell specific actin-bundling protein villin.\textsuperscript{82,90,91} There is also a distinct possibility that actin bundling proteins rapidly exchange between actin filaments within the filopodia to regulate filopodia turnover and dynamics.\textsuperscript{5,135} The types and numbers of F-actin bundling proteins and the number of actin filaments bundled would greatly affect the persistence length and/or stiffness of filopodia assembled by these proteins.\textsuperscript{11} These factors in turn, could influence how filopodia respond to chemical or mechanical cues and how downstream signals are activated in these cells. This would be consistent with the emerging concept that molecular characteristics of the filopodia may be determined by functional characteristics of the cell and the task executed by these cell surface protrusions.

### Comparison of Filopodia Assembled in Epithelial Cells by Villin and Fascin

In this review we highlight the function of epithelial cell-specific actin-bundling proteins like villin, which can assemble actin bundles in microspikes and filopodia of migrating epithelial cells that lack fascin.\textsuperscript{90,91} The role of villin in the assembly and maintenance of the microvilli has been known for over three decades.\textsuperscript{92,132} However, villin also regulates cell migration and in vivo villin is required for the reorganization of the actin cytoskeleton during changes in cellular plasticity, cell motility, cell morphogenesis and wound healing.\textsuperscript{99,106,136-142} Villin is unique among the actin-bundling proteins and in fact among all actin-binding proteins in that it can cap, sever, nucleate and bundle actin filaments. These unique properties of villin may contribute to the diverse functions performed by villin in epithelial cells. Furthermore, the actin regulatory functions of villin are modified in response to changes in calcium, tyrosine phosphorylation and ligand binding [phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)_2, PLC-γ, and actin] demonstrating villin’s ability to dynamically reorganize the actin cytoskeleton.\textsuperscript{106,137,138} Studies done in our laboratory have shown that villin’s ability to bind the plasma membrane [through its PtdIns(4,5)_2-binding domains] and simultaneously bundle actin filaments drives the emergence of filopodia (Fig. 1).\textsuperscript{143,144} Our recent findings illustrate that the convergence of different signaling cascades spatially restrict villin activity to areas of high PtdIns(4,5)_2 and high F-actin concentration to assemble filopodia.\textsuperscript{90} Furthermore, mutants of villin that fail to bind PtdIns(4,5)_2 or fail to bundle actin and assemble filopodia disrupt directional cell migration.\textsuperscript{90}

Fascin-1 is expressed most abundantly in brain, ovary and testis; at the cellular level it is found in neuronal and glial cells, microcapillary endothelial cells and antigen-presenting dendritic cells.\textsuperscript{145-147} However, fascin is absent from normal epithelial cells.\textsuperscript{38,8} Moreover fascin expression is restricted to specific tissues during development.\textsuperscript{150} By contrast villin is more widely expressed in epithelial cells (Table 2). Recently, expression of fascin has been reported in some transformed epithelial cell lines and in some carcinomas.\textsuperscript{151-152} However, not all transformed epithelial cell lines or carcinomas express fascin.\textsuperscript{8-10} Studies done in our laboratory suggest that in such cells, villin is the principal actin-bundling protein that assembles filopodia.\textsuperscript{90,91}

Both villin and fascin are associated with parallel actin bundles in the non-adherent more stable structures of the microvilli as well as in the adherent, more dynamic structures of the filopodia.\textsuperscript{8,91-95} However, villin is also associated with the less organized actin network in membrane ruffles and lamellipodia.\textsuperscript{8,9,91,342} Villin can cap, nucleate, sever and bundle actin while fascin only bundles actin filaments (Fig. 2). Villin can associate with PtdIns(4,5)_2 and the plasma membrane, another function not shared by fascin (Fig. 1).\textsuperscript{8,143} The diverse biochemical properties and the distinct intracellular distribution of villin and fascin suggests that it is unlikely that in epithelial cells that do express both these proteins they have redundant overlapping functions (Table 2). The most compelling data supporting this hypothesis comes from studies done in Drosophila where both fascin (singed) and villin (quail) have been shown to have non-overlapping essential functions and both proteins are required for actin bundle assembly (Table 1).\textsuperscript{135} Deletion of either fascin or villin in Drosophila results in abnormal egg chambers and female sterility due to abnormal assembly of actin bundles in the nurse cells demonstrating that both proteins are required for bundle assembly and have distinct roles in bundle organization.\textsuperscript{118} A model of fascin and villin in Drosophila predicts that villin crosslinks F-actin loosely and then fascin organizes

### Table 1. Multiple actin bundling proteins are required by D. melanogaster during oogenesis and bristle formation

| Protein             | Mutant phenotype                     |
|---------------------|-------------------------------------|
| Fascin (Singed)     | Short gnarled bristles; female sterility |
| Quail (Villin like) | Female sterility; disruption of follicle cells |
| Forked              | Gnarled bristles                     |
| α-Actinin           | Lethal; flightless, thoracic muscle, tubular flight muscle, leg muscle abnormalities |
| Filamin             | Female sterility                     |
organized actin bundles. Furthermore, recent studies with the villin knockout mice have demonstrated that rather than just assemble actin bundles, villin may be required to dynamically reorganize the actin bundles in response to cell signaling, cell injury and cell stress. It may be noted that unlike fascin, the actin bundling function of villin can be modified in response to them into larger more compact bundles. Consistent with that, biophysical studies have identified the extreme mechanical stiffness of actin bundles assembled by fascin-1 compared with other actin crosslinking proteins as well as identified the unique and unusual effects of fascin-1 on the rigidity of actin bundles. In contrast, villin is associated with the assembly of loose poorly organized actin bundles. Furthermore, recent studies with the villin knockout mice have demonstrated that rather than just assemble actin bundles, villin may in fact be required to dynamically reorganize the actin bundles in response to cell signaling, cell injury and cell stress. It may be noted that unlike fascin, the actin bundling function of villin can be modified in response to

**Figure 1.** In epithelial cells villin and fascin may have non-overlapping functions in filopodia assembly. Villin can nucleate, cap, sever and bundle actin filaments. Villin also binds PtdIns(4,5)P₂. Fascin is an actin-bundling protein that does not bind PtdIns(4,5)P₂. This predicts that filopodia assembled by villin and fascin could be molecularly and functionally distinct. In epithelial cells that do express villin and fascin, villin may be associated with more dynamic actin bundles that undergo rapid turnover in response to extracellular cues. By contrast, fascin could be associated with more stable and rigid actin bundles within the filopodia.

**Figure 2.** Model for filopodia assembly. This model describes the function of actin regulatory proteins including actin nucleating, capping, severing and bundling proteins during filopodia assembly. The figure on the right shows putative functions that could be attributed to tissue-specific actin capping, nucleating, severing and bundling proteins like villin during filopodia assembly.
cell signaling. This suggests that in epithelial cells that do express both villin and fascin, villin may be associated with more dynamic actin bundles that require rapid turnover in response to extracellular cues while fascin may be associated with more rigid, stable actin bundles within the filopodia. This predicts that filopodia assembled by villin and fascin could be not only molecularly but also functionally distinct. Further, this predicts that in carcinomas that do express villin and fascin, the dynamics of fascin and villin mediated filopodia might be significantly different and may be used by the cancer cells to assemble filopodia differing in dynamics, length and/or lifetime to regulate the different steps of tumorigenesis. Fascin and its actin bundling function are transcriptionally regulated, since fascin is generally absent from normal epithelial cells and is only expressed in some transformed epithelial cells. One can speculate then that in transformed epithelial cells that do express both villin and fascin, villin could be the first protein that is recruited to assemble filopodia to move cells prior to transcriptional upregulation of fascin.

EMT is strongly implicated in metastases but fascin is excluded from cells that display molecular signatures of EMT. Moreover, while some primary tumors are fascin 1-positive, all of the distant metastases have been found to be fascin 1-negative suggesting a tight and specific regulation of fascin 1 expression during tumorigenesis, which excludes cells that have undergone EMT. Collective cell migration is characteristic of certain types of cancers including high and intermediate differentiated lobular breast cancer, epithelial prostate cancer, large cell lung cancer and squamous cell carcinomas. The association of fascin with these types of cancers particularly squamous cell carcinomas and not those that have undergone EMT indicates that fascin may regulate collective cell migration in these types of cancers. Collective cell migration may represent an efficient migration strategy that allows translocation of heterogeneous sets of cells thereby disseminating cells of different clonality and function within one functional unit. This hypothesis is further supported by the observation that in transformed epithelial cell lines fascin is localized to areas of cell-cell contact and cell-cell adhesion. It is conceivable then that the more rigid filopodia assembled by fascin at cell-cell contacts function as mechanosensitive organelles while the more dynamic filopodia assembled by villin at the leading edge of cells function as chemosensitive organelles. It is worth noting that cell-cell junctions can form de novo and resolve again, demonstrating that individual and collective cell migration modes are interconvertible. Moreover cancer cell invasion modes can be altered by modifying cell-cell and cell-extracellular matrix adhesions. This would be congruent with the transcriptional regulation of fascin expression in some transformed epithelial cells. We suggest that such new models could help explain the adaptability of cell movement, the plasticity of epithelial cells, and significance of multiple actin bundling proteins villin and fascin in the filopodia of transformed epithelial cells.

Table 2. Comparison of villin and fascin and their role in filopodia assembly and turnover in normal and transformed epithelial cells

| Characteristics | Villin | Fascin |
|-----------------|--------|--------|
| **Expression**  | Expressed in epithelial cells of the gastrointestinal tract; urogenital tract; exocrine glands of endodermic lineage; brush cells, merkel cells, osteocytes, taste receptor cells. Expressed in all carcinomas and intestinal metaplasia. | Expressed in neuronal, glial, endothelial and dendritic cells. Not expressed in epithelial cells. Expressed in some transformed epithelial cell lines and some carcinomas such as squamous cell carcinomas. |
| **Expression in transformed epithelial cell lines** | Expression increases as cells become polarized and differentiated. | Expression decreases as cells become polarized and differentiated. |
| **Localization** | Associated with microvilli and terminal web in polarized epithelial cell lines. | Associated with cell-cell contacts in transformed epithelial cell lines. |
| **Metastasis** | Associated with EMT. | Not associated with EMT. |
| **Filopodia location** | Assembles filopodia at leading edge. | Assembles filopodia at leading edge of mesenchymal cells and in carcinomas may assemble filopodia at cell-cell contacts. |
| **Filopodia dynamics** | May regulate dynamics and turnover of filopodia. | May assemble rigid filaments in filopodia and regulate stability of filopodia. |
| **Actin Dynamics** | Can nucleate, cap, sever and bundle actin filaments. | Can bundle actin filaments. |
| **Capping activity** | Could cap filaments to regulate filopodia assembly and turnover. | Has no actin capping function. |
| **Nucleation activity** | Could nucleate actin assembly in filopodia. | Has no actin nucleating function. |

**Actin Bundling Proteins Like Villin That Can Also Nucleate and Cap Actin Filaments and their Role in Filopodia Assembly**

A major unanswered question in understanding filopodia assembly has been how they are initiated. Currently there are two models that describe how filopodia are assembled in all mammalian cells. Filopodia in migrating cells have been proposed to be formed primarily by reorganization of the dendritic actin network of the lamellipodium (Fig. 2). It has been suggested that...
Arp2/3-mediated nucleation of actin filaments plays a major role in generating the parallel bundles of the filopodia. Further, this model suggests that a subset of Arp2/3 nucleated filaments are capped and clustered by Ena/VASP proteins Dia2 and myosin-X and as these filaments rapidly elongate they are bundled by fascin to generate filopodia. However, Arp2/3 is absent from established filopodia, which suggests that Arp2/3 may participate in the initiation but not in the steady-state elongation of these structures. More importantly, mammalian cells depleted of Arp2/3 complex can assemble filopodia demonstrating that Arp2/3 is dispensable for filopodia formation. The second model proposes that filopodia are formed through the direct polymerization of parallel actin filaments by members of the formin family together with Ena/VASP. Such filopodia are not derived from the underlying lamellipodial network but are formed de novo. The synergistic role of Ena/VASP and formins in this model has not been adequately interpreted, although it is postulated that Ena/VASP proteins assemble filopodia via their F-actin bundling activity. Both these models may be relevant. Migrating cells also display parallel actin bundles in microspikes which remain embedded within the lamellipodia during cellular protrusion. Microspikes can evolve to filopodia when they protrude beyond the leading edge. Additionally, migrating cells display parallel actin bundles in retraction fibers which are left behind when lamellipodium retract. Generally speaking, microspikes, filopodia and the retraction fibers are inter-convertible organelles although the molecular origins of these structures are not well understood. It has been suggested that some of these structures could be assembled by the convergent-elongation mechanism while others may arise from formin-mediated actin nucleation, which would explain the existence of multiple mechanisms to initiate filopodia assembly in cells. As an alternative, it has been suggested that tissue specific actin nucleating proteins could regulate the assembly of parallel actin bundles in filopodia (Fig. 2). One such protein could be villin, which can nucleate and bundle actin filaments.

In intestinal cell lines as well as in enterocytes of the small and large intestine, the expression of villin increases as cells form long, well developed microvilli. In vitro, simple mixtures of villin and F-actin form uniformly polarized bundles similar to those seen in the ultrastructural analysis of microvilli. Overexpression of villin in fibroblasts and other villin-null cells reorganizes the actin cytoskeleton, which includes a loss of stress fibers and an assembly of parallel actin bundles in microvilli-like structures or in microspike and filopodia. Thus, villin has the ability to recruit actin from other microfilament structures to facilitate the formation of bundled actin structures. Villin is the first microvillar protein that is detected in the early stages of development in the immature chicken and mouse digestive tract and shows a transient elevation prior to the formation of the microvilli suggesting that villin plays a significant role in microvillar formation, maturation and/or maintenance. Moreover, the molecular basis for the localization of villin at this time, when there is little actin-based cytoskeleton, has led to the suggestion that villin interacts with the apical plasma membrane to nucleate actin and assemble the microvilli. As an actin filament nucleating and severing protein villin can accelerate actin assembly and actin-crosslinking role for villin has been hypothesized for several decades. Studies done in our lab and other have clearly demonstrated that in epithelial cells villin is a central player at the crossroads of signaling network that link the actin cytoskeleton with membrane dynamics. Most importantly, studies done in our lab have described how spatial organization of parallel actin bundles whose growth generates membrane deformation during cell motility are assembled by tissue-specific actin bundling proteins like villin. It is plausible then that villin's ability to nucleate and bundle actin filaments contributes to its function in filopodia assembly (Fig. 2).

Filopodia formation is thought to be dependent, in part, upon the relative activities of actin-capping and anti-capping proteins. It has also been shown that parallel actin bundle formation can be shifted to dendritic network formation by actin capping proteins. In fact, Mejillano and colleagues have demonstrated that depleting cells of capping proteins results in a dramatic explosion of filopodia while reinstating capping proteins result in lamellipodia assembly indicating the role of capping proteins on cellular protrusions. The localization of actin bundling proteins like villin to both the lamellipodia and the filopodia and the fact that villin can cap and bundle actin filaments adds to the complexity of filopodia assembly in epithelial cells and the role of actin crosslinkers like villin in the assembly of such structures (Fig. 2). While such new models are emerging, more detailed studies are required to describe the molecular mechanism of filopodia assembly in the majority of mammalian cells that either do not use Arp2/3 or do not express fascin or have more complex and heterogeneous filopodia that fulfill multiple functions such as the filopodia in epithelial cells.

### Filopodia Assembly by Actin Bundling Proteins That Can Also Interact with the Plasma Membrane

Recent studies have demonstrated that proteins that lack actin regulatory functions but interact with membrane lipids are sufficient to facilitate filopodia assembly. E.g., overexpression of the lipid phosphates related protein-1, LPR1 in HeLa and Cos-7 cells is associated with a large increase in the number of peripheral and dorsal filopodia. Similarly, the inverse BAR (I-BAR) domain of IRSp53 protein, which binds phosphoinositol-rich membranes with high affinity induces membrane protrusion. I-BAR/IM domains do not crosslink actin filaments but induce PtdIns(4,5) P₂ clustering which results in membrane bending due to electrostatic interactions. Moreover, these proteins remain dynamically associated with the inner leaflet of the membrane as a consequence they also stabilize these membrane protrusions. IRSp53 localizes to filopodia. The ectopic expression of IRSp53 induces filopodia and knockdown of IRSp53 diminishes the ability of cells to develop filopodia. These studies reveal that direct membrane deformations without actin bundles can form filopodia. Many of the I-BAR domain proteins, however, harbor protein-protein interaction domains indicating that a strong association between the plasma membrane and actin dynamics...
is nonetheless required to sustain these membrane protrusions. Moreover, actin filaments are present in the majority of I-BAR-induced filopodia and may be essential for their long-term maintenance. However, what is noteworthy is that actin filaments themselves do not drive the protrusion but only provide mechanical support to the membrane deformations and/or stabilize the membrane protrusions. These recent findings highlight how little we know still about the molecular mechanisms that assemble filopodia.

It has also been suggested that transient changes in membrane curvature such as those caused by PtdIns(4,5)P2 clustering can cause filopodia assembly by activating pathways that trigger actin polymerization. It is hypothesized that the concentration of the actin filaments assembled and bundled by PtdIns(4,5)P2 clustering would curve the membrane locally. It has also been speculated that if the barbed end of actin filaments are kept together by a crosslinker that stays close to the growing filament tips, then the filaments would grow in the direction of protrusion and the corresponding bending force could be generated by the polymerization ratchet mechanism. These could be alternative mechanisms for filopodia assembly that are employed by actin bundling proteins that also bind PtdIns(4,5)P2 such as α-actinin, villin and espin.

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Cell Adhesion & Migration 416

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