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Specialized intercellular communications via cytonemes and nanotubes.

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Abstract

In recent years, thin membrane protrusions such as cytonemes and tunneling nanotubes have emerged as a novel mechanism of intercellular communications. Protrusion-based cellular interactions allow specific communication between participating cells, and would have a distinct spectrum of advantages compared to secretion-diffusion-based intercellular communications. Identification of protrusion-based signaling in diverse systems suggests that this mechanism is a ubiquitous and prevailing means of communication employed by many cell types. Moreover, accumulating evidence indicates that protrusion-based intercellular communications are often involved in pathogenesis including cancers and infections. Here we review our current understanding of protrusion-based intercellular communications.

Introduction: discovery of thin membrane protrusions for intercellular communication.

Multicellular organisms represent a complex community of cells, whose concerted actions are essential for the development and maintenance of healthy tissues, organs and individuals. Essentially every single action of an individual cell depends on communication with its neighbors. The most critical aspect of cellular communication is specificity: who receives what kind of message from whom, and when?

Progress in recent years has revealed a new mode of intercellular communication, namely via thin membrane protrusions. These protrusions are proposed to provide specificity in signaling by allowing the direct physical contact between signaling-sending and -receiving cells. These protrusions have been found in different tissues from different organisms, with certain distinct functional/structural characteristics, leading to distinct naming such as cytonemes, tunneling nanotubes (TNTs), membrane nanotubes and microtubule-based nanotubes (MT-nanotubes). Investigators have continued to use distinct terms to describe

these structures, given the lack of concrete evidence that they represent the same organelle with the same function. To be more inclusive for similar structures and similar functions, generalized terms such as thin membrane protrusions, membrane extensions, TNT-like protrusions, cellular bridges, specialized filopodia, signaling filopodia and alike, have been employed as well. The efforts toward accuracy (e.g. not calling different structures by the same name), however, have created confusion in terminology. Furthermore, additional understanding of these protrusions has sometimes muddled existing definitions, especially when it was found that specific characteristics of certain protrusions, once used as defining features, are not stable and can change over time and under different conditions. In this review, we will use the term ‘thin membrane protrusions’ as a unified and generalized term, while maintaining original terms when referring to individual discoveries.

Filopodia are actin-based finger-like protrusions often observed at the leading edge of the migrating cells. Best known for their function in cell migration, filopodia are one of the earliest thin membrane protrusions recognized (Gardel et al 2010, Jacquemet et al 2015). Early studies suggested that filopodia enable cells to ‘sense’ the environment in deciding the directionality of cell migration: first, filopodia exhibited ‘scanning’ motion until they attach to the substratum, followed by extension of lamellopodia toward the stabilized filopodia (Albrecht-Buehler 1976, Trelstad et al 1967). This very notion of ‘environment sensing’ indicated filopodia’s role in signaling. However, much of the research on filopodia has focused on their cytoskeletal aspects and their role in cell migration and adhesion for a long time (Gardel et al 2010, Jacquemet et al 2015). However, it was found that the formation of thin filopodia did not always correlate with cell migration but instead correlate with intercellular signaling (Miller et al 1995), hinting at a role for filopodia in intercellular signaling (see below).

In the last two decades, the role of thin membrane protrusions as signaling devices has started coming into focus. Much has to be still learned about these thin membrane protrusions to establish their ‘natural history’ (how many kinds of thin membrane protrusions exist with similar/distinct structures and functions?) and functional relevance. One major challenge is the fact that, for most of these thin membrane protrusions, there is no simple way to specifically perturb their formation without affecting other biological processes of the cells, hampering critical assessment of their functionality. Yet, striking correlation has been observed between the presence of thin membrane protrusions and cells’ signaling ability. This review attempts to summarize our current knowledge that supports the signaling roles of these thin membrane protrusions, while drawing readers’ attention to the need of further critical assessments.

Cytonemes, defined as ‘filopodia of a special type’ that can reach up to 700 μm in length, were found to form in developing *Drosophila* imaginal discs (Ramirez-Weber & Kornberg 1999). These thin membrane protrusions harbor specific signaling ligand/morphogen in various cell types (Hsiung et al 2005, Huang & Kornberg 2015, Roy et al 2011, Roy et al 2014), leading to the idea that they allow specific signaling between specific combinations of cells. Cytonemes connect cells separated not only by distance but by other (non-participant) cells in the tissue, and are believed to deliver signaling molecules (e.g. morphogens) and their receptors between signal-sending and -receiving cells (Figure 1A).

Following the discovery of cytonemes, tunneling nanotubes (TNTs) were found to form between cells in culture cells, allowing trafficking of vesicular organelles (Rustom et al 2004). TNTs were shown to form de novo between previously unconnected cells by extension of protrusions (Rustom et al 2004) or by dislodgement of two previously-attached cells, which leaves TNTs behind (Sowinski et al 2008). It was shown that TNTs connect the cytoplasm of two cells, establishing continuity of their plasma membranes as well. Since then, many TNT-like structures have been observed in various cell types and contexts. Original TNTs were defined by their ‘tunneling’ characteristics: they are open-ended, thus connecting the cytoplasm of two cells (Figure 1B). Later studies have identified structures that strongly resembles TNTs, but have closed-end topology (Figure 1A). Other studies characterized similar structures with similar functions, yet their topology (open-vs. closed-ended) was not characterized. Therefore, it remains unclear whether all of these thin membrane protrusions should be categorized under the same header.

Recently, Microtubule-based nanotubes (MT-nanotubes) joined the group of ‘thin membrane protrusions (Figure 1C). MT-nanotubes are formed by *Drosophila* male germline stem cells (GSCs) and protrude into the major niche component, hub cells. The evidence supports that the surface of MT-nanotubes serves as a signaling interface where a receptor is specifically localized to receive a signaling ligand expressed by the niche cells (Inaba et al 2015). Unlike many other thin membrane protrusions, the major cytoskeletal component of MT-nanotubes appears to be microtubules. They are also different from many other thin membrane protrusions in that their function is to allow exclusive, short-range signaling, as opposed to long-range intercellular communication.

In this review, we attempt to summarize our current knowledge on the structure and function of various thin membrane protrusions, while paying particular attentions to two major challenges in the field, the difficulty in categorizing similar structures and the difficulty in proving their functionality.

Structures of thin membrane protrusions involved in signaling

Many distinct thin protrusions have been identified in many contexts and cell types. Perhaps the most challenging aspect of studying these protrusions is their fragile nature, which has hampered detailed characterization of individual structures. These thin membrane protrusions are often sensitive to experimental procedures such as mechanical stress and fixation, making it difficult to compare individual structures to each other. In addition, accumulating evidence suggests that the difficulty in categorizing these thin membrane protrusions arises not only because of technical challenges, but also because of their highly adaptive nature: these structures can change their cytoskeletal compositions upon stimulation, possibly making the same structure look distinct in different contexts.

Distinction based on cytoskeletal components

Cytonemes, originally found in *Drosophila* imaginal discs, mediate the delivery of specific signaling ligands (morphogens) and their receptors to allow specific signal transduction between cell types that are far apart from each other, often separated by large numbers of

intervening cells (Kornberg & Roy 2014). Like regular filopodia, cytonemes contain actin and require regulators of actin dynamics for their formation (Roy et al 2014).

Similarly, TNTs are also mostly actin-based thin membrane protrusions. TNTs were originally found in cultured cells, where they were observed to be ‘hovering’ in the media solution without any attachment to substratum, distinguishing them from cytonemes or filopodia, which adhere to substrata such as surfaces of other cells or the culture dish. This critical difference in the relationship between the thin membrane protrusions (cytonemes vs. TNTs) and their environments (other cells vs. media solution) caused a hesitation by researchers to view these structures as orthologous organelles, hence the use of distinct names. However, a recent study proposed that TNTs in culture may correspond to invadopodia in vivo, based on a number of similarities (Naphade et al 2015), indicating that TNTs do not necessarily ‘hover’ in the medium. Also, TNTs have been reported to form between immune cells (Sowinski et al 2008, Watkins & Salter 2005), in which case TNTs may indeed exist in fluid environments of blood/serum/lymph without adhering to any cells or substratum. Taken together, these observations suggest that different characteristics in different settings of the thin membrane protrusions may not necessarily reflect their distinct identity as an organelle, and suggest that many of thin membrane protrusions may represent orthologous organelles.

For some time, TNTs and related structures were classified based on their dimensions (thickness, length) and resident cytoskeletal components (all contains F-actin), with some TNTs containing microtubules as well (Gerdes & Carvalho 2008). When examined, the formation of TNTs was shown to depend on intact actin cytoskeleton (Rustom et al 2004). However, recent studies showed that microtubules can be induced in the TNTs upon stimulation (cellular stress such as UV irradiation), stabilizing TNTs and allowing for transfer of mitochondria, which rescues damaged cells from cell death (Wang & Gerdes 2015). Similarly, pseudorabies virus protein US3 induces TNTs that contain microtubules and thus are stabilized (Jansens et al 2017). TNTs formed in squamous cell carcinoma culture are reported to always contain microtubules, possibly indicating that these cells have adapted to constant ‘stress’ conditions, allowing better survival of tumor cells. Interestingly, microtubules were not required for TNT formation but required for TNT-mediated transfer of lysotracker-positive vesicles, suggesting that microtubule-dependent stabilization of TNTs is critical for trafficking (Saenz-de-Santa-Maria et al 2017). An important implication of having actin vs. microtubule is that the constituent cytoskeleton would determine the molecular motors (myosins, kinesins and dyneins), and thus the specific cargoes, to be trafficked between two cells.

Unlike cytonemes and TNTs, microtubule-based nanotubes (MT-nanotubes) mainly contain microtubules and do not require regulators of the actin cytoskeleton for their formation. Instead, MT-nanotubes utilize components of cilia formation (e.g. intraflagellar transport (IFT) proteins) although they lack other characteristics of cilia (such as acetylated tubulin) (Inaba et al 2015).

These results suggest that caution should be used in categorizing thin membrane protrusions based solely on cytoskeletal components, and that they might dynamically change cytoskeletal composition, possibly adjusting their functionality.

Distinction based on topology

Whereas cytonemes are considered to be closed-ended filopodia (Figure 1A), TNTs have been categorized based on their membrane topology. The original defining feature of TNTs was that they ‘tunnel’ two cells (Gerdes & Carvalho 2008, Rustom 2016, Rustom et al 2004) (Figure 1B): Open-ended TNTs are speculated to support continuity of cytoplasm between two cells as well as that of their membranes, leading to propagation of calcium signals and free diffusion of membrane proteins between two cells. Also, large organelles such as mitochondria have been observed to move from one cell to another via TNTs.

By contrast, some TNTs do not propagate calcium signals or allow free diffusion of membrane proteins, leading to the interpretation that these represent close-ended TNTs (Gerdes & Carvalho 2008). In some cases, TNTs were shown to clearly have a closed end, where the tip of TNT and the surface of the target cell adhere to each other (Figure 1A). Yet, these closed-end TNTs can mediate transmission of viruses from one cell to another (Jansens et al 2017), suggesting that virus transmission can occur through exocytosis and endocytosis at the surface of TNTs. Thus, the openness of TNTs may not necessarily be required for transmission of materials, and that transmission can be mediated by ‘synapse-like’ activity.

Open- or close-ended TNT structure might be the result of different mechanisms of their formation. TNTs can form *de novo* between two unconnected cells or as a remnant of cytokinesis. In the former case, cells must fuse two plasma membranes to create open-ended TNTs. However, genes specifically required for this membrane fusion step have not been identified, most likely owing to the difficulty of characterizing the topology of TNTs (open vs. closed). In this regard, a recent finding on the requirement of long, actin-based protrusions in myoblast fusion in *Drosophila* flight muscles may provide important insights into the mechanisms that underlie open-ended TNT formation. Existing myotubes extend multiple thin protrusions toward myoblasts prior to fusion events (Segal et al 2016). During this process, the actin regulator Enabled, which localizes to the tip of filopodia, controls filopodia formation and myoblast fusion. Duf, a protein required for myoblast-myotube fusion, also localizes to filopodia and likely promotes fusion at the point of contact between the filopodia and myoblast (Segal et al 2016). Thus, it is tempting to speculate that a similar mechanism drives TNT fusions.

Mechanisms of the formation of thin membrane protrusions.

Most cytonemes, TNTs and similar structures require the actin cytoskeleton and its regulators for their formation (Hanna et al 2017, Kornberg & Roy 2014, Sanders et al 2013), and are referred to as ‘specialized filopodia’. However, the mechanisms that promote their formation may be distinct (Delage et al 2016).

Cues that induce/guide the formation of thin membrane protrusions

While cytonemes provide a clear mechanism by which signal-sending cells and –receiving cells communicate with one another in a highly specific manner over a long distance, how cytonemes interact with cells along their path remains an open question. Do they squeeze in between non-target cells? Do they poke into the cells along the path? How do cytonemes know when they reach the correct target cells? A recent study provides critical insights into some of these questions. In *Drosophila*, air sac primordia (ASP) extend cytonemes to the wing discs to receive Dpp and FGF ligands. The planar polarity (PCP) system in the wing disc plays a critical role in guiding cytonemes from the ASP (Huang & Kornberg 2016). PCP components within the wing discs modify the extracellular environment (extracellular matrix components, e.g. glypicans and laminin), which in turn allows the directed growth of cytonemes from ASP (Figure 2A).

Stresses are common triggers for the formation or modification of TNTs. A broad range of stresses, including H₂O₂, UV, virus infection, prion aggregation, serum starvation and high-glucose concentration can trigger TNT formation (Thayanithy et al 2014, Wang & Gerdes 2015, Wang et al 2011). Typically, stressed cells form TNTs to reach out to healthy cells, indicating that TNT formation may be a mechanism that helps protect cells from death (see below). In the culture of rat hippocampal neurons and astrocytes, p53 and its downstream effectors (Egfr, Akt, phosphoinositide 3-kinase, and mTOR) are shown to be critical for TNT formation (Wang et al 2011), although its requirement is likely cell type-specific (Andresen et al 2013).

Receptor-ligand interactions mediate protrusion formation.

In many instances, ligand and receptor binding interactions regulate the formation of cytonemes/TNTs (Kimura et al 2013). For example, cytoneme formation relies on the receptor-ligand combinations (Ramirez-Weber & Kornberg 1999)(Roy et al 2011, Roy et al 2014, Sato & Kornberg 2002). In T cells, Fas (a TNF receptor family) and its ligand are critical for TNT formation, which in turn mediates the propagation of cell death signaling (Arkwright et al 2010, Luchetti et al 2012). In another example, it was shown that a small calcium binding protein, S100A4, and its receptor RAGE provide directional cues for TNTs (Sun et al 2012).

However, there is always a chicken-or-egg question as to whether the receptor-ligand interaction initiates protrusion formation, or whether the protrusion enables receptor signaling. The second possibility has been difficult to prove, particularly because the perturbation of protrusions without affecting other biological processes has been challenging as described above. However, if signaling is inducing the formation of the protrusions, one must ask, ‘for what purpose the signaling needs to induce the formation of protrusions?’. A possible explanation is that the initial signaling may be required to induce protrusion formation, but once formed, protrusions may contribute to maintaining, stabilizing or enhancing signaling. Furthermore, repeating cycles of signal activation and protrusion induction may enable cells to reach far beyond the neighboring cells, as is observed in the case of cytonemes in *Drosophila* imaginal discs. Such a mechanism could even enable cells to respond to a ‘morphogen gradient’: ‘naïve’ cell (without any protrusions) may extend

short cytonemes by sensing a low concentration of the morphogen nearby. Once extended, such short cytonemes would be in a better position to sense a higher concentration of the morphogen, further extending cytonemes toward the source of the morphogen. Once the cytoneme reaches the source of the morphogen, now it can engage in full activation of the signaling. This allows cells far from the morphogen source engage in the full degree of signaling activation. In this scenario, absolute concentration of morphogen along the ‘morphogen gradient’ can be too low to exert a particular signaling outcome, and the gradient may function solely to guide cytonemes to the signaling source. This would allow selective signal activation in cells far from the morphogen source, without activating the signaling in the intervening cells.

MT-nanotubes provides an example where this chicken-or-egg question has been partially resolved. In the *Drosophila* male GSC niche, Dpp (a BMP ligand) and Tkv (receptor) are required for MT-nanotube formation, yet MT-nanotubes are also required for Dpp-Tkv signaling. However, in this particular case, a dominant-negative form of Tkv receptor provided a clear answer to the chicken-or-egg question. When a dominant-negative form of Tkv (which can bind to the Dpp ligand, but cannot activate downstream signaling) is expressed in GSCs, it was sufficient to induce MT-nanotube formation (Inaba et al 2015). This result demonstrates that the induction of MT-nanotubes does not require downstream Tkv signaling, and suggests that the physical interaction of the ligand and receptor triggers the formation of the protrusions without requiring the downstream signaling. Once the protrusion is formed, it stimulates/promotes signal transduction (Figure 2B).

The function of specialized thin membrane protrusions

Earlier work in sea urchins noted that primary mesenchyme cells and secondary mesenchyme cells extend thick filopodia during gastrulation (Gustafson 1963, Gustafson & Wolpert 1961, Gustafson & Wolpert 1967). These protrusions were hypothesized to aid in the movement of individual cells. This idea was supported by additional work, which showed that the thick filopodia extending from secondary mesenchymal cells helped to physically pull cells to specific positions during development (Hardin 1988, Hardin 1987). In addition to thick filopodia, these early studies also noted the presence of thin filopodia, which they called “pseudopodia”. These cellular extensions were difficult to resolve using light microscopy, although ultrastructural studies confirmed their presence (Gibbins et al 1969, Tilney & Gibbins 1969). Follow-up studies found primary mesenchymal cells also formed these thin protrusions in vitro (Karp & Solursh 1985). Again, prevailing models at the time suggested that these pseudopodia played a role in cell migration. However, this view began to evolve with further investigations. In particular, it was found that the formation of these thin membrane protrusions did not correlate with cell movement (Miller et al 1995). Rather, these structures were present at times and at sites where signaling was known to take place. In addition, treating sea urchin embryos with nickel caused defects in thin filopodia formation, which correlated with defects in pattern formation (Miller et al 1995).

The potential function of thin membrane protrusions came into focus with the discovery that *Drosophila* cells form cytonemes during development (Ramirez-Weber & Kornberg 1999)

(Table 1 summarizes thin membrane protrusions and their proposed functions). Cytonemes exhibit polarized organization, suggested they orient in response to a chemoattractant. Further experiments showed FGF likely acts as a chemoattractant, at least in the specific contexts being studied (Ramirez-Weber & Kornberg 1999). The ability of cytonemes to form in response to at least one well-known signaling molecule hinted that thin membrane protrusions could potentially act as lines of communication between distant cells. More recent studies, using loss of function genetic approaches, have begun to provide further evidence that these structures actively mediate intercellular signaling in *Drosophila* tissues (Roy et al 2014) (Chen et al 2017). Further experiments designed to specifically change the frequency and size of cytonemes and other thin membrane protrusions, both in a positive and negative manner, without affecting other cellular processes, will test the functional significance of these structures in specific contexts.

Thin membrane protrusions have been conserved through multicellular evolution and a growing number of studies have begun to describe similar structures in a variety of vertebrate species. For example, epithelial cells in the zebrafish epiblast form long cellular projections, referred to as intracellular bridges, that link cells together, providing a potential avenue for material transfer between cellular partners (Caneparo et al 2011). In *Xenopus*, long thin filopodia span the blastocoel to connect cells to one another over relatively large distances (Danilchik et al 2013). Live cell imaging experiments show that vesicles travel along these filopodia, further suggesting they allow for transfer of cytoplasmic material between linked cells.

Protrusions as conduits for signaling at a distance

The characterization and functional analyses of specialized filopodia has challenged the notion that signaling molecules, including morphogens such as Wnt, FGF and Hh family members, passively diffuse through complex extracellular environments to influence cell fate over a distance in all *in vivo* contexts. Several in-depth reviews have discussed the merits of diffusion-based models over alternative modes of morphogen distribution (Muller et al 2013, Rogers & Schier 2011, Wolpert 2016). However, several of signaling molecules, such as hedgehog and Wnt family members, are post-translationally modified in such a way as to severely limit their predicted ability to travel through extracellular space (Fairchild & Barna 2014, Galli & Burrus 2011, Grover et al 2011, Mann & Beachy 2004, Miura & Treisman 2006, Pepinsky et al 1998, Resh 2006). In addition, recent experiments show that membrane tethered Wingless, *Drosophila* Wnt homolog, can still direct patterning in the wing disc (Alexandre et al 2014). Although this result might not prove the need of cytonemes, it at least urges us to think that ‘diffusion’ of the morphogen cannot solely be responsible for patterning. Given this background, specialized filopodia including cytonemes, tunneling nanotubes and MT-nanotubes have emerged as potentially important regulators of cell-cell communication over a range of distances, at least in certain contexts.

Cytonemes still remain one of the best characterized cellular protrusions involved in signaling. These structures are very sensitive to fixation, which has obscured their presence in some tissues and has somewhat limited their functional characterization (Hsiung et al 2005, Ramirez-Weber et al 2000, Ramirez-Weber & Kornberg 1999, Sato & Kornberg 2002,

Sigler & Congly 1990). However, recently developed preservation protocols should now facilitate the detailed examination of cytonemes using well-established cell biological techniques (Bodeen et al 2017). Despite these challenges, seminal studies using live cell imaging to study *Drosophila* imaginal discs and other larval structures reveal that these actin-based structures mediate the activity of a variety of major signaling pathways, including the Dpp (a BMP family member), Wingless (Wg) (a *Drosophila* Wnt homolog), Hedgehog, (Hh) and epidermal growth factor (EGF) pathways (reviewed in (Gradilla & Guerrero 2013, Kornberg & Roy 2014, Roy & Kornberg 2015, Stanganello & Scholpp 2016)). Although it has been difficult to prove the functionality of cytonemes and TNTs due to the difficulty in perturbing these structures without affecting other actin-dependent processes, spatially and temporally controlled inactivation of cytoneme formation provides evidence that these structures actively mediate Dpp signaling within the developing *Drosophila* air sac primordium (ASP) (Roy et al 2014). Cells within the ASP extend cytonemes towards Dpp producing cells, and activation of Tkv, the Dpp receptor, appears to take place at the tips of these cytonemes. A number of proteins, including Diaphanous (Dia), a *Drosophila* formin, Neuroglian (Nrg) and Capricious (Caps) localize to cytonemes, and RNAi knockdown of the corresponding genes results in decreased cytoneme numbers and reduced Dpp signaling within ASP cells, as assayed by phosphorylated Mad (pMad) and Daughters against dpp (Dad) expression. In addition, inactivation of the dynamin protein Shrbire (Shi) also disrupts cytoneme function and leads to decreased Dpp signal transduction. While these genetic manipulations perturb cytoneme formation and function, they do not appear to grossly disrupt the overall morphology of cells within the ASP. Moreover, these cells remain capable of receiving Dpp, based on their response to ectopic over-expression of Dpp within the ASP itself. Together, these results indicate that cytonemes function in transducing Dpp signaling within this tissue.

Additional studies show that signaling ligands can be observed moving along cytonemes in a directed manner, further suggesting the involvement of these thin membrane protrusions in signal transduction (Hsiung et al 2005) (see Table 1 for more comprehensive list of thin membrane protrusions and their suggested functions). Studies of *Drosophila* myoblast cytonemes reveal that recipient cells form protrusions that contain Wnt pathway signaling components (Huang & Kornberg 2015), highlighting that cytoneme formation is not limited to signal sending cells. Similarly, detailed analysis of hedgehog signaling components within *Drosophila* wing discs and abdominal epidermis, revealed that the Hh co-receptor Interference hedgehog (Ihog) localizes to cytonemes that lie between the anterior and posterior compartments (Gonzalez-Mendez et al 2017, Gradilla et al 2014). Interestingly, the Hh gradient, as reflected by Patched expression, correlates with cytoneme length and disrupting the formation of cytonemes, through RNAi-mediated knockdown of proteins that interact with the actin cytoskeleton, significantly shortened the Hh gradient (Chen et al 2017). Importantly, interfering with the actin cytoskeleton does not perturb overall Hh expression levels, just its distribution. The positive correlation between cytoneme formation and function, in regards to Hh signaling gradients, further suggests that these specialized filopodia regulate cell-to-cell communication (Chen et al 2017). Also it was observed that Wnt proteins are transported along cellular protrusions emanating from producing cells towards recipient cells in zebrafish (Luz et al 2014, Stanganello et al 2015).

Cytoneme-like structures that participate in hedgehog signaling have also been functionally characterized in vertebrate tissues (Sanders et al 2013). Similar to the behavior of signaling ligands in *Drosophila*, this study found that sonic hedgehog formed distinct particles that remained associated with the signal sending cell through long thin cytoplasmic protrusions that often transversed several cell diameters. Live-cell imaging showed that these particles moved along these cytoneme-like extensions in a net anterograde direction. Signal responding cells also formed thin filopodia-like structures that contained specific subsets of Shh co-receptors. These extensions associated with those emanating from Shh producing cells, further suggesting that these specialized filopodia assist with both sending and receiving signals over distances of many cell diameters. Further genetic experiments designed to test the extent to which disruption or enhancement of thin cytoplasmic protrusion formation influences signaling between cell populations will continue to resolve the functional significance of these structures across species.

Protrusions promote paracrine signaling, ensuring communication occurs at the level of a whisper

Besides helping to establish signaling gradients over distances of many cell diameters, cytonemes have also been implicated in paracrine signaling between stem cells and their supportive niche cells. For example, work in the *Drosophila* ovary suggests the cap cells, which form the germline stem cell niche, extend cytonemes to communicate with their somatic cell neighbors. The cytoneme found in the *Drosophila* ovary is not connecting two cells over a long distance. Instead, it extends from cap cells to a neighboring cell to deliver Hh signaling. Thus, it remains unlikely that these cytonemes help to form a morphogen gradient, and points to a possibility that these cytonemes might be employed to increase the specificity of paracrine signaling (Rojas-Rios et al 2012).

Such a possibility was strongly supported by a discovery of MT-nanotubes found in *Drosophila* male germline stem cells (GSCs). Signaling between stem cells and their niches must be kept intimate to limit the number of cells that retain a stem cell identity, while excluding non-stem cells from engaging in the niche signaling (Inaba et al 2015). The discovery of MT-nanotubes reveals at least one mechanism by which stem cells and the niche cells can communicate with one another at the level of a whisper. In the *Drosophila* testis, 8–10 GSCs surround a cluster of 10–15 somatic hub cells. These hub cells express a number of signaling molecules including Unpaired, a ligand that activates the JAK/STAT signaling pathway, and Dpp, the ligand for the BMP signaling pathway. Both pathways help to maintain GSCs in an undifferentiated and self-renewing state. MT-nanotubes extend from GSCs towards interior positions within the niche cell cluster. Thickveins (Tkv), a receptor in the *Drosophila* BMP pathway, travels towards the close-ended tips of MT-nanotubes. Tkv expressed in GSCs engages with Dpp expressed by hub cells at the tips of these MT-nanotubes. MT-nanotubes require IFT (intraflagellar transport) machineries for their formation. Because GSCs lack cilia and knockdown of IFT components does not noticeably change anything but MT-nanotube formation, it provided an ideal opportunity to test the function of MT-nanotubes. Genetic disruption of MT-nanotubes by RNAi-mediated knockdown of IFT components attenuates BMP signal transduction within GSCs. Reciprocally, enhancement of MT-nanotube size increases pathway activity within the stem

cell population, strongly suggesting that MT-nanotubes are critical for the niche-stem cell signaling. These observations also raise a number of interesting questions and possibilities. First, do other stem cell-based systems use cellular protrusions as a means to limit the number of cells that adopt and/or maintain a stem cell fate? Do niche cells employ specialized secretory machinery to ensure limited accessibility to ligands that promote self-renewal?

Open-ended TNTs allow for exchange of cytoplasmic components

Cytonemes and other close-ended protrusions promote signaling between cells by allowing for the formation of specialized juxtaposed membranes that allow for ligand/ receptor binding, much like two people reaching out to shake hands. By contrast, open-ended extensions such as tunneling nanotubes also allow for different types of exchange between connected cells (Table 1). The original characterization of TNTs in PC12 cells in culture noted vesicular objects moving through the lumen of these extensions in one direction (Rustom et al 2004). Further experiments revealed the additional presence of acidic organelles and small synaptic vesicles within TNTs. Fluorescent labeling experiments provided evidence that organelles were actively exchanged between cells connected by TNTs.

More recent studies show that cells in culture can also exchange mitochondria and other organelles through TNTs. The functional significance of this cytoplasmic transfer becomes clear in the context of cellular damage and stress. For example, PC12 cells treated with UV irradiation undergo apoptosis because of mitochondrial failure. However, damaged cells co-cultured with untreated cells send out TNTs towards healthy cells within the population. Intact mitochondria move from the healthy cells to the irradiated cells through TNTs, thus rescuing the UV-induced lethality (Wang & Gerdes 2015). In addition, Bone Marrow Stromal Cells transfer mitochondria to leukemic cells through TNTs in response to chemotherapy and oxidative stress (Marlein et al 2017, Moschoi et al 2016). TNTs also help elevate the phenotypes associated with cystinosis, a lysosomal storage disorder (Naphade et al 2015). TNTs often originate in the distressed cells, suggesting their formation may represent a type of stress response. Once these damaged cells connect with healthy cells, cytoplasmic transfer through TNTs can rescue many of their phenotypes and prolong survival. In addition to organelles, TNTs can also mediate cell survival by promoting the transfer of anti-apoptotic proteins to cells to would otherwise initiate. For instance, nanotubes directly connect CD4⁺ T cells to airway smooth muscle (ASM) cells (Al Heialy et al 2015). The anti-apoptotic protein Mcl-1 can be observed passing from ASM cells through these TNTs to CD4⁺ T cells and loss of Mcl-1 in specifically in ASM cells results in increased levels of apoptosis in CD4⁺ T cells.

However, counter-examples suggest that some forms of nanotubes may also be used to cull damaged cells in certain contexts, particularly in the immune system. For example, human natural killer (NK) cells interact with target cells over a distance through nanotubes (Chauveau et al 2010). These nanotubes formed when NK cells came in contact with target cells and moved apart. MHC class I chain-related protein (MICA), which functions as a ligand for the NK cell activating NKG2D receptor, accumulates at synapse-like structures in

these nanotubes. Physically severing nanotubes resulted in a decrease in target cell lysis, suggesting that these structures play an active role in NK cell function. In addition, T lymphocytes transfer the FAS ligand to one another through TNTs, resulting in cell death (Arkwright et al 2010). Further evidence suggests that apoptotic cells transfer phosphatidylserine (PS) containing membranes to other cells through TNTs (Bittins & Wang 2017). These PS membranes trigger a phagocytic response, indicating that cells undergoing cell death can actively use TNTs to recruit macrophages to promote their own clearing. How different types of TNTs mediate these various different stress responses in different contexts remains unclear.

TNTs can mediate electrical and chemical coupling

Besides organelle and protein transfer, TNTs also mediate electrical and chemical coupling between connected cells (Table 1). This function was first observed in dendritic cells of the immune system, which exhibit calcium fluxes upon exposure to bacterial products (Watkins & Salter 2005). Strikingly, these calcium fluxes could be propagated in adjacent cells. Further work showed that TNTs played an essential role in both the coupling of these calcium fluxes between dendritic cells, and in the functional and morphological responses Dendritic cells exhibit upon stimulation. Additional studies showed that TNTs mediated the spread of electric signals in a number of different cells in culture, suggesting this type of coupling may be widespread. Drug experiments suggested this electric coupling depends on low voltage calcium channels and gap junctions. The potential involvement of gap junctions was further supported by the localization of Connexin 43 to the end of TNTs. Similar localization of Connexin 43 is seen in short-lived TNTs that form between neurons and astrocytes (Wang et al 2012). These TNTs appeared to mediate calcium signaling between these cells at a distance. TNT-like microtubes also appear to couple calcium waves between different tumor cells within the brain (Osswald et al 2015). These microtubes appeared to participate in cellular repair and made tumor cells more resistant to radiation-induced cell death. Despite the increasing number of examples whereby TNTs or similar protrusions help to couple electrical signaling between cells at a distance, one recent study provides a cautionary tale regarding TNT-mediated calcium coupling (Lock et al 2016). HeLa cells engineered to upregulate TNTs could propagate calcium fluxes to nearby cells. However further experiments showed that these fluxes were not the result of direct cytoplasmic continuity between cells. Rather intercellular transfer of IP₃ drove the observed calcium fluxes, emphasizing the need to carefully interpret correlations between TNTs and various coordinated signaling events between cells.

Thin membrane protrusions in disease

Over the last couple of decades, several studies demonstrated that TNTs and similar thin cellular protrusions play roles in the progression of pathological conditions such as infectious diseases, neurodegenerative diseases and cancers (Table 2). In most of these cases, cells physically connect over a long distance utilizing these structures (either open-ended or close-ended) as the route for sending and/or receiving various cargoes involved in disease progression.

Virus infection

Viruses are classically described to spread out from the infected cells by forming virions that can infect other cells by recognizing surface receptors. Other than the virion-mediated spreading, direct cell-cell contact (called “virological or infections synapses”) has been observed and shown to mediate virus spreading much more efficiently (Jolly et al 2004, McDonald et al 2003). First described in Vaccinia virus infected cells, cellular protrusions mediating virus transfer have also been observed (Cudmore et al 1995). To date, numerous studies have shown that thin membrane protrusions direct transfer of virus and viral proteins between cells, including Human T-cell leukemia virus type I (HTLV-1) (Van Prooyen et al 2010), murine leukemia virus in living cells (Sherer et al 2007), Marburgvirus (Kolesnikova et al 2007), African swine fever virus (Jouvenet et al 2006) herpes viruses (Ady et al 2016, Favoreel et al 2005, Gill et al 2008), Influenza A viruses (Kumar et al 2017, Roberts et al 2015) and Human immunodeficiency virus type I (HIV-1). HIV-1 represents one of the best-studied examples of how viruses can hijack TNT conduits of host cells to promote their spread. HIV-1 moves between CD4+ T Cells (Sowinski et al 2008) as well as between macrophages (Eugenin et al 2009, Hashimoto et al 2016). Interestingly, virus infection itself often enhances TNT formation (Eugenin et al 2009, Hashimoto et al 2016). Both HTLV-1 and HIV-1 infection promote TNT formation directly by inducing the host cell protein, M-Sec (Sowinski et al 2008, Van Prooyen et al 2010). HIV-1 promotes TNT formation via the viral protein HIV-Nef (Hashimoto et al 2016). Importantly, M-Sec is later identified as the essential protein for TNT formation in physiological conditions (Hase et al 2009, Kimura et al 2016).

Why were these structures hijacked by the pathogens? Are there any qualitative/quantitative differences in the communication between TNT mediated virus transfer and conventional virion-based spreading? Do they influence the types of cells that can be infected, or the distance over which the virus can spread? Can TNT overcome the barrier that prevents invasion of virions? Several recent studies provide potential answers for these questions. For example, TNTs were observed to transfer Influenza viruses to the cells that lack the expression of receptor, hemagglutinin (HA) protein, resulting in efficient replication of viral genome (Kumar et al 2017). These findings indicate that viruses can overcome cell type specificity barriers by utilizing TNTs. Furthermore, direct transfer of the viral genome can escape the effect of anti-virus drug, Oseltamivir, which inhibits the release of the virus from infected cells (Kumar et al 2017). HIV-1 infected primary macrophages transfer the viral protein, HIV-Nef to B cells via TNTs and inhibit T cell-dependent immunoglobulin class switching, thus escaping the humoral immune response (Xu et al 2009). These studies suggest that TNT mediated pathogen propagation indeed overwrite qualitative barriers.

Recently, the effect of M-Sec inhibitor in human monocyte derived macrophages was semi-quantitatively examined. This treatment, which reduces TNT formation, cut HIV spreading by half (Hashimoto et al 2016). Further functional studies, especially those using *in vivo* systems, will be required to understand quantitative/qualitative features of TNT mediated virus transfer, as well as how viruses themselves may mediate TNT formation. Furthermore, structural analysis may also help further understanding of TNT function. Fascinatingly, recent PRD-1 (an internal membrane-containing virus) bacteriophage work using Cryo-

Electron Microscopy characterized the architecture of host bacteria membrane tubes induced by viral infection (Peralta et al 2013). Viral double strand DNA perfectly fit in these tubes and can be delivered between host cells. Such observations imply that TNT-mediated viral genome transfer factors into the evolution of viruses and their hosts.

Bacterial infection

TNT-like structure mediated transfer of bacteria (the *Listeria monocytogenes*) between macrophages was first described already in 1989 (Tilney & Portnoy 1989). Bacteria can surf along thin membrane protrusions aided by constitutive flow of the membrane surface. These bacteria are then phagocytosed upon reaching the cell body (Onfelt et al 2006). Like viruses (see above), bacteria species may also utilize TNTs to efficiently spread themselves between host cells. However, interestingly, a recent study provides the possibility of anti-pathogenic function of TNTs in the pathology of bacterial infection. Mesenchymal stromal cells (MSCs) have been reported to improve bacterial clearance in preclinical models of Acute Respiratory Distress Syndrome (ARDS) and sepsis. Between MSCs and macrophages isolated from mouse *E. coli* pneumonia model, TNTs transfer mitochondria from MSCs to macrophages, resulting in enhanced phagocytotic activity of macrophages (Jackson et al 2016). In the same model, transfer events were also observed *in vivo* from MSC to lung macrophages. Furthermore, inhibition of TNT formation significantly reduces the bacteria clearance (Jackson et al 2016). Proposing a positive role for TNTs in fighting bacterial infection, this work suggests that TNTs might not be just an Achilles' Heel.

Neurodegenerative diseases

Neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's diseases, ALS (amyotrophic lateral sclerosis) and Prion diseases, cause progressive loss of neurons in human central nervous system. Despite intensive study over the past three decades, the pathology of neurodegenerative diseases still remains poorly understood, and practical therapeutic strategies that can reverse the progressive loss of neurological function have not been developed. Increased intracellular or extracellular aggregates of misfolded proteins and their cell-to-cell propagation are the hallmarks of these conditions, and are linked with neurotoxicity and neuronal dysfunction. Misfolded proteins can spread into other cells via several proposed routes; axonal transmission, exosome mediate secretion/uptake (Arellano-Anaya et al 2015, Schneider & Simons 2013) and/or direct transfer via TNTs (Abounit et al 2016a, Costanzo et al 2013, Gerdes 2009). The role of TNTs in the transfer of protein aggregates was first described for Prion diseases (Gousset et al 2009). The Infectious form of a prion protein (PrP^{Sc}) acts as a seed, and is capable of converting naïve PrP^C into PrP^{Sc} upon direct interaction. Thus, the intercellular transmission of proteins is a key process for prion pathogenesis (Gousset et al 2009). This previous work and several recent *in vitro* studies have demonstrated the importance of TNT-mediated transmission of misfolded proteins, not for only PrP^{Sc}, but also for other pathogenic proteins found in broad neurodegenerative diseases such as Tau, β -amyloid and α -synuclein (Abounit et al 2016a, Costanzo et al 2013, Dieriks et al 2017, Tardivel et al 2016, Victoria et al 2016, Zhu et al 2015). Similar to many other examples, functional characterization of TNTs in neurodegenerative diseases still largely relies on *in vitro* co-culture systems, using primary cells or cell lines. Further *in vivo* studies will be necessary to more fully understand how

TNTs contribute to these disorders. Certainly, selective inhibition of TNTs could represent a novel therapeutic intervention that can potentially arrest or slower the disease propagation. Several studies have demonstrated that inhibition of TNT formation can block progression of these diseases (Abounit et al 2016a, Abounit & Zurzolo 2012, Costanzo et al 2013, Gousset et al 2009).

Tumor progression

Various types of cancer cells have been shown to communicate among themselves using TNTs as well as between cancer cells and surrounding non-cancer stromal cells, the latter contributing to the formation of microenvironments and cancer stem cell niches that support tumor growth (Table 2)(de Rooij et al 2017, Polak et al 2015). TNTs can promote tumor progression via transfer of different cargoes, such as mitochondria, microRNAs, exosomes and oncoviruses, influencing potential pathological outcome (Table 2, Reviewed in (Ariazi et al 2017, Vignais et al 2017). Although thin membrane protrusions on cancer cells were initially visualized using *ex vivo* culture systems, recent studies have observed similar structures in samples surgically resected from human patients (Table 2). TNTs were observed in malignant pleural mesothelioma and lung adenocarcinoma (Lou et al 2012). Another study showed TNT-like connections in human glioblastoma tumors when implanted in mouse brains (Osswald et al 2015). A TNT connection was also observed within the osteosarcoma sample resected from mice and also in ovarian tumors resected from human patients (Thayanithy et al 2014).

Through TNT-mediated material transfer, tumor cells often acquire new or altered features or advantages, such as metastasis, invasion, chemo-resistance and better survival in response to metabolic stress (Reviewed in (Sisakhtnezhad & Khosravi 2015, Vignais et al 2017). To make matters worse, the overall tumor environments, such as hypoxia/acidic and low nutrient and metabolically stressed conditions seem to favor TNT formation. These observations, again, suggest the possibility that TNT inhibition may represent a useful therapeutic approach to treat various cancers. It was shown that adding physical forces to separate TNT connection between co-cultured leukemia cells and mesenchymal stromal cells reverses the viability and chemo-resistance of the leukemic cells (Polak et al 2015). Identifying molecular components of TNTs and specific TNT inhibitors will accelerate the development of therapeutic applications. It was also shown that the mammalian target of Rapamycin (mTOR) inhibitor everolimus and the anti-diabetic drug metformin, which both exhibit anti-proliferative effects on cancer cells, can prevent TNT formation (Lou et al 2012). Similarly, focal adhesion kinase (FAK) inhibitor also inhibits TNT formation (Saenz-de-Santa-Maria et al 2017). Both cases, the mechanism of TNT inhibition is still unclear. Further studies will be needed to confirm the causal relationships of TNT inhibition and anti-proliferative effects of these drugs.

These examples demonstrate that TNTs underlie a plethora of pathological conditions, making the disease worsen and progress faster. Typically, it is interpreted as TNTs being 'hijacked'. Whereas TNT can be considered as a possible therapeutic target, it raises a fundamental question as to why such a vulnerable organelle would be favored and selected during the evolution.

Concluding remarks

We described recent findings on the structures, functions and disease implications of thin membrane protrusions, an emerging cellular organelle that plays fundamental roles in intercellular signaling and trafficking. Accumulating evidence suggests that thin membrane protrusions are a prevalent mechanism through which cells communicate in various contexts and in a broad range of organisms. This emerging form of intercellular communication may influence our understanding of how cellular community is formed in multicellular organisms.

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A Close-ended (e.g. cytonemes, TNT-like bridges)

B Open-ended (e.g. TNTs)

C Encapsulated (e.g. MT-nanotubes)

Figure 1. Structures of thin membrane protrusions

A. Close-ended thin membrane protrusions such as cytonemes and TNT-like protrusions. Ligand and receptor can interact at the interphase of protrusions. Alternatively, synapse-like transfer of information (including endocytosis and exocytosis) may occur.

B. Open-ended thin membrane protrusions such as tunneling nanotubes (TNTs), where cytoplasmic components (e.g. organelles, viruses) can be transferred from one cell to another.

C. Thin membrane protrusions encapsulated by neighboring cells such as MT-nanotubes. Ligand-receptor interaction can occur at the surface of the protrusions. In addition, endocytosis/exocytosis may happen, although not reported thus far.

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Figure 2. Mechanisms of thin membrane protrusion formation

A. Formation of cytonemes is guided by underlying epithelium. The underlying epithelium secretes glypicans (Dally and Dlp, components of extracellular matrix (ECM) that regulate signaling) in a manner dependent on planar cell polarity genes in the epithelium. Dally- and Dlp-containing ECM in turn instruct cytoneme formation.

B. Formation of MT-nanotubes is induced by ligand (Dpp)-receptor (Tkv) interaction without requiring downstream signal transduction of the receptor (Tkv) activation. Upon induction, MT-nanotube transmits Dpp-Tkv signaling to GSCs. In this system, hub cells,

which functions as a component of the stem cell niche, provide Dpp ligand to the neighboring GSCs.

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Table 1:

Thin membrane protrusions that participate in intercellular communications

Name of protrusions	System	Structure, cytoskeletal components	Proposed function/ mechanism of formation	references
cytonemes	Drosophila wing disc	Actin-based filopodia. Up to 700µm in length (in culture), 0.2µm in diameter. Up to 80µm in tissues. Likely close-ended.	Hh and Dpp (TGFβ) are required for cytoneme formation. Tkv (Dpp receptor) is trafficked along the cytonemes. Cytonemes are directed to the source of Dpp. Cytoneme formation depends on Dia (formin), SCAR (activator of actin assembly), Nrg (cell adhesion molecule), synaptobrevin (v-SNARE). Cytonemes are required for morphogen gradient formation.	(Ramirez-Weber & Kornberg 1999). (Hsiung et al 2005). (Roy et al 2011). (Chen et al 2017) (Bischoff et al 2013, Gonzalez-Mendez et al 2017, Gradilla et al 2014)
cytonemes	Drosophila air sac primordia (ASP)	Up to 50µm, extended toward the direction of ASP migration. Positive for actin, but not Tau (microtubule associated protein). ASP cytonemes synapse with wing disc cell (Dpp source). Dia (formin), Shi (dynamin), Nrg and Caps (cell adhesion molecules).	ASP forms two kinds of cytonemes: 1. containing FGF-receptor, and extend toward Fgf source. 2. Containing Dpp-receptor and respond to Dpp source (in the wing disc). Dia (formin), Shi (dynamin), Nrg and Caps (both putative cell adhesion molecules) are required for cytoneme formation and Dpp signaling. Components of PCP is required in guiding cytonemes.	(Roy et al 2011, Roy et al 2014, Sato & Kornberg 2002) (Huang & Kornberg 2016)
cytonemes	Drosophila eye disc	Actin-based filopodia	Cytonemes are formed in response to Egf. Egfr is required for cytoneme formation.	(Roy et al 2011)
cytonemes	Drosophila myoblast (for flight muscle)	Actin-based filopodia	Fz-containing cytonemes in myoblasts take up Wg from the wing disc. Delta-containing cytonemes in myoblasts activates Notch signaling in ASP. Dia, Nrg, Shi are required for cytoneme formation and signaling.	(Huang & Kornberg 2015)
cytonemes	Drosophila female germline stem cell (GSC) niche	Actin-based filopodia	Cytonemes deliver Hh from cap cells (niche component) to escort cells (another niche component juxtaposed to cap cells) to specify GSC identity. Dia and Wasp are required for cytoneme formation.	(Rojas-Rios et al 2012)
Specialized filopodia	Mesenchymal cells of developing limb bud in chicken embryo	Actin-based filopodia	Shh transport (both ligand and receptor are transported on the filopodia in ligand-sending and receptor-expressing cells, respectively). The effects of blocking filopodia formation remains untested.	(Sanders et al 2013)

Name of protrusions	System	Structure, cytoskeletal components	Proposed function/ mechanism of formation	references
Filopodia-like protrusions	Chicken embryo somite epithelial cells	Actin- and tubulin-positive	Transfer of Frizzled-7. Contains GPI-Aps (glycophosphatidylinositol-anchored proteins)	(Sagar et al 2015)
Filopodia	Zebrafish epiblast cells of the developing neural plate and PAC2 fibroblasts, neuroectoderm	Actin-based filopodia	Wnt8a is transported along the filopodia. Perturbation of actin cytoskeleton by drugs or by cdc42 depletion results in slight decrease in Wnt signaling activity.	(Luz et al 2014, Stanganello et al 2015)
Intercellular bridges	Epiblast cells in pre-gastrula stage embryos in zebrafish	Extends several cell diameter (~215µm on average). Forms between two sister cells after mitosis by retaining membrane tether: thus these bridges are likely open-ended. β-actin was observed throughout the bridges. α-tubulin was observed only at the base of bridges.	Unknown	(Caneparo et al 2011)
Filopodia, protrusions	Blastomeres in Xenopus embryo	~250µm, observed in extracellular space of the embryos. 0.2–0.7µm in diameter. Actin-based. Negative for tubulin.	Capable of transferring cytoplasmic vesicles (e.g. dextran dye). No biological relevance known.	(Danilchik et al 2013)
Tunneling nanotubes (TNTs)	Culture cells (PC12, HEK293), human retinal pigment epithelial cells, bone marrow-derived stromal cells, mesenchymal stem cells, macrophage, endothelial progenitor cells	50–200µm. Actin-based.	Small molecules, lysosomes, endosomes, ER, Golgi, mitochondria.	(Bukoreshtliev et al 2009, Islam et al 2012, Liu et al 2014, Naphade et al 2015, Patheja & Sahu 2017, Rustom et al 2004, Vallabhaneni et al 2012, Wittig et al 2012, Zhu et al 2005)
TNTs, MT-TNT	PC12 cells	Actin-based, MT can be induced upon UV treatment (MT-TNT).	Mitochondria transfer from healthy cells can rescue UV-treated apoptotic cells.	(Wang & Gerdes 2015)
TNTs	PC12 cells	Actin-based	Phagocytic signal	(Bittins & Wang 2017)
TNTs	Myeloid-lineage dendritic cells, monocytes, human retinal pigment epithelial cells, HeLa cells,	Actin-based	Propagation of calcium flux, electrical coupling	(Watkins & Salter 2005, Wittig et al 2012) (Hase et al 2009, Wang et al 2012, Wang &
	neurons/astrocytes, rat kidney cells			Gerdes 2012, Wang et al 2010)
Membrane nanotubes	Human monocyte-derived macrophages	Thin nanotubes contain only F-actin, thick nanotubes (>~0.7µm) contain F-actin and microtubules (MTs).	Endosomes, lysosomes, and mitochondria traffic within thick membrane nanotubes.	(Onfelt et al 2006)
Membrane nanotubes	T cells	Actin-based	Cell death signal (Fas/CD95)	(Arkwright et al 2010, Luchetti et al 2012)
Membrane nanotubes	Human natural killer (NK) cells	Actin-based	Promote target cell lysis by promoting MICA (ligand)-	(Chauveau et al 2010)

Name of protrusions	System	Structure, cytoskeletal components	Proposed function/ mechanism of formation	references
			NKG2D (NI receptor) interaction.	
MT-nanotubes	Drosophila male GSC niche	MT-based protrusion. IFT (intraflagellar transport) regulates its formation.	GSC forms MT-nanotubes, which is inserted into invagination formed by the niche cells (hub cells). Dpp receptor Tkv localizes to the tip of MT-nanotubes, where it colocalize with Dpp that is expressed by hub cells. Perturbation of MT- nanotube formation by knocking down IFT components reduced MT- nanotubes and Dpp signaling.	(Inaba et al 2015)

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Table 2:

TNTs in disease (if not specified by specific names, they are called TNTs)

Diseases (name of the protrusion)	Cargo/signal	Cell type	Functional outcome	References
Cancer				
Laryngeal squamous cell carcinoma, (“membranous tunneling tubes”)	Mitochondria and siRNAs Electrical and metabolic coupling	Primary culture of human laryngeal squamous cell carcinoma (LSCC) cells and surgical microsections		(Antanaviciute et al 2014)
Mesothelioma	Vesicles, proteins, and mitochondria	Mesothelioma cell lines and primary human mesothelioma cells, surgical specimens	Invasion, proliferation, and radioresistance	(Lou et al 2012)
Lung adenocarcinoma		Surgical specimens		(Lou et al 2012)
Ovarian or breast cancer	Mitochondria	Mesenchymal stromal cells (MSCs) or endothelial cells and cancer cells (ovarian or breast cancer cell lines)	Chemoresistance	(Pasquier et al 2013)
Astrocytomas (“tumour microtubes”)	Ca ²⁺ through Gap junctions	Astrocytomas	Invasion, proliferation and radioresistance	(Osswald et al 2015)
Breast cancer or Melanoma (“nanoscale membrane bridges”)	miRNAs	Metastatic cancer cells to the endothelium	Promote metastasis	(Connor et al 2015)
Mesothelioma	Herpes simplex virus and viral thymidine kinase activated drugs	Mesothelioma cell lines	Drug induced apoptosis of virus uninfected cells	(Ady et al 2016)
Squamous cell carcinoma (SCC)	Vesicles, mitochondria and autophagosomes	SCC derived cell lines, human SCC tissues		(Saenz-de-Santa-Maria et al 2017)
Acute lymphoblastic leukemia	ICAM1, Autophagosomes and mitochondria	Leukemic cell to MSCs	Signal to MSCs to promote pro-survival factor production, which subsequently influences leukemic cell survival and drug resistance	(de Rooij et al 2017, Polak et al 2015)
Bladder cancer	Mitochondria	Bladder cancer cells	Promote invasiveness	(Lu et al 2017)
Neuro degenerative diseases				
Huntington’s	Polyglutamine (polyQ), huntingtin (Htt)	Catecholaminergic mouse (CAD) neuron-like cells and primary neurons	Aggregates spreading	(Costanzo et al 2013)
Prion	Endosome/Prion aggregates	Prion infected neuronal cell line	Prion spreading	(Zhu et al 2015)
	Prion	Primary infected astrocytes and granule neurons or CAD cells	Glia mediated clearance?	(Victoria et al 2016)
Parkinson’s	alpha-synuclein fibrils inside lysosomal vesicles	CAD cells	Aggregates spreading	(Abounit et al 2016a)
	alpha-synuclein	SH-SY5Y cells and primary brain pericytes	Aggregates spreading	(Dieriks et al 2017)
Alzheimer’s	Amyloid A β peptide	CAD cells, primary neurons, astrocytes	Aggregates spreading, induce cytotoxicity	(Wang et al 2011)
	tau	CAD cells	Aggregates spreading	(Abounit et al 2016a, Abounit et al 2016b)
	tau	CAD cells, Rat primary embryonic cortical neurons	Aggregates spreading	(Tardivel et al 2016)
Viral infection				

Diseases (name of the protrusion)	Cargo/signal	Cell type	Functional outcome	References
The human immunodeficiency virus type1 (HIV-1) ("intercellular conduits")	HIV-1 protein Nef	Macrophages to B cells	Suppress immunoglobulin class switching	(Xu et al 2009)
HIV-1 ("membrane nanotubes")	HIV-1 protein Gag	Jurkat T cells		(Sowinski et al 2008)
HIV-1	Virus genome	Macrophages	Virus spreading	(Eugenin et al 2009)
HIV-1, ("filopodia")	Virions	Dendritic Cells to T-cells	Virus spreading	(Aggarwal et al 2012, Shrivastava et al 2015)
Influenza or parainfluenza virus ("intercellular connections")	Viral proteins	MDCK or A549 cells		(Roberts et al 2015)
Influenza A virus	Influenza A virus (IAV)	MDCK, A549 or primary bronchial epithelial cells (NHBE)	Bypass the Oseltamivir effect	(Kumar et al 2017)
Murine leukemia virus (MLV) ("filopodial bridges")	Virus genome	Cos-1, XC and HEK 293 cells	Virus spreading	(Sherer et al 2007)
Murine gammaherpesvirus-68 ("membrane fronds")	Virions	NIH-3T3 cells, BHK-21 cells or 293T cells	Virus spreading	(Gill et al 2008)
Bacterial infection ("membrane nanotubes")	Surfing Mycobacterium bovis bacillus CalmetteGuérin	Human monocytederived macrophages	Bacteria spreading	(Onfelt et al 2006)