The Ways of Actin: Why Tunneling Nanotubes Are Unique Cell Protrusions

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Abstract

Actin remodeling is at the heart of the cell's response to external or internal stimuli allowing a variety of membrane protrusions to form. Fifteen years ago, tunneling nanotubes (TNTs) were identified, bringing a novel addition to the family of actin-supported cellular protrusions. Their unique property as conduits for cargo transfer between distant cells, emphasizes the unique nature of TNTs among other protrusions. While TNTs in different pathological and physiological scenarios have been described, the molecular basis of how TNTs form is not well understood. This review addresses several actin regulators in the formation of TNTs and suggests potential players based on their comparison with other actin-based protrusions. New perspectives for discovering a distinct TNT formation pathway would enable us to target them in treating the increasing number of TNT-involved pathologies.

Actin processes generate a diverse array of cell protrusions

Actin, one of the key cytoskeletal polymers of the cell, forms helix-shaped **polar filaments** (see Glossary) that are further assembled into highly-organized actin networks, such as branched and linear.[1] The spatial and temporal control of these actin networks is crucial in maintaining the integrity of the cell, contributing to its mechanical properties, and driving cell shape changes that enable a cell's response to various processes.[1] Cell protrusions appear as the most prominent changes in cell shape, whose growth and characteristics mainly rely on actin cytoskeleton structure.[1] Filamentous actin (F-actin) is formed through the polymerization of globular actin (G-actin) monomers, a process governed by a broad pool of actin regulators and/or actin-binding proteins. In order for a cell protrusion to form into a mature structure, actin remodeling processes—that comprise the steps of initiation, actin polymerization and stabilization of the actin filaments—need to take place in the cell in a tightly controlled manner. Among these protrusions, **filopodia**, **microvilli** and **dendritic filopodia/spines** (see Glossary) comprise a family of morphologically similar structures (see Figure 1).

An addition to the family of cell protrusions, tunneling nanotubes (TNTs), were first documented in 2004 by Rustom and colleagues.[2] They described TNTs as membranous tubular extensions connecting two remote cells, thus providing cytoplasmic continuity between them. Similar to canonical protrusions, they are thin (up to 700 nm)[3] and comprised of F-actin, but in contrast, they are non-surface adherent, and have an ability to reach extraordinarily long distances (up to 100 μm).[4] First evidence of being actin-supported was shown by phalloidin staining in rat pheochromocytoma (PC-12) cells.[2]

More recently, an ultrastructural study employing correlative cryogenic electron microscopy (cryoEM), shed light on the actin organization within TNTs in two different neuronal cell lines.[3] This work uniquely showed straight and continuous, hexagonally packed actin bundles that appear to run parallel along the entire length of the TNTs. Apart from this actin-related feature, the morphological aspect of TNTs was further assessed; what was observed as a single TNT using low-resolution confocal microscopy, cryo-EM coupled with

tomography showed that it was instead a bundle formed of individual TNTs (iTNTs). This implicates a more complex structural organization than initially anticipated [3], and necessitates further structure-function studies addressing whether the diverse array of TNT-like protrusions reported in different physio-pathological contexts have conserved or instead cell-type dependent structures (see [5,6] for discussion of specific morphological features of TNT-like protrusions).

Another unique property of TNTs is their ability to transfer cargo of various sizes, encompassing small ions and molecules, but also larger organelles such as lysosomes and mitochondria.[5] Our work supports the identifying property of TNTs as being open-ended conduits that directly connect the cytoplasm of two cells for organelle transport.[3] Thus, a key requirement to distinguish TNTs from other cellular protrusions is to confirm their functional ability to transfer cargo. In the field, a common way of this assessment is by performing a transfer experiment where donor cells, containing labeled cargo, are cocultured with differently labeled acceptor cells that are further analyzed (flow cytometry, confocal microscopy) for the presence of cargo.[7,8]. Our cryo-EM data support this TNT feature that vesicular cargo is transported on actin tracks, likely through **myosin motors** (see Glossary).[3] This is in agreement with previous studies looking for example at the presence of molecular motors inside TNTs.[8,9] However more effort is needed to identify the organelle-specific motors mediating transfer inside TNTs.

All these aforementioned differences have raised important questions about their disparate nature and role in the cell. Since their discovery, many studies followed that were eager to address their role in physiological and pathological conditions.[4,5,10] In contrast, what we still lack is a fundamental understanding about how these structures form. As many reviews have already highlighted the types of cargoes transferred by TNTs and the physiological and pathological implications that TNTs represent[5,10], the focus of this review is to discuss the less understood, but equally important mechanism of TNT formation. There are two proposed mechanisms of TNT formation: 1) a cell dislodgement one, where two cells, initially attached to each other, move apart leaving a membrane thread that eventually matures into an actin-supported TNT; and 2) an actin-driven one, where a cell

forms through actin assembly a precursor protrusion that subsequently fuses with a recipient cell, thus forming a TNT.[2,11,12]

As the scope of this review is to understand how TNTs differ from known cell protrusions formed through actin polymerization, we will focus here on discussing exclusively TNTs formed by the actin-driven mechanism. While the current body of evidence supports TNTs as unique protrusions, it is still not clear how the cell utilizes a common pool of actin regulators (see Box 1) to build such different but morphologically similar protrusions such as TNTs, filopodia, microvilli and/or dendritic filopodia/spines. Therefore, comparing TNTs with better investigated cellular projections will help clarify the still enigmatic actin-driven mechanism of TNT formation and allow us to precisely distinguish them from other communicating protrusions, such as **cytonemes** (see Glossary) and other filopodia-like structures.

Outstanding questions still need to be addressed to understand this process. What are the actin regulators orchestrating the formation of TNTs? Certain actin elongators (formins, etc.; see Box 1) and actin bundlers (fascin, fimbrin, epsin, etc.; see Box 1), and others might play a role in their biogenesis. Is the actin-driven mechanism of TNT formation initiated as a *de novo* process, or are TNTs matured from a filopodia-like precursor?

To better understand their distinct nature, in this review we will address and compare mechanisms of formation of several other better-understood cell protrusions such as filopodia, dendritic spines and microvilli. This will give the field insight into understanding how TNTs may form and consequently galvanize new experiments that are key and necessary in elucidating TNT assembly.

Initiation of TNT protrusions: from membrane deformation to actin growth cascades

Globally, to form an F-actin based protrusion, two tightly regulated processes within the cell need to occur in concert—membrane deformation and growth of actin filaments at the membrane. What precedes the other is still not clearly defined; an outgrowth of a membrane protrusion can either be triggered by membrane deforming I-BAR proteins [13] (see Box 1) that would initiate and stabilize negative membrane curvature[14], or by

localized spots of newly formed actin filaments that can collectively generate enough force to overcome the membrane tension and initiate outward membrane extension.[15]

Actin cascades necessary for protrusion formation are triggered by a set of proteins called Rho GTPases (see Box 1).[16] For example, CDC42 (see Box 1) promotes filopodia formation.[17] Downstream of these signaling molecules, protrusion initiation by I-BAR proteins, such as IRSp53 (see Box 1) seems to be a ubiquitous process in the formation mechanism of filopodia, microvilli and immature dendritic spines.[18–21] CDC42 can release the autoinhibition of IRSp53 by binding to its partial Cdc42- and Rac-interactive binding (CRIB) domain. [20] Upon activation, IRSp53's **barbed end** (see Glossary) capping behavior is inhibited and a direct interaction with VASP (see Box 1) is enabled.[22] IRSp53 then advantageously clusters VASP (see Box 1) molecules at the initiation site to promote actin filament elongation through VASP's anti-capping behavior necessary for filopodia outgrowth.[22]

Importantly, acting within the same CDC42-dependent pathway, IRSp53 when in complex with Eps8 (see Box 1) localizes to the filopodia initiation site, where they synergize and enhance each other's likely role in the process of filopodia formation through Eps8's F-actin bundling activity[23–26] and IRSp53's ability to subsequently stabilize the topology of the initiated filopodia.[25–27] Recent super resolution microscopy work has revealed that I-BAR proteins, among them IRSp53, IRTKS and MIM (see Box1) were found to localize all along a filopodium's length.[27] This suggests that this family of proteins can assemble into higher order structures that can stabilize the formed protrusion. Indeed, recent in silico molecular dynamics simulations show such assembly of IRSp53 in tubular membranes.[28]

Apart from IRSp53's well-documented role in filopodia, MIM and IRTKS have important roles in both, dendritic spine and microvilli formation. In dendritic spines, IRSp53 was important in the maintenance of their morphogenesis.[29] It was shown to maintain proper spine density, as well as their shape, width and length, all of which were negatively affected upon depletion of IRSp53. This might implicate the role of IRSp53 in the formation of curved initiation sites where dendritic spines will assemble, and/or several other possible roles in elongation/stabilization. Furthermore, MIM accumulated to membrane domains in order to

initiate the formation of a dendritic spine by promoting outward membrane deformation.[18] Upon actin polymerization blockage, this MIM-induced proto-protrusion devoid of actin was unable to grow further into a mature spine suggesting the role of MIM in the initiation step.

In contrast to filopodia, where IRTKS was observed throughout the protrusion[27], a recent study unraveled an important role of IRTKS in microvilli formation, demonstrating its localization exclusively to the tip of microvilli.[21] IRTKS was shown to positively regulate the density and length of microvilli, suggesting its role in initiation and elongation, possibly through the recruitment of its downstream partners. For example, Eps8 was targeted to the tip of microvilli by binding to the **SH3 domain** (see Glossary) of IRTKS, where it positively influenced the elongation of microvilli, likely through its actin bundling activity.[21]

Initiation of TNTs might depend on the cell type. In Box 2 we summarize specifically the actin-related proteins that have an opposite effect on TNT formation in differing cell types, e.g., neuronal vs. immune origin. A sole study using neuronal cells showed IRSp53 had a negative impact on the amount of TNT-connected cells and the transfer of vesicles in a CDC42-dependent manner, suggesting that activation of CDC42-dependent pathways would preferentially lead to the formation of canonical filopodia, rather than TNTs that are functional for cargo transport.[7] Expression of an IRSp53 mutant defective in its SH3 domain binding to its ligands, had no significant effect on the amount of TNT-connected cells and the functional transport of cargo, suggesting VASP (and perhaps other downstream partners) in the process of negatively regulating TNT formation.[7] In contrast, Eps8's bundling activity was identified as a positive regulator of TNT formation[7], suggesting its potential role in protrusion initiation when in complex with an I-BAR protein.[26] Therefore, this result does not completely negate the role of IRSp53 or other I-BAR proteins in TNT formation in neuronal or other cell types, because it is known from in vitro binding studies that IRSp53 has a high, nanomolar binding affinity for Eps8.[26] It does raise several questions, such as if IRSp53 is regulated in a different manner, redirecting it towards TNT formation, or if another I-BAR protein is responsible in TNT biogenesis. Recent studies show that regulation of IRSp53 in protrusions is indeed more complex than being solely activated by Rho GTPases. It was demonstrated that the regulatory molecule **14-3-3**[30] (see Glossary) coordinates the activity of IRSp53 and thus filopodia formation.[19] The work

showed that 14-3-3 binds to AMPK-phosphorylated IRSp53 and blocks its activation by CDC42 or by its several effectors such as VASP and Eps8.[19] While in the presence of 14-3-3, even though IRSp53 was expressed together with CDC42, VASP or Eps8, filopodia formation was largely inhibited, implicating that 14-3-3 has a substantial role in the fine tuning of filopodia formation through the inhibition of IRSp53 binding to the membrane.[19,31]

Apart from CDC42 and IRSp53 involvement in filopodia formation, the less investigated IRTKS induced the growth of attached filopodia.[21] Furthermore, an interaction between an atypical Rho GTPase Rif (see Box1) and IRTKS promoted dorsal filopodia assembly, which, similar to TNTs, are not surface adherent structures.[32] Moreover, in dendritic filopodia, when inactive Rif mutants were overexpressed, the length of these protrusions was significantly reduced, emphasizing the role of Rif in the formation of long non-adherent filopodia-like structures.[33] Another atypical Rho GTPase, RhoD (see Box1), was found to induce the formation of long filopodia-like protrusions.[34] These alternate mechanisms of protrusion initiation share striking similarities with TNTs and give us an insight into other potential avenues to explore in the field of TNTs.

While several recent reviews on TNTs have more thoroughly discussed the role of **M-Sec** (see Glossary), in this review we want to only highlight two possibilities through which this protein might function. Firstly, an interaction of M-Sec with **RalA** (see Glossary) was found to be necessary for the formation of TNTs.[35] Furthermore, RalA has been shown to interact with **filamin** (see Glossary) to promote the formation of filopodia[36], suggesting similar complexes that would influence actin crosslinking or other processes related to actin reorganization within TNTs. Secondly, given that the **exocyst complex** (see Glossary) is well known to be involved in vesicle trafficking[37], it is possible that the directed delivery of membrane to a growing TNT is needed, however, the exact mechanism of its involvement in TNT formation needs to be further characterized.

How linear F-actin assembly leads to protrusion growth

TNTs as compared to other similar protrusions reach extraordinary long distances. Foundational theoretical work in the field of filopodia, for example, has shown that filopodia

growth is largely limited by the diffusion of G-actin to the polymerizing barbed end, which ultimately sets an upper threshold on the order of 5 µm or less for the maximum length a filopodia can reach.[15] As previously described, TNTs likely contain uninterrupted, straight actin filaments organized in hexagonally packed bundles.[3] This actin organization suggests the involvement of actin nucleators, elongators and efficient delivery mechanisms that may supply required actin regulators, G-actin, etc. to the growing barbed end. Several actin nucleators and elongators have been assessed for their ability to extend actin filaments within different protrusions, but not much is known for TNTs. Given their length and actin architecture, only a potent elongator would be able to assemble such long actin filaments. In this section we will describe the involvement of different actin nucleators and elongators known to form filopodia, microvilli and dendritic filopodia, as they can be predictive for the types of actin regulators involved in the formation of TNTs.

A highly likely candidate in the formation of TNTs that fulfills the criteria of generating linear actin filaments is the formin protein family (see Box1).[38] The ability of formins to processively incorporate G-actin to a growing filament is highly sensitive to both the applied forces on actin filaments where increasing loads can accelerate actin subunit incorporation[39], and geometrical constraints including filament bundling and formin attachment to membranes.[40] While no studies have explored the role of formins in microvilli, far more information has been obtained about their performance in the elongation of filopodia and dendritic filopodial precursors. Notably, mDia1-3 were all found to form filopodia, along with other formins such as formin-like protein 3 (FMNL3), and Disheveled-associated activator of morphogenesis 1 (DAAM1)[41–46]. Moreover, FMNL2 was also recently found to regulate dynamics of fascin in filopodia.[47] mDia3C was able to elongate extraordinarily long filopodia-like protrusions in a RhoD-dependent fashion where the protrusions reached lengths of 20-30 µm that are on the same order found for TNTs.[34] Regarding dendritic filopodia, their elongation was driven by a Rif effector protein, mDia2[33], as well as by DAAM1.[48] Subsequently to the formation of the immature spine by mDia2, Arp2/3 complex (see Box 1) enabled the maturation of the spine through the formation of a branched actin array necessary for the spine head enlargement.[33]

Interactions of formins with I-BAR proteins and/or Rho GTPases are also important in filopodia biogenesis. In particular, IRSp53 associates with mDia1[41], while Rif activates mDia1 and mDia2.[42,44] Considering Rif also interacts with IRTKS[32], a possible association of IRTKS with some formins is highly likely to occur during protrusion formation, possibly through establishment of a protrusion tip complex.

As previously discussed, I-BARs interact with the Ena/VASP protein family to assemble protrusions such as filopodia.[22,26] VASP's highly organized tetrameric structure enhances actin polymerization during filopodia formation[49] and dendritic spine formation[50], while at the same time preventing the binding of actin-capping proteins to the barbed end.[49,51,52] In vitro experiments on fascin-induced bundling of actin filaments showed an increase of VASP's processivity. [49,52] Knowing that fascin, a well-established actin bundler in filopodia[53], has been shown to have an opposite, negative role in TNT formation[8], this negative impact of VASP on TNTs might be further explained through a synergistic activity with fascin. Fascin can increase the processivity of VASP and decrease the processivity of mDia1.[40,49] Considering that both VASP and fascin are negative regulators of TNTs and positive of filopodia, we might conclude that one of the formins might be indeed involved in the formation of TNTs and therefore represent an interesting path to explore.

Even though TNTs have been known since their discovery to be actin-based, why is there still no evidence for an actin nucleator/elongator involved in TNT formation? Many directions originating from what we know concerning filopodia, microvilli and dendritic spines might guide the scientific community to intensify its efforts in discovering the molecule(s) in charge of the assembly of actin filaments within TNTs.

Stabilization of F-actin to give protrusions greater permanence

Actin filament crosslinking represents a mechanism that cells employ in order to organize actin into different architectures and to stabilize the collection of actin filaments within a protrusion.[1] Primarily unlike single actin filaments, bundled filaments (on the order of 10 to 30 filaments)[54][55] have sufficient structural rigidity to overcome the resistance

imposed by the tension of the membrane, and also confer stability against buckling, necessary for outward growth and proper protrusion length.[15]

The canonical actin bundler fascin[53,56] was initially found to tightly bundle straight actin filaments in a filopodium.[53] It enables packing of actin filaments into hexagonal arrangements, with inter-filament distances on the order of 8-10 nm[57][58], thus providing necessary stability for the protrusion. Similar to fascin, fimbrin and espin bundle individual filaments in hexagonal structures, providing near identical distances between individual filaments.[58,59] Recent in vitro work suggests that these crosslinkers do not exclude each other and may mutually reside within the same actin network in the cell.[57] Indeed, the crosslinkers espin and fimbrin were both identified in microvilli.[60] Even after their depletion, the formation of microvilli was not completely abolished, implicating other crosslinkers such as Eps8 in the stabilization of microvilli.[61] In contrast, fascin acts as a negative regulator of functional TNT formation, further implicating the opposite regulation of filopodia and TNTs.[8]

Eps8 was able to promote filopodia formation in some cell models, highly likely through its interaction with an I-BAR protein that induces Eps8's crosslinking activity.[25,26] In contrast, in cells of neuronal origin, Eps8 had a negative impact on filopodia formation, as well on density of immature dendritic filopodia, possibly through its capping activity.[7,26,62] Similarly, in microvilli, overexpression of another Eps8 family member, Eps8L1a, controlled their overall length, preventing their excessive elongation, whereas bundling activity of Eps8 was crucial for maintaining microvillar shape.[63] However, in TNTs, Eps8's crosslinking property was found to be an important positive regulator of TNT formation, whereas its capping activity had no impact on the formation of TNTs.[7] We might hypothesize then that it is the bundling role that is enhancing the formation of TNTs, but this does not exclude other potential crosslinkers, other than fascin[8], creating hexagonally packed actin filament networks in TNTs.

Further evidence supports the important role of actin bundling and stabilization in TNT biogenesis. Dephosphorylated **βCaMKII** (see Glossary) was shown to prolong the half-life of TNTs observed in cell culture, implicating it has a positive effect on the lifetime of these

protrusions.[64] It has been shown that βCaMKII stabilizes F-actin, but also binds to G-actin preventing its nucleation.[65][66] Phosphorylating βCaMKII leads to its detachment from F- and G-actin, consequentially enabling actin polymerization from the free G-actin pool.[66] Recently, it was demonstrated that βCaMKII can bind multiple actin filaments, aligning them in a mostly parallel manner.[67] In TNTs, when overexpressed, βCaMKII was localized at the base of the protrusion.[64] Considering βCaMKII assembles F-actin with an interfilament spacing of approximately 36 nm[67], and given that bundlers having different molecular sizes exclude one another within an actin network[57], we assume that βCaMKII will specifically segregate and will not co-localize with molecularly smaller actin bundlers that most likely give rise to the hexagonal arrangement of actin observed within the shaft of neuronal TNTs.[3] This segregation of βCaMKII is likely why it more resides at the base of TNTs where it might compete against **cofilin** (see Glossary) binding to the same hydrophobic pocket on F-actin[67], preventing **pointed end** (see Glossary) depolymerization and consequentially stabilizing and enhancing the permanence of linear F-actin within TNTs.[3,64]

Delivery of proteins necessary for the protrusion biogenesis and elongation

How do cell protrusions, such as TNTs, reach long distances? How do they overcome the diffusion limit within the growing protrusion? One of the unconventional motors, Myosin-X (Myo10), which can link the plasma membrane—through phosphatidylinositol (3,4,5)-triphosphate lipid binding —with actin filaments[68], has been vastly investigated in the formation of protrusions, but not as much in the context of its contribution to direct the delivery of elongators, bundlers, etc. during protrusion growth. It was shown that Myo10 can recognize only straight actin filaments, known to be present within several protrusions, linking this property with cargo delivery for protrusion elongation.[69]

Notably, Myo10 was shown to induce dorsal and attached filopodia formation, and dendritic filopodia.[70–73] Myo10's head domain was sufficient to initiate short, but unstable filopodia[71,72], whereas the full-length Myo10 (FL-Myo10) was likely indispensable for the cargo-binding tail domain to exert its function in supplying the factors imperative for their

elongation.[72,74] It was observed that Myo10's C-terminal tail-located **FERM domain** (see Glossary) was responsible for β_1-integrin binding and FL-Myo10 relocalization to the tip of a filopodium, implicating the importance of integrin activation in Myo10-induced filopodia.[74] Additionally, FL-Myo10 was able to form longer filopodia, a property F2- and/or F3-deleted Myo10 mutants did not have.[74] This suggested Myo10 could elongate and stabilize these protrusions, the latter explained through the ability of Myo10 to recruit β_1-integrin to form focal adhesions involved in the stabilization of filopodia, while the elongation was probably dependent on VASP[73,75] or other, still unknown binding partners of Myo10.

In TNTs, Myo10 was also found to be a positive regulator of their formation.[8,76] In contrast, neither of Myo10 head or tail mutants were able to induce the formation of functional TNTs.[8] Therefore, how FL-Myo10 induces TNT formation might be explained by Myo10's role as a cargo transporter, suggesting the delivery of necessary actin regulators to the growing tip of the TNT. Furthermore, it was demonstrated that the F2 lobe of the FERM domain was fundamental in the ability of Myo10 to induce TNTs.[8] In contrast, Myo10 completely devoid of the FERM domain was still able to form dorsal filopodia, while both F2 and F3 lobes were important in the formation of attached filopodia.[8,70,72,74] This highlights the subtle but important difference in the mechanism of formation and characteristics of dorsal filopodia and attached filopodia vs. TNTs. Furthermore, CDC42, a master regulator of filopodia[17], was found to act upstream of Myo10 to promote growth of dorsal filopodia.[70] While little is known about the F2 lobe and its cargo binding, one can still hypothesize that proteins specific for TNT formation might bind to the F2 lobe to be transported to the tip of a growing TNT. This avenue should be further explored, as it might lead to the identification of TNT-specific components.

Concluding remarks and future perspectives

TNTs were described over 15 years ago, bringing a novel addition to the family of F-actin-composed cellular protrusions. Importantly, TNTs have been functionally characterized as conduits for the direct transfer of various cargoes and organelles between cells, emphasizing a key difference between TNTs and other actin-based cell protrusions. The ongoing identification of TNTs and TNT-like structures in a variety of cell types in culture and ex vivo

tissue slices have strengthened their involvement in normal physiological processes such as signal transduction, apoptosis, development and immune responses[5]. Furthermore, TNTs are also implicated in diseases, serving as a novel route for the propagation of infectious bacteria[77], viruses such as HIV-1[11], misfolded proteins involved in neurodegenerative diseases[78–80], and even in aggressive cancers[10]. Because of their pathological involvement, TNTs represent novel therapeutic targets that could offer a unique strategy to supplement the current therapeutics employed against pathogens and to fight presently incurable neurodegenerative diseases and therapy-resistant cancers[10,81].

As highlighted herein, formation of a TNT through actin-driven processes encompasses yet unknown signaling cascades that recruit membrane deforming and actin-regulating molecules that must work in concert to drive outward growth of a developing TNT towards a neighboring cell (Figure 2). Table 1 compares the few presently known actin-related molecules that promote (or inhibit) the formation of TNTs as compared to filopodia. However, still little to nothing is known about their underlying formation from the standpoint of which specific molecules are involved, leaving several aspects about their biogenesis, fusion with the recipient cell, and final maturation for cargo transport unknown (see Outstanding Questions).

For example, the identification of these TNT-specific molecules is necessary for directly targeting and impairing TNT formation in disease pathways. Future characterizations of TNTs in a robust manner must address their transfer ability in order to substantially distinguish them from other cell protrusions. This will supply the TNT community with indispensable information and insights on how to better understand and approach essential questions of tunneling nanotube identity, formation and structure-function relationships.

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BOX 1 – The cell's molecular toolbox for generating actin-based protrusions

Here we summarize important actin-regulating molecules that participate in the generation of cellular protrusions. **Figure I** below schematically depicts an interaction network between these molecules, identifying the kind(s) of protrusion(s) generated between pairs of molecules and providing the appropriate reference(s).

I-BAR proteins: A five-member family of inverted Bin/Amphiphysin/Rvs domain proteins that initiate and stabilize negative membrane curvature. Notable members include:
IRSp53 (insulin receptor tyrosine kinase substrate of 53 kDa), IRTKS (insulin receptor tyrosine kinase substrate) and MIM (missing-in-metastasis).[13]

Rho GTPases: Signaling proteins that cycle between an 'on' and 'off' state depending on their GTP and GDP cycle, respectively, that trigger actin rearrangement. Notable members include CDC42 (cell division cycle 42), Rac1 (Ras-related C3 botulinum toxin substrate 1) involved in Arp2/3 activation, Rif (Rho in filopodia) and RhoD.[16]

Arp2/3: The actin-related protein 2/3 (Arp2/3) is a complex made of seven protein subunits that mediates the formation of dense, branched networks of filamentous actin. When properly activated by CDC42-stimulated **N-WASP** (Neural Wiskott-Aldrich syndrome protein), or by Rac-stimulated **WAVE** (WASp family verprolin-homologous protein), Arp2/3 binds on the side of a pre-existing actin filament and through its Arp2 and Arp3 subunits, which closely resemble the structure of monomeric actin, nucleates the formation of a new, 'daughter' filament oriented 70° relative to the pre-existing 'mother' filament.[82][83]

VASP: Vasodilator-stimulated phosphoprotein – member of the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family of actin nucleators that elongates straight actin filaments.[51]

Eps8: Epidermal growth receptor substrate 8 – actin-binding protein with a dual function – when it interacts with an adaptor protein Abl Interactor 1 (Abi1) it acts as a capper to limit

protrusion extension; when it interacts with an I-BAR protein, e.g., IRSp53, it promotes bundling of actin filaments and thus the stabilization of the formed protrusion.[23–26]

Formins: A family of fifteen actin nucleators that elongate straight actin filaments through the processive addition of G-actin to the growing barbed end. Formins are autoinhibited, a state reverted by their interaction with Rho GTPases. Most notable members include mDia1-3 also known as Diaphanous-related formins (Drfs) formins, FMNL2 and FMNL3 (formin-like protein 2 and 3) and DAAM1 (Disheveled-associated activator of morphogenesis 1).[38][84]

Fascin, Fimbrin, Espin: Common actin crosslinkers that bundle straight actin filaments hexagonally into parallel arrangements.[53,56,58,59]

Figure I. Direct interactions of actin-related proteins found to be indispensable in protrusion formation. These interactions were characterized by immunoprecipitation and/or fluorescence resonance energy transfer (FRET) assays. Note: This schematic does not show interactions of actin-related proteins in other cell processes (e.g., secretory pathways).

BOX 2 – TNT formation mechanism differs depending on the cell type

The same Rho GTPase pathways activated in neuronal cells might have a different role in TNTs depending on the cell type (e.g., cells of immune origin). For example, in macrophages, two pathways converging on Arp2/3, one that is dependent on CDC42-mediated activation of N-WASP (see Box 1), and the other dependent on Rac1 activation of WAVE2 (see Box 1), were found to participate in the formation of TNTs.[85] While the inhibition of Arp2/3 in macrophages led to a decrease in the number of TNTs[85], suggesting the importance of branched actin network formation during TNT biogenesis, in neuronal cells Arp2/3 blockage had an opposite effect—it increased the percent of TNT-connected cells and the vesicle transfer they conduct.[3] Consistent with these observation, in neuronal cells TNTs were found to be composed of exclusively straight actin filaments[3], suggesting that the inhibition of Arp2/3 probably led to the reorganization of actin cytoskeleton through redirection of available G-actin towards straight F-actin formation, subsequently inducing TNT biogenesis in these cells. Considering cells of immune and neuronal origin are different,

the conclusion for this discrepancy probably lies in cell-specific mechanisms utilized to form TNTs. In order to fully assess the role of these proteins in macrophages, it is crucial to elucidate the actin architecture within macrophage TNTs and to confirm their functionality by employing transfer-based experiments, similar to what was shown in neuronal cells[7].

A similar observation regarding Arp2/3 inhibition was observed in microvilli.[86] Blocking Arp2/3-dependent branched network assembly stimulated the growth of longer microvilli, highly likely through the reorganization the F-actin assembly from the cortical towards straight actin network in microvilli.[86] In contrast, inhibition of Arp2/3 led to a decrease in the formation of **tumor microtubes (TMs)** (see Glossary) between pancreatic cancer cells**,** implicating a similar regulation of TM biogenesis as compared to macrophage TNTs, but an opposite one as compared to neuronal TNTs.[87]

GLOSSARY

14-3-3: A family of regulatory molecules that bind phosphorylated serine/threonine motifs to protect phosphorylated residues from phosphatases, block downstream protein binding, and provide a scaffold for promoting direct protein-protein interactions, for example.

βCaMKII: A serine/threonine-specific Ca^{2+}-Calmodulin-dependent protein kinase II (CaMKII) enzyme highly expressed in the brain that mediates synaptic structures through binding and bundling of F-actin, and by sequestering G-actin.

Cofilin: A member of the actin depolymerizing factor (ADF) family that disassembles actin filaments at their pointed end through severing.

Cytonemes: Long (up to 700 µm) actin-based extensions that specifically allow for direct protein-protein interactions involved in growth factor and morphogen signaling over long distances.

Dendritic spines: Neuronal protrusions emerging from dendrites that receive excitatory inputs from axons. Immature ‘dendritic filopodia’ adopt the characteristic mushroom shape of the mature spine that is supported by branched actin.

Exocyst complex: An eight-subunit complex involved in vesicle trafficking where it facilitates the tethering of vesicles to the plasma membrane for exocytosis prior to membrane fusion.

FERM domain: Is a module originally identified in the four-point one/ezrin/radixin/moesin protein family that mediates plasma membrane binding by interacting with phosphatidylinositol (4,5) bisphosphate lipids.

Filamins: A family of actin-binding proteins that crosslink actin filaments into orthogonal networks.

Filopodia: Dynamic, closed-ended finger-like protrusions containing parallel bundles of F-actin reaching typical lengths on the order of 1−5 μm. They can be found on the dorsal side of cultured cells, but more commonly they are observed attached to the substrate.

Microvilli: Epithelial protrusions on the order of 1–2 μm in length that form a dense array known as the 'brush border.' Similar to filopodia they contain a core of bundled actin filaments.

M-Sec: Also known as Tumor necrosis factor alpha-induced protein 2 (TNFAIP2) acts as a platform that connects RalA (a Ral GTPase subfamily member) and the exocyst complex.

Myosins: Motor protein family that bind actin and move along actin filaments. Conventional class II myosins form microfilaments and produce contractile forces, while non-class II myosins comprise notable motors for organelle transport (e.g., Myosin-V, Myosin-X).

Polar filaments: Actin filaments are polarized, i.e. having different ends, referred to as the **barbed** (i.e., plus) and **pointed** (i.e., minus) end. Actin monomers preferentially incorporate at the barbed end, while filament disassembly occurs preferentially at the pointed end. In protrusions, the barbed end is oriented towards the plasma membrane such that polymerization can help outward growth of the protrusion.

SH3 domain: Src homology 3 domain is an adapter module that mediates the assembly of multi-protein complexes by recognizing short PXXP peptide motifs (P, proline; X, any amino acid) that adopt a polyproline type II helix.

Tumor microtubes (TMs): Membrane protrusions forming networks between cancer cells. TMs are thicker than TNTs and apart from actin they contain microtubules. They are close-ended protrusions with GAP junction channels at their ends that permit intercellular transfer of electrical signals and small molecules.

Table 1. Summary of actin-related proteins and their effect in the formation of TNTs vs. filopodia.

Actin and membrane-related proteins	TNT formation	Filopodia formation
IRSp53	Inhibits[7]	Promotes[19,20]
IRTKS	n.d.	Promotes[21,32]
CDC42	Inhibits/Promotes[7,85]	Promotes[17,19,20]
Rac1	Promotes[85]	n.d.
Rif	n.d.	Promotes[32]
RhoD	n.d.	Promotes[34]
VASP	Inhibits[7]	Promotes[7,22]
mDia1-3	n.d.	Promotes[41-44]
DAAM1	n.d.	Promotes[45]
FMNL3	n.d.	Promotes[46]
Fascin	Inhibits[8]	Promotes[53]
Eps8	Promotes[7]	Inhibits/Promotes[7,25,26]
Filamin	n.d.	Promotes[36]
Myosin-X	Promotes[8,76]	Promotes[70-72]

* n.d. – not defined

Figure legends

Figure 1. Schematics of canonical actin-based protrusions. **A.** Microvilli (1-2 μm in length) form a brush border on the apical surface of epithelial cells and contain straight actin filaments bundled by several actin crosslinkers. **B.** Various cells form dorsal and attached filopodia (< 10 μm in length) that are comprised of straight actin bundles crosslinked by fascin. **C.** Immature dendritic spines (i.e., dendritic filopodia) found in neurons share similarities with microvilli and filopodia; in contrast, apart from straight actin they are supported by Arp2/3-formed branched actin filaments. A common feature amongst these protrusions, apart from their morphological resemblance, is the presence of I-BAR proteins necessary for initial protrusion formation through membrane curvature sensing and induction.

Figure 2. Proposed model of TNT formation between two cells. A cell in its inactive state (1) is stimulated by various signals (2) that activate a Rho GTPase which subsequently releases I-BAR inhibition. Activated I-BAR proteins sense and induce negative membrane curvature necessary for TNT formation (2); actin polymerization is triggered to form an initial actin bundle that can overcome membrane resilience further enabling TNT extension for example by formins or another actin elongator (3). The growing TNT from cell 1 reaches the recipient cell 2 and fuses with its membrane through an unknown fusion mechanism (not depicted here) (4). A functional TNT, containing straight actin filaments bundled, for example by Eps8, is formed between cell 1 and cell 2 which can now exchange large cargo such as vesicles (by not yet known motors, not depicted here) (5).

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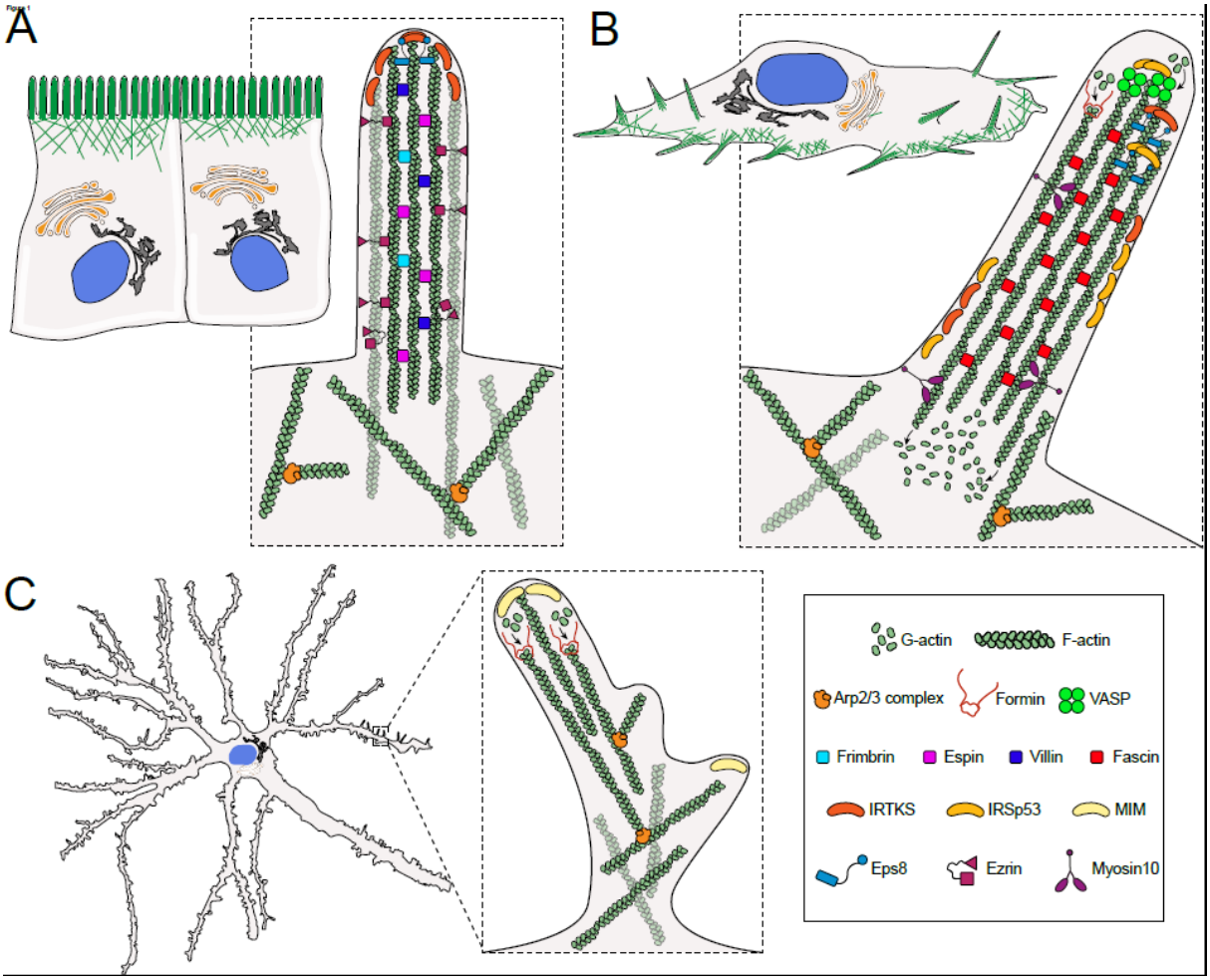
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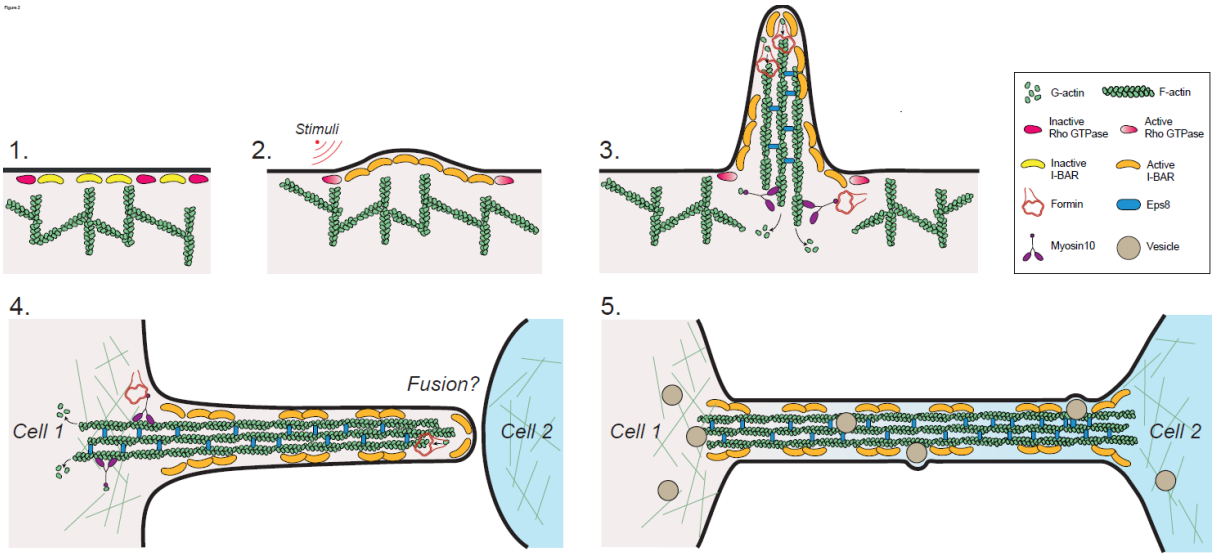
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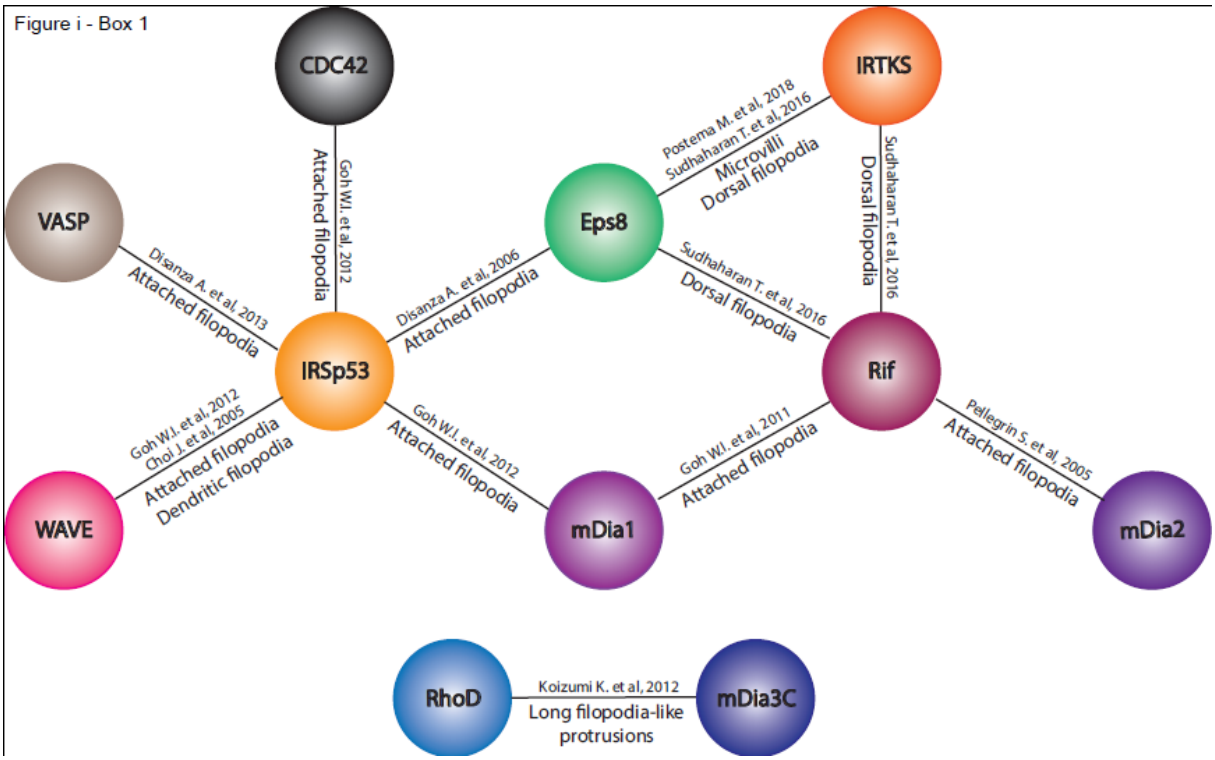
Figure i - Box 1
CDC42
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