Wiring through tunneling nanotubes – from electrical signals to organelle transfer

Saïda Abounit and Chiara Zurzolo*

Institut Pasteur, Unité de Trafic Membranaire et Pathogénèse, 25 rue du Docteur Roux, 75724 Paris, CEDEX 15, France

*Author for correspondence (zurzolo@pasteur.fr)

Journal of Cell Science 125, 1089–1098

doi: 10.1242/jcs.083279

Summary

Tunneling nanotubes (TNTs) represent a subset of F-actin-based transient tubular connections that allow direct communication between distant cells. Recent studies have provided new insights into the existence of TNTs in vivo, and this novel mechanism of intercellular communication is implicated in various essential processes, such as development, immunity, tissue regeneration and transmission of electrical signals. TNTs are versatile structures known to facilitate the transfer of various cargos, such as organelles, plasma membrane components, pathogens and Ca^{2+}. Recently, a new function of TNTs in the long-range transfer of electrical signals that involves gap junctions has been suggested. This indicates that different types of TNTs might exist, and supports the notion that TNTs might not be just passive open conduits but rather are regulated by gating mechanisms. Furthermore, TNTs have been found in different cell lines and are characterized by their diversity in terms of morphology. Here we discuss these novel findings in the context of the two models that have been proposed for TNT formation, and focus on putative proteins that could represent TNT specific markers. We also shed some light on the molecular mechanisms used by TNTs to transfer cargos, as well as chemical and electrical signals.

Key words: F-actin, Tunneling nanotubes, Filiopodia, Intercellular connections

Introduction

Cell-to-cell communication is essential for unicellular and multicellular organisms because it governs complex physiological processes, such as development and homeostasis. Intercellular communication is achieved by soluble factors by paracrine and endocrine signaling and by cell-to-cell contact that is mediated by synapses (neurological and immunological), gap junctions and plasmodesmata (in plants) (Lucas et al., 2009; Maeda and Tsukihara, 2011). Cellular projections represent another mode of communication that is used by various cell types and include dendrites, filopodia and cytonemes (in *Drosophila melanogaster* and viral cytonemes) (Ridley, 2011; Roy et al., 2011; Sherer and Mothes, 2008) (Fig. 1A). Recently, tunneling nanotubes (TNTs), which consist of thin F-actin-based membranous channels, have emerged as further means of intercellular communication between distant cells (Fig. 1) and were first discovered in cultured rat pheochromocytoma PC12 cells by Gerdes and colleagues (Rustom et al., 2004). These structures are distinct from filopodia and cytonemes (for details see Fig. 1) (Sherer and Mothes, 2008). Unlike other filamentous bridges (filopodia, cytonemes), TNTs mediate continuity between the cytoplasm of remote cells and in cell culture do not touch the substrate (Fig. 1). They are dynamic structures formed de novo within a few minutes and display in vitro lifetimes ranging from minutes up to several hours (Bukoreshtliev et al., 2009; Rustom et al., 2004). There are two main reasons for their relatively recent discovery. First, TNTs have very small diameters, ranging from 20 to 500 nm and can reach lengths up to several cell diameters. Second, in vitro, they appear to be transient connections; they are highly fragile and sensitive to light exposure, shearing force and chemical fixation (Rustom et al., 2004).

Based on the morphological features of TNTs described in PC12 cells, the presence of TNTs was subsequently observed in various cell types, including neurons (Gousset et al., 2009; Rustom et al., 2004; Wang et al., 2011), myeloid cells (Eugenin et al., 2009; Hase et al., 2009; Onfelt et al., 2006; Onfelt et al., 2004; Watkins and Salter, 2005), human and murine T cells (Sowinski et al., 2008), normal rat kidney (NRK) cells (Wang et al., 2010), rat cardiac myocytes (Koyanagi et al., 2005) and endothelial progenitor cells (Koyanagi et al., 2005; Yasuda et al., 2011). However, the fragility of TNTs and the lack of known molecular markers make it very difficult to observe these structures in their natural environment within tissues. Nevertheless, several studies indicate the presence of TNT-like bridges in vivo during certain developmental processes, such as blastocyst formation and neurulation in mice (Pyrgaki et al., 2010; Salas-Vidal and Lomeli, 2004), dorsal closure in *Drosophila* (Millard and Martin, 2008) and gastrulation in sea urchin (Miller et al., 1995) and zebrafish (Caneparo et al., 2011). TNTs were also found connecting dendritic cells in mouse cornea (Chinnery et al., 2008) and in other organisms, such as the malaria parasite *Plasmodium falciparum* (Rupp et al., 2011) and the bacterium *Bacillus subtilis* (Dubey and Ben-Yehuda, 2011). Therefore, TNTs might represent a sophisticated long distance cell-to-cell communication mechanism that is conserved from bacteria to mammals.

However, these studies have also highlighted the high degree of heterogeneity in these structures, which is reflected in their ability to transfer several types of cargo, such as vesicles derived from various organelles (early endosome, endoplasmic reticulum, Golgi complex and lysosome) (Kadiu and Gendelman, 2011a; Kadiu and Gendelman, 2011b; Smith et al., 2011; Wang et al.,

Journal of Cell Science

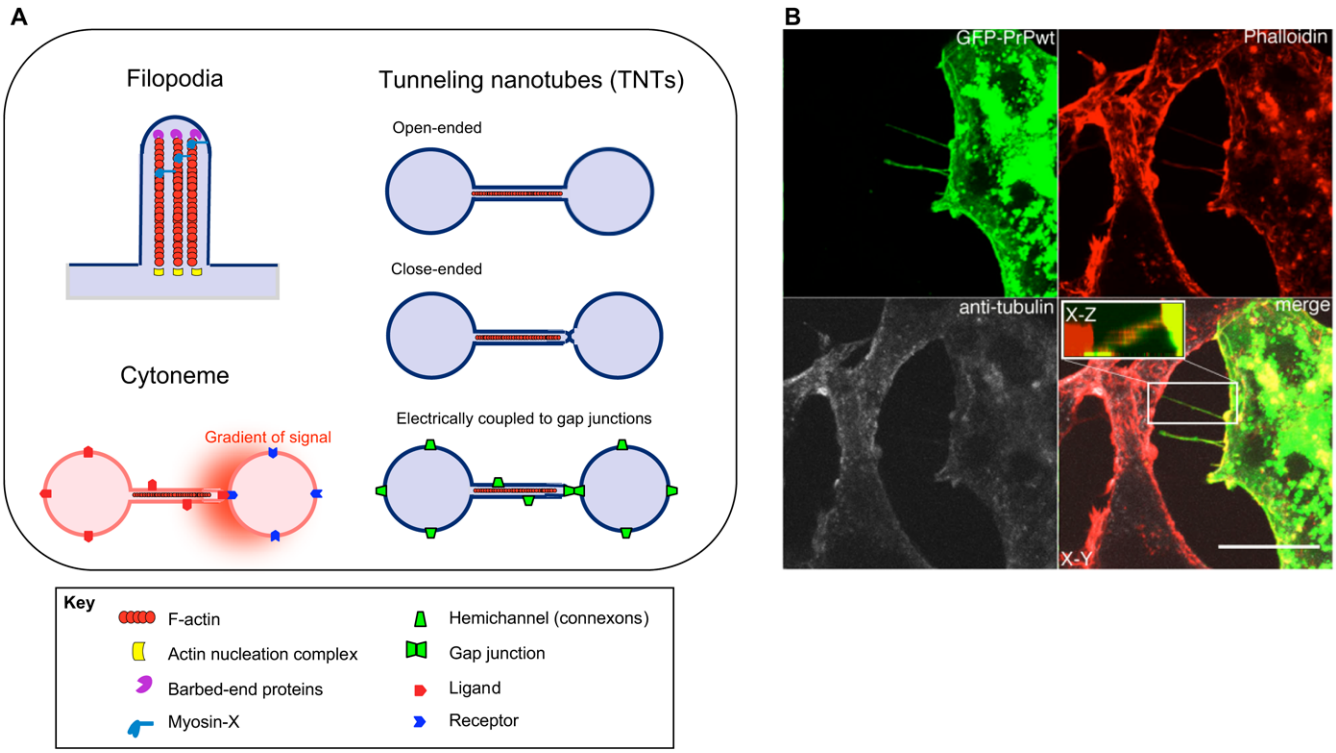

Fig. 1. Overview of different cytoplasmic extensions. (**A**) Schematic illustration of filopodia, cytonemes and tunneling nanotubes (TNTs). Filopodia (top left) are exploratory cytoplasmic projections containing parallel bundles of F-actin. The machinery involved in filopodia formation includes an actin nucleation complex that contains the specific small Rho GTPase CDC42. At the end of the F-actin tip, barbed-end proteins, such as capping proteins and Ena-VASP proteins regulate actin polymerization. Unlike filopodia, cytonemes and tunneling nanotubes (TNTs) are thin membrane bridges. Cytonemes (bottom left) are F-actin-containing cytoplasmic projections and were shown to mediate the transfer of surface-associated cargoes from cell to cell, which relies on specific ligand–receptor interaction between the tip of the cytoneme and the target cell. Because there is no cytoplasmic connection between the two cells, this type of interconnection is considered non-tubular. One specific feature of cytonemes is that the orientation of the outgrowth relies on a specific signal gradient triggered by the target cell as illustrated by the red shading to the left of the cell. TNTs (right) are F-actin-based tubular connections that allow direct communication between distant cells and are different from filopodia and cytonemes because they allow the exchange of cellular surface molecules and cytoplasmic content. Thus, they mediate continuity between the cytoplasm of remote cells without touching the substrate. Three morphologies of TNTs have been reported in the literature: open-ended TNTs, closed-ended TNTs, and TNTs electrically coupled to gap junctions (right). (**B**) TNTs in cultured cells. Two HEK293 cells [one transfected with a fluorescent prion protein construct (GFP-PrPwt; cell on right) and one untransfected (cell on left on each panel)] are shown to be connected by TNTs that contain actin but not tubulin. After fixation, cells were labelled with Alexa Fluor 546 Phalloidin for actin filaments (top right panel) and with an antibody against α-tubulin (bottom left panel), and imaged by confocal microscopy (Zeiss LSM 510). Tubes with diameters of less than 500 nm (shown 300±30 nm) were found to contain actin filaments, but no microtubules. The inset shown in the bottom right panel represents an *X-Z* reconstruction, showing that the tubes are not attached to the substratum. Panels displayed here are Z-projections of several confocal planes. Scale bar: 10 µm.

2011), plasma membrane components (Rustom et al., 2004), cytoplasmic molecules, Ca^{2+} (Smith et al., 2011; Watkins and Salter, 2005), as well as pathogens such as bacteria (Onfelt et al., 2006), HIV particles (Sowinski et al., 2008) and prions (Gousset et al., 2009; Gousset and Zurzolo, 2009) (Fig. 2). Furthermore, bigger organelles such as mitochondria have been shown to transfer through TNTs from cardiac myocytes to endothelial primordial stem cells (Koyanagi et al., 2005) (Fig. 2A). In addition, TNTs have recently been shown to associate with gap junctions, allowing electrical coupling between remote cells (Wang et al., 2010) (Fig. 1A). Therefore TNTs could constitute a dedicated route to mediate intercellular signaling required during development, immune responses and regeneration processes, as well as relaying electrical conduction (i.e. allowing the passage of charged ions) between distant cells. Moreover, TNTs can be hijacked by pathogens to facilitate their spreading in an infected host (Gousset et al., 2009; Gousset and Zurzolo, 2009) (Fig. 2). In this Commentary, we will address the diversity of TNT structures by discussing the mechanism of TNT formation and of cargo and signal transfer.

Mechanisms of TNT formation

The mechanism of TNT formation is not completely understood. Below we discuss the two models that have been proposed.

TNT formation by actin-driven protrusions

The first proposed model for TNT formation (Rustom et al., 2004) is based on the ability of either one or both of the cells involved in cell–cell contact to induce the outgrowth of filopodia-like protrusions that contain F-actin (Fig. 3A, left panel). Although the molecular basis for the formation of these protrusions remains unclear, if they share the same underlying mechanism as classical filopodia, their extension might involve the activation of CDC42, a member of the Rho-family GTPases, to initiate the actin nucleation complex (Faix and Rottner, 2006; Ridley, 2011) (Fig. 1). The filopodia-like protrusion could then

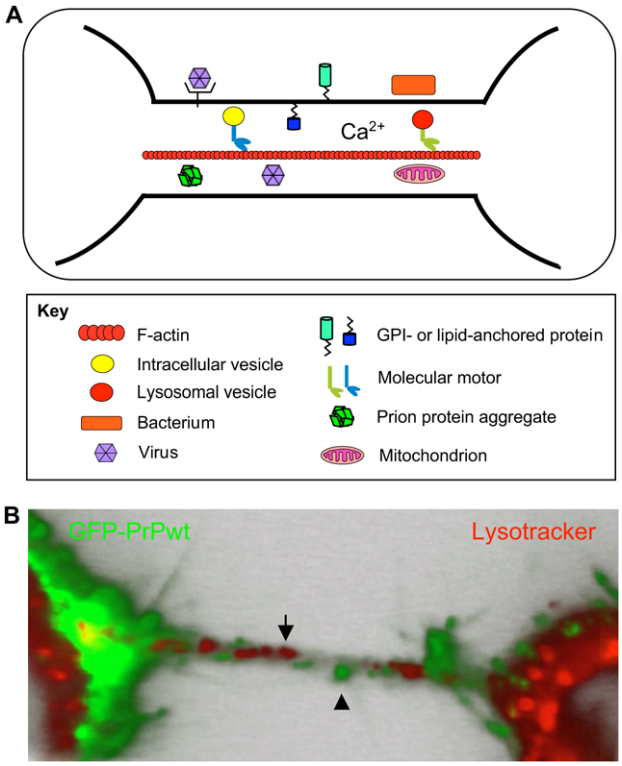

Fig. 2. TNTs transfer different cargoes. (**A**) Schematic of cargoes transferred by TNTs. Schematically shown here are the different cargoes that have been shown to be transferred by TNTs, such as lysosomal and intracellular vesicles (early endosomes, endoplasmic reticulum, Golgi) carrying different types of molecule and transported with the help of molecular motors; glycosylphosphatidylinositol (GPI)-anchored proteins; organelles (e.g. mitochondria); pathogens (e.g. viruses, bacteria, aggregates of prion proteins). (**B**) TNTs connecting CAD cells enable vesicular transfer. Shown are two CAD cells that are connected by one TNT, which contains vesicles that have been labeled with Lysotracker (see arrow) and vesicles carrying GFP-tagged prion proteins (GFP-PrPwt; arrowhead).

elongate by actin polymerization toward a target cell with a precise orientation (Fig. 3A, left panel). This might rely on a chemical gradient induced by the target cell (e.g. chemotaxis) as in the case for cytonemes (Ramirez-Weber and Kornberg, 1999) (Fig. 1). Indeed, in vitro and in vivo studies have demonstrated that the outgrowth of cytonemes is guided by gradients of different growth factors produced by the target cell (Hsiung et al., 2005; Ramirez-Weber and Kornberg, 1999; Tabata and Takei, 2004) (Fig. 1).

After elongation, the tip of the filopodia-like extension makes physical contact with the targeted cell, which might involve adhesion molecules (Rustom et al., 2004) (Fig. 3A). However, to establish an open TNT connection, membrane fusion needs to occur upon cell–cell contact to allow membrane continuity (Fig. 3B). Membrane fusion, the merging of two lipid bilayers, typically requires energy to allow close apposition of the two membranes and to induce membrane curvature. Usually, this energy is provided by fusion molecules, such as SNARE proteins and viral fusion proteins, which are able to induce bilayer disturbance and membrane curvature (Jena, 2011; Martens and McMahon, 2008). It is therefore likely that membrane-fusion molecules are located at the tip of the nascent TNT (Fig. 3B). In addition, the lipid bilayer composition might favor membrane fusion because, depending on its molecular structure, a lipid will adopt a specific curvature that could result in spontaneous membrane fusion. Thus, depending on the lipid composition, and in cases in which there is a high degree of curvature, it is possible that membrane fusion occurs spontaneously between the TNT tip and the targeted cell (Chernomordik and Kozlov, 2003).

TNT formation by actin-driven protrusion has so far been demonstrated in neuronal cells (PC12 and mouse catecholaminergic neuronal CAD cells) and normal rat kidney (NRK) cells (Bukoreshtliev et al., 2009; Gousset et al., 2009; Rustom et al., 2004). However, open-ended TNTs have rarely been observed by electron microscopy (Miyazawa et al., 2010; Rustom et al., 2004). Consequently, membrane fusion at the tip of TNTs is likely to be highly dynamic and transient (Miyazawa et al., 2010), as also supported by our observations on TNTs formed between CAD cells (Gousset et al., 2009) (Fig. 2). This fact makes the identification of fusion components more difficult and should be considered by researchers when devising experiments.

Cell-dislodgment mechanism of TNT formation

The second proposed mechanism of TNT formation is based on cell dislodgment and was shown to apply to TNTs found in immune cells (Onfelt et al., 2006; Onfelt et al., 2004; Sowinski et al., 2008; Watkins and Salter, 2005), as well as in cell lines of different origin, such as NRK, human embryonic kidney 293 (HEK293), neural crest cell (NCC) cell lines and primary human umbilical vein endothelial cells (HUVECs) (Wang et al., 2010). In this model, when two cells come into contact, they can either form an immune synapse as in the case of immune cells (Dustin et al., 2010) or can fuse (Fig. 3A, right panel). The subsequent migration of the cells in opposite directions draws out a nanotube, which could originate from either only one or from both cells involved (Davis and Sowinski, 2008; Rustom et al., 2004) (Fig. 3A, right panel). Whether this process requires adhesion molecules or fusogenic factors remains unknown, as are the mechanisms promoting initial cell contact and then cell migration. Likewise, little is known about the factors that are involved in cell–cell fusion, except that there is little conservation from insects to mammals (Chen et al., 2007). In mammals, few candidate proteins involved in cell–cell fusion have been identified. Among the cell–cell fusion processes investigated (such as trophoblast and macrophage cell–cell fusion), fertilization has been shown to rely on two proteins that mediate the fusion of the egg and the spermatozoid (Rubinstein et al., 2006). CD9, a member of the transmembrane-4 superfamily is present on the microvilli of the egg (Jegou et al., 2011; Kaji et al., 2000; Le Naour et al., 2000; Miyado et al., 2000; Miyado et al., 2008). By contrast, Izumo (IZUMO1), a transmembrane-domain protein with an extracellular immunoglobulin (Ig)-like domain is localized on the sperm surface (Inoue et al., 2005; Inoue et al., 2011). Interestingly, proteins of the Ig superfamily, such as the macrophage fusion receptor (MFR) (Saginario et al., 1998) and its ligand CD47, a transmembrane glycoprotein (Han et al., 2000), have been implicated in macrophage fusion (Vignery, 2000). Furthermore, proteins that mediate myoblast fusion in *Drosophila melanogaster* (Dworak and Sink, 2002), such as dumbfounded protein (DUF), roughest protein (RST) and stick-and-stone protein (SNS) all contain Ig-like domains (Bour et al., 2000; Ruiz-Gomez et al., 2000; Strunkelnberg et al., 2001). Therefore, it is tempting to speculate that a protein containing an Ig-like domain could be a potential

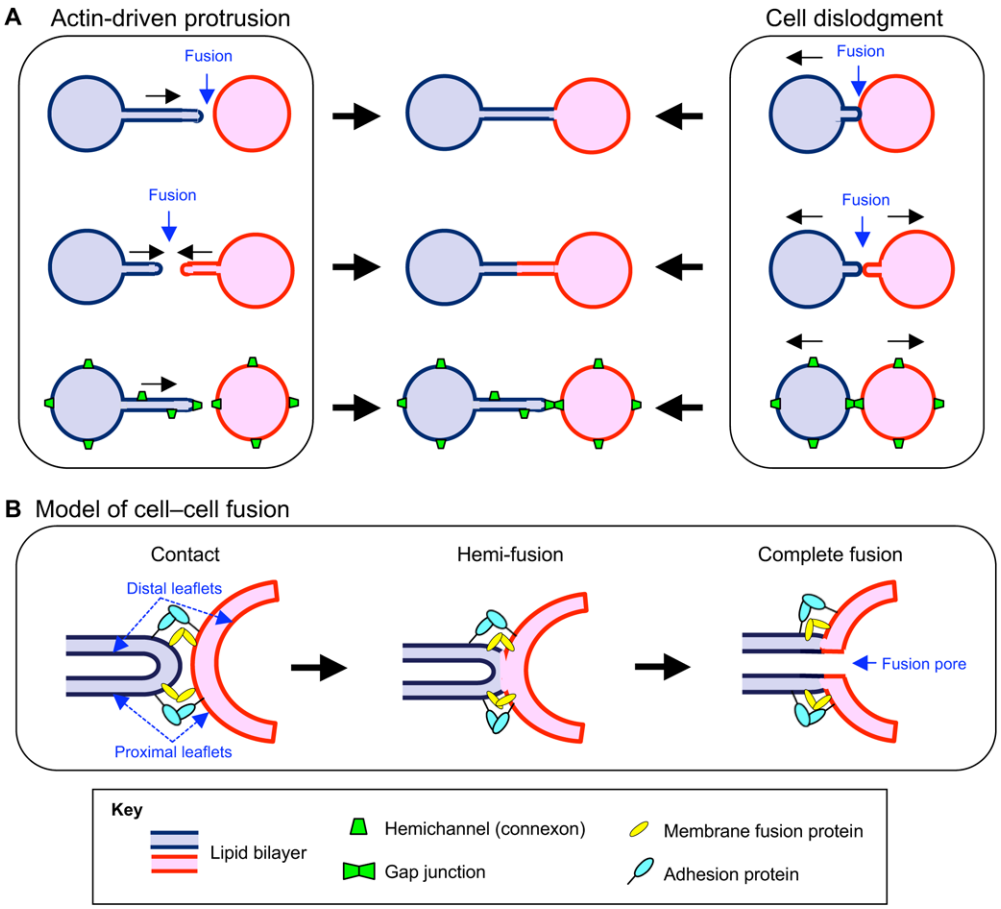

Fig. 3. Model of TNT biogenesis. (**A**) Two different models for TNT formation. In the actin-driven protrusion model (left), one or both cells extend filopodia-like protrusion towards the target cell (or towards each other) in a specific orientation. Once the tip of the protrusion establishes physical contact with the targeted cell, membrane fusion can occur either spontaneously or with the help of fusion factors, leading to the formation of open-ended TNTs, which allows cytoplasmic continuity between cells. In the cell dislodgment model (right), the two cells are in close contact, so that membrane fusion can occur. Subsequently, the cells migrate away from each other, drawing out membrane tethers, leading to the formation of TNTs, which could belong entirely to one cell or to both cells (as illustrated in the middle panel). In the case of immune cells (not shown), this contact can ultimately lead to the formation of an immune synapse, and therefore, might be mediated by adhesion molecules. In the case of electrically coupled TNTs, gap junctions mediate the contact between the two juxtaposed membranes at the tip of TNTs (bottom row). Gap junction formation might rely on recruitment of connexons (hemichannels) to the tip of the protruding filopodia (left), which could then dock at the target cell with receiving connexons. This mechanism should require high expression levels of connexins to increase the probability of connexons docking. Alternatively, upon contact between two cells, docking of opposite connexons takes place (right). During cell dislodgment of the docked cells, TNTs are drawn out, but only a few resist and form TNTs that are coupled to gap junctions (middle). (**B**) Model for cell–cell fusion. During TNT formation it is likely that membrane fusion occurs either in between the tip of the protruding TNT and the target cell, between the two tips of TNTs or between two cells that come in close contact. Membrane fusion comprises the merging of two lipid bilayers into one and requires energy to allow close apposition of the two membranes and to induce membrane curvature. The close contact between the two lipid bilayers might rely on the presence of adhesion molecules, such as cadherins (shown in light blue). In addition, membrane fusion proteins also need to be present in the contact area to induce distortion of the lipid packing and to curve the membrane. The membrane curvature and the juxtaposition of the two bilayers ultimately lead to hemi-fusion of the membranes, which consists of fusion of proximal leaflets only, whereas the distal leaflets are not fused. Fusogenic proteins might drive the hemi-fusion and possibly complete the fusion. At the late stage, a fusion pore forms, allowing complete cytoplasmic continuity between the two cells.

membrane-fusion protein involved in TNT formation (Fig. 3B). Furthermore, the actin cytoskeleton was found to have a role in myoblast fusion (Chen et al., 2007), which raises the question of the involvement of actin in TNT formation in the cell dislodgment model.

The two models for TNT formation described here are not mutually exclusive because they could occur in the same cell type. An interesting open question concerns actin polymerization, because it is unknown whether this process continues in only one or both cells after the establishment of the TNT. Analogous to filopodia, actin polymerization at the TNT tip could be regulated by proteins, such as capping proteins (CAPZA1 and CAPZA2) and members of the Ena-vasodilator-stimulated phosphoprotein (VASP) family (Fig. 1A). Capping proteins are considered to be major barbed-end terminators in filopodia because they prevent the loss of actin monomers (Schafer, 2004), whereas Ena-VASP proteins are found at their tips where they exert an anti-capping function to enable actin polymerization into longer filaments (Bear et al., 2002). However, the role of these proteins in TNT formation remains unexplored.

Open-ended, close-ended and electrically coupled TNTs – variants on the same theme?

Although TNTs by definition are open-ended bridges, experiments carried out in immune cells reveal that TNTs connecting T cells are close-ended (Sowinski et al., 2008) (Fig. 1A). After cell contact and migration, TNTs are ruptured and therefore close-ended, but the tips are still in contact, as observed by electron microscopy (Sowinski et al., 2008). In this study, the authors observed the transfer of viral HIV-1 particles through TNTs from infected to uninfected T cells. Interestingly, the mode of transfer is receptor dependent because the loss of contact between the viral protein Env and its host receptor CD4 at the tip of the nanotube impairs particle transfer, but not the occurrence of TNTs (Sowinski et al., 2008). Although the molecular mechanism of transfer was not addressed in this study, it is likely that receptor–ligand interactions promote phagocytosis of the TNT tip by the uninfected cell. Such a mechanism has been recently described for the transfer of melanosomes from melanocytes to keratinocytes through filopodial extensions, whereby the tip of filopodia containing melanosomes is phagocytosed by the keratinocytes (Singh et al., 2010). Interestingly, the authors demonstrated that this mechanism requires myosin-X, which was shown previously to enhance filopodia formation (Berg and Cheney, 2002) and more recently to increase TNT formation in neuronal cells (K. Gousset, L. Marzo and C.Z., unpublished results).

One important question raised by the study of Sowinsky and colleagues is whether the close-ended nanotubes they observe in T cells fit the definition of TNTs, or rather are viral cytonemes (Sherer and Mothes, 2008) (Fig. 1A). Although cytoneme formation requires the interaction between the viral protein Env and its specific host receptor CD4, the close-ended nanotubes described by the authors occur in the absence of Env. They can therefore be considered to be TNTs (Sowinski et al., 2008).

Close-ended TNTs were also found to form by cell–cell dislodgment between natural killer (NK) cells and mouse mastocytoma P815 cells (Chauveau et al., 2010). This study revealed that proteins involved in immune synapse formation in NK cells, such as the activating receptor, NKG2D, its signaling adaptor DAP10 and its ligand MICA, accumulate at nanotube junctions. This suggests that membrane nanotubes in NK cells contain an immune synapse at submicrometer scale that might mediate their cytotoxicity (Chauveau et al., 2010).

In addition to immune cells, TNTs were shown to form by cell dislodgment in numerous cell types (Wang et al., 2010). It is striking that many of these TNTs are shown to transfer electrical signals (see below) and are characterized by the presence of the gap junction protein connexin-43 at one side of the nanotube (Fig. 1A). Therefore, gap junctions could be the preferred sites for TNT formation by cell dislodgment (Fig. 3A, right panel). Because gap junction formation was shown to require association between connexins and N-cadherin (Wei et al., 2005), connexons (hemichannels composed of six connexins that forms a gap junction when docking with another connexon), along with cadherins, might be able to support the force that is generated by the pulling of the nanotube and thus maintain cell contact. When the TNT membranes pull out of the cell, some of the connexons might break, whereas some might remain at the TNT tips, allowing the formation of electrically coupled TNTs (Wang et al., 2011). Indeed about 20% of TNT-connected NRK cells are not electrically coupled and do not possess connexin-43 (Wang et al., 2010). The loss of connexons that are associated with TNTs might be caused by an internalization of connexins because they have a short half-life (Berthoud et al., 2004). Alternatively, this might indicate that a different mechanism of TNT formation (or the presence of different TNT-like structures) occurs in the same cell type (Wang et al., 2010). Electrically coupled TNTs might also be formed by actin-driven protrusion. This would imply the recruitment of connexons to the tip of the protruding filopodia, which could then dock with receiving connexons located at the target cell (Fig. 3A, left panel). Also, in this case, the presence of cadherins could facilitate the fusion process and the establishment of stronger homophilic interactions that are needed for the fusion to occur (Fig. 3B).

TNT-dependent electrical coupling appears to be cell-type specific. Nevertheless, it is not possible to assign a unique function to TNTs according to cell type. Indeed, PC12 cells do not spread electrical signals, but are able to form TNTs and transfer vesicles, in contrast to NRK cells, which are able to transfer both vesicles and electrical signals through TNTs. Furthermore, it is possible that some TNTs are involved in the transfer of both vesicles and electrical signals (Wang et al., 2010).

Factors involved in TNT formation and regulation

Several studies have aimed to identify the specific conditions and factors that induce or enhance TNT formation in various cell types. Chinnery and co-workers demonstrated that inflammation increases the formation of TNTs in putative dendritic cells in the mouse cornea, suggesting a role of TNTs in the immune response (Chinnery et al., 2008). Recently, the M-Sec protein was demonstrated to be a marker of TNTs and a promoter of TNT formation (Hase et al., 2009) (Fig. 4). M-Sec was initially described as tumor necrosis factor-α induced protein 2 (or B94) (Sarma et al., 1992) and subsequently shown to share homology with Sec6 (Hase et al., 2005), a component of the exocyst complex (Hsu et al., 2004). In another study, Ohno and co-workers showed that the lack of M-Sec protein expression in cultured macrophages reduces the formation of TNTs and is responsible for the impairment of intercellular Ca^{2+} flux, suggesting that M-Sec is essential for the formation of functional TNTs (Hase et al., 2009). In addition, there is evidence suggesting that an interaction between M-Sec and the active form of the Ras-like small GTPase Ral-A is required for TNT formation in HeLa cells (Hase et al., 2009) (Fig. 4). Interestingly, Ral-A is known to bind to filamin (a protein cross-linking actin filaments) in fibroblasts and to promote filopodia formation (Ohta et al., 1999) (Fig. 4). Furthermore, through its interaction with Ral binding protein 1 (RalBP1), Ral-A is able to activate CDC42, leading to actin remodeling and filopodia formation (Ikeda et al., 1998; van Dam and Robinson, 2006) (Fig. 4). Importantly, M-Sec-induced TNTs were shown to be associated with F-actin but not with microtubules (Hase et al., 2009). Therefore, it is likely that M-Sec induces the formation of TNTs by promoting actin cytoskeleton remodeling. In addition, the authors also demonstrate that a dominant-negative form of CDC42, which selectively binds to GDP, inhibits the formation of long TNTs, thereby suggesting that CDC42 has an important role in M-Sec-mediated elongation of TNTs (Hase et al., 2009) (Fig. 4). Interestingly, they also showed that blocking the interaction of Ral-A with its downstream effector, the exocyst complex, reduces the formation of TNTs (Hase et al., 2009).

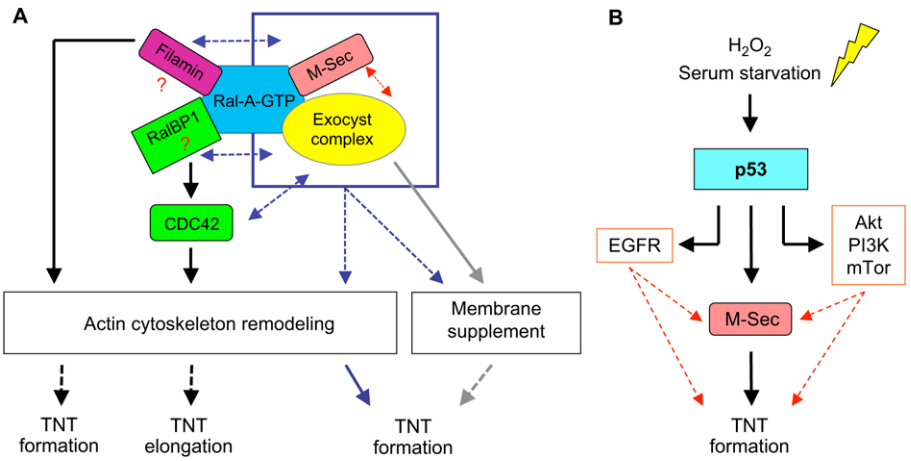

Fig. 4. Molecular machinery involved in TNT formation and regulation. (**A**) M-Sec induces TNT formation through its binding to the active form of Ral-A. This is dependent on the interaction of Ral-A with its downstream effector, the exocyst complex whose function is to target secretory vesicle to site of rapid membrane expansion. Furthermore, through its interaction with Ral binding protein 1 (RalBP1), Ral-A is able to activate CDC42, which leads to actin remodeling. Because of the role of Ral-A in actin remodeling and the role of the exocyst complex, it is likely that M-Sec induces TNT formation by actin cytoskeleton remodeling and membrane supplement. Likewise, the association of Ral-A with filamin, an actin-filament crosslinking protein, is directly implicated in the regulation of actin remodeling in filopodia. Therefore, filamin might be another candidate involved in TNT formation. (**B**) Stress conditions (H_2O_2 and serum starvation) can also induce TNT formation in astrocytes and hippocampal neurons through activation of the transcription factor p53, the EGF receptor (EGFR), and the Akt–PI3K–mTor pathway. M-Sec is also induced by the activation of p53. Whether EGFR and the Akt–PI3K–mTor pathway can directly trigger M-Sec-mediated TNT formation remains unknown. Black arrows, previously described pathways; dotted lines, hypothetical pathways. Blue color depicts interaction with the M-Sec–Ral-A–exocyst complex (blue square). Grey color shows exocyst-complex-mediated pathway. Single arrows indicate pathways, double arrows depict putative interactions.

Importantly, considering that one of the roles of the exocyst is to insert new cell membrane through the delivery of cytoplasmic vesicles (Burgoyne and Morgan, 2003) and that Ral-A is able to bind to the exocyst complex (Sugihara et al., 2002) and to M-Sec (Hase et al., 2009), M-Sec might further enhance TNT formation by promoting the supply of membranes (Fig. 4). Further insight into the mode of TNT formation was provided in T cells, where TNT formation was shown to be induced by stimulation of the Fas signaling pathway and was strictly dependent on Rho GTPases (Arkwright et al., 2010).

TNT formation is also induced in neuronal cells in response to oxidative stress (H_2O_2) and serum starvation (Wang et al., 2011) (Fig. 4). Here, TNT formation was dependent on the transcription factor p53 (Wang et al., 2011). Among the p53 target genes, epidermal growth factor receptor (EGFR) and components of the mitotic activated protein kinase pathway – Akt (AKT1), phosphoinositide-3-kinase (PI3K) and mTOR – were shown to be involved in inducing TNTs (Fig. 4). These data suggest that the ability to develop TNTs could be a cell defense in response to stress and used to transfer substances or energy from damaged to healthy cells (Wang et al., 2011).

Clearly, a better understanding of the molecular mechanisms underlying TNT formation is necessary to identify specific TNT markers (e.g. specific proteins that are not also involved in filopodia formation) to establish the physiological and pathological implications of TNTs in vivo.

Mechanisms of TNT-mediated transfer

Because the main function of TNTs is to mediate intercellular communication by supporting the wiring of various cargos and signals between remote cells, we will discuss here the different mechanisms of TNT-mediated transfer.

Molecular-motor-mediated transport

Similar to mammalian cells, plant cells are also connected by cytoplasmic bridges termed plasmodesmata, which allow the transfer of nutrients and signals necessary for growth and development (Lee and Lu, 2011). However, in contrast to TNTs, which are open conduits containing F-actin, plasmodesmata traverse the cell walls and contain three major layers. The plasma membrane defines the outer boundaries of the plasmodesmata, and the central axis is composed of the desmotubule, which is an appressed endoplasmic reticulum that connects adjacent cells. Between the plasma membrane and the desmotubule lies the cytoplasmic sleeve, which is the major conduit containing actin and myosins (Cilia and Jackson, 2004). Because both TNTs and plasmodesmata represent membrane conduits that lack microtubules (except for TNTs in macrophages and a subset of NK cells), this raises the possibility that TNTs might also use an actomyosin-dependent mechanism of transfer. Consistent with this hypothesis, studies in PC12 cells reveal a partial colocalization of endocytic-related organelles (small synaptic-like microvesicles) with the molecular motor myosin-Va inside TNTs (Rustom et al., 2004) (Fig. 2A). Furthermore, HIV-1 viral particles and lysosomal vesicles were found to transfer through TNTs in T cells and CAD cells, respectively, with a velocity that is consistent with that of actin-driven molecular motors (Gousset et al., 2009; Sowinski et al., 2008) (Fig. 2). In addition, it was shown that the transfer of GFP-tagged prion protein in vesicles of endosomal origin and vesicles containing the misfolded prion protein are transmitted through TNTs with the same velocity as that of actin molecular motors (Gousset et al., 2009) (Fig. 2B). Another study carried out with NRK cells and using myosin-specific inhibitors demonstrated that the transfer of endocytic organelles is actomyosin dependent (Gurke et al., 2008). Taken together, these studies suggest that

vesicular transfer through TNTs (in some cell lines at least) could be mediated by an actomyosin mechanism. It remains to be addressed whether this mechanism is similar to the mechanism of transfer in plasmodesmata in molecular terms.

As mentioned above, TNTs that are formed between macrophages and a subset of NK cells have been shown to contain both F-actin and microtubules (Chauveau et al., 2010; Onfelt et al., 2006), and this raises the question whether microtubules might be involved in the mode of transfer in these cells. Indeed, vesicular transport through thick TNTs (>0.7 μm in diameter) containing actin and microtubules was shown to be energy dependent and to require microtubule integrity, suggesting the involvement of microtubule molecular motors such as kinesins (Onfelt et al., 2006). Strikingly, in the same study, the authors revealed that thin TNTs that contain only actin enable bacteria to surf on their surface in an ATP-dependent manner (Fig. 2A). Recent studies reported the presence of TNT-like structures called bridging conduits connecting macrophages in vitro (Kadiu and Gendelman, 2011a; Kadiu and Gendelman, 2011b; Xu et al., 2009). These F-actin- and microtubule-based structures were found to transfer endocytosed HIV-1 particles between macrophages within endosomes and ER- and Golgi-derived vesicles in actomyosin-dependent manner (Kadiu and Gendelman, 2011a; Kadiu and Gendelman, 2011b). Whether the microtubule cytoskeletal machinery is involved in the transfer remains unknown. Therefore depending on the cytoskeletal component present in the TNT, the mechanism of transport could involve different molecular motors. However, it is not clear whether F-actin- and microtubule-mediated means of transport can occur simultaneously.

Calcium and electrical transfer

In addition to allowing the transfer of organelles, TNTs have been shown to be involved in various cell types in the transfer of Ca^{2+} fluxes between cells (Smith et al., 2011; Watkins and Salter, 2005) (Fig. 2A). Watkins and Salter were the first to demonstrate efficient Ca^{2+} flux transfer through TNTs that are formed between dendritic cells and THP-1 monocytes after either chemical (i.e. with bacterial products) or mechanical stimulation (Watkins and Salter, 2005). Importantly, they clearly demonstrated that this effect does not involve gap junctions or a release of ATP and, based on the kinetics of Ca^{2+} transfer, they also ruled out the possibility that TNT-mediated transfer of Ca^{2+} was driven by the action potential (Watkins and Salter, 2005). However, their observation that microinjected dye was able to transfer efficiently inside TNTs implies that Ca^{2+} could simply diffuse passively through TNTs. Alternatively, the increase of Ca^{2+} in the cell could result from the transfer of the secondary messenger inositol 1,4,5-trisphosphate (IP3) through TNTs, which induces Ca^{2+} release from the endoplasmic reticulum. This possibility was recently addressed by Smith and colleagues who observed that transfer of Ca^{2+} through TNTs in both human dopaminergic neuronal SH-SY5Y and HEK293 cells does not occur by passive diffusion, but involves IP3 receptors (Smith et al., 2011). Based on their findings, the authors propose that an IP3 receptor might actively propagate intercellular Ca^{2+} signals along TNTs by Ca^{2+}-induced calcium release (CICR).

Gap junctions, which are important for mediating electrical conductivity and Ca^{2+} flux, were found to have a role in TNT-mediated Ca^{2+} transfer (Wang et al., 2010). Indeed, TNTs associated with gap junctions (Fig. 1A), were shown to support the bidirectional spread of electrical signals leading to the activation of low-voltage-gated Ca^{2+} channels in the connected cells (Wang et al., 2010). The strength of the current appears to be inversely proportional to TNT length and is directly correlated with the number of TNTs. Interestingly, as in the case of gap junctions, electrical coupling is voltage sensitive and dependent on the presence of connexons (specifically connexin-43), which were mostly found at the junction of the TNT and the connected cell (Fig. 1A). However, compared with conventional gap junctions, electrical coupling through TNTs is likely to be more selective as it has much lower amplitude, thus allowing the coupling of the acceptor cell only and does not result in the spreading of the signal to the neighboring cells (Wang and Gerdes, 2011; Wang et al., 2010).

Functional significance of electrical wiring

The significance of cargo transfer through TNTs has been analyzed elsewhere (Davis and Sowinski, 2008; Gerdes and Carvalho, 2008; Gousset and Zurzolo, 2009), so we will discuss here the possible physiological relevance of TNT-mediated electrical transfer.

Although a sophisticated mechanism of electrical signal transfer (synaptic transmission) is strictly restricted to neuronal cells, TNTs were shown to spread electrical information between various cell types (e.g. NRK, HEK293, HUVEC cells) (Wang et al., 2010). Thus, electrical connection between distant non-neuronal cells is a new concept that could have implications for many physiological processes. For instance, TNT-mediated electrical coupling might be involved in the wound-healing process. This mechanism involves cytoplasmic extensions that are enriched in F-actin and connect opposite cells, as well as the occurrence of membrane depolarization at the leading edge of the wound (Chifflet et al., 2005; Wood et al., 2002; Zhao et al., 2006). Therefore, by spreading electrical signals, TNTs could synchronize the actin-remodeling activity of cells during the healing process (Wang and Gerdes, 2011).

However, TNTs could also reinforce neuronal activity by allowing communication between neurons, between neurons and astrocytes, and even between the branches in dendritic trees. Additionally, based on the fact that neuronal crest cells are connected by thin cellular protrusions in vivo (Teddy and Kulesa, 2004) and by TNT-dependent electrical coupling in vitro (Wang et al., 2010), TNTs could play a key role in synchronizing the migration of cells through the spreading of electrical signals (Wang and Gerdes, 2011).

Furthermore, communication through gap junctions has been shown to be a major signaling mechanism during early brain development, when neural progenitors are not excitable by chemical synapses owing to the lack of expression of ligand-gated channels (Rozental et al., 2000). For example, gap junctions play an essential role in neocortex development (Elias et al., 2010; Elias et al., 2007). During corticogenesis, the neuronal precursor cells that compose the radial glia differentiate into excitatory glutamatergic neurons and inhibitory γ-aminobutyric acid (GABA)ergic interneurons. Depending on the differentiation fate, stem cells that are located in different brain regions have to migrate to reach the appropriate cortical regions (Rakic, 2003). The gap junctions and connexin-43 and connexin-26 are required for proper glial guidance (Elias et al., 2007). In this process, the radial glia extends radial fibers to

guide the migration of embryonic neurons from the ventricular region to the cortical plate. Interestingly, the migration of inhibitory interneurons starts tangentially from the ventral telencephalon and then switches to radial migration to reach their laminar position (Rakic, 2003). The switch between these migratory directions is mediated by connexin-43 (Elias et al., 2010). Further to the recent findings that TNTs are coupled electrically with gap junctions, we can speculate that glial guidance might be mediated by TNTs that are electrically coupled to gap junctions.

Interestingly, during neuronal differentiation, the expression pattern of connexins changes, suggesting that there is specificity in the electrical coupling, which appears to persist in different areas of the brain even after birth and after formation of neuronal circuits in the adult brain (Rozental et al., 2000). In particular, connexin-36-dependent dye coupling has been shown to occur between mature neurons both in vivo and in rat mid-brain slices (Allison et al., 2006). Interestingly, the authors report the absence of membrane-impermeable dye coupling (using Alexa Fluor 488) in adjacent neurons, whereas they observe a spreading of the dye to distant neurons that are separated by more than 100 μm (Allison et al., 2006). Because TNTs might be able to cover such a distance, it will be interesting to analyze the presence of connexin-36 in TNT-like structures in the brain and to investigate their physiological implications.

Importantly, TNT-mediated electrical coupling could affect downstream pathways by modulating the activity of small-molecule transporters and by activating specific enzymes, such as voltage-sensitive phosphatase, PI3K and protein kinase A (Wang and Gerdes, 2011).

Conclusions

We have attempted to provide a comprehensive review of the current knowledge of the mechanism(s) of TNT formation and transfer. Although the occurrence of TNTs has been observed in many cell types in vitro, it remains to be determined whether the TNT transfer mechanisms and their cargos are cell-type specific. Furthermore, it is not clear whether the different mechanisms of TNT formation highlighted here lead to different types of connections. Another important issue is whether the different types of TNTs sustain different or similar functions (depending on the type of cargo or signal transferred). As discussed here, there is mounting evidence suggesting that TNTs represent a new mode of intercellular communication that play a role in a wide range of physiological processes, such as the immune response, development, cell migration, self organization, cell repair, regeneration and pathogen spreading. Furthermore, the recent discovery of TNT-dependent electrical coupling implicates these nanotubes in additional physiological processes.

The lack of specific TNT markers hampers progress towards the elucidation of the molecular mechanisms that govern their formation (e.g. membrane fusion) as well as the visualization or identification of these structures in vivo. For example, the ability of TNT structures to persist in the bloodstream is a key question that remains to be addressed.

A better understanding of the molecular mechanisms underlying the formation of TNTs and their mechanisms of transfer might provide further information regarding the physiological processes in which TNTs participate and their relevance for cellular and organismal function. This could then lead to therapeutic targets and the development of drugs to limit or eradicate the spreading of diseases and infections in the body. The development of screens for proteins that are involved in TNT formation and regulation might help to further elucidate the underlying mechanisms and resulting TNT composition. To this end, in silico modeling approaches could also be very useful. Because the biophysical characteristics of nanotube formation and behavior can be described in detail, their simulations might help to predict the biological processes, in which they might operate and, in turn, aid the search for relevant players. Clearly, transport through TNTs represents an unanticipated but fundamental means for intercellular communication that warrants further exploration.

Acknowledgements

We thank all the members of the Zurzolo lab for stimulating discussion, in particular Karine Gousset for sharing unpublished data, and Ludovica Marzo and Edwin Schiff for TNT pictures shown in Figs 1 and 2. We also thank Chris Bowler for critical reading of the manuscript.

Funding

The work on TNTs in Zurzolo lab is supported by Agence Nationale de Recherche [grant number ANR-09-BLAN-0122-01, PRIONTRAF]; and the EU FP7 program [grant number N.222887, PRIORITY].

References

Allison, D. W., Ohran, A. J., Stobbs, S. H., Mameli, M., Valenzuela, C. F., Sudweeks, S. N., Ray, A. P., Henriksen, S. J. and Steffensen, S. C. (2006). Connexin-36 gap junctions mediate electrical coupling between ventral tegmental area GABA neurons. *Synapse* **60**, 20-31.

Arkwright, P. D., Luchetti, F., Tour, J., Roberts, C., Ayub, R., Morales, A. P., Rodriguez, J. J., Gilmore, A., Canonico, B., Papa, S. et al. (2010). Fas stimulation of T lymphocytes promotes rapid intercellular exchange of death signals via membrane nanotubes. *Cell Res.* **20**, 72-88.

Bear, J. E., Svitkina, T. M., Krause, M., Schafer, D. A., Loureiro, J. J., Strasser, G. A., Maly, I. V., Chaga, O. Y., Cooper, J. A., Borisy, G. G. et al. (2002). Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell* **109**, 509-521.

Berg, J. S. and Cheney, R. E. (2002). Myosin-X is an unconventional myosin that undergoes intrafilopodial motility. *Nat. Cell Biol.* **4**, 246-250.

Berthoud, V. M., Minogue, P. J., Laing, J. G. and Beyer, E. C. (2004). Pathways for degradation of connexins and gap junctions. *Cardiovasc. Res.* **62**, 256-267.

Bour, B. A., Chakravarti, M., West, J. M. and Abmayr, S. M. (2000). Drosophila SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. *Genes Dev.* **14**, 1498-1511.

Bukoreshtliev, N. V., Wang, X., Hodneland, E., Gurke, S., Barroso, J. F. and Gerdes, H. H. (2009). Selective block of tunneling nanotube (TNT) formation inhibits intercellular organelle transfer between PC12 cells. *FEBS Lett.* **583**, 1481-1488.

Burgoyne, R. D. and Morgan, A. (2003). Secretory granule exocytosis. *Physiol. Rev.* **83**, 581-632.

Caneparo, L., Pantazis, P., Dempsey, W. and Fraser, S. E. (2011). Intercellular bridges in vertebrate gastrulation. *PLoS ONE* **6**, e20230.

Chauveau, A., Aucher, A., Eissmann, P., Vivier, E. and Davis, D. M. (2010). Membrane nanotubes facilitate long-distance interactions between natural killer cells and target cells. *Proc. Natl. Acad. Sci. USA* **107**, 5545-5550.

Chen, E. H., Grote, E., Mohler, W. and Vignery, A. (2007). Cell-cell fusion. *FEBS Lett.* **581**, 2181-2193.

Chernomordik, L. V. and Kozlov, M. M. (2003). Protein-lipid interplay in fusion and fission of biological membranes. *Annu. Rev. Biochem.* **72**, 175-207.

Chifflet, S., Hernandez, J. A. and Grasso, S. (2005). A possible role for membrane depolarization in epithelial wound healing. *Am. J. Physiol. Cell Physiol.* **288**, C1420-C1430.

Chinnery, H. R., Pearlman, E. and McMenamin, P. G. (2008). Cutting edge: Membrane nanotubes in vivo: a feature of MHC class II+ cells in the mouse cornea. *J. Immunol.* **180**, 5779-5783.

Cilia, M. L. and Jackson, D. (2004). Plasmodesmata form and function. *Curr. Opin. Cell Biol.* **16**, 500-506.

Davis, D. M. and Sowinski, S. (2008). Membrane nanotubes: dynamic long-distance connections between animal cells. *Nat. Rev. Mol. Cell Biol.* **9**, 431-436.

Dubey, G. P. and Ben-Yehuda, S. (2011). Intercellular nanotubes mediate bacterial communication. *Cell* **144**, 590-600.

Dustin, M. L., Chakraborty, A. K. and Shaw, A. S. (2010). Understanding the structure and function of the immunological synapse. *Cold Spring Harb. Perspect. Biol.* **2**, a002311.

Dworak, H. A. and Sink, H. (2002). Myoblast fusion in Drosophila. *BioEssays* **24**, 591-601.

Elias, L. A., Wang, D. D. and Kriegstein, A. R. (2007). Gap junction adhesion is necessary for radial migration in the neocortex. *Nature* **448**, 901-907.

Elias, L. A., Turmaine, M., Parnavelas, J. G. and Kriegstein, A. R. (2010). Connexin 43 mediates the tangential to radial migratory switch in ventrally derived cortical interneurons. *J. Neurosci.* **30**, 7072-7077.

Eugenin, E. A., Gaskill, P. J. and Berman, J. W. (2009). Tunneling nanotubes (TNT) are induced by HIV-infection of macrophages: a potential mechanism for intercellular HIV trafficking. *Cell. Immunol.* **254**, 142-148.

Faix, J. and Rottner, K. (2006). The making of filopodia. *Curr. Opin. Cell Biol.* **18**, 18-25.

Gerdes, H. H. and Carvalho, R. N. (2008). Intercellular transfer mediated by tunneling nanotubes. *Curr. Opin. Cell Biol.* **20**, 470-475.

Gousset, K. and Zurzolo, C. (2009). Tunnelling nanotubes: a highway for prion spreading? *Prion* **3**, 94-98.

Gousset, K., Schiff, E., Langevin, C., Marijanovic, Z., Caputo, A., Browman, D. T., Chenouard, N., de Chaumont, F., Martino, A., Enninga, J. et al. (2009). Prions hijack tunnelling nanotubes for intercellular spread. *Nat. Cell Biol.* **11**, 328-336.

Gurke, S., Barroso, J. F., Hodneland, E., Bukoreshtliev, N. V., Schlicker, O. and Gerdes, H. H. (2008). Tunneling nanotube (TNT)-like structures facilitate a constitutive, actomyosin-dependent exchange of endocytic organelles between normal rat kidney cells. *Exp. Cell Res.* **314**, 3669-3683.

Han, X., Sterling, H., Chen, Y., Saginario, C., Brown, E. J., Frazier, W. A., Lindberg, F. P. and Vignery, A. (2000). CD47, a ligand for the macrophage fusion receptor, participates in macrophage multinucleation. *J. Biol. Chem.* **275**, 37984-37992.

Hase, K., Ohshima, S., Kawano, K., Hashimoto, N., Matsumoto, K., Saito, H. and Ohno, H. (2005). Distinct gene expression profiles characterize cellular phenotypes of follicle-associated epithelium and M cells. *DNA Res*. **12**, 127-137.

Hase, K., Kimura, S., Takatsu, H., Ohmae, M., Kawano, S., Kitamura, H., Ito, M., Watarai, H., Hazelett, C. C., Yeaman, C. et al. (2009). M-Sec promotes membrane nanotube formation by interacting with Ral and the exocyst complex. *Nat. Cell Biol.* **11**, 1427-1432.

Hsiung, F., Ramirez-Weber, F. A., Iwaki, D. D. and Kornberg, T. B. (2005). Dependence of Drosophila wing imaginal disc cytonemes on Decapentaplegic. *Nature* **437**, 560-563.

Hsu, S. C., TerBush, D., Abraham, M. and Guo, W. (2004). The exocyst complex in polarized exocytosis. *Int. Rev. Cytol.* **233**, 243-265.

Ikeda, M., Ishida, O., Hinoi, T., Kishida, S. and Kikuchi, A. (1998). Identification and characterization of a novel protein interacting with Ral-binding protein 1, a putative effector protein of Ral. *J. Biol. Chem.* **273**, 814-821.

Inoue, N., Ikawa, M., Isotani, A. and Okabe, M. (2005). The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature* **434**, 234-238.

Inoue, N., Ikawa, M. and Okabe, M. (2011). The mechanism of sperm-egg interaction and the involvement of IZUMO1 in fusion. *Asian J. Androl.* **13**, 81-87.

Jegou, A., Ziyyat, A., Barraud-Lange, V., Perez, E., Wolf, J. P., Pincet, F. and Gourier, C. (2011). CD9 tetraspanin generates fusion competent sites on the egg membrane for mammalian fertilization. *Proc. Natl. Acad. Sci. USA* **108**, 10946-10951.

Jena, B. P. (2011). Role of SNAREs in membrane fusion. *Adv. Exp. Med. Biol.* **713**, 13-32.

Kadiu, I. and Gendelman, H. E. (2011a). Human immunodeficiency virus type 1 endocytic trafficking through macrophage bridging conduits facilitates spread of infection. *J. Neuroimmune Pharmacol.* **6**, 658-675.

Kadiu, I. and Gendelman, H. E. (2011b). Macrophage bridging conduit trafficking of HIV-1 through the endoplasmic reticulum and Golgi network. *J. Proteome Res.* **10**, 3225-3238.

Kaji, K., Oda, S., Shikano, T., Ohnuki, T., Uematsu, Y., Sakagami, J., Tada, N., Miyazaki, S. and Kudo, A. (2000). The gamete fusion process is defective in eggs of Cd9-deficient mice. *Nat. Genet.* **24**, 279-282.

Koyanagi, M., Brandes, R. P., Haendeler, J., Zeiher, A. M. and Dimmeler, S. (2005). Cell-to-cell connection of endothelial progenitor cells with cardiac myocytes by nanotubes: a novel mechanism for cell fate changes? *Circ. Res* **96**, 1039-1041.

Le Naour, F., Rubinstein, E., Jasmin, C., Prenant, M. and Boucheix, C. (2000). Severely reduced female fertility in CD9-deficient mice. *Science* **287**, 319-321.

Lee, J. Y. and Lu, H. (2011). Plasmodesmata: the battleground against intruders. *Trends Plant Sci.* **16**, 201-210.

Lucas, W. J., Ham, B. K. and Kim, J. Y. (2009). Plasmodesmata-bridging the gap between neighboring plant cells. *Trends Cell Biol.* **19**, 495-503.

Maeda, S. and Tsukihara, T. (2011). Structure of the gap junction channel and its implications for its biological functions. *Cell. Mol. Life Sci.* **68**, 1115-1129.

Martens, S. and McMahon, H. T. (2008). Mechanisms of membrane fusion: disparate players and common principles. *Nat. Rev. Mol. Cell Biol.* **9**, 543-556.

Millard, T. H. and Martin, P. (2008). Dynamic analysis of filopodial interactions during the zippering phase of Drosophila dorsal closure. *Development* **135**, 621-626.

Miller, J., Fraser, S. E. and McClay, D. (1995). Dynamics of thin filopodia during sea urchin gastrulation. *Development* **121**, 2501-2511.

Miyado, K., Yamada, G., Yamada, S., Hasuwa, H., Nakamura, Y., Ryu, F., Suzuki, K., Kosai, K., Inoue, K., Ogura, A. et al. (2000). Requirement of CD9 on the egg plasma membrane for fertilization. *Science* **287**, 321-324.

Miyado, K., Yoshida, K., Yamagata, K., Sakakibara, K., Okabe, M., Wang, X., Miyamoto, K., Akutsu, H., Kondo, T., Takahashi, Y. et al. (2008). The fusing ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice. *Proc. Natl. Acad. Sci. USA* **105**, 12921-12926.

Miyazawa, K., Emmerling, K. and Manuelidis, L. (2010). Proliferative arrest of neural cells induces prion protein synthesis, nanotube formation, and cell-to-cell contacts. *J. Cell. Biochem.* **111**, 239-247.

Ohta, Y., Suzuki, N., Nakamura, S., Hartwig, J. H. and Stossel, T. P. (1999). The small GTPase RalA targets filamin to induce filopodia. *Proc. Natl. Acad. Sci. USA* **96**, 2122-2128.

Onfelt, B., Nedvetzki, S., Yanagi, K. and Davis, D. M. (2004). Cutting edge: Membrane nanotubes connect immune cells. *J. Immunol.* **173**, 1511-1513.

Onfelt, B., Nedvetzki, S., Benninger, R. K., Purbhoo, M. A., Sowinski, S., Hume, A. N., Seabra, M. C., Neil, M. A., French, P. M. and Davis, D. M. (2006). Structurally distinct membrane nanotubes between human macrophages support long-distance vesicular traffic or surfing of bacteria. *J. Immunol.* **177**, 8476-8483.

Pyrgaki, C., Trainor, P., Hadjantonakis, A. K. and Niswander, L. (2010). Dynamic imaging of mammalian neural tube closure. *Dev. Biol.* **344**, 941-947.

Rakic, P. (2003). Developmental and evolutionary adaptations of cortical radial glia. *Cereb. Cortex* **13**, 541-549.

Ramirez-Weber, F. A. and Kornberg, T. B. (1999). Cytonemes: cellular processes that project to the principal signaling center in Drosophila imaginal discs. *Cell* **97**, 599-607.

Ridley, A. J. (2011). Life at the leading edge. *Cell* **145**, 1012-1022.

Roy, S., Hsiung, F. and Kornberg, T. B. (2011). Specificity of Drosophila cytonemes for distinct signaling pathways. *Science* **332**, 354-358.

Rozental, R., Srinivas, M., Gokhan, S., Urban, M., Dermietzel, R., Kessler, J. A., Spray, D. C. and Mehler, M. F. (2000). Temporal expression of neuronal connexins during hippocampal ontogeny. *Brain Res. Brain Res. Rev.* **32**, 57-71.

Rubinstein, E., Ziyyat, A., Wolf, J. P., Le Naour, F. and Boucheix, C. (2006). The molecular players of sperm-egg fusion in mammals. *Semin. Cell Dev. Biol.* **17**, 254-263.

Ruiz-Gomez, M., Coutts, N., Price, A., Taylor, M. V. and Bate, M. (2000). Drosophila dumbfounded: a myoblast attractant essential for fusion. *Cell* **102**, 189-198.

Rupp, I., Sologub, L., Williamson, K. C., Scheuermayer, M., Reininger, L., Doerig, C., Eksi, S., Kombila, D. U., Frank, M. and Pradel, G. (2011). Malaria parasites form filamentous cell-to-cell connections during reproduction in the mosquito midgut. *Cell Res.* **21**, 683-696.

Rustom, A., Saffrich, R., Markovic, I., Walther, P. and Gerdes, H. H. (2004). Nanotubular highways for intercellular organelle transport. *Science* **303**, 1007-1010.

Saginario, C., Sterling, H., Beckers, C., Kobayashi, R., Solimena, M., Ullu, E. and Vignery, A. (1998). MFR, a putative receptor mediating the fusion of macrophages. *Mol. Cell. Biol.* **18**, 6213-6223.

Salas-Vidal, E. and Lomeli, H. (2004). Imaging filopodia dynamics in the mouse blastocyst. *Dev. Biol.* **265**, 75-89.

Sarma, V., Wolf, F. W., Marks, R. M., Shows, T. B. and Dixit, V. M. (1992). Cloning of a novel tumor necrosis factor-alpha-inducible primary response gene that is differentially expressed in development and capillary tube-like formation in vitro. *J. Immunol.* **148**, 3302-3312.

Schafer, D. A. (2004). Cell biology: barbed ends rule. *Nature* **430**, 734-735.

Sherer, N. M. and Mothes, W. (2008). Cytonemes and tunneling nanotubules in cell-cell communication and viral pathogenesis. *Trends Cell Biol.* **18**, 414-420.

Singh, S. K., Kurfurst, R., Nizard, C., Schnebert, S., Perrier, E. and Tobin, D. J. (2010). Melanin transfer in human skin cells is mediated by filopodia-a model for homotypic and heterotypic lysosome-related organelle transfer. *FASEB J.* **24**, 3756-3769.

Smith, I. F., Shuai, J. and Parker, I. (2011). Active generation and propagation of Ca2+ signals within tunneling membrane nanotubes. *Biophys. J.* **100**, L37-L39.

Sowinski, S., Jolly, C., Berninghausen, O., Purbhoo, M. A., Chauveau, A., Kohler, K., Oddos, S., Eissmann, P., Brodsky, F. M., Hopkins, C. et al. (2008). Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. *Nat. Cell Biol.* **10**, 211-219.

Strunkelnberg, M., Bonengel, B., Moda, L. M., Hertenstein, A., de Couet, H. G., Ramos, R. G. and Fischbach, K. F. (2001). rst and its paralogue kirre act redundantly during embryonic muscle development in Drosophila. *Development* **128**, 4229-4239.

Sugihara, K., Asano, S., Tanaka, K., Iwamatsu, A., Okawa, K. and Ohta, Y. (2002). The exocyst complex binds the small GTPase RalA to mediate filopodia formation. *Nat. Cell Biol.* **4**, 73-78.

Tabata, T. and Takei, Y. (2004). Morphogens, their identification and regulation. *Development* **131**, 703-712.

Teddy, J. M. and Kulesa, P. M. (2004). In vivo evidence for short- and long-range cell communication in cranial neural crest cells. *Development* **131**, 6141-6151.

van Dam, E. M. and Robinson, P. J. (2006). Ral: mediator of membrane trafficking. *Int. J. Biochem. Cell Biol.* **38**, 1841-1847.

Vignery, A. (2000). Osteoclasts and giant cells: macrophage-macrophage fusion mechanism. *Int. J. Exp. Pathol.* **81**, 291-304.

Wang, X. and Gerdes, H. H. (2011). Long-distance electrical coupling via tunneling nanotubes. *Biochim. Biophys. Acta*. (in press)

Wang, X., Veruki, M. L., Bukoreshtliev, N. V., Hartveit, E. and Gerdes, H. H. (2010). Animal cells connected by nanotubes can be electrically coupled through interposed gap-junction channels. *Proc. Natl. Acad. Sci. USA* **107**, 17194-17199.

Wang, Y., Cui, J., Sun, X. and Zhang, Y. (2011). Tunneling-nanotube development in astrocytes depends on p53 activation. *Cell Death Differ.* **18**, 732-742.

Watkins, S. C. and Salter, R. D. (2005). Functional connectivity between immune cells mediated by tunneling nanotubules. *Immunity* **23**, 309-318.

Wei, C. J., Francis, R., Xu, X. and Lo, C. W. (2005). Connexin43 associated with an N-cadherin-containing multiprotein complex is required for gap junction formation in NIH3T3 cells. *J. Biol. Chem.* **280**, 19925-19936.

Wood, W., Jacinto, A., Grose, R., Woolner, S., Gale, J., Wilson, C. and Martin, P. (2002). Wound healing recapitulates morphogenesis in Drosophila embryos. *Nat. Cell Biol.* **4**, 907-912.

Xu, W., Santini, P. A., Sullivan, J. S., He, B., Shan, M., Ball, S. C., Dyer, W. B., Ketas, T. J., Chadburn, A., Cohen-Gould, L. et al. (2009). HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via long-range intercellular conduits. *Nat. Immunol.* **10**, 1008-1017.

Yasuda, K., Khandare, A., Burianovskyy, L., Maruyama, S., Zhang, F., Nasjletti, A. and Goligorsky, M. S. (2011). Tunneling nanotubes mediate rescue of prematurely senescent endothelial cells by endothelial progenitors: exchange of lysosomal pool. *Aging* **3**, 597-608.

Zhao, M., Song, B., Pu, J., Wada, T., Reid, B., Tai, G., Wang, F., Guo, A., Walczysko, P., Gu, Y. et al. (2006). Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-gamma and PTEN. *Nature* **442**, 457-460.