HOKKAIDO UNIVERSITY

Title	Tunneling nanotubes: Emerging view of their molecular components and formation mechanisms
Author(s)	Kimura, Shunsuke; Hase, Koji; Ohno, Hiroshi
Citation	Experimental Cell Research, 318(14), 1699-1706 https://doi.org/10.1016/j.yexcr.2012.05.013
Issue Date	2012-08-15
Doc URL	http://hdl.handle.net/2115/50688
Type	article (author version)
File Information	ECR318-14_1699-1706.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Title

Tunneling nanotubes: emerging view of their molecular components and formation mechanisms

Shunsuke Kimura[1, 2], Koji Hase[3, 4, 5], and Hiroshi Ohno[2, 5]

[1]Laboratory of Histology and Cytology, Graduate School of Medicine, Hokkaido University, North 15, West 7, Kita-ku, Sapporo 060-8638, Japan

[2]Laboratory for Epithelial Immunobiology, Research Center for Allergy and Immunology, RIKEN, 1-7-22 Suehirocho, Tsurumi, Yokohama, Kanagawa 230-0045, Japan

[3] Laboratory for Bioenvironmental Epigenetics, Research Center for Allergy and Immunology, RIKEN, 1-7-22 Suehirocho, Tsurumi, Yokohama, Kanagawa 230-0045, Japan

[4]PRESTO, Japan Science and Technology Agency, Tokyo102-0075, Japan.

[5]Division of Immunobiology, Department of Supramolecular Biology, Graduate School

of Nanobioscience, Yokohama City University, 1-7-29 Suehiro, Tsurumi, Yokohama 230-0045, Japan

Correspondence to: Hiroshi Ohno, M.D., Ph.D., ohno@rcai.riken.jp

Keywords

Tunneling nanotubes; membrane nanotubes; cytonemes; M-Sec (TNFaip2); intercellular communication

Acknowledgements

We thank Dr. Peter D. Burrows for critical reading of the manuscript.

Abstract

Cell-to-cell communication is essential for the development and maintenance of multicellular organisms. The tunneling nanotube (TNT) is a recently recognized distinct type of intercellular communication device. TNTs are thin protrusions of the plasma membrane and allow direct physical connections of the plasma membranes between remote cells. The proposed 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 in a wide variety of cell types. Moreover TNT and TNT-related structures are thought to facilitate the intercellular spreading of virus and/or pathogenic proteins. Despite their contribution to normal cellular functions and importance in pathological conditions, virtually nothing is known about the molecular basis for their formation. We have recently shown that M-Sec (also called TNFaip2) is a key molecule for TNT formation. In cooperation with the RalA small GTPase and the exocyst complex, M-Sec can induce the formation of functional TNTs, indicating that the remodeling of the actin cytoskeleton and vesicle trafficking are involved in M-Sec-mediated TNT formation. Discovery of the role of M-Sec will accelerate our

understanding of TNTs, both at the molecular and physiological levels.

Introduction

To develop and maintain homeostasis, cells of a multicellular organism must communicate with each other in various ways. Communication between remote cells is mediated mainly by secretion of signaling molecules, such as cytokines, from the stimulatory cells and their subsequent binding to specific receptors on the responding cells. Another form of communication with remote cells is mediated by small membrane vesicles, exosomes, secreted from a broad range of cell types as a consequence of fusion of multivesicular late endosomes/lysosomes with the plasma membrane [1].

By contrast, adjacent cells often communicate with each other via junctional complexes, such as gap junctions and synaptic junctions [2]. The gap junctions are narrow channels directly connecting the cytoplasm of neighboring cells, which 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. 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 junctions [3].

In addition to these well-established examples, tunneling nanotubes (TNTs), sometimes referred to as membrane nanotubes, and related structures are newly-emerged distinctive mechanisms for cell-cell communication in a wide variety of cell types. These structures can directly connect cells even over relatively long distances [4][5]. While accumulating evidence from recent studies indicates their importance for a variety of cellular functions, we still have a limited molecular understanding of TNTs regarding their structures and biogenesis. TNTs have become a very active research area, however, and new evidence on molecular components and formation mechanisms has been forthcoming. Here we summarize current findings on molecular aspects of TNTs and related structures.

What are TNTs?

TNTs can be recognized as thin membranous structures connecting two or more cells and, importantly, these structures are not attached to the substratum (Figure 1 A to F)[6][7]. A different sort of cytoplasmic connection is observed after cell division as a structure containing the midbody; it persists temporarily as a tether between the two

daughter cells, contains dense matrix material, and is visible by light microscopy. By contrast, TNTs do not contain the midbody. Gerdes and colleagues 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 [7]. They discovered these structures in rat pheochromocytoma PC12 cells and rat kidney NRK cells, but subsequent studies have identified TNTs or similar structures in various types of cells, including T24 urothelial cells, Jurkat T cells, THP-1 human monocytes and human primary natural killer (NK) cells (Table 1).

The detailed morphological and structural characteristics of TNTs are substantially different among cell types [4]. TNTs of PC12 cells are 50 – 200 nm in diameter, and they can be up to several cell diameters [7]. 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 [8]. TNTs contain an F-actin backbone and lack microtubules in most cell types [4][7]; however, some exceptions do exist [4]. For example, NK cells have TNTs containing microtubules [9]. Macrophages have two type of TNTs, and the thicker ones

(>0.7 μm in diameter) contains both F-actin and microtubule backbones [10]. This heterogeneity probably represents cell-specialized functions and features of TNTs as described below. More recent studies have also shown that the termini of some TNTs and associated structures are not continuous with the plasma membrane of connected cells but instead have junctions [9][11][12][13][14], as we will also describe later.

Proposed functions of TNTs

Transmission of intercellular signaling via TNTs

Calcium flux is the best-characterized signal transmitted between remote cells via TNTs. Upon mechanical or chemical stimulation, myeloid-linage dendritic cells (DCs) and monocytes propagate their calcium signals within seconds to other cells connected by TNTs (Figure 1G)[12]. TNT-medicated intercellular transmission of calcium signals induced morphological changes such as lamelipodia extension in recipient DCs, one of the earliest responses seen in phagocytes following stimulation [12]. This observation demonstrates that the cell-cell interaction via TNTs facilitates transduction of signals between remote cells (Table 1).

Watkins and Salter [12] performed a rigorous examination to exclude the possibility that the propagation of calcium flux between DCs is dependent on ATP released from these cells following mechanical stimulation as described previously[15]. The Ca^{2+} flux between DCs was not induced by addition of BzATP, a stabilized form of ATP, and not blocked by suramin, an antagonist of P2 purinergic receptors responsible for ATP-mediated calcium responses in mast cells. In addition, physical disruption of the TNT-network by scraping with a micropipette tip resulted in abrogation of the intracellular propagation of the calcium flux (Figure 1). These investigators further demonstrated that the small cytoplasmic dye Lucifer yellow could readily transfer via TNTs between THP-1 myeloid cells, whereas the larger cytoplasmic molecule dextran could not [12]. Thus, there must be some junctions and/or gating mechanisms for TNT-based transport of intercellular material, which may imply the association of a gap-junction that can pass low molecular weight molecules but not macromolecules. Intriguingly, however, calcium transmission via TNTs in THP-1 cells was insensitive to conventional gap-junction inhibitors, suggesting the presence of previously unknown gating mechanisms. On the other hand, Gerdes and colleagues recently showed that in

various cell types, including HEK293, NRK, human umbilical vein endothelial cells (HUVEC) and neural crest cells, TNTs have gap junctions at their distal ends which mediate electrical coupling between distant cells through the interposed gap junction channels [11]. Furthermore they also showed that the electrical signals transferred from one cell to another via TNTs are sufficient to induce a transient calcium elevation in the recipient cell by activating low voltage-gated Ca^{2+} channels [11]. These studies suggest that different mechanisms of intercellular calcium signaling are likely to exist ant may reflect the diversity of TNT structures and functions in specific cell types.

Another type of TNT-mediated signal transduction was reported in NK cells. NK TNTs contain a submicron scale junction similar to the intercellular lytic synapse between NK cells and target cells, where a proximal signaling protein of NK-cell activating receptor NKG2D and its ligand MHC Class I chain-related protein A (MICA) accumulates [9]. One function of NK cells is recognition and elimination of cells undergoing different forms of stress, such as microbial infection and malignant transformation [16]. This study raises the intriguing possibility that NK cells might eliminate target cells via an immunological synapse located at the terminus of their

TNTs; thus, TNTs could aid the lysis of distant cells by NK cells. NK-cell cytotoxicity involves the secretion of cytolytic effector molecules from specialized organelles known as lytic granules. Intracellular trafficking of the lytic granule is dependent on microtubules [16], and the NK-cell TNTs contain a microtubule backbone. Thus, the microtubule within NK-cell TNTs may act as a railway for delivering lytic granules to the distal end of the TNTs.

Organelle transfer via TNTs

A striking function of TNTs is that they can transfer membrane-bound components such as organelles. Gerdes and colleagues first reported organelle transfer via TNTs both in PC12 and NRK cells [7]. Subsequent studies have shown that the TNT-mediated organelle transfer is common in a variety of cell types (Table 1) [4].

The organelle transfer in PC12 and NRK cells is unidirectional and dependent on actin filaments [7], and is inhibited by ATP depletion and the general myosin inhibitor 2,3-butanedione monoxime [17]. In addition, the actin-based molecular motor, myosin-Va, co-localizes with organelles inside the TNTs of PC12 and rat primary

astrocytes cells [7] [18], suggesting that this motor protein is an interesting potential candidate to mediate movement of organelles along TNT. On the other hand, thicker TNTs in macrophages, whose diameter is over 0.7 μm, transfer organelles by bidirectional movement, indicating the association of microtubule-dependent motor proteins [10]. Conceivable mechanisms of organelle-transfer might involve exo- and endocytic events at the membrane interface, or transient fusion of the TNT membrane with the target-cell membrane, resulting in an open-ended TNT structure as reported in PC12 cells [7][11]. The mechanism and significance of organelle transfer via TNTs are of considerable interest for future studies.

Implications in Disease

Infection with the human immunodeficiency virus type I (HIV-1) retrovirus, causes the gradual and progressive loss of $CD4^+$ T cells, leading to a severe immunodeficiency, acquired immune deficiency syndrome (AIDS). HIV-1-infected human T cells were recently found to be interconnected by TNTs [13]. Expression of the envelope glycoprotein and the virus capsid protein are features of the late stage of productive

viral replication. Both molecules have been detected on conduits connecting HIV-1-infected and uninfected T cells, suggesting that TNTs may be one mechanism for the transfer of HIV from infected to uninfected T cells.

Intriguingly, when two populations of Jurkat T cells labeled with membrane dyes, DiD or DiO, are mixed together, neither DiD nor DiO seamlessly traffics between connected cells by TNTs, revealing the existence of a junction also in the T-cell TNTs (Figure 1 F)[13]. This is consistent with electron microscopic analysis that showed junctional structures in TNT between T cells [13]. It is still unclear how HIV-1 overrides this junction and transfers to the bystander cells. Transfer of HIV-1 via TNTs is dependent on the existence of the CD4 virus receptor on recipient cells, whereas formation of T cell TNTs is independent from the interaction between envelop protein and CD4 [13]. Thus, one possibility is that, like NK-cell TNTs, HIV-infected T cells might have a similar structure with virological synapses at the ends of the TNTs.

The frequency of TNT formation by T cells is not affected by HIV-1 infection [13]. On the other hand, TNTs are induced in macrophages infected with HIV-1 [19][20], and HIV-1 negative factor (Nef) protein is thought to be responsible for this induction

[20]. The Nef protein can block the generation of effective neutralizing antibodies against HIV-1 by antibody-producing plasma cell progeny of B cells in AIDS patients [21]. However, it had been unclear how Nef is transferred to B cells, which are not themselves infected by HIV. Cerutti and colleagues reported that ectopic expression of Nef alone in monocyte THP-1 cells induced TNT-like conduits bridging THP-1 cells with each other and also with B cells, and that Nef protein was transferred to B cells via the conduits [20]. The Nef protein is myristoylated at its N-terminus and is recruited to the plasma membrane. This anchoring of Nef to the plasma membrane and probably subsequent actin remodeling by Nef are essential to stimulate formation of TNT-like conduit in macrophages [20]. In addition, these investigators showed that the invasion of Nef into B cells is dependent on clathrin-dependent endocytosis and actin remodeling in Nef-donor macrophages [20]. It is not yet known whether junctions exist in TNTs formed between macrophages and B cells and, if so, how endocytic vesicles containing Nef proteins are transferred from macrophages to B cells.

Another pathogenic protein, the self-propagating aggregated isoform of prion protein PrP^{Sc}, also exploits TNTs as an intercellular route for spreading across cells [22].

PrP^{Sc} is the infectious agent that causes spongiform encephalopathies such as scrapie in sheep, bovine spongiform encephalopathy in cattle and Creutzfeldt-Jakob disease in humans. According to the widely accepted prion hypothesis, infectious PrP^{Sc} is a conformational isomer of the normal cellular prion protein PrP^{C}. The direct interaction of non-pathogenic PrP^{C} with pathogenic PrP^{Sc} is thought to drive conversion of PrP^{C} to the infectious form of the protein. The precise mechanisms for the transport of Prp^{Sc} from their peripheral entry sites, i.e. the gut and the skin, to the brain where they cause disease is poorly understood, PrP^{Sc} is initially transported by dendritic cells to lymphoid tissues. Thereafter, PrP^{Sc} enters the peripheral nervous system and spreads toward the central nervous system by retrograde axonal transport. TNTs are thought to be important for the transfer of PrP^{Sc} from dendritic cells to neurons, as well as between neurons [22]. PrP^{C} is a GPI-anchored glycoprotein predominantly localized on the outer leaflet of the plasma membrane and endosomal/lysosomal membranes, whereas the pathogenic PrP^{Sc} is located on the luminal side of lysosomal structures. Therefore, the transfer of PrP^{Sc} along TNTs may be accomplished by vesicular transport inside TNTs.

TNT-like structures that mediate cell-cell communication

There are other structures similar to, but somewhat different from, TNTs in their functions and molecular components.

Cytonemes and viral cytonemes (filopodial bridges)

Cytonemes are actin-rich filopodia-like structures extending up to 100 μm connecting anterior and posterior compartments of the imaginal disc in fruit flies [23]. Morphogen receptors such as Decapentaplegic migrate along the length of the cytoneme and make morphogen gradients that determine the body pattern. In mammalian cells, thin membrane tubes called filopodial bridges, also referred to as viral cytonemes, connect retrovirus-infected cells and uninfected target cells, in a fashion similar to cytonemes [24]. Like filopodia, these structures contact the substratum, but the filopodial bridges are longer than filopodia, with average length of 5.8 μm; filopodia, which do not connect cells, are normally around 2.37 μm in length. Both structures are induced by retrovirus infection [24]. COS-1, XC and HEK293 cells form filopodial bridges only when there is reciprocal expression of a retroviral envelope glycoprotein on the surface

of infected cells and appropriate viral receptors on the uninfected cells [24]. Viruses move directionally along the outer surface of these filopodial bridges to infect neighboring cells. Their contact with substratum and dependence of their formation on envelop protein-receptor interaction are the main features that distinguish filopodial bridges from the TNTs also used by retrovirus for intracellular transfer [6][13][24].

Klein and colleagues reported long tubulovesicular extensions (TVE) on human neutrophils, also structures similar to cytonemes. Interestingly, they showed that human neutrophils demonstrate long-range extracellular catching and holding of bacteria by the TVE [25].

EP bridges

More recently, a novel form of tubular cellular bridge was discovered, termed the epithelial (EP) bridge. In contrast to the numerous cell types in which TNTs interconnect, EP bridges have only been found in primary cultures of human bronchial epithelial cells and A549 human alveolar basal carcinoma cells [26]. There are two types of EP bridges; similarly to TNTs, the type I EP bridge facilitates cellular material

transport between remote cells, whereas the type II EP bridge is involved in cell migration [26]. Compared to TNTs, the diameters of EP bridges are greater, ranging from 1 to 20 μm, and EP bridges can extend from 25 μm to over a millimeter in length [26]. An interesting characteristic common to TNTs and EP bridges is that these structures make no contact with the underlying substratum, hovering freely above it. Strikingly, EP bridges contain both F-actin and microtubules, and are structurally stable for longer periods of time, remaining intact for up to 2 days as opposed to the transient, i.e. minutes to several hours, integrity of most TNTs.

Molecular requirements for TNT formation

Cell adhesion molecules and receptor-ligand interaction

At least two distinct pathways in TNT formation have been reported [4]. One is *de novo* protrusion from a given cell, ultimately making contact with a neighboring cell, which results in TNT formation, as seen in PC12 cells and T24 cells [7][14]. In the other pathway, cells first come into contact and then draw out TNTs as they subsequently move apart, which is observed in many types of immune cells [6][9][10][13]. These two

processes are not necessarily mutually exclusive and can even occur at the same time during TNT formation.

In either case, adhesion molecules are required for the initiation of TNT formation and/or stabilization of the formed TNTs. Veranič and colleagues reported that N-cadherin and β-catenin, adherence junction proteins, accumulate in TNTs of urothelial T24 cells [14]. They 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 [14][27]. On the other hands, it has been reported that an initial contact of an adequate duration is generally required for TNT formation in the case of macrophage, NK cells and T cells[4][6][8][9][10]. As described above, TNT formation between NK cells and target cells requires interaction of an NK cell activating receptor and its ligand on the target cell [9].

Membrane lipids composition of TNTs

Iglič and colleagues recently provides an insight into membrane lipids composition of

TNTs. They showed the enrichment of cholesterol-sphingomyelin membrane nanodomain along TNT in T24 cell lines, whereas there were no other signals of membrane nonodomain markers, caveolin-1 and flotillin-1, and very little signals of GM1 ganglioside [28]. Moreover depletion of choresterol from plasma membrane by treatments of methyl- ocyclodextrin led to significant reduction of the density of TNT in T24 cell surface[28]. Their study suggests the role of cholesterol-sphingomyelin membrane nanodomains in the stability of TNTs.

M-Sec, a clue for understanding the molecular mechanisms of TNT formation

The identity of molecules involved in the membrane protrusion of TNTs had until recently not been elucidated. Our recent study has revealed that M-Sec is a central factor for membrane protrusion during TNT formation [29]. M-Sec was first identified as tumor necrosis factor alpha-induced protein 2 (TNFaip2, also called B94), but its function has long been unknown [30]. M-Sec induces membrane protrusions extending out from the plasma membrane, some of which tether onto adjacent cells to form TNT-like structures (Figures 2A) [29].

Although TNT-like structures are observed upon transient expression of M-Sec in HeLa cells, functional TNTs mediating intracellular Ca^{2+} flux only form when M-Sec is stably expressed (Figure 2B) [29]. This observation could be explained if functional TNT formation and/or its maintenance requires the expression of M-Sec in both of the cells connected by TNT. In contrast to the stable M-Sec-expressing HeLa cell clone, in which virtually all the cells should express M-Sec, a substantial portion of the cells are devoid of M-Sec expression in the case of a HeLa cell population transiently transfected with an *M-Sec* transgene. As a result, one of the cells would fail to express M-Sec and so, even though the other cell expressing M-Sec extends TNT-like membrane protrusion to that cell, there would be formation of filopodial bridges instead of complete TNT. Alternatively, sustained expression of M-Sec might be required for M-Sec to collaborate with other participants in TNT formation to constitute functional TNTs. Given the association of a junctional complex with functional TNTs as reported in many types of cells, over-expression of M-Sec alone could induce excess formation of incomplete TNTs that lack other molecules essential for the formation of functional TNTs. This is consistent with our finding that stably expressed M-Sec could induce

functional TNTs in cooperation with the Ras-related small GTPase RalA and the exocyst complex. RalA is involved in controlling actin cytoskeletal remodeling and vesicular transport by interacting with the exocyst complex [31][32]. M-Sec preferentially interacts with the active-form of RalA, although it is unclear at this moment whether this interaction is direct or not. M-Sec likely coordinates with RalA and the exocyst complex to initiate TNT formation and elongation by regulating actin polymerization and membrane vesicle trafficking to the region of cell surface protrusion for the generation of TNTs (Figure 2C) [29]. In addition, Cdc42 also seems to be required for TNT elongation, since short protrusions of membrane tubules accumulate without further elongation in the presence of dominant negative Cdc42 [29]. Involvement of actin remodeling in M-Sec-mediated TNT formation is consistent with a previous observation that TNTs are associated with F-actin. Indeed, M-Sec-induced TNTs are associated with F-actin.

Steady-state expression of M-Sec is essentially restricted to myelomonocytic lineage cells and some epithelial organs in normal tissues [30]. Intriguingly, human T-cell leukemia virus type 1 is known to induce M-Sec expression in T cells [33].

Therefore, it is possible that HIV-1 may also induce M-Sec expression to promote TNT-like structures in T cells, resulting in the enhancement of intercellular HIV-1 transmission. In addition, recent studies showed that treatment of rat hippocampal astrocytes with hydrogen peroxide increases the expression of M-Sec mRNA in a p53-dependent manner, resulting in TNT formation [34].

M-Sec induced only F-actin-containing TNT formation, suggesting that formation of TNTs with microtubules may require as yet unknown factor(s) in combination or apart from M-Sec. In any case, there is no doubt that the discovery of M-Sec as a marker for TNTs and promoter of TNT formation will help clarify the mechanisms of formation of these structures as well as their structural and functional properties.

Outlook for the future

Recent wide-ranging research has revealed that TNT is a common communication device utilized by diverse cells. Moreover, such studies have made the morphological and molecular features of TNTs increasingly clear. Although these findings are mainly

based on studies *in vitro*, they have helped make us aware of the existence of similar structures *in vivo*. For example, osteocytes, which are present sparsely and embedded in the bone matrix, are connected to each other by thin cell protrusions located in the small bony tubes of the canaliculi [35]. The osteocyte protrusions in chick bone are 104 ± 69 nm in the diameter and have an actin filament backbone [35]. Intriguingly, these protrusions have a gap-junction and are thought to transmit chemical and electrical signals [36]. In ovarian follicles, numerous extensions from cumulus cells traverse the zona pellucida and come to interact with the oocyte surface by a gap junction before ovulation [37].

These structures have morphological features and functions similar to TNTs. The relationship of these structures to TNTs will become clear when the molecular components involved in their structure and formation are clarified. The identification of TNT-associated molecules also will make clearer the existence of TNTs in immune cells *in vivo*, and their physiological significance. As discussed, a piece of the answer is now being elucidated. Studies of M-Sec, the RalA-exocyst complex and junctional-associated proteins in TNTs will shed light on the molecular structures of

TNTs and molecular mechanisms of their formation. Especially, M-Sec might be a key player in TNT formation. M-Sec-knockout mice should be a useful model to uncover the physiological role of M-Sec induced TNTs. In addition, the identification of M-Sec-targeted drugs may lead to a new strategy for containment of viral infections such as with HIV-1.

Figure Legends

Figure 1

(A to D) Architecture of TNTs between cultured PC12 cells. Wheat germ agglutinin-stained PC12 cells were analysed by 3D live-cell microscopy. Cells were connected via one (A), several TNTs (B) or branched TNTs with surrounding cells. TNT structures are not attached to the substratum (D). (E) PC12 cells were analyzed with scanning electron microscopy. Higer magnification images are shown (E1 to E3). Scale bars: (A and B), 15 μm; (E), 10 μm; (E1), 200 nm. (Reprinted with permission from Ref [7]. Copyright 2004 AAAS.) (F) Long membrane tethers, or membrane nanotubes, readily form between primary human T-cell labelled with DiO (green) and DiD red. T-cell nanotubes contain a junction revealed by a distinct border between DiO (green) and DiD (red) membrane dyes (Reprinted with permission from Ref [13]. Copyright 2008 Nature Publishing Group). (G) Calcium Flux Propagates along TNT in DC. Each panel shows a pseudocolored intensity image for the calcium signal (see panel 1 for intensity values) on the left and the corresponding differential interference contrast image on the right. The red arrow indicates the position of the microinjection needle used to touch the cell. This series shows DC at high magnification; a relatively large

(approximately 250 nm diameter) TNT can be seen to interconnect cells in the DIC image in panel 1. Each frame (1–4) is separated by 1 s following stimulation in panel 1. The calcium flux can be clearly seen moving along the TNT connecting the cells. Panels 5 and 6 are taken at 10 and 20 s, respectively. At 10 s, significant calcium flux has occurred in all cells. In panel 5, the TNT has a beaded appearance; this is more clearly seen in panel 6 at 20 s. Scale bar equals 10 μm. (Reprinted with permission from Ref [12]. Copyright 2005 Elsevier)

Figure 2

(A) M-Sec-GFP cDNA transfection into HeLa cells can induce TNT-like structures. HeLa cells were transfected with plasmid for expressing GFP-M-Sec (upper panel) or GFP as control (lower panel). After 48 hours from transfection, cells were fixed, and DIC (left panels) images and GFP signals (right panels) were taken with confocal microscopy (FV300, Olympus). (A1) Higher magnification image of HeLa expressing GFP-M-Sec in squared region 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 which do not (dashed line). Scale bar is 20 μm. (B) Physical connection is required for the intracellular propagation of a calcium flux in HeLa cells expressing M-Sec. A culture of HeLa cells stably expressing M-Sec was scratched with a micropipette tip to physically disrupt membrane nanotubes connecting cells. After this treatment, cells were labeled with a calcium indicator and a single cell (arrowhead) on the right side of the scratch was mechanically stimulated. This stimulation evoked calcium flux transmission, which was restricted to cells on the same side of the scratch. One example of representative data from 10 repeated experiments is shown. Scale bar: 20 μm. Reprinted from Nature Cell Biology, Ref [29]. (C) Model of M-Sec induced membrane protrusion in cooperation with RalA and the exocyst complex. M-Sec interacts with RalA GTP binding form. RalA−exocyst effecter complex may regulate transport of secretory vesicles to plasma membrane for supplying membrane components to M-Sec induced membrane nanotubes. M-Sec−RalA interaction may also regulate actin polymerization in coordinating with Cdc42 for protruding and/or stabilizing membrane nanotubes.

References

[1] S. Keller, M.P. Sanderson, A. Stoeck, P. Altevogt, Exosomes: from biogenesis and secretion to biological function, Immunol. Lett. 107 (2006) 102-108.

[2] E. Oviedo-Orta, W. Howard Evans, Gap junctions and connexin-mediated communication in the immune system, Biochim. Biophys. Acta 1662 (2004) 102-112.

[3] T. Saito, T. Yokosuka, Immunological synapse and microclusters: the site for recognition and activation of T cells, Curr. Opin. Immunol. 18 (2006) 305-313.

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

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

[6] S. Sowinski, J.M. Alakoskela, C. Jolly, D.M. Davis, Optimized methods for imaging membrane nanotubes between T cells and trafficking of HIV-1, Methods 53 (2011) 27-33.

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

[8] B. Önfelt, S. Nedvetzki, K. Yanagi, D.M. Davis, Cutting edge: Membrane nanotubes connect immune cells, J. Immunol. 173 (2004) 1511-1513.

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

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

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

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

[13] S. Sowinski, C. Jolly, O. Berninghausen, M.A. Purbhoo, A. Chauveau, K. Kohler, S. Oddos, P. Eissmann, F.M. Brodsky, C. Hopkins, B. Önfelt, Q. Sattentau, D.M. Davis, Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission, Nat. Cell Biol. 10 (2008) 211-219.

[14] M. Lokar, A. Iglič, P. Veranič, Protruding membrane nanotubes: attachment of tubular protrusions to adjacent cells by several anchoring junctions, Protoplasma 246 (2010) 81-87.

[15] Y. Osipchuk, M. Cahalan, Cell-to-cell spread of calcium signals mediated by ATP receptors in mast cells, Nature 359 (1992) 241-244.

[16] J.S. Orange, Formation and function of the lytic NK-cell immunological synapse, Nat. Rev. Immunol. 8 (2008) 713-725.

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

[18] D. Zhu, K.S. Tan, X. Zhang, A.Y. Sun, G.Y. Sun, J.C. Lee, Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes, J. Cell Sci. 118 (2005) 3695-3703.

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

[20] W. Xu, P.A. Santini, J.S. Sullivan, B. He, M. Shan, S.C. Ball, W.B. Dyer, T.J. Ketas, A. Chadburn, L. Cohen-Gould, D.M. Knowles, A. Chiu, R.W. Sanders, K. Chen, A. Cerutti, HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via long-range intercellular conduits, Nat. Immunol. 10 (2009) 1008-1017.

[21] X. Qiao, B. He, A. Chiu, D.M. Knowles, A. Chadburn, A. Cerutti, Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells, Nat. Immunol. 7 (2006) 302-310.

[22] K. Gousset, E. Schiff, C. Langevin, Z. Marijanovic, A. Caputo, D.T. Browman, N. Chenouard, F. de Chaumont, A. Martino, J. Enninga, J.C. Olivo-Marin, D.

Mannel, C. Zurzolo, Prions hijack tunnelling nanotubes for intercellular spread, Nat. Cell Biol. 11 (2009) 328-336.

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

[24] N.M. Sherer, M.J. Lehmann, L.F. Jiménez-Soto, C. Horensavitz, M. Pypaert, W. Mothes, Retroviruses can establish filopodial bridges for efficient cell-to-cell transmission, Nat. Cell Biol. 9 (2007) 310-315.

[25] S.I. Galkina, J.M. Romanova, E.E. Bragina, I.G. Tiganova, V.I. Stadnichuk, N.V. Alekseeva, V.Y. Polyakov, T. Klein, Membrane tubules attach Salmonella Typhimurium to eukaryotic cells and bacteria, FEMS Immunol. Med. Microbiol. 61 (2011) 114-124.

[26] B.G. Zani, L. Indolfi, E.R. Edelman, Tubular bridges for bronchial epithelial cell migration and communication, PloS ONE 5 (2010) e8930.

[27] P. Veranič, M. Lokar, G.J. Schütz, J. Weghuber, S. Wieser, H. Hägerstrand, V. Kralj- Iglič, A. Iglič, Different types of cell-to-cell connections mediated by nanotubular structures, Biophys. J. 95 (2008) 4416-4425.

[28] M. Lokar, D. Kabaso, N. Resnik, K. Sepčić, V. Kralj-Iglič, P. Veranič, R. Zorec, A. Iglič, The role of cholesterol-sphingomyelin membrane nanodomains in the stability of intercellular membrane nanotubes, Int. J. Nanomedicine 2012 (2012) 1891-1902.

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

[30] F.W. Wolf, V. Sarma, M. Seldin, S. Drake, S.J. Suchard, H. Shao, K.S. O'Shea, V.M. Dixit, B94, a primary response gene inducible by tumor necrosis factor-alpha, is expressed in developing hematopoietic tissues and the sperm acrosome, J. Biol. Chem. 269 (1994) 3633-3640.

[31] S. Moskalenko, D.O. Henry, C. Rosse, G. Mirey, J.H. Camonis, M.A. White, The exocyst is a Ral effector complex, Nat. Cell Biol. 4 (2002) 66-72.

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

[33] T. Ruckes, D. Saul, J. Van Snick, O. Hermine, R. Grassmann, Autocrine antiapoptotic stimulation of cultured adult T-cell leukemia cells by overexpression of the chemokine I-309, Blood 98 (2001) 1150-1159.

[34] Y. Wang, J. Cui, X. Sun, Y. Zhang, Tunneling-nanotube development in astrocytes depends on p53 activation, Cell Death Differ.18 (2011) 732-42.

[35] L.D. You, S. Weinbaum, S.C. Cowin, M.B. Schaffler, Ultrastructure of the osteocyte process and its pericellular matrix, Anat. Rec. 278 (2004) 505-513.

[36] B.D. Stephen, Morphological Evidence of Gap Junctions Between Bone Cells, Calcif. Tissue Res. 33 (1981) 509-512.

[37] N.B. Gilula, M.L. Epstein, W.H. Beers, Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex, J. Cell Biol. 78 (1978) 58-75.

[38] D. Wittig, X. Wang, C. Walter, H. H. Gerdes, R. H. Funk, C. Roehlecke, Multi-Level Communication of Human Retinal Pigment Epithelial Cells via Tunneling Nanotubes.,PLoS One 7 (2012) e33195.

[39] K. He, X. Shi, X. Zhang, S. Dang, X. Ma, F. Liu, M. Xu, Z. Lv, D. Han, X. Fang, and Y. Zhang, Long-distance intercellular connectivity between cardiomyocytes and cardiofibroblasts mediated by membrane nanotubes, Cardiovasc. Res. 92 (2011) 39-47.

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

[41] E. Lou, S. Fujisawa, A. Morozov, A. Barlas, Y. Romin, Y. Dogan, S. Gholami, A.L. Moreira, K. Manova-Todorova, M.A. Moore, Tunneling nanotubes provide a unique conduit for intercellular transfer of cellular contents in human malignant pleural mesothelioma, PLoS One 7 (2012) e33093.

[42] I.F. Smith, J. Shuai, I. Parker, Active generation and propagation of Ca2+ signals within tunneling membrane nanotubes, Biophys J. 100 (2011) L37-9.

[43] N. Van Prooyen, H. Gold, V. Andresen,O. Schwartz, K. Jones, F. Ruscetti, S. Lockett, P. Gudla, D. Venzon, G. Franchini, Human T-cell leukemia virus type 1 p8 protein increases cellular conduits and virus transmission, Proc. Natl. Acad. Sci. U S A. 107 (2010) 20738-43.

Figure 1

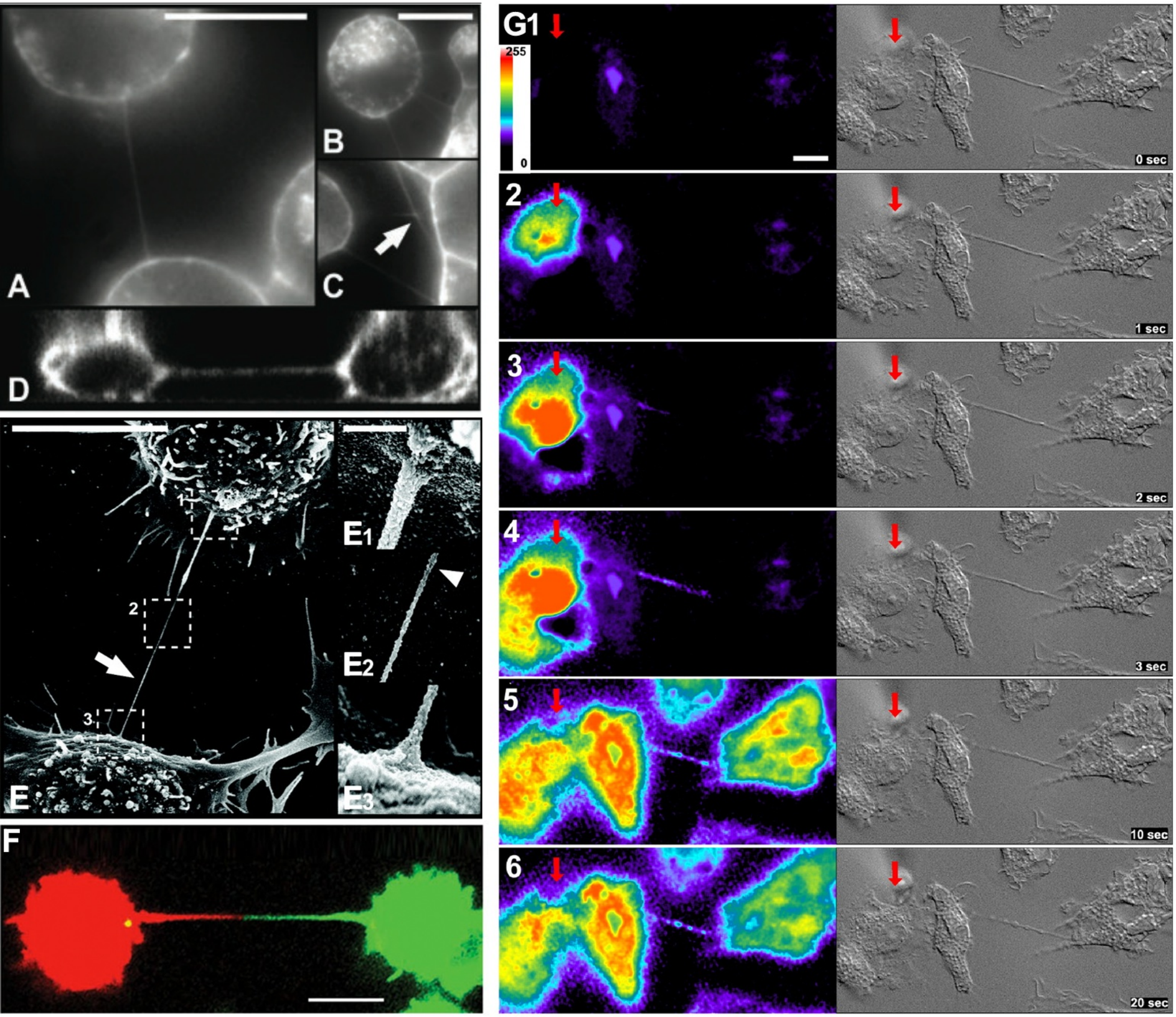
A
B
C
D
E
E1
E2
E3
1
2
3
F
G1
255
0
2
3
4
5
6
0 sec
1 sec
2 sec
3 sec
10 sec
20 sec

Figure 2

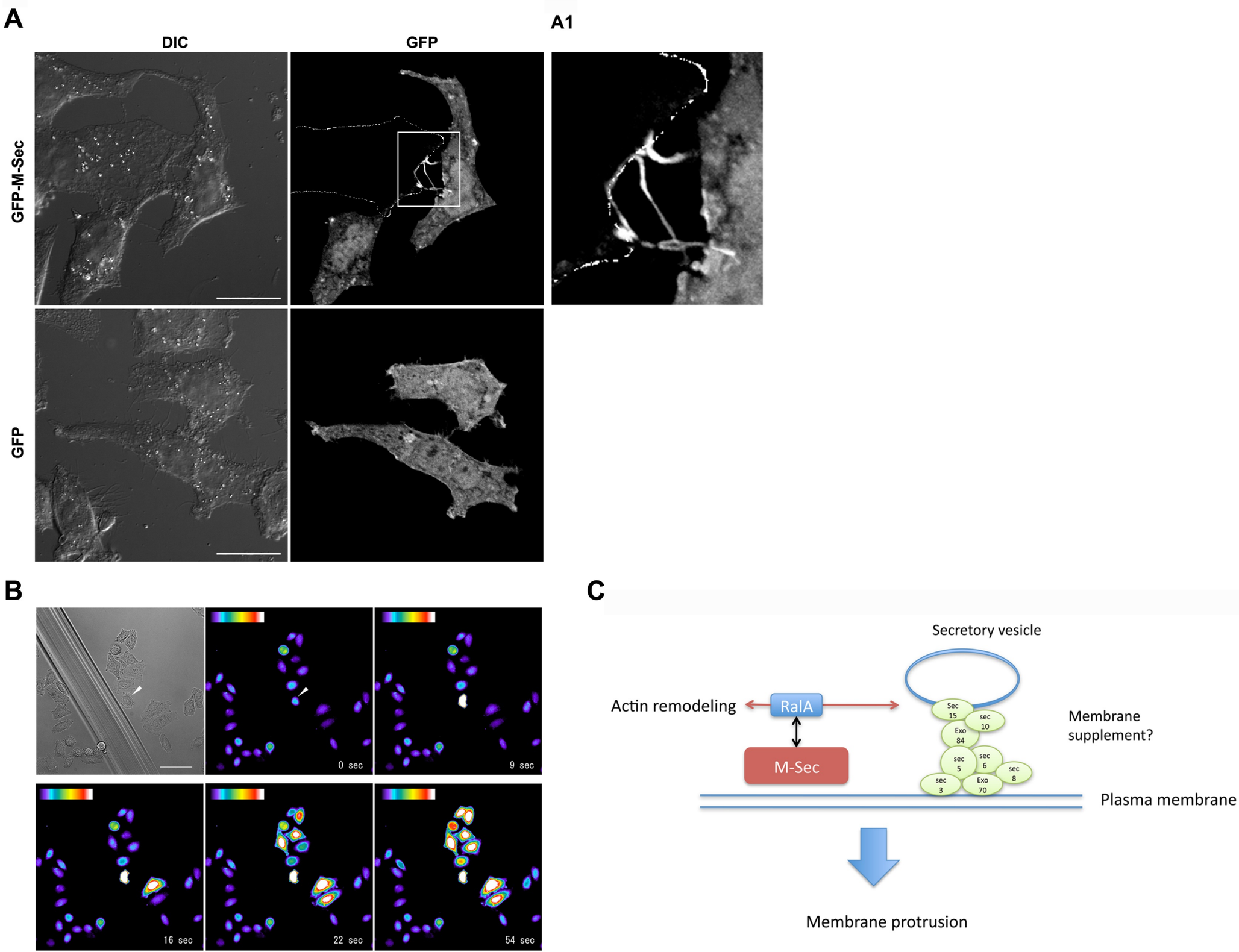
A
A1
DIC
GFP
GFP-M-Sec
GFP
B
0 sec
9 sec
16 sec
22 sec
54 sec
C
Secretory vesicle
Actin remodeling
RalA
M-Sec
Sec 15
sec 10
Exo 84
sec 5
sec 6
sec 8
sec 3
Exo 70
Membrane supplement?
Plasma membrane
Membrane protrusion

Table 1 Proposed functions of TNTs and TNT-like structures

Proposed Functions Cargos	Studied Cells
Transmission of intercellular signaling	
Ca2+ flux	ARPE-19 [38], HEK293[11], THP-1[12], Human dendritic cells[12], Cardiomyocytes and cardiofibroblasts[39], Rat hippocampal astrocytes and neurons[34], Raw264.7[29], HeLa cells stably expressing M-Sec[29]
Electric coupling	ARPE-19[38], HEK293[11], PC12[11], NRK[11], NCC[11], HUVEC[11]
NK cell activating signals	NK cells and its target cells[9]
Fas signaling	T cell[40]
Intercellular transfer of organelle or protein	
Mitochondria	Mesothelioma cell lines[41], Cardiomyocytes and cardiofibroblasts[39], SH-SY5Y[42]
Endosome-related vesicles	PC12[1, 2, 3][7], NRK[1][7][17], T24[1][27], RT4[1][27], J774[1][8]
Membrane anchored proteins	PC12[4][7]
Implications in Disease	
Intercellular virus transmission	
Human Immunodeficiency Virus-1 (HIV-1)	Jurkat T cell[13], Human T cell [13]
Human T-cell Lymphotropic Virus (HTLV)	Jurkat T cell [43]
Murine leukemia virus (MLV)	HEK293[24], XC cell[24], Cos-1[24]
Pathogenic protein transmission	
Pathogenic prion protein PrP^{Sc}	CAD cell[22], CAD cells to bone marrow-derived dendritic cells[22]
HIV-1 Nef protein	HIV-infected macrophage to B cells[20]
Amyloid-β	Rat primary astrocytes and neuron[34]
Other cellular functions	
Cell migration	NK cells to target cells[9], Bronchial epithelial cells[26]
Capture bacteria	Human monocyte derived macrophage (Mycobacterium bovis bacillus Calmette-Guerin (BCG))[10], Human neutrophils (Salmonella enterica serovar Typhimurium)[25]

ARPE-19, human Retinal Pigment Epithelial cell line; CAD cells, mouse neuronal cell line; Cos-1, monkey kidney cell line; HEK293, human embryonic kidney cell line; HUVEC, Normal Human umbilical vein endothelial cells; J774, murine macrophages cell line; Jurkat T cell, Human T cell lymphoblast-like cell line; NCC, neuronal crest cells; NRK, Normal rat kidney cell; PC12, rat pheochromocytoma cell; Raw264.7, mouse monocyte/macrophage cell line; SH-SY5Y, human neuroblastoma cell line; T24 and RT4, human urothelial carcinoma cell lines; THP-1, Human acute monocytic leukemia cell line; XC, Rat sarcoma cell line

[1] membrane vesicles labeled by lipophilic membrane fluorescent dyes such as DiO, DiI, DiD

[2] Acidic organelles labeled by lysotracker

[3] Early endosomes and/or endosome-derived microvesicle marked by GFP-fused synaptophysin.

[4] Membrane lipids labeled with EGFP-fused farnesylation signal of c-Ha-Ras