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Formation principles of tunneling nanotubes

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Contents

Abstract

Tunneling nanotubes (TNTs) are thin membranous tubes that connect cells, providing a route for cell-to-cell communication and pathogen spread. TNTs form between a variety of cell types, but the mechanisms by which they form are largely unknown. We review general concepts related to the formation and stability of membranous tubular structures, with a focus on a membrane nanodomain deviatoric elasticity model.

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We examine experimental evidence that tubular structures form as a result of local membrane bending aided by laterally distributed proteins or anisotropic membrane nanodomains. The numerical results of several theoretical and simulation models of nanodomain segregation that suggest mechanisms of TNT inception and stability are also discussed. We address the relationship between the segregation of nanodomains and the protruding cytoskeletal forces, which are primarily produced in eukaryotic cells by the polymerization of actin. We also review the latest cell biological processes for the origin of TNTs in association with motor proteins in normal and cancer cells.

1. Introduction

1.1 Early experiments

Tunneling nanotubes (TNTs) are thin and hollow membranous protrusions connecting two cells at a distance of 10–250 μm. TNTs role is to mediate cell-to-cell communication. In a series of experiments in 2002 and 2003, Kralj-Iglič and colleagues observed long thin protrusions attached to giant unilamellar vesicles (GUVs), reporting on a movement of gondola-like cargo between them (Fig. 1A, E, F) [1,2]. Since the discovery of TNTs in 2004 in cells in vitro, its existence was later demonstrated in 3D in vitro models and in vivo tissue explants from animals and humans, mostly from cancer biopsies [3–5]. Previous studies have examined TNTs in various eukaryotic cell types, including red blood cells, neurons, immune cells, epithelial cells, and tumor cells [2,6–22] (Fig. 1B, C, D). It has been discovered that these TNTs not only conduct molecular or electrical signals [23–26], but are also capable of forming a cytoplasmic continuum between connected cells through which organelles, vesicles, proteins, nucleic acids, lipid droplets, prions, viruses, and nanoparticles can be transported (reviewed in [27]). Furthermore, these TNTs may be arranged into a larger network of tens or more, particularly in the case of connections between cancer urothelial cells [2,6], glioblastoma cells [28], and between mesenchymal stem and stromal cells in spheroids [29]. A very intense molecular and vesicular transport could be seen via nanotubes between immune cells, despite the rarity of such long networks between immune cells [12,24,30,31]. The active transport in TNTs requires microtubule- and actin-based transport machinery.

The definition of TNTs is still up for debate because there are so many other terms for similar protruding structures: cytonemes [32], membrane tethers [8], nanotubes (NTs) [11], or tumor microtubes (TMs) [33], intercellular membranous nanotubes [34], membrane nanotubes (MNTs)

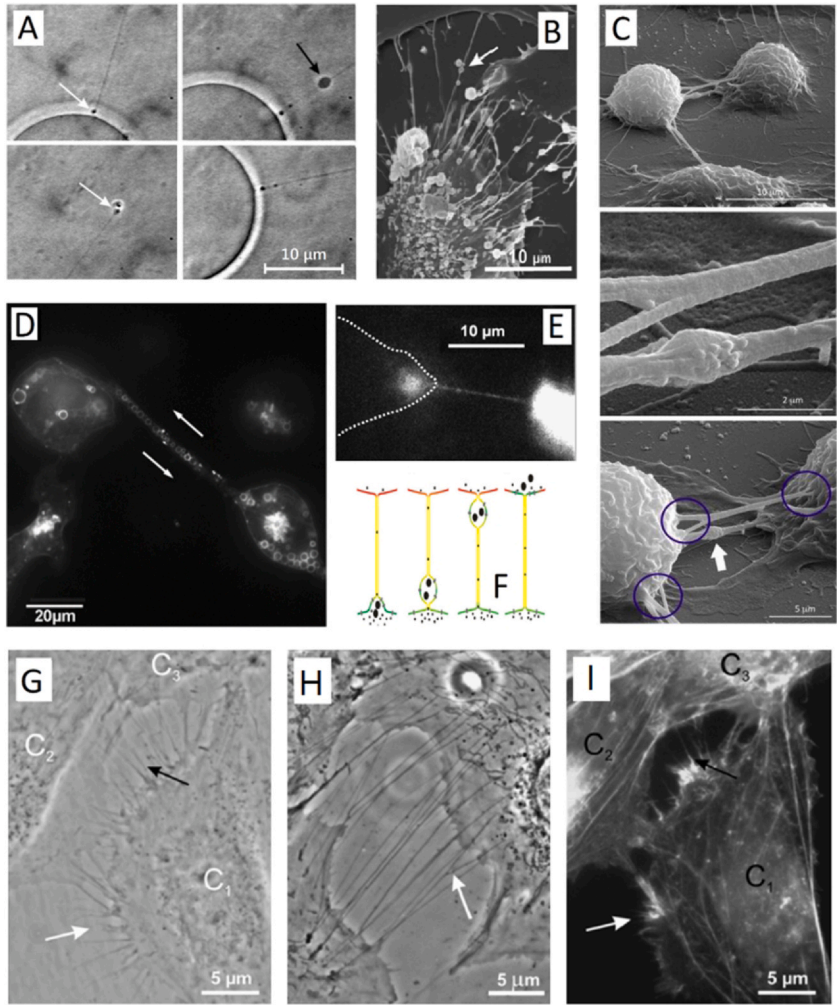

Fig. 1 (A) Photographs showing the movement of a small gondola (black arrow) inside a GUV (spherical, white arrow). The traveling gondola and the GUV's membrane fused together in the final phase (white arrow). (B) Under physiological conditions, scanning electron microscopy shows membrane nanotubes with gondolas (white arrows) found between cells in the human urothelial cell line. (C) A set of SEM pictures of RAW cells highlight the presence of various points of connectivity (circled, bottom panel), as well as a gondola inside a TNT (white arrow, bottom panel). (D) High-resolution photos of eukaryotic (B cell) nanotubes taken in vitro, white arrows indicate bidirectional cargo transfer. (E) Exchange of actin-GFP via a TNT between two T24 cells (cell borders are indicated by a dashed line). (F) A schematic illustration of TNT-directed transport between cells. Note that the gondola is the integral part of the membrane. (G, H) A phase contrast image of live T24 cells with type I TNTs. (I) After

(Continued)

[35] and epithelial bridges [36]. TNTs have few distinct characteristics, according to recent review by Cervantes and Zurzolo: (i) they must connect at least two cells; (ii) they must not touch the substrate; and (iii) they must include actin filaments, and (iv) enable cell to cell transfer of cargo [37]. Filopodia, which also contain actin filaments but do not fulfill other criteria, are not subject of this review. Our recent studies uncovered the tricytoskeletal composition in TNTs; normal and cancer urothelial cells were predominantly tricytoskeletal, and contained actin filaments, intermediate filaments, and microtubules [38,39].

1.2 The function of TNTs in spreading of pathogens

As evidence grows regarding the role of TNTs in cancer and neurodegenerative diseases, they are increasingly seen as a potential therapeutic target [42]. TNTs, which exhibit various forms, have been implicated in the transportation of bacteria, prions, and viruses [12,43,44]. An increasing number of studies highlight the significance of TNTs in the development of neurodegenerative diseases and cancer. The first documentation of viral transmission via TNTs was observed in the spread of human immunodeficiency virus (HIV) between infected and uninfected T cells [12]. This novel HIV transmission pathway was subsequently verified in vivo within the lymph nodes of mice [45]. The exploitation of TNTs by HIV accelerates the spread of the virus to numerous cells, playing a crucial role in HIV neuropathogenesis and the formation of viral reservoirs [46]. Additionally, the HIV accessory protein Nef has been found to encourage actin remodeling and stimulate TNT formation [43,47].

1.3 The growth of TNTs relies on specific regulatory proteins

There are indications that various regulatory proteins play a role in specific TNT growth scenarios. MSec was one of the first proteins revealed to be responsible for the formation of TNTs [48], even in

Fig. 1—Cont'd being fixed in paraformaldehyde for 15 min, the identical cells as in (G) are actin-labeled in the fluorescence micrograph. Cells C2 and C3 are being approached by cell C1. The white arrows show a brief, dynamic membrane protrusion that the approaching cell uses to scout its surroundings. The black arrow in G points at protrusions that have already connected to the target cell. Actin filaments can be seen in each of these numerous tubular connections (arrows in I). *(A) Adapted from [2]. (B) Adapted from [6]. (C) Adapted from [40]. (D) Adapted from [41]. (E) Adapted from [6]. (F) Adapted from [2]. (G), (H), (I) Adapted from [1].*

neurons that do not express MSec endogenously [49,50]. TNT formation is promoted by MSec interaction with RalA [48] and Rac1–exocyst pathway [51]. To prevent the creation of an imperfect close-ended TNT known as a filopodial bridge, MSec expression must be reciprocated by the target cell [11]. Close association of MSec with actin filaments was found in cell cortex and inside TNTs, though exact impact on TNT formation is not yet known [48]. However, actin filaments plays a central role in the formation of TNT [43]. Hanna et al. showed that the downstream Rho GTPase effectors mediate actin polymerization through Arp2/3 nucleation, Wiskott–Aldrich syndrome protein (WASP) and WASP family verprolin-homologous 2 (WAVE2) [51]. Both pathways led to actin polymerization and act together during TNT biogenesis [51]. A network of CDC42/IRSp53/VASP that promotes filopodia in neuronal cells may have a detrimental impact on TNT synthesis and hinder TNT-mediated intercellular vesicle transfer, according to recent study [52]. In contrast, an increase in Eps8, an actin regulating protein, enhances TNT creation while decreasing the proliferation of filopodia [52]. Treatment with latrunculin B, a toxin that depolymerizes actin filaments, prevented TNT development in PC 12 cells, derived from a rat adrenal medullary tumor (pheochromocytoma) [3]. Actin polymerization appears to be a nearly universal characteristic of eukaryotic TNT formation, but the specifics of the inception and timeline of TNT production remain unclear.

1.4 Is there a universal mechanism driving TNT growth?

This paper's goal is to review some recent TNT formation processes from both a biological and a computational modeling standpoint. Given the wide diversity of eukaryotic TNT morphology and structure described in recent reviews [11,37,53–56], it is critical to pinpoint some overarching factors governing their development. It is evident that the contractile stresses exerted on membrane-bound filaments by molecular motors and localized polymerization of cytoskeletal elements play a crucial role in the formation of TNT [46]. Studying in vitro trials of cellular membrane shape changes that require the recruitment of the cytoskeleton has proven to be particularly challenging thus far [57]. Combining these with protrusive pressures from the cytoskeleton, such as the polymerization of actin, may result in an as-yet-unidentified but potentially all-encompassing mechanism for TNT development.

2. Stability of TNT-like structures

Tubular membrane structures are widespread in most cellular contexts with a high surface-to-volume ratio and are structurally delicate [58]. Membrane continuity is a characteristic shared by the majority of tubular membrane structures [59].

It is generally agreed upon that TNT formation occurs in one of two ways: either the protrusion is entirely fueled by actin polymerization (type I) or cells that come into contact draw out nanotubes as they move apart (type II) [1] (Fig. 2).

These two processes can happen simultaneously among cells in vitro and are not mutually exclusive. Type I TNTs start to branch out dynamically from the donor cell and grow like filopodia as they seek connections with acceptor cells (Fig. 1E–G). Dagar et al. proposed that the process of TNT direction, elongation and attachment to donor cells is likely to be dictated by secreted chemotactic molecules and their sensors on TNTs [27]. One of identified chemotactic molecules is extracellular molecule S100A4 from calcium binding S100 protein family. S100A4 mediates TNT guidance through RAGE, a putative cell surface receptor for S100A4, through which TNT direction is guided [60]. Our study revealed that cancer urothelial cells with enriched in cholesterol/sphingomyelin membrane domains form TNTs that are attracted by normal urothelial cells

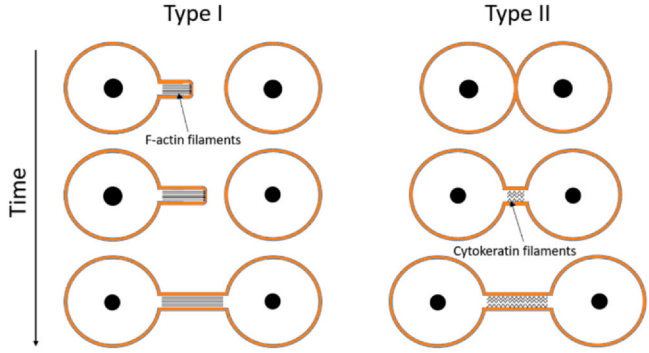

Fig. 2 (A) A diagram illustrating the formation of TNTs. Type I TNTs consist of actin filaments and initiate growth in a similar manner to filopodia. These protrusions often emerge in clusters of multiple tubes, actively seeking connections with adjacent cells. Type II TNTs begin to develop as nearby cells separate. In the context of urothelial cell lines RT4 and T24, some actin is still detectable at the entry point of the Type II tubes during the initial stages of tube development [1]. As the tube extends, actin progressively vanishes, leaving only cytokeratin filaments behind.

without these domains and vice versa, which supports soluble nature of chemoattractant for TNT guidance [39]. However, the compatibility of junctional proteins at TNT membrane and donor cell membrane is likely to be one of the necessities for TNT attachment. Cadherin family proteins N-cadherin and E-cadherin and gap junctional protein Cx43 have been shown to be involved [39,61–63] as well as receptor-ligand interactions [64]. Stability of mature TNTs depends also on cytoskeletal composition of TNTs. In urothelial cells, type 1 TNTs are shorter and more dynamic, and they contain actin filaments, whereas type 2 TNTs are longer and more stable than type I, and have cytokeratin filaments [1]. Triple labeling technique revealed that the cytoskeletal composition in urothelial TNTs is more complex [38]. TNTs with mono-, bi- and tricytoskeletal composition were disclosed. The evaluation showed that tricytoskeletal composition of TNTs, having actin filaments, microtubules and intermediate filaments, predominates [39] and is found also in co-culture of cancer and normal urothelial cells (Fig. 3). If the stability of TNTs increases with the presence of different combinations of cytoskeletal elements, especially intermediate filaments with strong mechanical stability, has to be evaluated. Optical tweezers assay showed that cancer urothelial TNTs are more deformable than normal urothelial TNTs, and significantly more tethers were pulled out of the TNT in cancer urothelial cells than in normal urothelial cells.

Frenkel et al. examined the impact of area expansion on membrane tension by removing tubes from GUVs using optical tweezers [65]. There ought to be a discernible rise in membrane tension with the lengthening of a short membrane tube. The tension should theoretically increase by about

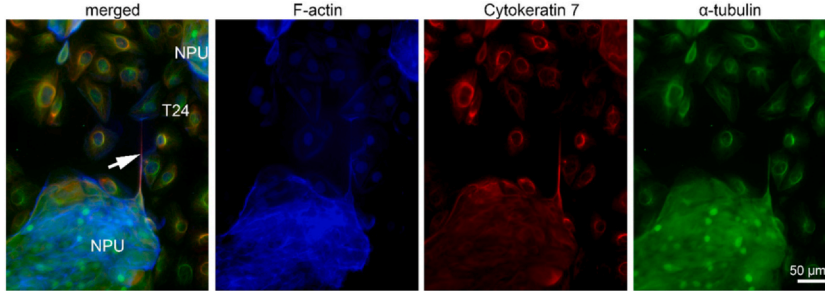

Fig. 3 TNTs with tricytoskeletal compositions in co-cultures of cancer and normal urothelial cells. Merged image shows TNT (arrow) between cancer (T24) urothelial cells and normal urothelial cell (NPU), which is composed of actin filaments (F-actin), intermediate filaments (cytokeratin 7), and microtubules (α-tubulin).

a factor of 10 and the plateau force by about a factor of 10, respectively, for an increase in area caused by pulling out a 10 m of radius 100 nm [66]. The absence of this increase, however, lends further credence to the idea that a membrane reservoir maintains constant surface tension. This reservoir may be caused by a partial membrane separation from the cover slip surface or may be composed of minute membrane inclusions [67]. The force barrier for membrane tube production increases linearly with the area that the force is applied to [65]. Additionally, according to numerical projections, the motor proteins must exert an initial force 13% greater than what is required to pull a long membrane tube in order for a tube to form, demonstrating that tube development is all-or-nothing [68]. However, due to variations in the cytoskeletal cortex, tubes produced from various sides of the cell exhibit various overshoot forces [69]. On the membrane of cells, lipid subdomains a few hundred nm in size and protein clusters can be seen [70]. Force generators (such kinesin and polymerizing cytoskeletal components) may not be able to counteract a force applied to one of these patches necessary for initial tube construction [65]. The acquisition of proteins or lipids, on the other hand, may be essential to reduce the overshoot force [71].

3. Changes in local curvature are related to membrane protrusions

Any binding of proteins or other substances to the membrane causes some curvature and disrupts the symmetry. The membrane develops a local spontaneous curvature as a result of this action [72]. The analysis of such passive systems revealed that, absent direct attraction between the inclusions, the entropic cost inhibits the aggregation of membrane inclusions in the limit of a nearly flat membrane with modest height deformations [73]. The hypothesis that these proteins play a role in promoting membrane curvature was first proposed in response to the identification of membrane-associated proteins with curved, lipid-facing surfaces (e.g., those with the so-called BAR protein domains) [6,74–76].

3.1 Curvature-sensing membrane inclusions

There are reports that describe the observation of curvature-sensing proteins without any presumptions regarding scaffolding or desirable protein-protein interactions. Prévost and colleagues discovered that the tagged

IRSp53 I-BAR protein's fluorescence signal was extremely weak on the tube that was removed from the GUV using optical tweezers in a recent work, indicating that the I-BAR domain has a poor affinity for positive curvature [77]. In a second experiment, the GUVs were grown with IRSp53 I-BAR dimers attached to both of their leaflets before being put into a buffer to separate the proteins on the outer leaflet. The protein interacted with the nanotube's negatively curved inner leaflet when a tube was extracted from one of these GUVs, dramatically enhancing the I-BAR domain on the tube. The proteins' area fraction was close to 5%. Thermodynamic explanations that take into consideration the energies involved in membrane bending and stretching, protein mixing entropy, and the energetic interaction between proteins and membranes can explain why this sorting is dependent on the tube curvature [78]. The configurational entropy of lateral ordering of membrane components describes the degree of disorder or randomness in the spatial arrangement of lipid molecules within the membrane. A high degree of configurational entropy indicates that the lipids are arranged in a relatively disordered or random fashion, while a low degree of configurational entropy indicates that the lipids are arranged in a more ordered or structured manner.

3.2 Anisotropic membrane components models

Additionally, the components of cellular membranes can be thought of as intrinsically anisotropic: cholesterol addition to POPC GUVs demonstrated that lipids with the opposite ratio create negative curvature, while lipids with a large area ratio of polar head groups to acyl chains create positive curvature. The membrane in this model is viewed as a self-assembling structure made of nanodomains, and it is possible to simulate a nanodomain's inherent form using the deviatoric elasticity model by selecting one of the two main intrinsic curvatures (Fig. 4A) [76,79]. The membrane nanodomains in the deviatoric elasticity model energetically favor a local geometry that matches the two intrinsic principal curvatures' descriptions of the appropriate local curvature (C_{1m} and C_{2m}). The nanodomain is isotropic if the two curvatures are same, and anisotropic if they differ (Fig. 4A).

By directly minimizing the membrane's free energy under the restrictions of constant volume, surface area, and the number of nanodomains, the morphologies of closed vesicles can be calculated computationally [84]. The accumulation of anisotropic components in the thin protruding sections of the vesicles is demonstrated to be favored by the creation of

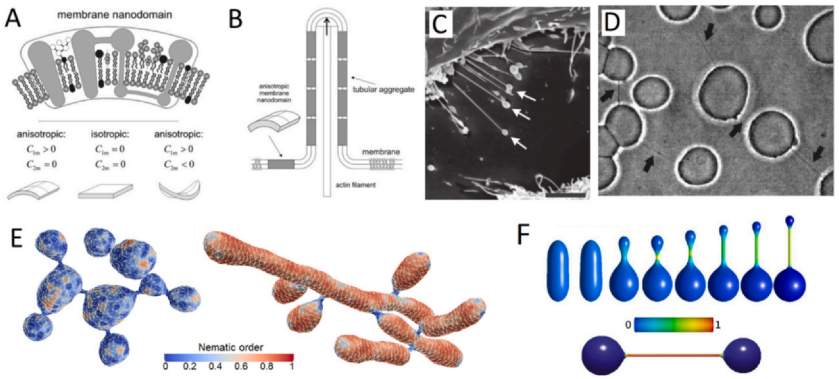

Fig. 4 (A) A diagram illustrating three distinct forms of flexible membrane nanodomains: partially cylindrical, flat, and saddle-shaped. Nanodomains exhibiting C_{1m} greater than 0 and C_{2m} less than 0 tend to adopt a saddle-like membrane structure, which is typically observed in the membrane neck that connects a daughter vesicle to its parent membrane. (B) A diagram demonstrating the stabilization of membrane protrusions through the accumulation of anisotropic membrane nanodomains in the tubular area. When cylindrical-shaped anisotropic membrane domains gather in the membrane section of a nanotubular membrane protrusion, they maintain the protrusion's mechanical stability even after the disintegration of cytoskeletal elements, such as actin filaments. (C) Some nanotubes forming between neighboring RT4 urothelial cells have vesicles at their free tips, as indicated by the arrows. Bar = 10 μm. (D) Giant unilamellar vesicles (GUVs) composed of POPC, cholesterol, and cardiolipin are linked by slender nanotubular structures (highlighted by black arrows). The GUVs were created using a modified electroformation technique [81,82]. (E) Numerical simulations of elongated membranous structures with anisotropic inclusions present everywhere ($C_{1m} > 0$ and $C_{2m} = 0$). The white lines show the direction of C_{1m}. If nematic ordering is not enforced between neighboring inclusions, the total bending energy of the shape is minimized by pearling structures (left), resulting in connected spherical vesicles. Conversely, nematic ordering between inclusions results in smooth and long membranous tubes (right). (F, top) Numerically calculated equilibrium cell shapes for a two-component membrane comprising both isotropic and anisotropic nanodomains, while maintaining a constant volume constraint. As the intrinsic mean curvature of isotropic membrane components increases from left to right, a more pronounced segregation between isotropic and anisotropic components is observed, as indicated by the color map. The color signifies the area fraction occupied by isotropic components, with fully blue representing a membrane made up solely of isotropic nanodomains. As the color transitions towards green and red, this indicates a higher lateral density of anisotropic components. (F, bottom) The calculated equilibrium closed membrane shape obtained in a system of isotropic components and anisotropic components, drawn without the inclusion of the entropy term. *(A) and (B) Modified from Reference [1]. (C) Adapted from [80] under the Creative Commons 3.0 License. (D) Adapted from Reference [74]. (F, top) Adapted from Reference [79]. (F, bottom) Adapted from Reference [83].*

equilibrium vesicle forms, providing structural stability (Fig. 4B). According to research by Perutkova et al., the attachment of flexible rod-like proteins can reduce the membrane's overall free energy and stabilize tubular protrusions through lateral sorting. This occurs if the decline in the proteins' orientational free energy is sufficient to outweigh the rise in free energy brought on by the decline in configurational entropy [74]. Other studies that used a deviatoric elasticity model to account for the anisotropic membrane inclusions verified the same findings [2,8,84–86].

A two-component membrane having isotropic and anisotropic components, each with a different intrinsic curvature, is shown in Fig. 4F in its axisymmetric equilibrium shape. By accumulating the anisotropic components in the tubular protrusion, the deviatoric energy of the anisotropic component reaches its lowest value. The mismatch between the membrane's curvature and the anisotropic component's spontaneous curvature causes this segregation [70]. These structures are typical of vesicular systems (Fig. 4D). The length of cylindrical protrusions increases with an increase in the intrinsic mean curvature of the anisotropic component when the intrinsic mean curvature of the isotropic component is increased (for fixed constant volume), resulting in shapes that lack up-down symmetry and resembling type II TNTs seen in vitro for RT4 urothelial cells (Fig. 4C, F). Numerical Monte Carlo results show that vesicles completely covered with anisotropic cylindrical curvature-preferring nanodomains ($C_{1m} > 0$, $C_{2m} = 0$) can lead to smooth tubular structures if the inclusions are bound by nematic ordering (Fig. 4E, right). Conversely, vesicles can form a network of spherical caps connected by thin necks when inclusions have no effect on orientational ordering of their neighbors (Fig. 4E, left). This may cause the tip vesicle to separate and become an extracellular vesicle [87] (Fig. 4E, left). When the vesicle's membrane is only partly covered by anisotropic membrane components, numerical results constrained by a rotational symmetry show that the spherical vesicle at the tip of the nanotube is considered to be stabilized by isotropic nanodomains with a convex (positive) curvature, whereas the nanotube is assumed to be generated from anisotropic cylindrical curvature-preferring nanodomains ($C_{1m} > 0$, $C_{2m} = 0$) (Fig. 4F) [79]. Only at lower values of the reduced volume are up-down symmetrical shapes stable [79].

3.3 Alternate theories: Protein crowding

There are also questions, though, about the idea that only innately bent proteins may bend membranes [88,89]. Epsin is not necessary for the

production of profoundly invaginated clathrin-coated pits, according to in vitro findings provided by Dannhauser and Ungewickell [90]. Clathrin alone can cause membrane deformation.

Recently, Stachowiak et al. in 2012 described another method of protein crowding, by which membrane bending is induced even in the absence of any protein insertion into the lipid layer in vitro [91]. They demonstrated that tubulation could be accomplished using either the ENTH domain of epsin or the ANTH domain of AP180 using GUVs that contained phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) as the membrane substrate (another protein found in endocytic clathrin-coated pits and vesicles). Thus, the authors demonstrated that, independent of how the proteins are recruited on the membrane, excessive local protein concentrations (crowding) adsorbed on the membrane can cause membrane curvature and tubule formation. Fluorescence-lifetime tubules efficiently produced when the epsin ENTH domain covered around 20% of the membrane, according to Förster resonance energy transfer (FRET). Such crowding has not yet been proven in vivo under typical protein expression circumstances.

4. Membrane protrusions coupled with cytoskeletal forces

There is proof that the mentioned curved membrane nanodomains are associated with active cytoskeleton-derived forces that cause the membrane to bend [92–94]. Owing to the highly non-equilibrium characteristics of membrane protein dynamics and associated membrane distortions, formations that would be unattainable under thermodynamic equilibrium can emerge. Curved protein complexes and lipid domains gather in areas where their inherent curvature aligns with the local membrane curvature, responding to the membrane's shape. A comparable adsorption mechanism for curved molecules (such as membrane proteins) may also occur for molecules that are absorbed from the cytoplasm onto the membrane [95]. TNT initiation may be significantly influenced by proteins that can control actin dynamics as well as sense or induce membrane curvature [16].

4.1 Deformation of membranes and protrusive forces: A positive-feedback mechanism

According to a model put forth by Gov and Gopinathan, this kind of self-organization that results in TNT growth can happen through feedback

mechanisms between three components: cell membrane deformation, protrusive forces of the cytoskeleton, and curved membrane activators of the cytoskeleton that correspond to the membrane shape [96]. A minor fluctuation in membrane shape triggers a flow of curved membrane components (CMCs) toward the most protruding part of the undulation. This flow results in a higher local CMC concentration at the protruding tip, subsequently increasing the force exerted by the cytoskeleton at that location and pushing it further outward in comparison to the surrounding area. As the protrusion forms, more convex CMCs move toward the tip through a self-reinforcing feedback mechanism. In this model, protrusive forces are attributed to actin polymerization near the membrane. Since the forces generated by the cytoskeleton stem from an energy-consuming process, they cannot be derived from the Helmholtz free energy; instead, they must be incorporated into the equations of motion for the membrane shape [97]. A conservation equation that includes the current of CMC on the membrane can adequately represent this active flow of CMCs [97]. The entropic cost prohibits aggregation in the absence of direct attraction between CMCs caused by this current [73]. A straightforward modeling of these dynamics assumes that heat fluctuations cause the activating membrane proteins, which have their own spontaneous curvature, to freely diffuse in the flat membrane. The actin polymerization is more widespread where the activators are concentrated, increasing the membrane's usual speed. It was hypothesized that the ensuing membrane motion would enhance the production of tubular protrusions by the interaction between the dynamics of the membrane and the resident activators with convex spontaneous curvature [96]. Even for length scales down to about 10 nm, which is very close to the molecular size, the use of continuum theory is appropriate and confirmed by in vitro tests [97].

A typical instance of an actin-attracting protein possessing these characteristics is the 53 kDa insulin receptor tyrosine kinase substrate. Evidence from structural, biochemical, and cellular biology studies corroborates the exceptional function of this protein family in transmitting signals that connect the protruding membrane to the underlying actin cytoskeleton [52]. Insulin receptor substrate of 53 kDa, or IRSp53, has been demonstrated to be able to sense membrane curvature and deform the membrane [52,77]. Additionally, IRSp53 is found endogenously in the human plasma membrane at modest densities [98]. Its association with the VASP protein family, which is involved in a number of cytoskeletal remodeling-related activities, encourages high-density clustering, which is necessary for active filament

elongation, which is typical of the early phases of TNT production in mammalian cells [99]. According to a recent study, the human Formin-like protein 1 dimer forms a convex surface when Cdc42, a protein involved in controlling the cell cycle, binds to it, assembling into an umbrella-shaped structure that encourages the proliferation of filopodia [100].

Another instance of this type of linked recruitment is seen with pentasaccharides with a strong hydrophilic nature called gangliosides, or GM1s, which dock onto the outer leaflet of cell membranes. The extremely curved borders of caveolae, where the large positive curvature is sustained by hydrogen bonds between the sugar moieties of adjacent GM1s, are where it has been demonstrated that GM1s congregate [101]. The early proliferation of TNTs may be mediated by positive membrane curvature produced by such an aggregation of GM1 molecules. I-BAR domain proteins are drawn to the negative curvature at the inner leaflet of a cell membrane caused by the positive and negative curvatures at the outer and inner leaflets caused by a GM1 aggregation. The actin nucleation machinery is activated by the I-BAR domain proteins, which further flex the membrane. The membrane protrusion then grows longer as a result of the actin filaments' subsequent nucleation (Fig. 5A) [13].

4.2 Anisotropic membrane components coupled with protrusive forces

The deviatoric elasticity model's modeling of actin activation forces entails minimization of the free energy, which is made up primarily of two contributions: bending energy and entropic mixing of isotropic and anisotropic components, with the actin filaments force modeled as a rigid rod restricting maximum vesicle size. The isotropic and anisotropic membrane elements must mix due to the entropy of mixing. However, a protrusion cannot form without the application of actin force because the lateral segregation of the components is insufficient. The isotropic and anisotropic membrane components segregate as the inner rod-like structure lengthens, permitting the creation of a smooth tubular protrusion (Fig. 5B). The anisotropic components heavily accumulate in the tubular protrusion when it is thin enough, which causes a virtually complete lateral separation of the isotropic and anisotropic membrane constituents. Only when actin force is applied can this happen, even for high values of vesicle volumes. The equilibrium axisymmetric forms can only produce undulated (necklace-like) protrusions without the rod's mechanical actin force (Fig. 5C) [102].

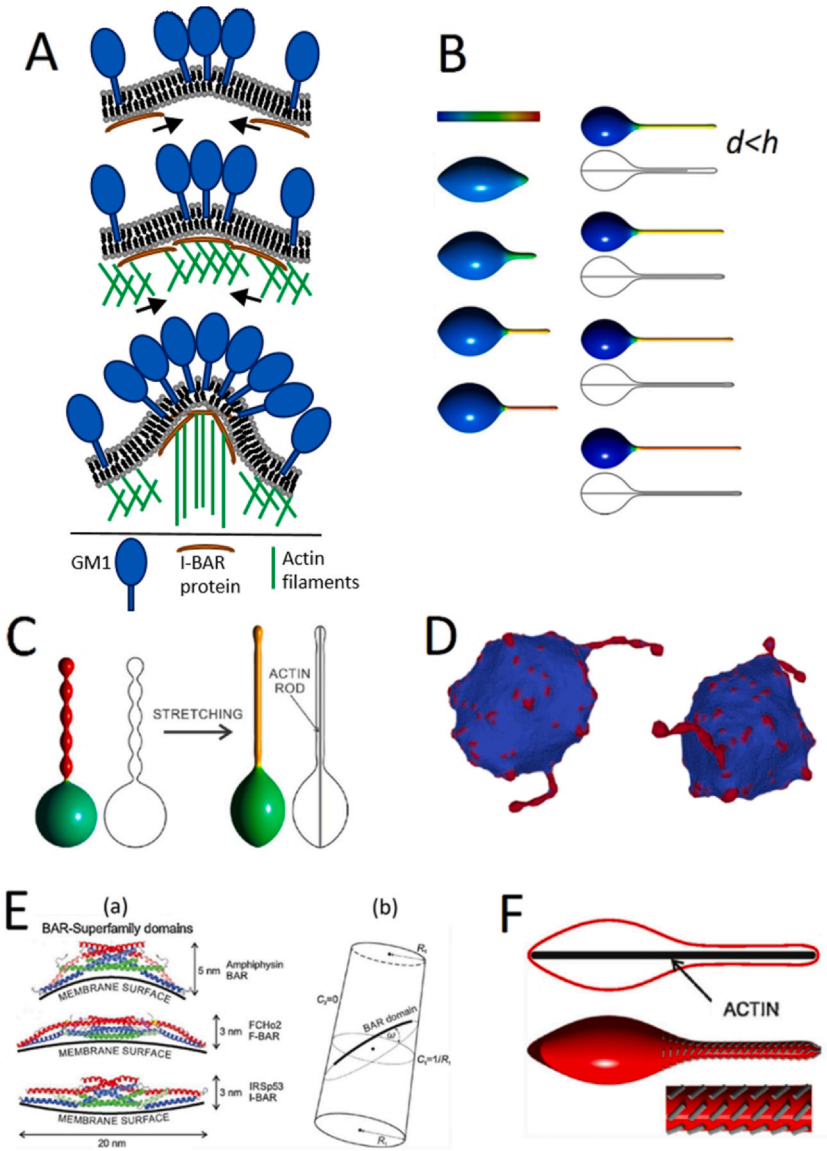

Fig. 5 (A) A graphic representation illustrating the process by which GM1 aggregates attract I-BAR domain proteins and actin filaments. (B) Computed equilibrium cell shapes for varying lengths *d* of the actin rod-like structure contained within the vesicle of height *h*. All vesicles share an identical relative volume (0.9). Excluding the top-right vesicle shape, $d = h$. The mixing entropy term was taken into account solely for the left column. The color indicates the area fraction occupied by isotropic components. A completely blue color signifies a membrane composed solely of isotropic nanodomains, while yellow and red colors represent high concentrations of

(Continued)

A recent study by Fošnarič et al. [104] also examined the theoretical connection between curvature and activity beyond the scope of axisymmetric shapes. Using Monte Carlo simulations, the vesicle membrane is depicted as a triangulated, self-avoiding network where each node can be empty (with no intrinsic curvature) or filled by an isotropic protein (with a constant intrinsic curvature) that can "diffuse" laterally between nodes during each algorithmic time step. Numerical equilibrium shapes without actin forces display only bead-like undulating protrusions where protein domains with intrinsic curvature gather on the protrusions (Fig. 5D). Consistent with experimental findings on the dependency of F-actin polymerization on membrane-bound proteins, this protrusive force is directed outward along the membrane's normal at each site occupied by a protein node. The findings predict the development of tubular protrusions when cytoskeletal forces are present at protein concentrations of approximately 15% of total nodes [104].

A system of flexible rod-like objects linked to the closed membrane that mimic BAR domains can receive a similar course of treatment (Fig. 5E(a)) [105]. The angle between the principal curvature and the flexible rod-like BAR domains' orientation-dependent energy is provided by this angle (Fig. 5E(b)). A rotational degree of freedom that depends on the area density of the BAR domains is connected to the orientational entropy of those domains. A single BAR domain is not sterically restricted by its neighbors at low area densities, making all orientational states equally likely. In contrast, at high area density, steric interactions with nearby domains prevent a single domain from rotating, which causes the overall configuration of BAR

Fig. 5—Cont'd anisotropic components. (C) Computed vesicle shapes for a two-component membrane, in which one component exhibits a high positive intrinsic curvature. The red hue signifies the maximum concentration of the membrane component possessing a high inherent curvature. (D) Results from the Monte Carlo simulation for non-axisymmetric vesicle forms in the absence of an actin cytoskeleton. (E) Diagrammatic representations of the BAR superfamily domains and the cylindrical surface with the connected rod-shaped BAR domain. (a) The BAR domains are shown with their characteristic dimensions and curvature preferences. (b) The flexible rod-like BAR domain is connected to the membrane surface of a cylindrical shape, where R_1 represents the cylinder's radius. Angle ω is the angle between the normal plane of the first principal curvature C_1 and the normal plane where the BAR domain is positioned. C_2 refers to the second principal curvature. (F) The influence of external force on the alignment of BAR domains. The sideways orientation of membrane-bound BAR domains (depicted as gray lines) undergoes change when the vesicle is stretched by an external force, potentially stemming from the growth of the cytoskeleton within the vesicle. *(B) and (C) Modified from Reference [103]. (D) Modified from Reference [104]. (E) Adapted from Reference [105]. (F) Modified from Reference [105].*

domains to resemble a close-packing configuration with nematic order [105]. When the vesicle is stretched by an actin force from within the vesicle, this lateral orientational ordering also occurs (Fig. 5F). For a non-zero angle, lateral BAR domains may form a chiral structure. The promotion of TNT synthesis by chiral amphiphile self-organization has been demonstrated [106], suggesting that such a process may be responsible for the emergence of TNTs.

4.3 Experimental evidence of membrane clustering

IRSp53 forms small clusters about one second before VASP recruitment at the same location, followed by protrusion growth at the same location a few seconds later [99]. Similar clustering was recently observed in full-length MIM [108], implying that this is a common feature of I-BAR domain proteins. Based on the I-BAR domain's ability to be locally enriched and constrict a weakly curved membrane, the cytoskeleton's local and transient fluctuation of the membrane could be stabilized and amplified via phase separation into coexisting domains of different curvatures. The IRSp53 cluster at the deformation's tip is expected to generate a domain that aids in the recruitment of Cdc42. Furthermore, it collects actin-related proteins like VASP, which leads to the growth of TNTs [109]. Tubular extension occurs as a result of actin polymerization, and the positive feedback between membrane deformation caused by actin polymerization and actin nucleator recruitment can also aid TNT growth [77].

IRSp53 clustering was also observed in live-cell imaging experiments in U2OS cells, demonstrating that TNT growth is frequently preceded by the formation of an IRSp53 cluster at the membrane. Similar I-BAR domain protein clustering has been observed before filopodia elongation in mouse embryonic fibroblasts and rat primary neurons [99,108].

In summary, anisotropic membrane components [102,110] or localized external forces acting on the membrane [103] are required for the stability of tubular membrane protrusions that develop on the surface of parent vesicles (refer to Figs. 5 and 6). However, from a theoretical perspective, stable free tubular lipid bilayer vesicle structures at small relative vesicle volumes do not necessarily require external local forces or anisotropic membrane components (Fig. 6). Theoretically, stable cylindrical vesicle shapes can be predicted at fixed vesicle area and volume by minimizing the local isotropic membrane bending energy for small values of the area difference between the outer and inner lipid layers of the membrane bilayer (Fig. 6) [74,107,111]. The same sequence of elongated vesicle shapes shown in Fig. 6 can be calculated within the spontaneous curvature model [111].

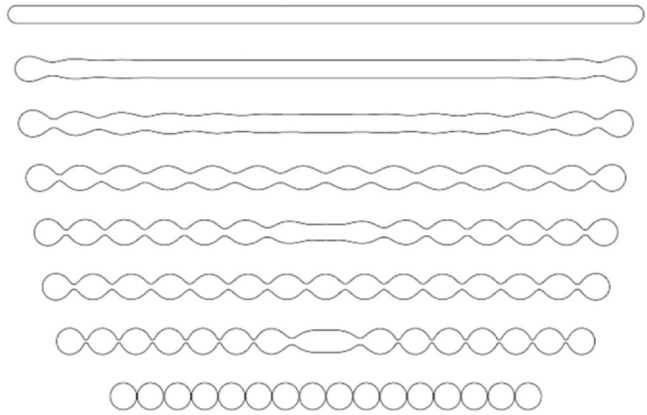

Fig. 6 Analytically determined symmetrical vesicle contours at low relative volume, obtained by minimizing local membrane bending energy [107]. *The series of calculated shapes is restricted by two extreme forms: tubular vesicles on top and necklace-like configurations on the bottom of the series of derived shapes (modified from reference [74]).*

The solutions to the variational problem, which determine the limiting forms of the computed sequence of elongated vesicle shapes at low relative volumes (Fig. 6), rely on the extreme average curvature deviator and extreme average mean curvature for a given area and volume of the vesicle [74,107,111]. The calculated limiting forms for the series of elongated vesicle shapes at small relative volumes can be either necklace-like, corresponding to the maximum average mean curvature (bottom limiting shape in Fig. 6), or a tubular structure with spherical caps, corresponding to the maximum possible average curvature deviator (top limiting shape in Fig. 6) [74,102,107,112]. A continuous shape transition from the limiting tubular to the limiting necklace-like vesicle shape (a series of spherical vesicles connected by extremely thin necks) can be theoretically predicted, driven by increasing the area difference between the outer and inner layers, where the latter is directly proportional to the average mean curvature, or by increasing the membrane's spontaneous curvature [74,107,111].

5. Motor proteins as key regulators of membrane protrusion formation and transport

The action of motor proteins is required for force generation to pull the protruding tubular structures along the cytoskeletal tracks in TNT formation [113] (Fig. 7). The actin-associated molecular motor myosin-X

(Myo10) was indicated as TNT promoting motor protein [114]. Expression of Myo10 increased the number of TNTs and accelerated the unidirectional transfer of vesicles between neuronal cells [114]. On the other hand, myosin 2 (Myo 2) motor activity was shown to control the outgrowth of membranous protrusions. Suppression of Myo 2 activity by inhibition of Rho-kinase with Y-27632, enhanced (ca. threefold) the TNT formation in a concentration dependent manner by inhibiting the inward force on the actin filaments [31]. Similar effects of myosin inhibition on TNT growth were demonstrated in normal rat kidney cells [115] and Jurkat T cells [116].

The molecular motors of microtubules and actin filaments are indispensable actors in cargo transport in a cellular context. Transport in TNTs depends on the molecular motors expressed in TNTs based on the presence of plus-end and minus-end processive motor proteins in the tube. Three classes of molecular motors transport cargoes along the actin filaments or the microtubules in a particular direction: the myosin motors that move along actin filaments unidirectionally; the kinesin motors that move along microtubules, predominantly towards the microtubule plus-ends; and the dynein motors that move towards the microtubule minus-ends. In microtubule- based transport, the kinesin and dynein motors can make the cargo move back and forth [117]. Although unidirectional [115,118] or bidirectional [30,31,119] transport of cargo has been observed in TNTs, mainly actin-based myosins have been detected in TNTs. Recently, we used immunofluorescence and showed combinations of actin and microtubule-based motor proteins in urothelial TNTs between cancer cells, between normal cells and between cancer and normal cells [39] which is the first demonstration of microtubule-based motor proteins kinesin 5b and dynein in TNTs.

Although the molecular mechanisms that regulate the formation of TNT remain unclear, there is more evidence of external stimuli that facilitate their formation. The formation rate of TNTs in vitro can be promoted by conditions that are not optimal for cells, but rather stressful. TNTs are more frequently formed under metabolic stress i.e. serum deprivation [120,121], whole nutrient starvation [120], and environmental stress such as exposure to hydrogen peroxide [9,121], hypoxia [120,122,123], acidic pH [120], temperature [13] or inflammation [124]. Interestingly, TNTs originate from stressed cells and associate with unstressed cells and vice versa [120], revealing cell detrimental and cell rescue role of TNTs.

Actin polymerization appears to be a nearly common characteristic of all eukaryotic TNTs and TNT-like structures, but the exact beginning and chronology of these structures formation are yet unknown. In addition to

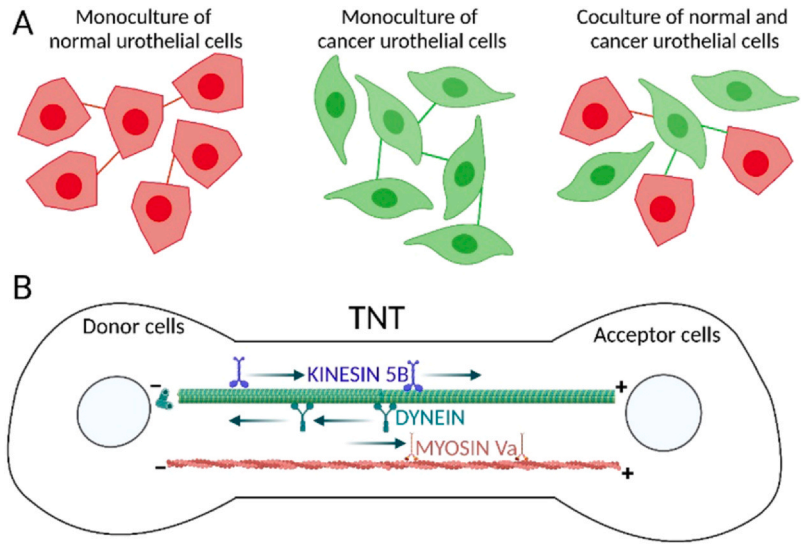

Fig. 7 Motor proteins in urothelial cells. (A) TNTs are formed between cancer cells, between normal cells in monocultures and between normal and cancer cells in co-cultures. The color of the TNT in co-cultures indicates, which cell is the donor of the TNT. (B) The scheme represents transport machinery in urothelial TNTs according to the results of immunofluorescence of motor proteins in monocultures and co-cultures published in Resnik et al. [39]. The presence of the microtubule-associated motor protein kinesin 5B and dynein in TNTs indicates bidirectional transport from the donor cell (TNT forming) to the acceptor cell (TNT attaching). The kinesins have the plus-end direction, and the dyneins have the minus-end direction. Myosin Va mediates transport on actin filaments. The TNT in the scheme is magnified and not drawn to scale.

actin filaments, microtubules are required for the formation of TNTs in the first stage (<16 h in culture), while in the second stage (>16 h in culture), microtubules are related to the morphological features of TNTs and mitochondrial transfer in human mesenchymal stem cells and neonatal mouse cardiomyocytes [35]. In contrast, in astrocytes the microtubule-disrupting agent nocodazole and the microtubule-stabilizing agent paclitaxel did not alter the induction of TNTs [120]. These studies suggest that proteins and pathways involved in TNT formation are cell type dependent.

6. Conclusion

Tunneling nanotubes appear to be a common mode of cellular communication and trafficking. The TNT field necessitates the

standardization of terminology for their definition, as well as tools designed for the detection and monitoring of these common structures, especially in 3D in vitro models and in a more complex in vivo tissue. The main challenge remains the identification of molecular markers for studying TNTs as well as principle of TNT formation from mechanistic point of view. TNTs' fragility makes them difficult to manipulate, and new devices, such as microfluidic systems, are needed. Understanding the basic workings of their inception mechanisms may help to overcome the enormous challenges posed by resolving their diversity in morphology and structure.

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Conflict of interest

The authors declare no conflicts of interest in this paper.

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