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## **Title:** 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.

## 1 **Actin processes generate a diverse array of cell protrusions**

2

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

17

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

25

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

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

38

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

52

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

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

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

75

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

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

86

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

88

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

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

96

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

108

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

118

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

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

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

137

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

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

163

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

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

#### 184 **How linear F-actin assembly leads to protrusion growth**

185

186 **TNTs as compared to other similar protrusions reach extraordinary long distances.**

187 Foundational theoretical work in the field of filopodia, for example, has shown that filopodia



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

200

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

218

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

224

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

237

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

243

#### 244 **Stabilization of F-actin to give protrusions greater permanence**

245

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

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

252

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

265

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

278

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

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

297

### 298 **Delivery of proteins necessary for the protrusion biogenesis and elongation**

299

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

308

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

313 elongation.[72,74] It was observed that Myo10's **C-terminal tail-located FERM domain** (see  
314 **Glossary**) was responsible for  $\beta_1$ -integrin binding and FL-Myo10 relocalization to the tip of a  
315 filopodium, implicating the importance of integrin activation in Myo10-induced  
316 filopodia.[74] Additionally, FL-Myo10 was able to form longer filopodia, a property F2-  
317 and/or F3-deleted Myo10 mutants did not have.[74] This suggested Myo10 could elongate  
318 and stabilize these protrusions, the latter explained through the ability of Myo10 to  
319 recruit  $\beta_1$ -integrin to form focal adhesions involved in the stabilization of filopodia, while the  
320 elongation was probably dependent on VASP[73,75] or other, still unknown binding partners  
321 of Myo10.

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

337

### 338 **Concluding remarks and future perspectives**

339

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

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

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

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

368

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376



378 **BOX 1 – The cell’s molecular toolbox for generating actin-based protrusions**

379

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

384

385 **I-BAR proteins:** A five-member family of inverted Bin/Amphiphysin/Rvs domain proteins that  
386 initiate and stabilize negative membrane curvature. Notable members include:

387 IRSp53 (insulin receptor tyrosine kinase substrate of 53 kDa), IRTKS (insulin receptor tyrosine  
388 kinase substrate) and MIM (missing-in-metastasis).[13]

389

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

394

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

402

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

406

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



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

411

412 **Formins:** A family of fifteen actin nucleators that elongate straight actin filaments through  
413 the processive addition of G-actin to the growing barbed end. Formins are autoinhibited, a  
414 state reverted by their interaction with Rho GTPases. Most notable members include mDia1-  
415 3 also known as Diaphanous-related formins (Drfs) formins, FMNL2 and FMNL3 (formin-like  
416 protein 2 and 3) and DAAM1 (Disheveled-associated activator of morphogenesis 1).[38][84]

417

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

420

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

425

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

427

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

441 the conclusion for this discrepancy probably lies in cell-specific mechanisms utilized to form  
442 TNTs. In order to fully assess the role of these proteins in macrophages, it is crucial to  
443 elucidate the actin architecture within macrophage TNTs and to confirm their functionality  
444 by employing transfer-based experiments, similar to what was shown in neuronal cells[7].  
445 A similar observation regarding Arp2/3 inhibition was observed in microvilli.[86] Blocking  
446 Arp2/3-dependent branched network assembly stimulated the growth of longer microvilli,  
447 highly likely through the reorganization the F-actin assembly from the cortical towards  
448 straight actin network in microvilli.[86] **In contrast, inhibition of Arp2/3 led to a decrease in  
449 the formation of tumor microtubes (TMs) (see Glossary) between pancreatic cancer cells,  
450 implicating a similar regulation of TM biogenesis as compared to macrophage TNTs, but an  
451 opposite one as compared to neuronal TNTs.[87]**

452

### 453 **GLOSSARY**

454

455 **14-3-3:** A family of regulatory molecules that bind phosphorylated serine/threonine motifs  
456 to protect phosphorylated residues from phosphatases, block downstream protein binding,  
457 and provide a scaffold for promoting direct protein-protein interactions, for example.

458

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

462

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

465

466 **Cytonemes:** Long (up to 700  $\mu\text{m}$ ) actin-based extensions that specifically allow for direct  
467 protein-protein interactions involved in growth factor and morphogen signaling over long  
468 distances.

469

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

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

475

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

479

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

482

483 **Filopodia:** Dynamic, closed-ended finger-like protrusions containing parallel bundles of F-  
484 actin reaching typical lengths on the order of 1–5  $\mu\text{m}$ . They can be found on the dorsal side  
485 of cultured cells, but more commonly they are observed attached to the substrate.

486

487 **Microvilli:** Epithelial protrusions on the order of 1–2  $\mu\text{m}$  in length that form a dense array  
488 known as the 'brush border.' Similar to filopodia they contain a core of bundled actin  
489 filaments.

490

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

493

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

497

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

503

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

507

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

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

538

<b>Actin and membrane-related proteins</b>	<b>TNT formation</b>	<b>Filopodia formation</b>
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

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552 **Figure legends**

553

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

564

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

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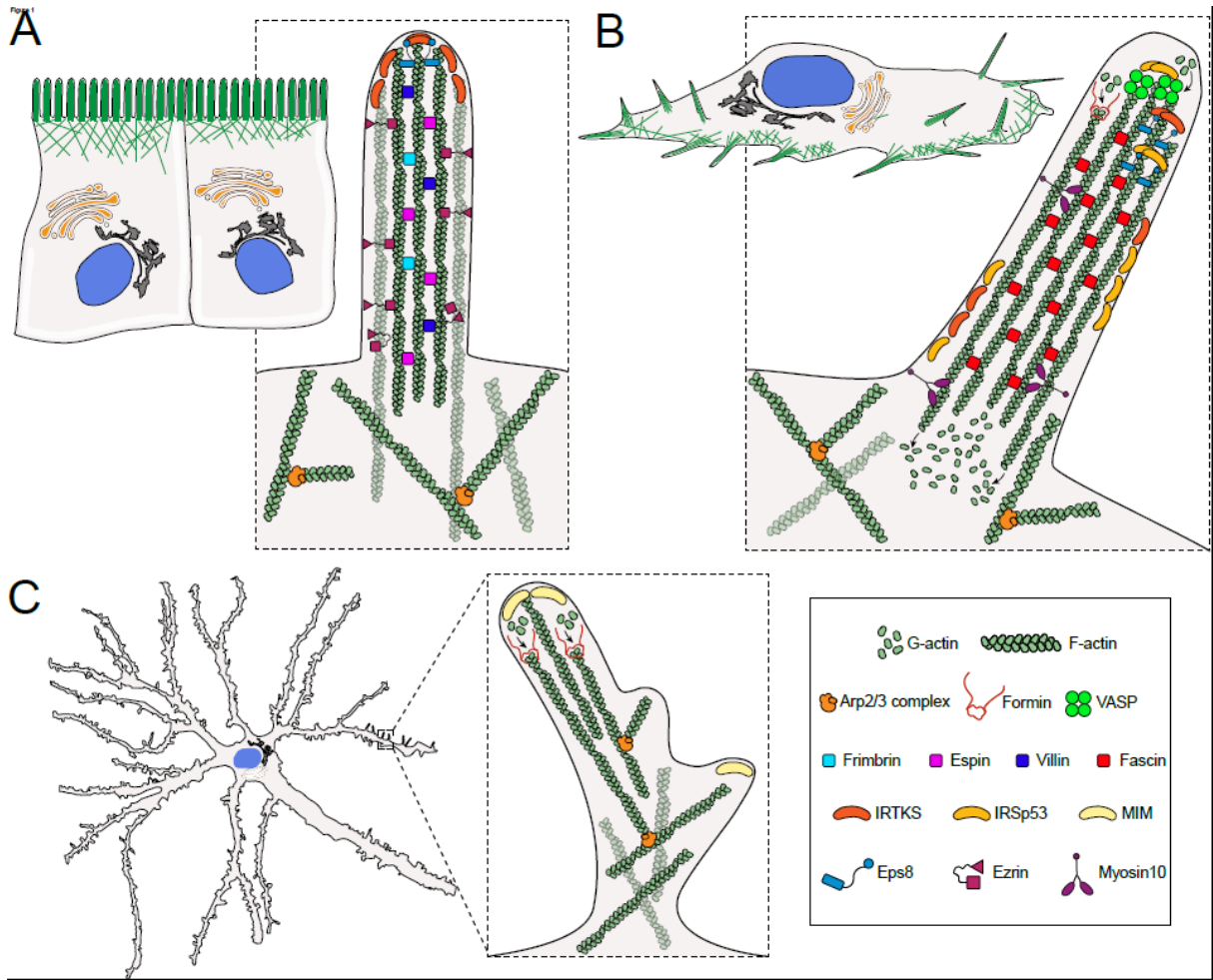


Figure 1

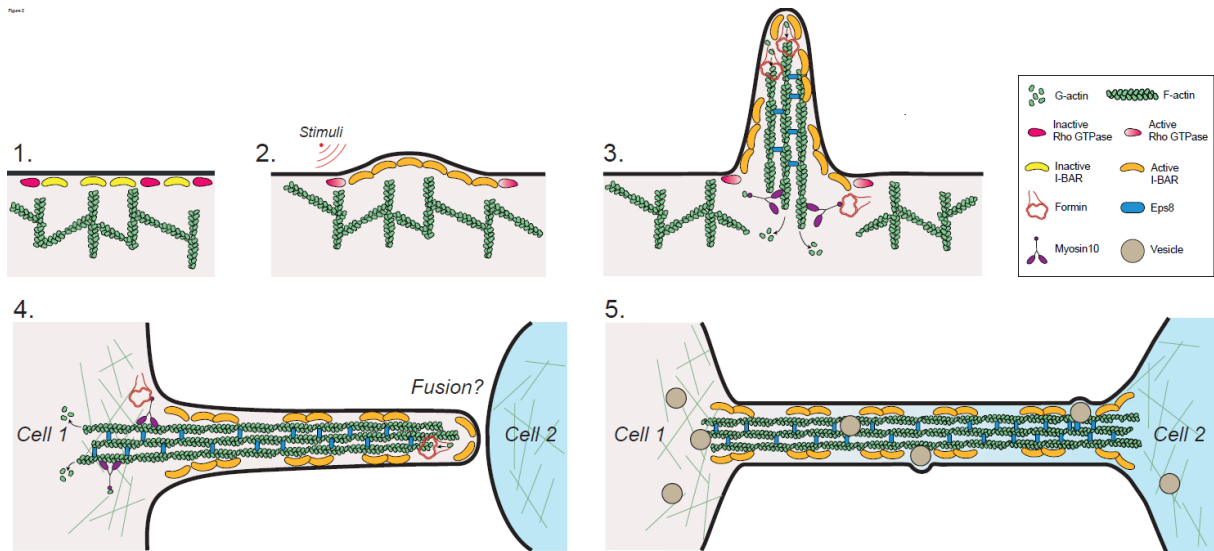


Figure i - Box 1

