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Actin-based protrusions at a glance

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ABSTRACT

Actin-based protrusions are at the base of many fundamental cellular processes, such as cell adhesion, migration and intercellular communication. In recent decades, the discovery of new types of actin-based protrusions with unique functions has enriched our comprehension of cellular processes. However, as the repertoire of protrusions continues to expand, the rationale behind the classification of newly identified and previously known structures becomes unclear. Although current nomenclature allows good categorization of protrusions based on their functions, it struggles to distinguish them when it comes to structure, composition or formation mechanisms. In this Cell Science at a Glance article, we discuss the different types of actin-based protrusions, focusing on filopodia, cytonemes and tunneling nanotubes, to help better distinguish and categorize them based on their structural and functional differences and similarities.

KEY WORDS: Cytonemes, Filopodia, Tunneling nanotubes

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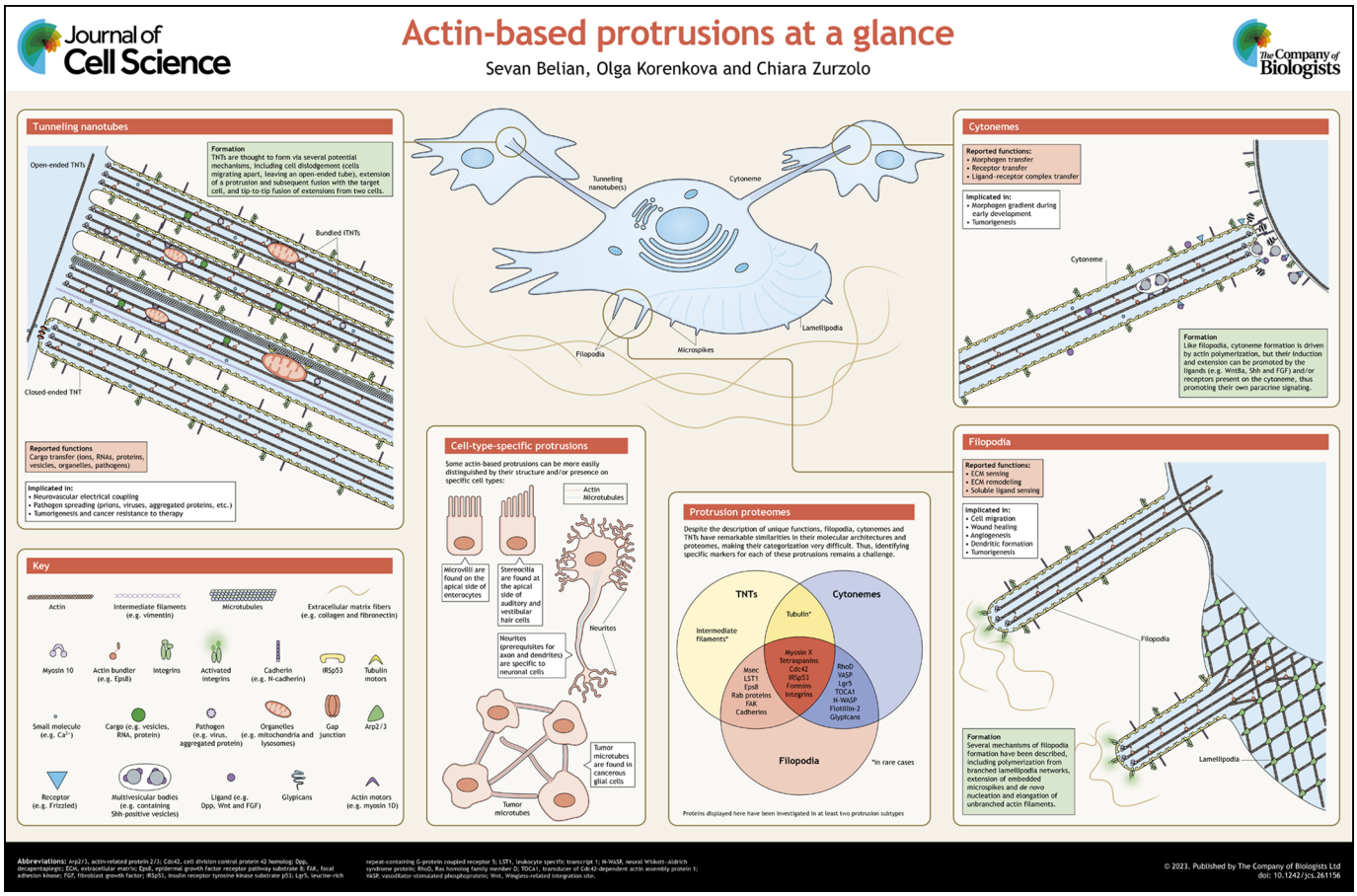
Introduction

Owing to their central role in a wide variety of cellular functions, such as cell migration, environment sensing and cell–cell signaling, actin-based protrusions are of great interest to researchers in many fields. Filopodia were the first thin actin-based protrusion described in the late 19th century by Ramón y Cajal, who observed membrane extensions spanning along the growth cones of commissural neurons in fixed chicken embryos (Cajal, 1890). Filopodia were then later described in 1961 in live sea-urchin larva cells (Gustafson and Wolpert, 1961). Before the development of the current terminology, the generic term filopodia encompassed every thin actin-based protrusion observed.



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Following the discovery of unique structures or functions in different models, new names appeared to categorize them. As such, the term 'cytoneme' was introduced in 1999 (Ramirez-Weber and Kornberg, 1999) to discriminate long specialized filopodia in *Drosophila* wing imaginal disc that mediate cell–cell signaling. Later, the term 'tunneling nanotube' (TNT) was coined in 2004 to describe long intercellular connections observed between PC12 neuronal cells that were open-ended at both ends, enabling cytoplasmic continuity between cells and organelle transfer, unlike any other protrusions known to date (see poster) (Rustom et al., 2004).

Since then, the classification of these three structures has been primarily based on their distinct functions: filopodia participate in probing of the environment and cell migration, cytonemes allow ligand–receptor interactions and TNTs are able to transfer intracellular material between cells (Casas-Tintó and Portela, 2019; Mattila and Lappalainen, 2008; Rustom et al., 2004). However, the field is lacking specific markers that can molecularly discriminate between them. Although it has been shown that certain factors are located in each protrusion type, these are not specific and are often also found in other protrusions (see poster). There is also heterogeneity in the composition of each of these structures, complicating their categorization; for example, the first report of TNTs outlined them as tubulin-devoid structures open-ended at both ends. Nonetheless, further studies have shown that, in other cell types, they can also contain tubulin (Onfelt et al., 2006), or can be closed-ended with gap junctions at their tips (Wang et al., 2010). Similarly, cytonemes were initially described as straight actin-based signaling filopodia in *Drosophila*, but were later observed in vertebrates and have been shown to have more complex curved geometries and contain tubulin in some models (Hall et al., 2021; Sagar et al., 2015; Wood et al., 2021). Moreover, recent reports have shown that cytonemes can not only allow ligand–receptor interactions but also transport ligand-containing vesicles or functional ligand–receptor complexes from donor cell to acceptor cell (Hall et al., 2021; Zhang et al., 2022 preprint), a type of transfer originally thought to be specific to TNTs. Although some actin-based protrusions (such as microvilli, stereocilia, neurites and tumor microtubes) can be identified and distinguished more easily due to unique structures or cell-type specificities (see Box 1), the architectures of filopodia, cytonemes and TNTs appear to be heterogeneous across different model systems. Furthermore, their functions can overlap, making it difficult to categorize them. Thus, with the uncovering of many new functions and types of protrusions, some clarification of their definitions is required. In this Cell Science at a Glance article, we focus on filopodia, cytonemes and TNTs, aiming to describe these three types of protrusions on the basis of the most recent and compelling data about their structure(s) and function(s). We also highlight the molecular machinery involved in their formation and discuss both common and distinct features between them.

Filopodia

Filopodia can be found in the majority of eukaryotic cells and are the most studied actin-based protrusions. They contain 10 to 30 actin filaments cross-linked by actin-bundlers, such as fascin or villin proteins, and rarely exceed 10 µm of length, with a diameter typically ranging from 100 to 300 nm (Jacquemet et al., 2015; Leijnse et al., 2022).

Filopodia exert a sensory function by probing the environment for the detection of soluble factors or sensing extracellular matrix (ECM) composition and rigidity via membrane receptors, such as activated integrins (Albuschies and Vogel, 2013; Heckman and Plummer, 2013; Miihkinen et al., 2021). In response, they can induce remodeling of the microenvironment of the cell through the secretion of proteases near the base of the filopodial shaft or via myosin-driven force generation (Kim et al., 2015; Leijnse et al., 2022). Filopodia also play central roles in adhesion, cell migration, wound healing and dendritic spine formation. They are also upregulated in many cancer types, where they favor metastasis, as well as proliferation and survival (reviewed in Jacquemet et al., 2015; Mattila and Lappalainen, 2008).

Several formation mechanisms have been identified for filopodial protrusions. Firstly, concomitant bundling of the lamellipodial branched actin network by fascin into short embedded structures referred to as microspikes (Svitkina et al., 2003) and clustering of the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family of actin-polymerizing proteins at the barbed end of microspikes. These compete with actin cappers and enable linear actin polymerization (Bear et al., 2002; Damiano-Guercio et al., 2020). Secondly, *de novo* actin nucleation and elongation by formins (Breitsprecher and Goode, 2013; Schirenbeck et al., 2005). Finally, via activation of the Rho GTPase Cdc42, which in turn recruits the IBAR protein IRSp53 (also known as BAIAP2) to the membrane in complex with the actin bundler and capper epidermal growth factor receptor pathway substrate 8 (Eps8), leading to VASP recruitment and processive actin growth (Disanza et al., 2013, 2006). Interestingly, these formation mechanisms seem to co-exist within the cell, with different regulatory mechanisms. Pathways favoring the lamellipodial formation, characteristic of motile cells, promote microspike formation, yet do not prevent filopodia from forming outside of the lamellipodial sheet. These filopodia remain following CRISPR/Cas9-mediated deletion of members of the Ena/VASP family (Evl, VASP and Mena), which, opposingly, abolishes microspike formation (Damiano-Guercio et al., 2020). Of particular interest, isoforms of tropomyosin, which are dimeric fibrous proteins stabilizing F-actin, have recently been suggested to differentially segregate to actin filaments, conferring distinct functionalities by enabling or preventing the attachment of specific myosin motors or actin-severing factors (Gateva et al., 2017; Reindl et al., 2022).

Furthermore, filopodia are characterized by an important heterogeneity of the tip complex, even between protrusions of an individual cell, which has not been associated with functional differences (Jacquemet et al., 2019). Such heterogeneity of the protein enrichments at the filopodial tips was revealed in tracheal cells of *Drosophila* embryos (Dobramysl et al., 2021). In addition, those authors used a cell-free model system producing filopodia-like structures to demonstrate that different pairs of proteins exhibited strong intra-tip complex correlations, such as Ena–VASP with transducer of Cdc42-dependent actin assembly protein (TOCA-1; also known as FNBP1L)–diaphanous 3 (Diaph3) and VASP–Cdc42-GTP with neuronal Wiskott–Aldrich syndrome protein (N-WASP; also known as WASL)–Cdc42-GTP. Overall, they observed a positive correlation between most proteins at the tip, leading them to the proposal of a stochastic model for filopodial formation, where the cooperative assembly of proteins at the tip into heterogeneous complexes can converge into the formation of structurally similar protrusions (Dobramysl et al., 2021).

Cytonemes

Cytonemes were first characterized as thin and long actin-based membranous protrusions in *Drosophila* wing imaginal disc (Ramirez-Weber and Kornberg, 1999). They could be up to 700 µm long with a diameter below 0.2 µm and were suggested to contain actin, but not tubulin. These protrusions were uniformly

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Box 1. Cell-type-specific actin-based protrusions

Although filopodia, cytonemes and TNTs show overlapping morphologies, other actin-based protrusions are more easily identified by their characteristics and/or cell type specificity. For example, neurites are small processes, specific to neuronal cells, that develop into dendrites and axons (Sainath and Gallo, 2015). Unlike dendrites, axons are tubulin positive and can be myelinated, yet both are specialized in conducting action potentials within the neuronal network. Also tubulin-positive are tumor microtubes (TMs), specific to cancerous glial cells, which can reach hundreds of microns in length and persist for hundreds of days (see poster) (Osswald et al., 2015). Two subtypes of TMs have been described – interconnecting TMs that enable Ca^{2+} coupling through gap junctions, and non-connecting TMs promoting cancer cell invasion (Wang et al., 2022).

Microvilli were initially described in the Ascaris intestinal brush border by electron microscopy (Bretschneider, 1954). Intestinal microvilli are found on the apical side of enterocytes and have a very specific morphology, with tight regulation of length and filamentous (F)-actin content, a consistent set of actin cross-linking proteins (fimbrin, villi, and espin family proteins) and cadherin-enriched tip links (Sauvanet et al., 2015). Also found on the apical side of cells, stereocilia are mechanosensory organelles of the auditory and vestibular hair cells, which have specific stair-like morphology and unique actin turnover (see poster) (Lin et al., 2005). Despite unique features of microvilli, description of similar structures in other cell types, such as trophoblast and immune cells (Booth and Vanderpuye, 1983; Polliack et al., 1973), raises the question of how strict the classification should be. For example, placental trophoblast microvilli are more dynamic and variable in length than intestinal microvilli and contain fimbrin and α-actinin but no villin (reviewed in Sauvanet et al., 2015). Likewise, recent reviews suggest categorizing stereocilia as a subtype of microvilli, even though they were initially described as different structures (Sauvanet et al., 2015; Sharkova et al., 2023).

oriented from lateral flanks towards the disc midline, where the morphogen signaling protein Decapentaplegic (Dpp) is expressed, which suggested that cytonemes were formed in response to a chemoattractant and could play a role in morphogen distribution (Ramirez-Weber and Kornberg, 1999).

Indeed, cytonemes were further shown to participate in signal transduction for Dpp and other morphogens, including fibroblast growth factor (FGF), epidermal growth factor (EGF), Wingless (Wg), Hedgehog (Hh) and Delta–Notch pathways in *Drosophila* (Roy and Kornberg, 2015). In vertebrates, this has also been shown for Wnt and its receptor frizzled 7, Sonic hedgehog (Shh) and bone morphogenetic protein 7 (BMP7) (Sagar et al., 2015; Sanders et al., 2013; Schlueter and Mikawa, 2018; Stanganello et al., 2015). During embryogenesis, cytonemes play an important role in tissue patterning by enabling morphogen transfer, and are especially crucial for gradient formation of membrane-bound morphogens, such as Wnt, Shh or Delta–Notch, that cannot be effectively distributed by passive diffusion (Sanders et al., 2013; Stanganello and Scholpp, 2016). Apart from embryogenesis, cytonemes have also been implicated in tumor progression by promoting proliferative signaling, including signaling mediated by Delta–Notch, Dpp and Wnt (Boukhatmi et al., 2020; Fereres et al., 2019; Routledge et al., 2022).

Different mechanisms of cytoneme-based communication have been reported: (1) ligand-bearing cytonemes extending towards receptor-expressing cells; (2) cytonemes expressing receptors and contacting ligand-producing cells; and (3) both producing and receiving cells extending cytonemes that connect tip-to-tip (González-Méndez et al., 2019). Moreover, cells can extend several types of cytonemes, each type segregating different receptors and oriented towards corresponding ligand-producing cells (Roy et al., 2011). This feature can be used to define specific subtypes of cytonemes by the presence of certain ligands and/or receptors, such as Wnt8-positive cytonemes or Wnt3-positive cytonemes (Routledge et al., 2022; Stanganello and Scholpp, 2016).

Ligands can be transferred via cytonemes in membrane receptor-bound forms or associated with vesicles in multivesicular bodies within cytonemes. Myosin X is suggested to be implicated in CD9/CD81-positive vesicle transfer, being the only motor protein shown to play a role in cytoneme function (Hall et al., 2021). In the receiving cells, ligands can induce specific receptor activation or can be transferred to the receiving cell in the complex with the receptor (González-Méndez et al., 2019; Zhang et al., 2022 preprint).

Several mechanisms regulating cytoneme formation have been described. In *Drosophila*, FGF activates two targets: Cut, which responds to low levels of FGF and suppresses protrusion formation, and Pointed, which responds to high levels of FGF and enhances cytoneme formation. Consequently, receiving cells respond to higher levels of FGF by inducing receptor-bearing cytonemes and further signaling, or to lower levels of FGF by suppressing signaling (Du et al., 2018). In mouse mesenchymal cells, FGF was shown to induce cytoneme formation through the activation of the small GTPase RhoD and the formin mDia3C, promoting actin polymerization in an N-WASP-independent manner (Koizumi et al., 2012). In zebrafish, Wnt8a has been shown to induce its own propagation via cytonemes; in producing cells, Wnt8a binds receptor tyrosine kinase like orphan receptor 2 (Ror2) and activates the Wnt/planar cell polarity (PCP) pathway and Cdc42 to induce protrusion formation, whereas in receiving cells it activates the canonical Wnt/β-catenin pathway (Mattes et al., 2018). Vangl2, a member of the PCP pathway and a downstream effector of Ror2, has also been shown to be involved in Wnt8a-containing cytoneme formation in zebrafish (Brunt et al., 2021). Zebrafish Wnt8a-bearing cytonemes also harbor filopodia-inducing proteins such as Myosin X, Cdc42, IRSp53, N-WASP and TOCA1 (see poster) (Stanganello et al., 2015). Similarly, flotillin-2, which is known to promote filopodial formation in various contexts, has been shown to synergistically enhance Wnt cytoneme formation when associated with Ror2 and thus to promote Wnt/PCP signaling (Routledge et al., 2022). In *Drosophila*, cytonemes are affected by the perturbation of expression of actin-binding formins, adhesion molecules, dynamin (Roy et al., 2014), integrins and PCP pathway components (Huang and Kornberg, 2016). Interestingly, Huang and Kornberg suggested an indirect effect of PCP signaling on cytonemes through the extracellular matrix components glypican and laminin (Huang and Kornberg, 2016). Recent studies have confirmed that glypicans, cell membrane-anchored proteoglycans, can regulate cytoneme orientation (Aguirre-Tamaral et al., 2022; Hu et al., 2021). Likewise, the distribution of adhesion protein Interference hedgehog (Ihog) and the glypicans Dally and Dally-like-protein (Dlp) is sufficient to predict Hh cytoneme orientation in the *Drosophila* wing imaginal disc (Aguirre-Tamaral et al., 2022). In HEK cells, the stem cell marker leucine-rich repeat-containing G-protein coupled receptor 5 (Lrp5) can induce cytoneme-like protrusions bearing myosin X, VASP and fascin proteins. Interestingly, Lgr5-induced cytonemes in this system seemed to emerge from underlying lamellipodia, similar to what is seen for canonical filopodia (Snyder et al., 2015). Overall, the vast majority of proteins shown to be involved in the formation of cytonemes have also been shown to play roles in filopodial formation, while some additional regulatory mechanisms were only identified in functional cytonemes (e.g. implication of glypicans in cytoneme orientation).

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However, it is important to note that these mechanisms can only be suggested to be cytoneme-specific before assessing their absence in other protrusion subtypes.

Tunneling nanotubes

TNTs are the latest addition to actin-based protrusions. First described in rat PC12 cells (Rustom et al., 2004), they allow intercellular communication by connecting cells via an open-ended tube, conferring them the unique property of transferring material (ions, RNAs, proteins, vesicles and organelles) via both active and passive transport (Rustom et al., 2004). In 2D culture, they are found to be non-adherent to the substrate. TNTs have a diameter comparable to previously described actin-based protrusions (50–900 nm) but are capable of reaching far longer distances than canonical filopodia, up to several hundreds of micrometers depending on the cell type. Indeed, TNTs have been found in many cell lines (Cordero Cervantes and Zurzolo, 2021) and can also form between cells of different lineages. For example, these structures have been observed between macrophages and breast tumor cells, where they mediate transfer of Dil-stained vesicles and promote tumor invasion in an EGF-dependent pathway (Hanna et al., 2019). TNTs have also been observed between neuronal and glial cells, where they mediate transfer of α-synuclein aggregates (a pathological hallmark of Parkinson's disease) primarily from neurons to microglia, as well as mitochondria from microglia to neurons. These observations hint towards a TNT-mediated protective role of microglia towards neurons in neurodegenerative diseases (Chakraborty et al., 2023).

TNTs have been shown to be upregulated in pro-inflammatory conditions, suggesting that these structures have a role in the maintenance of homeostasis and control of inflammation (Omsland et al., 2017). In cancer cells, they enable resistance to cytotoxic treatments, such as radio and chemotherapy, through mitochondria exchange with the surrounding cells (Burt et al., 2019; Pasquier et al., 2013). TNTs also promote the transfer and spreading of various pathogens (e.g. viruses) or aggregated proteins found in neurodegenerative diseases (Chakraborty et al., 2023; Eugenin et al., 2009; Pepe et al., 2022; Victoria and Zurzolo, 2017). Several subtypes of TNTs have been described; in addition to actin, they can contain tubulin and/or, to a lesser extent, intermediate filaments (IFs) (Resnik et al., 2019). Interestingly, IFs have not been described in filopodia or cytonemes. The different cytoskeletal composition of TNTs appears to be cell type dependent and is thought to regulate their stability and enable different molecular motors to transport cargoes (Onfelt et al., 2006). Importantly, both microtubule-positive and microtubule-negative TNTs have been reported to actively transfer material in different cell types (Onfelt et al., 2006; Rustom et al., 2004; Sartori-Rupp et al., 2019), but so far, only two actin-dependent motor proteins have been reported to be involved in TNT-mediated transfer – myosin X and myosin 1D (Duan et al., 2023; Gousset et al., 2013). Finally, 'closed-ended TNTs' have also been described, presenting gap junctions at their tips, allowing electrical coupling between cells through Ca^{2+} signaling in the mouse retina (Alarcon-Martinez et al., 2020).

As is the case for cytonemes, the structures of TNTs and filopodia are remarkably similar. In fact, one of the challenges of the field is the absence of known specific markers for TNTs. The molecular actors that have been shown to be involved in their formation [Msec (also known as TNFAIP2), LST1, Cdc42, Eps8, Rab35, Myo10, IRSp53, FAK (also known as PTK2), CD9 and CD81; see poster] are known to promote filopodial formation (Dagar et al., 2021; Hanna et al., 2017; Henderson et al., 2022 preprint; Ljubojevic et al., 2021; Notario Manzano et al., 2022 preprint; Sáenz-de-Santa-María et al., 2017). Thus, it remains uncertain whether TNTs differentiate from filopodial protrusions, similar to what has been hypothesized for cytonemes, or whether they are initiated by distinct signaling mechanisms and molecular actors. Cryo-correlative light and electron microscopy (cryo-CLEM) in murine neuronal cells has revealed a highly similar arrangement of F-actin within filopodia and TNTs, suggesting similar scaffolding proteins, but actin filaments in filopodia were found to be more discontinuous when compared to the ones in TNTs (Sartori-Rupp et al., 2019). This could imply that different processive actin polymerizers are involved early during formation of these structures, or that F-actin is differentially protected from actin-severing factors in TNTs compared to filopodia. Another interesting observation is that TNTs seen in confocal microscopy as single protrusions consist of bundles of thinner protrusions (averaging 123 nm in diameter) called individual TNTs (iTNTs) and are linked to each other by N-cadherin, unlike adherent filopodia for which such bundles could not be observed (Sartori-Rupp et al., 2019).

Several mechanisms have been proposed for TNT formation: cell dislodgement, with the separation of two cells from one another enabling the extension of a membrane tube in which actin polymerizes, and protrusion elongation, where a cell extends a long protrusion towards a neighbor cell before fusion occurs. Recently, another mechanism involving tip-to-tip connections has been proposed for the formation of close-ended TNTs, which allows bi-directional ionic transfer between cells, similar to what is observed for some cytonemes (Chang et al., 2022).

Conclusions and challenges

The expanding field of actin-based protrusions faces new limitations we need to overcome with the development of novel approaches. Some of the limitations are technical, such as the fact that cytonemes and TNTs are fragile structures damaged by classical fixation protocols, which has led to the publication of new optimized protocols (Abounit et al., 2015; Hall et al., 2022; Rogers and Scholpp, 2021). The low frequency of transfer via these structures also makes it difficult to reconcile live imaging and quantitative approaches. Other limitations are more theoretical; because of the central role filopodia play in many different cellular processes, their study can be challenging owing to the complexity of pathways regulating their formation, and the broad consequences perturbing their initiation or maturation can have for the cells. Despite the description of unique functional roles, studies on TNTs and cytonemes are facing the same issue due to the high redundancy of actors involved in the formation of these structures and the absence of specific markers to distinguish them (Korenkova et al., 2020).

A third type of limitation is more difficult to address and relates to the expectations of the field. Inherited approaches typically aim to differentiate subtypes of protrusions with the description of specific molecular pathways leading to the formation of defined structures with expected functions. The discovery of unique functions for TNTs and cytonemes started a race to uncover the molecular specificity behind their formation. This hypothesis can certainly not be rejected, especially with the growing pool of evidence suggesting tropomyosin isoforms give different functions and identities to actin filaments that were previously indistinguishable (Gateva et al., 2017; Reindl et al., 2022). However, another model could emerge to tackle this question, in which molecular specificity is not mandatory for functionally distinct protrusions to form. Increasing evidence suggests that different structures can converge into having similar functions, and that local protein enrichment can drastically affect functionality of structures (Dobramysl et al., 2021; Jacquemet et al.,

2019). More generally, we observe that intracellular processes stochastically emerge from a multiplicity of actors with high redundancy, enabling flexibility in the complexes that can form and favoring robustness of the cellular machinery. Growing evidence now hints towards a new vision of subcellular protrusions, where the association of probabilistic events leads to the creation of a wide range of structures with various probabilities of functions. This exciting hypothesis might help us to better understand how the cell regulates the formation of this diversity of protrusions with such a wide variety of functions that continue to be uncovered.

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Competing interests
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High-resolution poster and poster panels
A high-resolution version of the poster and individual poster panels are available for downloading at https://journals.biologists.com/jcs/article-lookup/doi/10.1242/jcs.261156#supplementary-data.

References
Abounit, S., Delage, E. and Zurzolo, C. (2015). Identification and characterization of tunneling nanotubes for intercellular trafficking. *Curr. Protoc. Cell Biol.* **67**, 1-21. doi:10.1002/0471143030.cb1210s67

Aguirre-Tamaral, A., Cambón, M., Poyato, D., Soler, J. and Guerrero, I. (2022). Predictive model for cytoneme guidance in Hedgehog signaling based on Ihog-Glypicans interaction. *Nat. Commun.* **13**, 5647. doi:10.1038/s41467-022-33262-4

Alarcon-Martinez, L., Villafranca-Baughman, D., Quintero, H., Kacerovsky, J. B., Dotigny, F., Murai, K. K., Prat, A., Drapeau, P. and Di Polo, A. (2020). Interpericyte tunnelling nanotubes regulate neurovascular coupling. *Nature* **585**, 91-95. doi:10.1038/s41586-020-2589-x

Albuschies, J. and Vogel, V. (2013). The role of filopodia in the recognition of nanotopographies. *Sci. Rep.* **3**, 1658. doi:10.1038/srep01658

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. doi:10.1016/S0092-8674(02)00731-6

Booth, A. G. and Vanderpuye, O. A. (1983). Structure of human placental microvilli. *Ciba Found Symp.* **95**, 180-194.

Boukhatmi, H., Martins, T., Pillidge, Z., Kamenova, T. and Bray, S. (2020). Notch Mediates Inter-tissue Communication to Promote Tumorigenesis. *Curr. Biol.* **30**, 1809-1820.e4. doi:10.1016/j.cub.2020.02.088

Breitsprecher, D. and Goode, B. L. (2013). Formins at a glance. *J. Cell Sci.* **126**, 1-7. doi:10.1242/jcs.107250

Bretschneider, L. H. (1954). Die submikroskopische Struktur der Darmzelle von Ascaris Suilla: eine elektronenoptische Analyse: I und II: North-Holland.

Brunt, L., Greicius, G., Rogers, S., Evans, B. D., Virshup, D. M., Wedgwood, K. C. A. and Scholpp, S. (2021). Vangl2 promotes the formation of long cytonemes to enable distant Wnt/β-catenin signaling. *Nat. Commun.* **12**, 2058. doi:10.1038/s41467-021-22393-9

Burt, R., Dey, A., Aref, S., Aguiar, M., Akarca, A., Bailey, K., Day, W., Hooper, S., Kirkwood, A., Kirschner, K. et al. (2019). Activated stromal cells transfer mitochondria to rescue acute lymphoblastic leukemia cells from oxidative stress. *Blood* **134**, 1415-1429. doi:10.1182/blood.2019001398

Cajal, S. R. (1890). Notas anatomicas. Sobre la aparición de las expansiones celulares en la médula embrionaria.

Casas-Tintó, S. and Portela, M. (2019). Cytonemes, their formation, regulation, and roles in signaling and communication in tumorigenesis. *Int. J. Mol. Sci.* **20**, 5641. doi:10.3390/ijms20225641

Chakraborty, R., Nonaka, T., Hasegawa, M. and Zurzolo, C. (2023). Tunnelling nanotubes between neuronal and microglial cells allow bi-directional transfer of α-Synuclein and mitochondria. *Cell Death Dis.* **14**, 329. doi:10.1038/s41419-023-05835-8

Chang, M., Lee, O. C., Bu, G., Oh, J., Yunn, N. O., Ryu, S. H., Kwon, H. B., Kolomeisky, A. B., Shim, S. H., Doh, J. et al. (2022). Formation of cellular close-ended tunneling nanotubes through mechanical deformation. *Sci. Adv.* **8**, eabj3995. doi:10.1126/sciadv.abj3995

Cordero Cervantes, D. and Zurzolo, C. (2021). Peering into tunneling nanotubes-The path forward. *EMBO J.* **40**, e105789. doi:10.15252/embj.2020105789

Dagar, S., Pathak, D., Oza, H. V. and Mylavarapu, S. V. S. (2021). Tunneling nanotubes and related structures: molecular mechanisms of formation and function. *Biochem. J.* **478**, 3977-3998. doi:10.1042/BCJ20210077

Damiano-Guercio, J., Kurzawa, L., Mueller, J., Dimchev, G., Schaks, M., Nemethova, M., Pokrant, T., Brühmann, S., Linkner, J., Blanchoin, L. et al. (2020). Loss of Ena/VASP interferes with lamellipodium architecture, motility and integrin-dependent adhesion. *Elife* **9**, e55351. doi:10.7554/eLife.55351

Disanza, A., Bisi, S., Winterhoff, M., Milanesi, F., Ushakov, D. S., Kast, D., Marighetti, P., Romet-Lemonne, G., Muller, H. M., Nickel, W. et al. (2013). CDC42 switches IRSp53 from inhibition of actin growth to elongation by clustering of VASP. *EMBO J.* **32**, 2735-2750. doi:10.1038/emboj.2013.208

Disanza, A., Mantoani, S., Hertzog, M., Gerboth, S., Frittoli, E., Steffen, A., Berhoerster, K., Kreienkamp, H.-J., Milanesi, F., Fiore, P. P. D. et al. (2006). Regulation of cell shape by Cdc42 is mediated by the synergic actin-bundling activity of the Eps8–IRSp53 complex. *Nat. Cell Biol.* **8**, 1337. doi:10.1038/ncb1502

Dobramysl, U., Jarsch, I. K., Inoue, Y., Shimo, H., Richier, B., Gadsby, J. R., Mason, J., Szałapak, A., Ioannou, P. S., Correia, G. P. et al. (2021). Stochastic combinations of actin regulatory proteins are sufficient to drive filopodia formation. *J. Cell Biol.* **220**, e202003052. doi:10.1083/jcb.202003052

Du, L., Sohr, A., Yan, G. and Roy, S. (2018). Feedback regulation of cytoneme-mediated transport shapes a tissue-specific FGF morphogen gradient. *Elife* **7**, e38137. doi:10.7554/eLife.38137

Duan, Q., Zhang, Q., Nie, K., Huang, R., Yang, J., He, P., Tie, Z., Huang, H., Ma, G., Zhang, Y. et al. (2023). Myo1d promotes alpha-synuclein transfer from brain microvascular endothelial cells to pericytes through tunneling nanotubes. *iScience* **26**, 107458. doi:10.1016/j.isci.2023.107458

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. doi:10.1016/j.cellimm.2008.08.005

Fereres, S., Hatori, R., Hatori, M. and Kornberg, T. B. (2019). Cytoneme-mediated signaling essential for tumorigenesis. *PLoS Genet.* **15**, e1008415. doi:10.1371/journal.pgen.1008415

Gateva, G., Kremneva, E., Reindl, T., Kotila, T., Kogan, K., Gressin, L., Gunning, P. W., Manstein, D. J., Michelot, A. and Lappalainen, P. (2017). Tropomyosin isoforms specify functionally distinct actin filament populations In Vitro. *Curr. Biol.* **27**, 705-713. doi:10.1016/j.cub.2017.01.018

González-Méndez, L., Gradilla, A. C. and Guerrero, I. (2019). The cytoneme connection: direct long-distance signal transfer during development. *Development* **146**, dev174607. doi:10.1242/dev.174607

Gousset, K., Marzo, L., Commere, P. H. and Zurzolo, C. (2013). Myo10 is a key regulator of TNT formation in neuronal cells. *J. Cell Sci.* **126**, 4424-4435. doi:10.1242/jcs.129239

Gustafson, T. and Wolpert, L. (1961). Cellular mechanisms in the morphogenesis of the sea urchin larva. The formation of arms. *Exp. Cell Res.* **22**, 509-520. doi:10.1016/0014-4827(61)90127-6

Hall, E. T., Daly, C. A., Zhang, Y., Dillard, M. E. and Ogden, S. K. (2022). Fixation of embryonic mouse tissue for cytoneme analysis. *J. Vis. Exp.* **184**, 10.3791/64100. doi:10.3791/64100

Hall, E. T., Dillard, M. E., Stewart, D. P., Zhang, Y., Wagner, B., Levine, R. M., Pruett-Miller, S. M., Sykes, A., Temirov, J., Cheney, R. E. et al. (2021). Cytoneme delivery of Sonic Hedgehog from ligand-producing cells requires Myosin 10 and a Dispatched-BOC/CDON co-receptor complex. *Elife* **10**, e61432. doi:10.7554/eLife.61432

Hanna, S. J., McCoy-Simandle, K., Leung, E., Genna, A., Condeelis, J. and Cox, D. (2019). Tunneling nanotubes, a novel mode of tumor cell-macrophage communication in tumor cell invasion. *J. Cell Sci.* **132**, jcs223321. doi:10.1242/jcs.223321

Hanna, S. J., McCoy-Simandle, K., Miskolci, V., Guo, P., Cammer, M., Hodgson, L. and Cox, D. (2017). The Role of Rho-GTPases and actin polymerization during Macrophage Tunneling Nanotube Biogenesis. *Sci. Rep.* **7**, 8547. doi:10.1038/s41598-017-08950-7

Heckman, C. A. and Plummer, H. K. (2013). Filopodia as sensors. *Cell. Signal.* **25**, 2298-2311. doi:10.1016/j.cellsig.2013.07.006

Henderson, J. M., Ljubojevic, N., Chaze, T., Castaneda, D., Battistella, A., Gianetto, Q. G., Matondo, M., Descroix, S., Bassereau, P. and Zurzolo, C. (2022). Arp2/3 inhibition switches Eps8's network associations to favour longer actin filament formation necessary for tunneling nanotubes. *bioRxiv*, 2022.08.24.504515. doi:10.1101/2022.08.24.504515

Hu, B., Rodriguez, J. J., Kakkerla Balaraju, A., Gao, Y., Nguyen, N. T., Steen, H., Suhaib, S., Chen, S. and Lin, F. (2021). Glypican 4 mediates Wnt transport between germ layers via signaling filopodia. *J. Cell Biol.* **220**, e202009082. doi:10.1083/jcb.202009082

Huang, H. and Kornberg, T. B. (2016). Cells must express components of the planar cell polarity system and extracellular matrix to support cytonemes. *Elife* **5**, e18979. doi:10.7554/eLife.18979

Jacquemet, G., Hamidi, H. and Ivaska, J. (2015). Filopodia in cell adhesion, 3D migration and cancer cell invasion. *Curr. Opin. Cell Biol.* **36**, 23-31. doi:10.1016/j.ceb.2015.06.007

Jacquemet, G., Stubb, A., Saup, R., Miihkinen, M., Kremneva, E., Hamidi, H. and Ivaska, J. (2019). Filopodome mapping identifies p130Cas as a mechanosensitive regulator of filopodia stability. *Curr. Biol.* **29**, 202-216.e7. doi:10.1016/j.cub.2018.11.053

Kim, M. C., Whisler, J., Silberberg, Y. R., Kamm, R. D. and Asada, H. H. (2015). Cell Invasion Dynamics into a Three Dimensional Extracellular Matrix Fibre Network. *PLoS Comput. Biol.* **11**, e1004535. doi:10.1371/journal.pcbi.1004535

Koizumi, K., Takano, K., Kaneyasu, A., Watanabe-Takano, H., Tokuda, E., Abe, T., Watanabe, N., Takenawa, T. and Endo, T. (2012). RhoD activated by fibroblast growth factor induces cytoneme-like cellular protrusions through mDia3C. *Mol. Biol. Cell* **23**, 4647-4661. doi:10.1091/mbc.e12-04-0315

Korenkova, O., Pepe, A. and Zurzolo, C. (2020). Fine intercellular connections in development: TNTs, cytonemes, or intercellular bridges? *Cell Stress* **4**, 30-43. doi:10.15698/cst2020.02.212

Leijnse, N., Barooji, Y. F., Arastoo, M. R., Sønder, S. L., Verhagen, B., Wullkopf, L., Erler, J. T., Semsey, S., Nylandsted, J., Oddershede, L. B. et al. (2022). Filopodia rotate and coil by actively generating twist in their actin shaft. *Nat. Commun.* **13**, 1636. doi:10.1038/s41467-022-28961-x

Lin, H. W., Schneider, M. E. and Kachar, B. (2005). When size matters: the dynamic regulation of stereocilia lengths. *Curr. Opin. Cell Biol.* **17**, 55-61. doi:10.1016/j.ceb.2004.12.005

Ljubojevic, N., Henderson, J. M. and Zurzolo, C. (2021). The ways of actin: why tunneling nanotubes are unique cell protrusions. *Trends Cell Biol.* **31**, 130-142. doi:10.1016/j.tcb.2020.11.008

Mattes, B., Dang, Y., Greicius, G., Kaufmann, L. T., Prunsche, B., Rosenbauer, J., Stegmaier, J., Mikut, R., Ozbek, S., Nienhaus, G. U. et al. (2018). Wnt/PCP controls spreading of Wnt/beta-catenin signals by cytonemes in vertebrates. *Elife* **7**, e36953. doi:10.7554/eLife.36953

Mattila, P. K. and Lappalainen, P. (2008). Filopodia: molecular architecture and cellular functions. *Nat. Rev. Mol. Cell Biol.* **9**, 446-454. doi:10.1038/nrm2406

Miihkinen, M., Grönloh, M. L. B., Popović, A., Vihinen, H., Jokitalo, E., Goult, B. T., Ivaska, J. and Jacquemet, G. (2021). Myosin-X and talin modulate integrin activity at filopodia tips. *Cell Rep* **36**, 109716. doi:10.1016/j.celrep.2021.109716

Notario Manzano, R., Chaze, T., Rubinstein, E., Matondo, M., Zurzolo, C. and Brou, C. (2022). Proteomic landscape of tunneling nanotubes reveals CD9 and CD81 tetraspanins as key regulators. *bioRxiv* 2022.12.21.521537. doi:10.1101/2022.12.21.521537

Omsland, M., Bruserud, Ø., Gjertsen, B. T. and Andresen, V. (2017). Tunneling nanotube (TNT) formation is downregulated by cytarabine and NF-κB inhibition in acute myeloid leukemia (AML). *Oncotarget* **8**, 7946-7963. doi:10.18632/oncotarget.13853

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. doi:10.4049/jimmunol.177.12.8476

Osswald, M., Jung, E., Sahm, F., Solecki, G., Venkataramani, V., Blaes, J., Weil, S., Horstmann, H., Wiestler, B., Syed, M. et al. (2015). Brain tumour cells interconnect to a functional and resistant network. *Nature* **528**, 93-98. doi:10.1038/nature16071

Pasquier, J., Guerrouahen, B. S., Al Thawadi, H., Ghiabi, P., Maleki, M., Abu-Kaoud, N., Jacob, A., Mirshahi, M., Galas, L., Rafii, S. et al. (2013). Preferential transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance. *J. Transl. Med.* **11**, 94. doi:10.1186/1479-5876-11-94

Pepe, A., Pietropaoli, S., Vos, M., Barba-Spaeth, G. and Zurzolo, C. (2022). Tunneling nanotubes provide a route for SARS-CoV-2 spreading. *Sci. Adv.* **8**, eabo0171. doi:10.1126/sciadv.abo0171

Polliack, A., Lampen, N., Clarkson, B. D., De Harven, E., Bentwich, Z., Siegal, F. P. and Kunkel, H. G. (1973). Identification of human B and T lymphocytes by scanning electron microscopy. *J. Exp. Med.* **138**, 607-624. doi:10.1084/jem.138.3.607

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. doi:10.1016/S0092-8674(00)80771-0

Reindl, T., Giese, S., Greve, J. N., Reinke, P. Y., Chizhov, I., Latham, S. L., Mulvihill, D. P., Taft, M. H. and Manstein, D. J. (2022). Distinct actin-tropomyosin cofilament populations drive the functional diversification of cytoskeletal myosin motor complexes. *iScience* **25**, 104484. doi:10.1016/j.isci.2022.104484

Resnik, N., Erman, A., Veranič, P. and Kreft, M. E. (2019). Triple labelling of actin filaments, intermediate filaments and microtubules for broad application in cell biology: uncovering the cytoskeletal composition in tunneling nanotubes. *Histochem. Cell Biol.* **152**, 311-317. doi:10.1007/s00418-019-01806-3

Rogers, S. and Scholpp, S. (2021). Preserving cytonemes for immunocytochemistry of cultured adherent cells. *Methods Mol. Biol.* **2346**, 183-190. doi:10.1007/7651_2020_305

Routledge, D., Rogers, S., Ono, Y., Brunt, L., Meniel, V., Tornillo, G., Ashktorab, H., Phesse, T. J. and Scholpp, S. (2022). The scaffolding protein flot2 promotes cytoneme-based transport of wnt3 in gastric cancer. *Elife* **11**, e77376. doi:10.7554/eLife.77376

Roy, S., Hsiung, F. and Kornberg, T. B. (2011). Specificity of Drosophila cytonemes for distinct signaling pathways. *Science* **332**, 354-358. doi:10.1126/science.1198949

Roy, S., Huang, H., Liu, S. and Kornberg, T. B. (2014). Cytoneme-mediated contact-dependent transport of the Drosophila decapentaplegic signaling protein. *Science* **343**, 1244624. doi:10.1126/science.1244624

Roy, S. and Kornberg, T. B. (2015). Paracrine signaling mediated at cell-cell contacts. *BioEssays* **37**, 25-33. doi:10.1002/bies.201400122

Rustom, A., Saffrich, R., Markovic, I., Walther, P. and Gerdes, H. H. (2004). Nanotubular highways for intercellular organelle transport. *Science* **303**, 1007-1010. doi:10.1126/science.1093133

Sagar, Pröls, F., Wiegreffe, C. and Scaal, M. (2015). Communication between distant epithelial cells by filopodia-like protrusions during embryonic development. *Development* **142**, 665-671. doi:10.1242/dev.115964

Sainath, R. and Gallo, G. (2015). Cytoskeletal and signaling mechanisms of neurite formation. *Cell Tissue Res.* **359**, 267-278. doi:10.1007/s00441-014-1955-0

Sanders, T. A., Llagostera, E. and Barna, M. (2013). Specialized filopodia direct long-range transport of SHH during vertebrate tissue patterning. *Nature* **497**, 628-632. doi:10.1038/nature12157

Sartori-Rupp, A., Cordero Cervantes, D., Pepe, A., Gousset, K., Delage, E., Corroyer-Dulmont, S., Schmitt, C., Krijnse-Locker, J. and Zurzolo, C. (2019). Correlative cryo-electron microscopy reveals the structure of TNTs in neuronal cells. *Nat. Commun.* **10**, 342. doi:10.1038/s41467-018-08178-7

Sauvanet, C., Wayt, J., Pelaseyed, T. and Bretscher, A. (2015). Structure, regulation, and functional diversity of microvilli on the apical domain of epithelial cells. *Annu. Rev. Cell Dev. Biol.* **31**, 593-621. doi:10.1146/annurev-cellbio-100814-125234

Schirenbeck, A., Bretschneider, T., Arasada, R., Schleicher, M. and Faix, J. (2005). The Diaphanous-related formin dDia2 is required for the formation and maintenance of filopodia. *Nat. Cell Biol.* **7**, 619-625. doi:10.1038/ncb1266

Schlueter, J. and Mikawa, T. (2018). Body cavity development is guided by morphogen transfer between germ layers. *Cell Rep.* **24**, 1456-1463. doi:10.1016/j.celrep.2018.07.015

Sharkova, M., Chow, E., Erickson, T. and Hocking, J. C. (2023). The morphological and functional diversity of apical microvilli. *J. Anat.* **242**, 327-353. doi:10.1111/joa.13781

Snyder, J. C., Rochelle, L. K., Marion, S., Lyerly, H. K., Barak, L. S. and Caron, M. G. (2015). Lgr4 and Lgr5 drive the formation of long actin-rich cytoneme-like membrane protrusions. *J. Cell Sci.* **128**, 1230-1240.

Stanganello, E., Hagemann, A. I., Mattes, B., Sinner, C., Meyen, D., Weber, S., Schug, A., Raz, E. and Scholpp, S. (2015). Filopodia-based Wnt transport during vertebrate tissue patterning. *Nat. Commun.* **6**, 5846. doi:10.1038/ncomms6846

Stanganello, E. and Scholpp, S. (2016). Role of cytonemes in Wnt transport. *J. Cell Sci.* **129**, 665-672.

Svitkina, T. M., Bulanova, E. A., Chaga, O. Y., Vignjevic, D. M., Kojima, S., Vasiliev, J. M. and Borisy, G. G. (2003). Mechanism of filopodia initiation by reorganization of a dendritic network. *J. Cell Biol.* **160**, 409-421. doi:10.1083/jcb.200210174

Sáenz-de-Santa-María, I., Bernardo-Castiñeira, C., Enciso, E., García-Moreno, I., Chiara, J. L., Suarez, C. and Chiara, M. D. (2017). Control of long-distance cell-to-cell communication and autophagosome transfer in squamous cell carcinoma via tunneling nanotubes. *Oncotarget* **8**, 20939-20960. doi:10.18632/oncotarget.15467

Victoria, G. S. and Zurzolo, C. (2017). The spread of prion-like proteins by lysosomes and tunneling nanotubes: Implications for neurodegenerative diseases. *J. Cell Biol.* **216**, 2633-2644. doi:10.1083/jcb.201701047

Wang, X., Liang, J. and Sun, H. (2022). The Network of Tumor Microtubes: An Improperly Reactivated Neural Cell Network With Stemness Feature for Resistance and Recurrence in Gliomas. *Front Oncol.* **12**, 921975. doi:10.3389/fonc.2022.921975

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. doi:10.1073/pnas.1006785107

Wood, B. M., Baena, V., Huang, H., Jorgens, D. M., Terasaki, M. and Kornberg, T. B. (2021). Cytonemes with complex geometries and composition extend into invaginations of target cells. *J. Cell Biol.* **220**, e202101116. doi:10.1083/jcb.202101116

Zhang, C., Brunt, L., Rogers, S. and Scholpp, S. (2022). Cytoneme-mediated transport of active Wnt5b/Ror2 complexes in zebrafish gastrulation. *bioRxiv* 2022.04.07.487468. doi:10.1101/2022.04.07.487468