Title

The molecular basis of induction and formation of tunneling nanotubes

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Abstract

Tunneling nanotubes (TNTs) and associated structures are recently recognized structures for intercellular communication. They are F-actin containing thin protrusions of the plasma membrane of a cell and allow a direct physical connection to the plasma membranes of remote cells. TNTs and associated structures serve as mediators for intercellular transfer of organelles as well as membrane components and cytoplasmic molecules. Moreover, several pathogens were shown to exploit these structures to spread among cells. Because of their contribution to normal cellular functions and importance in pathological conditions, studies on TNTs and related structures have accelerated over the past few years. These studies have revealed key molecules for their induction and/or formation; HIV Nef and M-Sec can induce the formation of TNTs in coordination with the remodeling of the actin cytoskeleton and vesicle trafficking.

Introduction

Cells within a multicellular organism must communicate and coordinate with each other for tissue development and homeostasis. Extracellular signaling molecules, such as cytokines, growth factors and hormones, and their receptors mediate intercellular communication between both remote cells and neighboring cells. Adjacent cells often communicate with each other via junctional complexes. Gap junctions are channel-forming junctions which create passageways linking the cytoplasms of adjacent cells, and allow inorganic ions and small water-soluble molecules to pass from the cytoplasm of one cell to another, thereby coupling the cells both electrically and metabolically (Oviedo-Orta and Howard, 2004). Neurological and immunological synapses also transmit cell-cell signals through the extracellular space, relying on mechanisms of ligand–receptor signaling across the closely apposed cell-cell junction (Saito and Yokosuka, 2006).

Tunneling nanotubes (TNTs), also referred to as membrane nanotubes or intercellular nanotubes, and related structures are newly emerging mechanisms for cell-cell communication in a wide variety of cell types (Gerdes and Carvalho, 2008;

Davis and Sowinski, 2008). These structures can directly connect cells even over a long distance. TNTs can be recognized as thin membranous structures connecting two or more cells (Figure 1). Rustom et al. first described TNTs as a structure that provides plasma membrane continuity between connected cells and facilitates the selective transfer of membrane vesicles and organelles to neighboring cells (Rustom et al., 2004). They discovered these structures in rat pheochromocytoma PC12 cells and rat kidney NRK cells. Subsequent studies have identified TNTs or similar structures in various types of cells, and have also revealed variations in the length, diameter and cytoskeletal components of these structures among different cell types (Rustom et al., 2009; Kimura et al., 2012). For examples, TNTs of PC12 cells are 50 – 200 nm in diameter, and they can be up to several cell diameters long (Rustom et al., 2004). In the case of immune cells such as macrophages, Epstein Barr Virus-transformed B cells and human peripheral blood NK cells, the average length of TNTs reaches 30 μm with some measuring over 140 μm (Önfelt et al., 2004). TNTs contain an F-actin backbone and lack microtubules in most cell types; however, some exceptions do exist. For example, human NK cells have TNTs containing microtubules. Macrophages have two type of

TNTs, and the thicker ones (>0.7 μm in diameter) contains both F-actin and microtubule backbone (Önfelt et al., 2006).

The proposed physiological functions for TNTs include the cell-to-cell transfer of large cellular structures such as membrane vesicles and organelles as well as signal transduction molecules such as calcium. Myeloid-linage dendritic cells (DCs) and monocytes propagate their calcium signals within seconds to other cells connected by TNTs, and this intercellular transmission of Ca^{2+} signals induced morphological changes such as lamelipodia extension in recipient cells, one of the earliest responses seen in phagocytes following stimulation (Watkins and Salter, 2005). Since then, calcium flux has been shown to propagate via these structures in many other cell types (Kimura et al., 2012).

Several pathogens are also shown to utilize TNTs for cell–cell transmission and spreading. Human immunodeficiency virus type I (HIV-1) spreads from infected to uninfected T cells via TNTs (Sowinski et al., 2008). The self-propagating aggregated isoform of prion protein PrP^{Sc}, and cytotoxic amyloid beta also exploit TNTs as an intercellular route for spreading across cells (Gousset et al., 2009; Wang et al., 2011).

TNTs are also associated with the intercellular transfer of P-glycoprotein, a 170-kDa transmembrane transporter protein that can pump many chemotherapeutic drugs out of cells. The transfer of P-glycoprotein from non-tumor cells to tumor cells allows the whole tumor to eventually gain multi-drug resistance (Pasquier et al., 2012).

Accumulating evidence from recent studies indicates importance of TNT for a variety of cellular functions. Gradually, we also have learned about the TNT-induction and -formation mechanisms. Here we summarize recent findings about the induction and formation process of TNTs.

Factors inducing TNT formation

Inflammatory conditions

Formation of TNTs and TNT-like structures is promoted by inflammatory conditions. Gram-negative bacterial endotoxin lipopolysaccharide (LPS) induces acute inflammatory responses in mammals, a typical host reaction to tissue injury or infection. In mice cornea, thin membrane bridges with features of TNTs were occasionally observed interconnecting two or more MHC class II-positive cells (Chinnery et al.,

2008). Frequency of these nanotubes was significantly increased in the cornea subjected to trauma and LPS (Chinnery et al., 2008).

Administration of LPS has been also used as an animal model of sepsis-related lung injury associated with inflammation. It is known that the lung injury in mice is ameliorated by intranasal administration of mesenchymal stem cells (MSCs), however the mechanism has been unknown (Ortiz et al., 2003; Ortiz et al., 2007). Islam et al. have recently shown that TNT-like structures were formed between administrated MSCs and the LPS-injured lung alveolar cells, and mitochondrial transfer from MSCs through these structures contributes to tissue repair (Islam et al., 2012). MSCs form connexin 43-containing gap junctional channels with the alveolar epithelia, and the physical association between MSCs and the alveolar epithelia through the gap junction is prerequisite the TNT formation. The authors have further demonstrated that intracellular Ca^{2+}-chelated MSCs successfully attached to the alveolar wall but fail to form TNTs, suggesting the possibility that gap junctional Ca^{2+} communication initiates the formation of TNTs (Islam et al., 2012).

Other instances of TNT formation induced by pro-inflammatory stimuli have

been reported, including microglia activated with phorbol myristate acetate or calcium ionophore, monocytes/macrophages treated with LPS plus interferon γ, and human primary peritoneal mesothelial cells treated with TNF-α (Martinez et al., 2002; Eugenin et al., 2003; Ranzinger et al., 2011).

Fas ligand

Fas ligand-receptor interaction induces programmed cell death through caspase activation, and plays an important role in the regulation of the immune system and the progression of cancer. Stimulation by the Fas ligand can induce TNT formation in T cells, and promotes the propagation of cell death signals between connected cells. This TNT formation is dependent on Rho GTPases (Luchetti et al., 2012; Arkwright et al., 2011). This is consistent with a previous report showing that Fas signaling activates Rho GTPase family, and this activation facilitates Fas-induced cell death (Subauste et al., 2000). Intriguingly, the formation process of TNT by Fas ligand is not dependent on caspase activation, revealing that Fas signaling induces TNT formation via a different mechanism from the main pathway for programmed cell death (Arkwright et al., 2011).

Cellular stress

TNT formation is also induced in astrocytes and hippocampal neurons in response to oxidative stress. Zhu et al. have initially shown that oxidative stress induced by H_2O_2 changes membrane fluidity, induces cytoskeletal reorganization, and increases the formation of TNTs through activation of the p38 MAPK pathway (Zhu et al., 2005). A subsequent study of Wang et al. has confirmed the oxidative stress-induced TNT formation in astrocytes and hippocampal neurons, and further demonstrated that this TNT formation is dependent on activation of the transcription factor p53, epidermal growth factor receptor and the Akt/PI3K/mTor pathway (Wang et al., 2011).

Klein and colleagues have reported that microbial alkaloid staurosporine and nitric oxide induce long tubulovesicular extensions (TVEs) in human neutrophils. TVEs are also a structure similar to TNTs. These authors further showed that human neutrophils demonstrate long-range extracellular catching and holding of bacteria by the TVEs (Galkina et al., 2012, Galkina et al., 2011, Galkina et al. 2009, Galkina et al., 2010). A similar example has been reported in TNTs by Önfelt et al., who have shown

by time-lapse observation that a thin membrane nanotube could be formed when migrating macrophages separate from each other after contact, and that *Mycobacterium bovis* BCG could be trapped and transported along this nanotubes for phagocytosis at the macrophage cell body (Önfelt et al., 2006).

Molecules contributing to initiation of TNT formation

Cell adhesion molecules and receptor-ligand interaction

Adhesion molecules and receptor-ligand interactions are required for the initiation of TNT formation and/or stabilization of the formed TNTs. Veranič and colleagues have reported that N-cadherin and β-catenin, adherence junction proteins, accumulate in TNTs of urothelial T24 cells (Lokar et al., 2010). They have also observed that TNTs grow out of the upper area of cell surface distinct from filopodia; subsequently the TNTs reach adjacent cells and are then stabilized to adhere with the adherence junctions containing N-cadherin and β-catenin (Lokar et al., 2010; Veranič et al., 2008). On the other hand, it has been reported that an initial contact of an adequate duration is generally required for TNT formation in the case of macrophages, NK cells

and T cells (Sowinski et al., 2011; Chauveau et al., 2010). TNT formation between NK cells and target cells requires interaction of an NK cell activating receptor and its ligand on the target cells (Chauveau et al., 2010).

Filopodial bridges, also referred to as viral cytonemes, are thin membrane tubes that connect retrovirus-infected cells and uninfected target cells. Filopodial bridge formation requires initial contact of retrovirus-infected cells with uninfected cells, and a strong association of the viral envelope glycoprotein (Env) on an infected cell with the receptor molecules on a target cell generates a stable bridge (Lehmann et al., 2005; Sherer et al., 2007).

M-Sec/TNFaip2/B94

M-Sec was first identified as tumor necrosis factor alpha-induced protein 2 (TNFaip2, also called B94) cloned from TNFα stimulated endothelium (Sarma et al., 1992). The M-Sec gene encodes a 73-kDa cytosolic protein that has homology to Sec6, a component of the exocyst complex (Hase et al., 2009).

Until recently, the function of M-Sec has been unknown. Our study has

revealed that M-Sec is a central factor in the indution of plasma membrane protrusion during TNT formation (Hase et al., 2009). Depletion of M-Sec by RNA interference greatly reduces TNT formation as well as the intercellular propagation of a calcium flux in a macrophage cell line, Raw264.7. On the other hand, ectopic expression of M-Sec in HeLa cells induces membrane protrusions extending out of the plasma membrane, some of which tether onto adjacent cells and subsequently fuse with their plasma membrane (Figure 1). These membrane conduits contain actin-filaments and can transmit a Ca^{2+} flux to the adjacent cells. Three-dimensional imaging revealed that the membrane nanotubes induced by M-Sec protruded from elevated regions of the cell surface and were not in contact with the substratum. The length of membrane nanotubes induced by M-Sec is 17.7 ± 8.3 μm (mean ± SEM), although they sometimes extend to 40 μm. They are not restricted to connecting pairs of cells via a single nanotube, but instead interconnect several cells, probably forming local networks. These features of M-Sec-induced membrane nanotubes are consistent with that of previously reported TNTs (Sowinski et al., 2011).

The M-Sec-induced formation of TNTs was observed within 6 h

post-transfection and had increased further by 18 h (Hase et al., 2009). Time-lapse analysis of cells expressing GFP-M-Sec showed that a short membrane nanotube gradually extends outwards from a bright spot of GFP-M-Sec signal on the plasma membrane (Figure 1 and video 1), and eventually contacts the plasma membrane of a neighboring cell (Hase et al., 2009). This observation illustrates that M-sec-induced TNT formation occurs as a consequence of enhanced membrane protrusive activity and the resulting membrane extension is involved in TNT formation. These findings do not exclude the possibility that M-Sec also mediates the other mechanism of TNT formation, where short membrane protrusions tether the plasma membranes of adjacent cells and these membrane tethers elongate into long, thin nanotubes as the cells move apart.

The expression of M-Sec mRNA seems to be up-regulated under conditions known to enhance TNT formation. For example, TNFα and LPS, which activate M-Sec expression, are reported TNT-induction factors (Chinnery et al., 2008; Ranzinger et al., 2011). Treatment of rat hippocampal astrocytes with hydrogen peroxide increases the expression of M-Sec mRNA, resulting in TNT formation (Wang et al., 2011). Infection with Human T-cell leukemia virus type 1 (HTLV-1), which increases TNT-like cellular

conduits, is also known to induce M-Sec expression in T cells (Ruckes et al., 2001; Van Prooyen et al., 2010).

HIV Nef

Intercellular spreading of virus causes progression of infection and thus is one of important issues in infectious diseases. Several publications have reported intercellular virus transfer via TNTs and/or TNT-like structures (Aggarwal et al., 2012; Eugenin et al., 2009; Nikolic et al., 2011; Nobile et al., 2010; Sowinski et al., 2008; Xu et al., 2009). HIV-1 infects vital cells in the human immune system such as helper T cells (specifically CD4+ T cells), macrophages, and dendritic cells. Infection with HIV-1 causes gradual and progressive loss of $CD4^+$ T cells, leading to a severe immunodeficiency, acquired immune deficiency syndrome (AIDS). Sowinski et al. have shown that TNTs physically connect HIV-1-infected $CD4^+$ T cells (Sowinski et al., 2008), and the virus uses TNTs as a route to uninfected cells. Subsequent studies confirmed the intercellular HIV transfer via TNT and/or TNT-like membrane conduits, and have indicated that HIV-1 negative factor (Nef) protein is responsible for the

formation of TNTs and/or TNT-like structures in infected cells (Aggarwal et al., 2012; Eugenin et al., 2009; Nikolic et al., 2011; Nobile et al., 2010; Xu et al., 2009).

Nef is a 27–35-kDa HIV-1 accessory protein that alters the actin cytoskeletal organization and endocytic activity in T lymphocytes and dendritic cells. Xu et al. reported that ectopic expression of Nef alone in monocyte THP-1 cells induced TNT-like conduits bridging THP-1 cells each other and also with B cells, and that Nef protein was transferred to B cells via the conduits (Xu et al., 2009). The Nef-dependent membrane protrusion was dependent on both N-terminus region and proline-rich motif in the central region of Nef (Xu et al., 2009). The N-terminus region is myristoylated, and is required for recruiting Nef to the plasma membrane and actin cytoskeleton (Kaminchik et al., 1994; Fackler et al., 1997). The proline-rich motif mediates the interaction with the SH3 domain of members of the Src family kinase family and Vav (Saksela et al., 1995), and the interaction induces actin cytoskeleton remodeling, endosome formation and signaling. Taken together, the anchoring of Nef to the plasma membrane and probably subsequent actin remodeling may be essential to induce formation of TNT-like conduits in HIV-1-infected THP-1 cells (Xu et al., 2009). Nobile

et al. confirmed this study in HIV-1-infected primary $CD4^+$ T cella and Jurkat cells; infection of nef-deleted HIV-1 did not induce thin filopodium-like protrusions, and ectopic expression of Nef gene was sufficient for the induction (Nobile et al., 2010). In addition, the effect of HIV-1 Nef was also dependent on its myristoylated motif and SH3-binding domain (Nobile et al, 2010).

Aggarwal et al. reported F-actin rich filopodial like structures in HIV-1-infected DCs, and defined the structuer as HIV-1 viral filopodia (VF) (Aggarwal et al., 2012). In infected DC, budding HIV-1 but not mature HIV particles concentrated at the ends of largely freely moving untethered VFs. Once $CD4^+$ T cells were tethered by VFs, they are subsequently repositioned and converge to become the DC-T cell viral synapse. The authors further demonstrated that VF formation was dependent on HIV Nef protein and the Diaphanous 2, a key regulator of long filopodia and an actin regulator enriched in cells of myeloid lineage (Aggarwal et al., 2012).

HTLV p8

HTLV-1 is a human RNA retrovirus that is known to cause adult T-cell

leukemia/lymphoma and tropical spastic paraparesis/HTLV-1-associated myelopathy. This retrovirus is barely secreted from infected cells, but is efficiently transmitted by cell-to-cell contacts such as virological synapses (Bangham et al., 2003). Recently, Van Prooyen et al. have shown that ectopic expression of HTLV p8 in Jurkat cells induces the formation of TNT-like cellular conduits, allowing rapid transfer of the virus and of p8 itself into neighboring cells (Van Prooyen et al., 2010). Formation of these conduits is dependent on actin cytoskeleton organization. HTLV-1 p8 overexpression also increases virological synapse formation and viral transmission. The authors concluded that HTLV-1 could be transmitted upon contact with the target cell, via the virological synapse, and through cellular conduits. HTLV-1 p8 down-regulates proximal TCR signaling and causes T-cell anergy (Fukumoto et al., 2007; Fukumoto et al., 2009). They propose a model in which p8 would invade neighboring cells to favor rapid transfer of virus, and at the same time, induce T-cell anergy to protect the infected cells from immune recognition (Van Prooyen et al., 2010).

Molecules associated with protrusion steps of TNTs

Rho family small GTPases

Small GTPases are a family of hydrolases with molecular masses usually in the range of 20-25 kDa. They can bind and hydrolyze guanosine triphosphate (GTP) and function as molecular switches in intracellular signaling to control a wide variety of cellular functions. Members of the Rho family, a subfamily of the Ras superfamily, play a role in actin cytoskeleton organization, membrane traffic, and multiple other cellular functions (Burridge and Wennerberg, 2004).

Cdc42 is a member of the Rho small GTPase family and is required for TNT and/or TNT-like nanotube formation. This protein is partially localized to M-Sec-induced nanotubes (Hase et al., 2009) and the expression of Cdc4217N, a dominant negative form of Cdc42, led to a decrease in the number of long membrane protrusions accompanied by an increase in short protrusions. This resulted in a slight suppression of both TNT formation and the propagation of calcium flux (Hase et al., 2009). Nikolic et al. have shown that HIV-1 induces membrane extensions in immature DCs through activation of Cdc42 (Nikolic et al., 2011). Silencing of Cdc42 by siRNA treatment or treatment with a specific Cdc42 inhibitor, Secramine A, dramatically

reduces the number of membrane protrusions and decreased HIV-1 transfer via infectious synapses. They further demonstrated that these extensions were induced after attachment of HIV-1-infected DC and T cells by interaction of HIV-1 envelop with DC-SIGN, which is a C-type lectin receptor expressing in DCs, and responsible for the formation of the infectious synapse. They have proposed a 2-step model for HIV-1 transfer from immature DCs to T cells that involves HIV-1 envelop engagement of the DC-SIGN receptor, leading to Cdc42 activation and formation of membrane extensions, followed by the transfer of virus to the T cell (Nikolic et al., 2011).

Rho GTPase family also facilitates Fas-induced nanotube formation in T cells as described above. The general inhibitor of Rho GTPases, toxin B of *Clostridium difficile*, and a Cdc42-specific inhibitor secramine A are effective in blocking formation of these structures (Arkwright et al., 2011).

Ral/exocyst effector complex

Ral is a member of the Ras family of small GTPases and consists of two highly similar RalA and RalB isoforms (sharing 82% identity) (Ohta, et al., 1999;

Sugihara, et al., 2002). Ral GTPases were found to be highly colocalized with M-Sec-positive membrane nanotubes (Hase et al., 2009). Furthermore, RalA28N, which selectively binds to GDP and thereby functions as a dominant negative mutant, almost completely abrogated the induction of long membrane nanotubes by M-Sec, although a limited number of immature, short protrusions were observed (Hase et al., 2009). As a result, propagation of a calcium flux was drastically reduced. Consistent with these observations, we found that lentivirus-mediated expression of the RalA28N mutant in Raw264.7 cells attenuated their spontaneous formation of TNTs (Hase et al., 2009).

The active Ral GTPase interacts with the exocyst complex and acts as an upstream effector (Moskalenko et al., 2002; Sugihara et al., 2002). The exocyst complex has been reported to be involved in the tethering, docking and fusion of post-Golgi vesicles with the plasma membrane (He and Guo, 2009). It is composed of eight subunits that are conserved from yeast to mammalian cells, and includes Sec3, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 in addition to Sec5. Two subunits of the exocyst complex, Exo84 and Sec5, are effector targets for active Ral GTPases (Moskalenko et al., 2002; Sugihara et al., 2002). Structural and biochemical studies further revealed that

Sec5 and Exo84 competitively bind to active RalA, suggesting that RalA plays an important role in regulating exocyst assembly (Jin et al., 2005).

The exocyst complex is also involved in TNT formation. RNAi-mediated depletion of Sec5 significantly impairs M-Sec-induced TNT formation, and this was associated with a reduction in the propagation of a calcium flux. Depletion of another exocyst component, Sec6, has a similar inhibitory effect on M-Sec-inducible TNT formation. Overexpression of RalA48W or RalA38R mutants, which are unable to bind to the Exo84 or Sec5 exocyst complex components, respectively, resulted in a significant reduction in the formation of TNTs, indicating that the interaction of RalA and the exocyst is important for TNT formation (Hase et al., 2009).

Mukerji et al. have recently reported that the exocyst complex interacts with HIV Nef protein by proteomic analysis of Nef immuno-complexes from Jurkat cells expressing Nef protein (Mukerji et al., 2012). Nef associates with five components of the exocyst complex. Furthermore, depletion of Sec5 by shRNA treatment in Jurkat cells also abrogates Nef-mediated enhancement of TNT formation (Mukerji et al., 2012).

Concluding remarks

Recent researches have shown that several stress conditions can induce TNT-associated structures, and found molecules associated with formation of these structures (Table I and II). TNTs and TNT-like structures are not static but dynamic structures; the formation of these structures is induced by exposure to several kinds of environmental conditions, such as inflammation, infection, and oxidative stress. Future studies on common signaling pathways stimulated by these conditions might provide some clues to understanding TNT formation mechanisms. For example, a number of signaling pathways can be activated by LPS, including phosphatidylinositide 3-kinase (PI3K) pathway (Arbibe et al., 2000). PI3K generates phosphatidylinositol 3,4,5-triphosphate (PIP3), which recruits several proteins regulating cell survival, actin cytoskeleton organization and vesicular transport, to the plasma membrane. The PI3K pathway is also activated by IFN-gamma and Nitric oxide (Nguyen et al., 2001; Ciani et al., 2002), and is associated with pathogenic role of HIV Nef, which downregulates MHC class I via PI3K-regulated endocytic pathway to escape from adaptive immunity

(Blagoveshchenskaya et al., 2002; Hung et al., 2007). In addition, Wang et al. have shown that PI3K pathway is involved in TNT formation by oxidative stress (Wang et al., 2011). Taken together, the PI3K pathway is possibly involved in TNT formation. It is also interesting that Rho small GTPase family and Ral/exocyst complex seems to be commonly involved in HIV-1 Nef- and M-Sec-induced membrane protrusions. These molecules are known to be associated with filopodia/lamelipodia formation (Liu, et al., 2012; Sugihara et al., 2002), suggesting the possibility that actin cytoskeleton remodeling and vesicular traffic during TNT formation relies on mechanisms similar to that of filopodia/lamelipodia formation.

Recent studies have indicated that intercellular organelle transfer could make up for damaged organelle, resulting in recovery of cellular functions (Spees et al., 2006; Yasuda et al., 2011). It is noteworthy that mitochondrial transfer via TNTs and/or microvesicles rescues injured cells in a mouse model as mentioned above (Islam et al., 2012). Although transfer of membrane-bound components such as mitochondria is known as a unique feature of TNTs (Kimura et al., 2012), its significance *in vivo* has not been verified. The intercellular mitochondrial transfer from MSC to injured cells could

potentially be important, although further studies are required to support this concept. It is still unclear the association of the microvesicles with TNTs, and that whole cell fusion and/or partial cell fusion between human MSCs with recipient cells was also involved in mitochondrial transfer.

There are other examples of intercellular transfer of organelles *in vivo*, including melanosomes, which are transferred from melanocytes to keratinocytes, and ribosomes from Schwann cells to axons (Scott et al., 2002; Twiss et al., 2009). The elucidation of the mechanisms of TNT-mediated organelle transfer might provide wide-ranging insights into the process of intercellular transport, and vice versa.

Figure Legend

Figure 1

(a) Raw264.7 cells are labeled with Alexa488 conjugated wheat germ agglutinin. TNTs are thin membranous structures connecting two or more cells and are indicated by arrows. Bar, 20 μm. (b) M-Sec-GFP cDNA transfection into HeLa cells can induce TNT-like structures. HeLa cells were transfected with expression plasmid for GFP-M-Sec (b, b' and b'') or GFP as control (b''' and b''''). Forty-eight hours after transfection, cells were fixed, and DIC (b and b''') images and GFP signals (b', b'' and b'''') were taken with confocal microscopy (FV300, Olympus). (b'') A higher magnification image of HeLa expressing GFP-M-Sec (area in the square in panel b') is shown. M-Sec signals are detected throughout the cytoplasm, and strong signals are detected in the TNT-like membrane protrusion. M-Sec positive membrane protrusions tether the surface of a neighboring cell (dashed line). Scale bar is 20 μm. (Reprinted with permission from Ref. Kimura et al., 2012. (Copyright 2012 Elsevier) (c) Time-lapse video microscpy of GFP-M-Sec-transfected HeLa cells. GFP signals can be observed within 6 hours post-tranfection (time = 0 s), and a short membrane nanotube

gradually extends outwards from a bright spot of GFP-M-Sec signal on the plasma membrane. Bar, 10 μm. See supplemental video 1.

Supplemental video 1

Time-lapse video microscpy of GFP-M-Sec-transfected HeLa cells.

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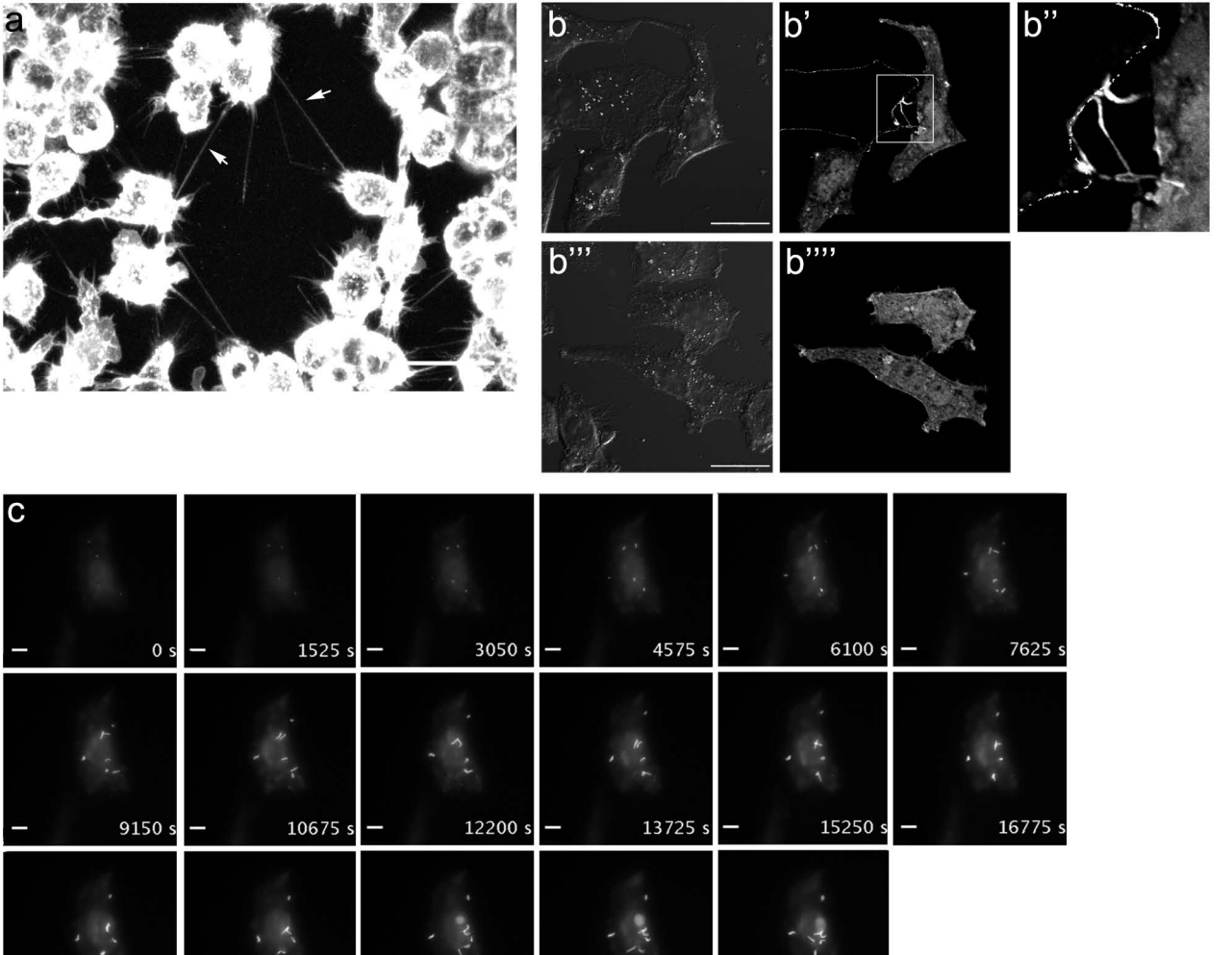
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Table I Induction stimuli and/or condition for formation of TNTs and associated structures

Stimuli or condition	Organ or cells	Reference
LPS	Mouse Cornea	(Chinnery et al., 2008)
	Mouse Lung	(Islam et al., 2012)
LPS plus IFNγ	Human monocytes/macrophages	(Eugenin et al., 2003)
TNFα	Human primary peritoneal mesothelial cells	(Ranzinger et al., 2011)
Phorbol myritate acetate	Newborn rat microglia	(Martinez et al., 2002)
Calcium ionophore	Newborn rat microglia	(Martinez et al., 2002)
Fas-FasL interaction	Jurkat cells	(Arkwright et al., 2011; Luchetti et al., 2012)
Oxidative stress	Rat astrocytes	(Zhu et al., 2005)
	Rat astrocytes and hippocampal neurons	(Wang et al., 2011)
Nitric oxide	Human Neutrophil	(Galkina et al., 2009)
Microvial alkaloid	Human Neutrophil	(Galkina et al., 2010)
Expression of M-Sec	HeLa, HEK293, Raw264.7	(Hase et al., 2009)
HIV infection	Mouse macrophages	(Eugenin et al., 2009)
	THP-1	(Xu et al., 2009)
	CD4+ T cells and Jurkat cells	(Nobile et al., 2010)
	Immature human DCs	(Nikolic et al., 2011)
	Human DCs	(Aggarwal et al., 2012)
Expression of HIV Nef	Jurkat cells	(Mukerji et al., 2012)
HTLV-1 infection	Jurkat cells	(Van Prooyen et al., 2010)

Table II Molecules associated with protrusion steps of TNTs

Experimental model	Experimental methods	Reference
Ral/Exocyst complex		
Jurkat cells expressing Nef	Co-immunoprecipitation with Nef and knockdown of sec5 by siRNA	(Mukerji et al., 2012)
HeLa cells expressing M-Sec	Immunocytochemistry of RalA and overexpression of RalA28N[*1], and knockdown of sec5 and sec6 by siRNA	(Hase et al., 2009)
Raw264.7 cells	Overexpression of RalA28N[*1]	(Hase et al., 2009)
Rho small GTPase family		
T cell treated with FasL	Treatment with toxin B of Clostridium and secramine A[*2]	(Arkwright et al., 2011)
HIV-infected immature DC	Knockdown of cdc42 by siRNA and overexpression of cdc4217N[*3]	(Nikolic et al., 2011)
HeLa cell expressing M-Sec	Immunocytochemistry of cdc42 and overexpression of cdc4217N[*3]	(Hase et al., 2009)
Diaphanous2		
HIV Nef	Knockdown of Diaphanous2 by siRNA	(Aggarwal et al., 2012)

[*1] Dominant negative form of RalA
[*2] General inhibitor of Rho GTPases family and Cdc42-specific inhibitor, respectively
[*3] Dominant negative form of cdc42