



# Study of the role of Rab proteins and their effectors on Tunneling nanotubes

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# ***Study of the role of Rab proteins and their effectors on Tunneling nanotubes***

Thèse de doctorat de l'Université Paris-Saclay

préparée à l'Université Paris-Sud

École doctorale n°577 structure et dynamique des systèmes vivants (SDSV)

Spécialité de doctorat: sciences de la vie et de la santé

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**Titre :** Etude du rôle des protéines Rab et de leurs effecteurs sur les Tunneling nanotubes

**Mots clés:** Tunneling nanotubes, Rab8a, Rab11a, Rab35

**Résumé :** Les nanotubes de tunnellation (TNT) sont des structures riches en F-actine qui relient des cellules distantes, permettant ainsi le transport de nombreux composants cellulaires, notamment des vésicules, des organites et différents types de molécules. Les TNT sont impliqués dans des processus cellulaires clés, tels que le développement, l'immunité et la régénération des tissus, mais également dans la transmission de divers agents pathogènes.

Plusieurs facteurs moléculaires ont été identifiés pour participer à la régulation de la formation de TNT. Le complexe de l'exocyste est l'un des premiers facteurs moléculaires impliqués dans la formation de TNT. Ce complexe est également impliqué dans la fixation des vésicules sécrétoires pendant la sécrétion, ce qui suggère que les protéines qui régulent le trafic vésiculaire pourraient jouer un rôle dans la formation de TNT. Nous avons émis l'hypothèse que la formation de TNT est modulée par des protéines qui participent à la fois à la régulation du trafic vésiculaire et au remodelage du cytosquelette d'actine, et que ces deux processus sont essentiels pour la formation de ces structures.

Comme les GTPases de Rab sont les principaux régulateurs du trafic vésiculaire et participent également à la régulation du cytosquelette d'actine, nous avons examiné le rôle de cette famille de protéines dans la formation de TNT. Tout d'abord, nous avons effectué un criblage de plusieurs protéines de Rab différentes pour son effet sur le transfert de vésicule dépendant de TNT. Nous avons constaté que Rab8a, Rab11a et Rab35 ont un effet positif sur le transfert de vésicule. Des études supplémentaires ont démontré que la surexpression de Rab8a et Rab11a augmentait également le nombre de cellules connectées au TNT. Lors de la surexpression de VAMP3 (une autre protéine impliquée dans le trafic vésiculaire), nous avons également observé une augmentation du nombre de cellules connectées au TNT. Une analyse plus poussée a montré que les trois protéines, à savoir Rab11a, Rab8a et VAMP3, ont un effet sur la formation de TNT de manière cascade. Pour établir une relation entre Rab11a et Rab8a, nous avons vérifié le rôle de Rabin8 sur la formation de TNT (une protéine qui interagit avec Rab11 et qui active Rab8) et

nous avons constaté qu'elle n'avait aucun rôle dans la formation de TNT.

De plus, nous avons vérifié une autre protéine dont la fonction est similaire à Rabin8, à savoir GRAB (facteur d'échange de nucléotide de guanine pour Rab3A) et son rôle dans la formation de TNT. Les résultats montrent que la surexpression de GRAB augmente la formation de TNT, mais qu'elle agit de manière indépendante de Rab11 et Rab8a pour réguler la formation de TNT. L'analyse de Rab35, une protéine impliquée dans le recyclage des endocytos, la cytokinèse et la croissance des neurites, a montré que la forme liée au GTP-Rab35 augmente également la formation de TNT. La croissance des neurites est un processus essentiel pour établir la connectivité neuronale et le recyclage des vésicules joue un rôle crucial dans ce processus. Rab35 interagit avec plusieurs protéines impliquées dans le trafic vésiculaire, telles que ACAP2 (agit en tant que GAP de ARF6), MICAL-L1 (molécule interagissant avec CasL-like 1, qui joue un rôle dans le recyclage des vésicules) EHD1 (un ciseau moléculaire) qui joue un rôle dans la scission de la vésicule). Sur les endosomes positifs pour ARF6, Rab35 recrute ACAP2 et MICAL-L1 et forme un complexe qui se lie à EHD1 pour réguler la croissance des neurites. Nos données suggèrent fortement que ces effecteurs pourraient également être impliqués dans la formation de TNT. Individuellement, ACAP2, EHD1 et ARF6-GDP régulent la formation de TNT de manière positive. Mais la surexpression de MICAL-L1 dans les cellules ne montre aucun effet sur les TNT. En outre, des données préliminaires indiquent que Rab35 et EHD1 agissent dans un mécanisme en cascade pour réguler la formation de TNT. Ceci indique que la formation de TNT et la croissance des neurites peuvent agir de manière similaire, mais pas exactement. Les molécules identifiées ici qui jouent un rôle dans la formation de TNT constituent des cibles moléculaires potentielles pour les thérapies visant à bloquer la propagation d'agents pathogènes transférés à travers les TNT.

Cette étude prouve que les protéines jouant un rôle dans le trafic vésiculaire et la croissance des neurites, telles que les protéines de Rab, participent également à la formation de TNT.



**Title:** Study of the role of Rab proteins and their effectors on Tunneling nanotubes

**Keywords:** Tunneling nanotubes, Rab8a, Rab11a, Rab35.

**Abstract:** Tunneling nanotubes (TNTs) are F-actin rich structures that connect distant cells, allowing the transport of many cellular components, including vesicles, organelles, and different kind of molecules. TNTs are implicated in key cellular processes, such as development, immunity, and tissue regeneration, but also in the transmission of various pathogens.

Several molecular factors have been identified to participate in the regulation of TNT formation. One of the early molecular factors that is implicated in TNT formation is the exocyst complex. This complex is also involved in the tethering of secretory vesicles during secretion, which suggest that proteins that regulate vesicle trafficking could have a role in TNT formation. We have hypothesized that the formation of TNTs is modulated by proteins that participate in both, the regulation of vesicle trafficking and the remodelling of the actin cytoskeleton, and that these two processes are key for the formation of these structures.

Since Rab GTPases are the major regulators of vesicle trafficking and also participate in actin cytoskeleton regulation, we examined the role of this protein family in TNT formation. First, we performed a screening of several different Rab proteins for its effect on TNT-dependent vesicle transfer. We found that Rab8a, Rab11a and Rab35 have a positive effect on vesicle transfer. Additional studies demonstrated that Rab8a and Rab11a overexpression also increase the number of TNT connected cells. Upon overexpression of VAMP3 (another protein involved in vesicle trafficking), we also observed an increase in the number of TNT connected cells. Further analysis showed that all three proteins, i.e. Rab11a, Rab8a and VAMP3, show an effect on TNT formation in a cascade dependent manner. To establish a relationship between Rab11a and Rab8a, we checked the role on TNT formation of Rabin8 (a protein that interacts with Rab11a and activates Rab8a)

and we found that it has no role in TNT formation. Additionally, we checked another protein whose function is similar to Rabin8, i.e. GRAB (guanine nucleotide exchange factor for Rab3A) and its role in TNT formation. The results show that GRAB overexpression increases TNT formation, but it acts in a pathway independent of Rab11 and Rab8a to regulate TNT formation. The analysis of Rab35, a protein involved in endocytic recycling, cytokinesis, and neurite outgrowth, showed that the GTP-Rab35 bound form also increases TNT formation. Neurite outgrowth is an essential process in order to establish neural connectivity and vesicle recycling plays a crucial role in this process. Rab35 interacts with several proteins, that are involved in vesicle trafficking such as such as ACAP2 (acts as GAP of ARF6), MICAL-L1 (molecule interacting with CasL-like 1, which plays a role in vesicle recycling) EHD1 (a molecular scissor that has a role in vesicle scission). At the ARF6 positive endosomes, Rab35 recruits ACAP2 and MICAL-L1, and forms a complex that binds to EHD1 to regulate neurite outgrowth. Our data strongly suggest that these effectors may also be involved in the formation of TNTs. Individually, ACAP2, EHD1 and ARF6-GDP regulate TNT formation in a positive manner. But MICAL-L1 overexpression in cells shows no effect on TNTs. Also, preliminary data, indicates that Rab35 and EHD1 acts in a cascade mechanism to regulate TNT formation. This indicates that TNT formation and neurite outgrowth may act in a similar, but not exact pathway. The molecules identified here that have a role in TNT formation, constitute potential molecular targets for therapies aiming to block the spreading of pathogens that transfer through TNTs.

This study proves that proteins that have a role in vesicle trafficking and neurite outgrowth, such as Rab proteins, are also involved in TNT formation.



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*To,*

*Amma, Papa and Ashachikki*

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# ***Section1***

## **Introduction**



**Preamble:** The introduction is divided in three chapters. Chapter 1 will introduce Tunnelling nanotubes and summarize the current findings on their regulation and role in intercellular communication. Chapter 2 will give an overview on vesicle trafficking pathways and on the role of Rab proteins, as the focus of my thesis has been the investigation of the role of Rab proteins in the formation of TNTs. Finally, in Chapter 3, I will discuss more in depth the Rabs and downstream pathways that I have found, during my PhD thesis work, involved in TNT regulation.

## **Chapter 1: Introduction to TNTs.**

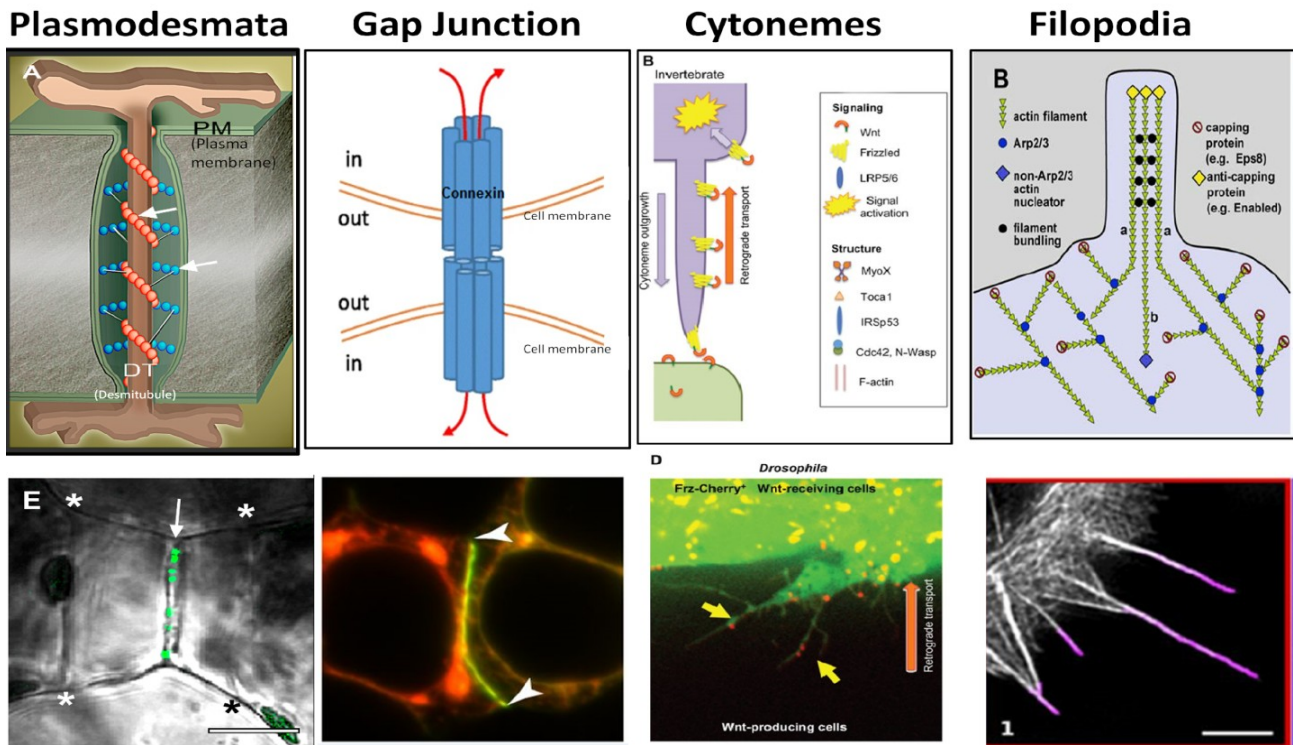
### **1) Overview of TNTs:**

#### **a) Intercellular connections and introduction to TNTs:**

Cell-cell communication is important for development and function of multicellular organisms. One of the many mechanisms for cell communication is via cell-cell interactions through different mechanisms between adjacent or distant cells. Intercellular connecting structures have been shown in a wide variety of living organisms like plasmodesmata in plants, cytonemes in *Drosophila* and gap junctions in mammals. These structures allow transport of molecules, ions, metabolites, and hormones.

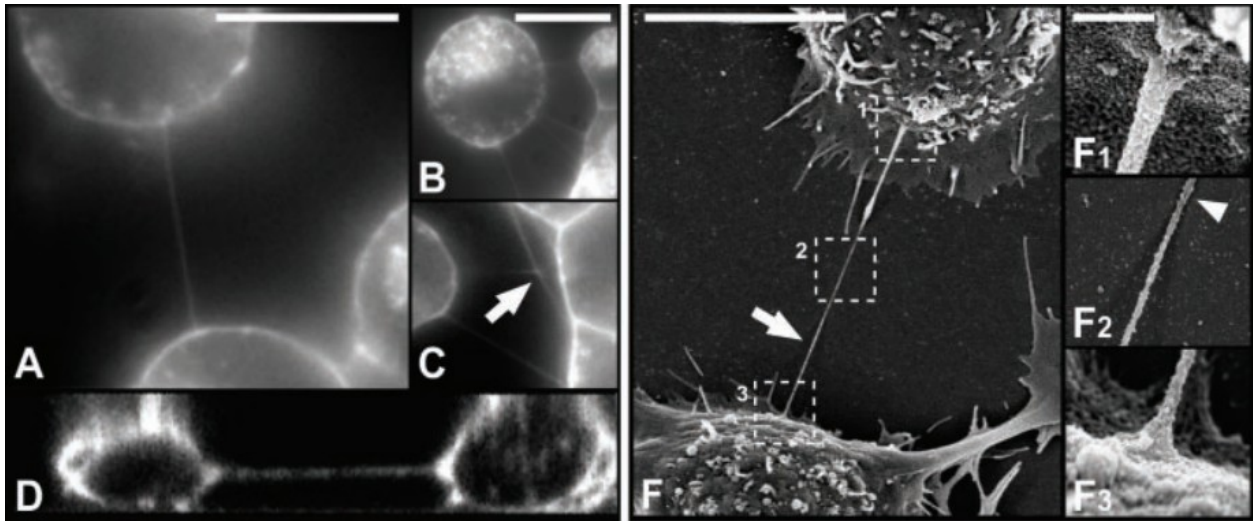
Plasmodesmata are cylindrical channels about 40 nm. in diameter, which are connected to the endoplasmic reticulum (ER) of the adjacent cells through the desmitubule (Tilney et. al., 1991). They can transport ribonucleoprotein complexes, which dictate whole organism co-ordination for development or can be used by viruses to spread infection. Gap junctions are made of integral membrane protein assemblies, primarily of connexins. They have molecular cutoff values of around 1kDa. They allow the passage of different molecules, ions, and electrical impulses, therefore are involved in calcium ( $\text{Ca}^{+2}$ ) signalling between neural cells, healthy functioning of the heart etc. Cytonemes and filopodial bridges are membranous protrusions rich in filamentous actin (F-actin) shown to connect neighbouring cells with the help of adhesion and ligand receptor interaction between the two cells. These structures are observed during development and generally transport morphogens which regulate development of the organism (Figure 1).

(Lucas et. al., 2001, Ramirez-Weber et. al., 1999, Maeda and Tsukihara, 2011, Sherer and Mothes, 2008, Roy et. al., 2011, Goodenough et. al., 1996)



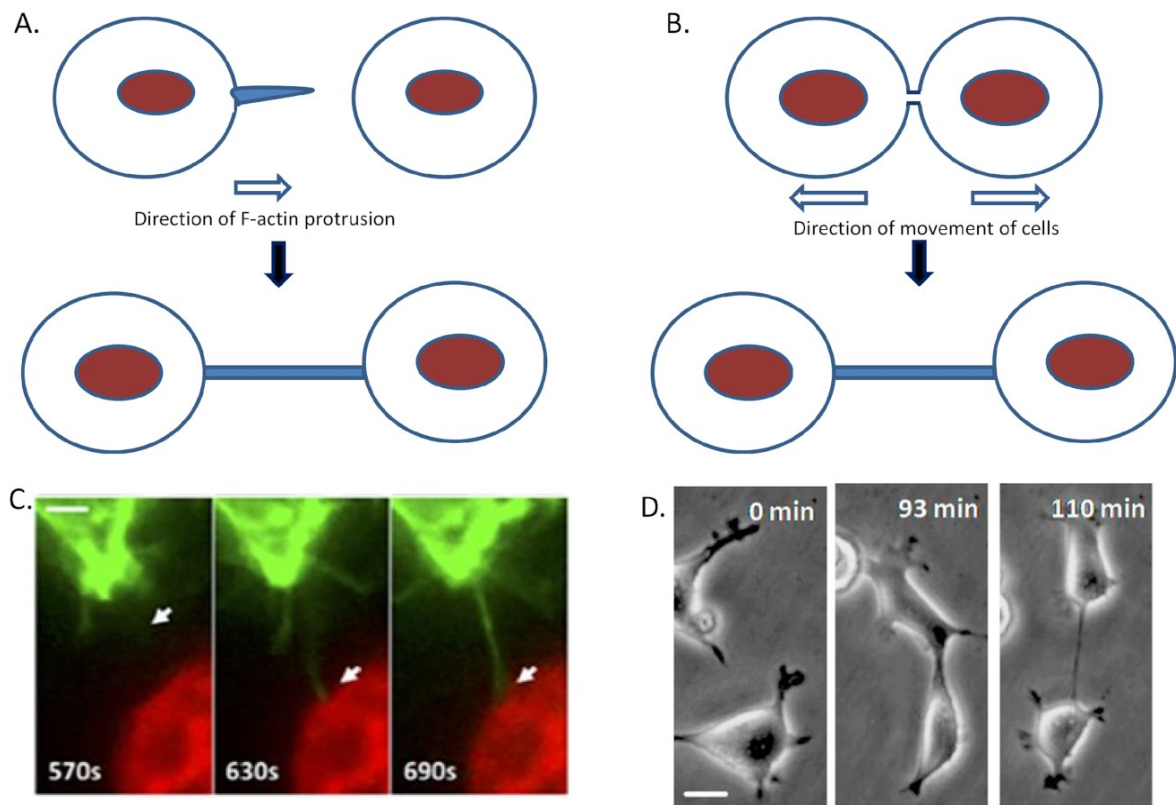
**Figure 1:** Structures involved in cell to cell interaction: **Plasmodesmata:** Top panel- cartoon of a plasmodesmata seen in PM (plasma membrane) lined channel through the cell wall containing central ER-derived rod shaped desmitubule. Proteinaceous components in the channel represented by arrows. Bottom panel- Punctate distribution of plasmodesmata protein (green) along the cell wall in *Arabidopsis*. **Gap junction:** Top panel: Cartoon showing the docking of two hemichannels from two cells forming a gap junction. Bottom panel: Cell labeled for Connexin 43 (green) and TMR (Tetramethyl rhodamine) label (red). **Cytonemes:** Top panel: Schematic showing cytonema extending from one cell to another. Bottom panel: Frizzled-cherry expressing myoblasts (Marked with CD86) sending out cytonemes in *Drosophila*. **Filopodia:** Top panel: Schematic diagram of filopodia with actin. Bottom panel: Filopodia (highlighted with magenta) and cell stained for actin (gray) along the cell edge in DCIS.COM (Ductal carcinoma in situ) cells (Thomas et. al., 2008, Wu and Wang, 2019, Wang et. al., 2015, Stanganello and Scholpp, 2016, E Lundquist, 2009, Jacquemet et. al., 2017).

A novel form of cell-cell connecting structures were discovered by Rustom et. al., in 2004 in the rat PC12 (pheochromocytoma) cell line and named Tunneling nanotubes (TNTs) (Figure 2). Tunneling nanotubes are primarily F-actin based intercellular membrane protrusions that transport cytoplasmic contents, cargoes, and organelles. They were originally described as cell to cell connecting bridges made of continuous membranous connections, that are not adherent to the substrate. Their diameter ranges from 50-150 nm. and their length can extend up to hundreds of micrometers (Rustom et. al., 2004, Sartori-Rupp et. al., 2019). They are extremely fragile in nature, sensitive to light, chemical and mechanical fixation. Since their discovery TNTs have been described under several different names as “T membrane nanotubes”, “intercellular’ or ‘epithelial’ bridges” or “cytoplasmic extensions” (Figure 2) (Lou et. al., 2012, Arkwright et. al., 2010, Abounit et. al., 2015), or the closed membranous tubes are named as “nanotubes”. While all these connections should be called TNT-like connections, only the open-ended tubes are real “tunneling nanotubes” (Sartori-Rupp et. al., 2019, Lou et. al., 2012, Arkwright et. al., 2010, Abounit et. al., 2015, Korenkova et. al., 2019, submitted).



**Figure 2:** Overview of TNTs in PC12 cells: A to D) TNTs between cultured PC12 cells. Cells are connected via one (A) or several TNTs (B) with surrounding cells. Rarely, branched TNTs were observed [(C), arrow]. In (D) a selected (x-z) section obtained from a confocal 3D reconstruction of TNT. Ultrastructure of TNTs. PC12 cells analyzed by SEM (Scanning Electron Microscope) (F) from the boxed areas higher magnification images are shown Open arrowhead show secretory granule. Scale bars: (A to E), 15 µm; (F), 10 µm; F<sub>1</sub> to F<sub>3</sub>, 200 nm (Rustom et. al., 2004).

TNTs may be formed by two different mechanisms; a) By the extension of cellular protrusion like actin dependent filopodia, to form an initial connection which results in TNT formation (Rustom et. al., 2004, Yang et. al., 2016). b) Or they can form by cell to cell contact and then dislodgement of the cells which results in a functional TNT connection between the cells (Figure 3) (Onfelt et. al., 2004, Reichert et. al., 2016, Pedicini et. al., 2018).



Formation of functional TNT between two cells

**Figure 3:** Cellular mechanism of TNT formation: A) One cell forms an F-actin driven protrusion (Dark Blue) to another cell which fuses and forms a functional TNT. B) Two cells come in contact with each other and then move away which eventually gives rise to a functional TNT. C) RAW/LR5 macrophages transfected with GFP-CAAX and co-cultured with cells transfected with mcherry-CAAX forming TNT like protrusion. D) Cells forming TNT like connections by cell dislodgement mechanism in MCF7 cells subsequent to cell division (Shaarvari Bhat, Hanna et. al., 2017b, Patheja and Sahu, 2017).



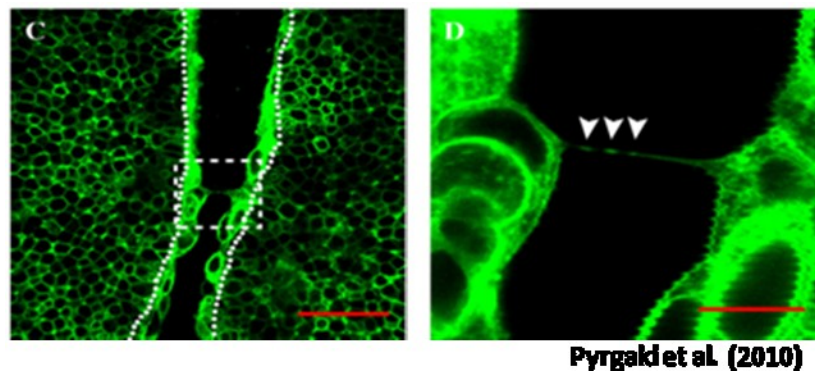
The cytoskeletal composition of TNTs described is slightly varied with a vast majority containing F-actin, but a subpopulation also contained microtubules. For example, in laryngeal carcinoma cells TNTs are made up of both actin and alpha-tubulin. (Antanaviciute et. al., 2014). In astrocytes TNTs were mostly microtubule positive (Wang et. al., 2012).

Treating cells with drugs affecting cytoskeleton showed an effect on TNTs. F-actin affecting agents such as cytochalasin D, latrunculin A (disrupts F-actin) or tolytoxin (a cyanobacterial nucleoside which affects F-actin polymerization), show reduction in TNTs in AML (Acute Myeloid Leukemia) cells, neurons, astrocytes, PC12, bone marrow derived mesenchymal stem cells (MSCs), SW13 (epithelial cell line) and SH-SY5Y cells (human neuronal cell line). But the microtubule affecting agents as nocodazole (which disrupts the microtubule), and paclitaxel (microtubule stabilizing agent) had no effect on TNTs in RAW 264.7 (mouse macrophage cell line), PC12, PC3 (human prostate cell line) cells (Marlein et. al., 2017, Wang et. al., 2011, Rustom et. al., 2004, Han et. al., 2016, Dilsizoglu Senol et. al., 2019, Zhang et. al., 2018, Hanna et. al., 2017a, Kretschmer et. al., 2019). Detailed explanation of cytoskeleton composition in TNTs is shown in Table 1.

The differences in the molecular and cytoskeletal composition of TNTs might indicate different structures (see below), therefore we proposed to define the different protrusions identified in vitro as “TNT-like structures” unless they fulfill four specific criteria (see below). In the last 10 years TNT-like structures have been found in multiple cell types including human peripheral blood NK (natural killer) cells, macrophages, and EBV (Epstein Barr Virus) -transformed B cells and murine macrophage J774 cells, human embryonic kidney (HEK) or normal rat kidney (NRK) cells, MSCs, dendritic cells and monocytes, prostate cancer cells (Yin et. al., 2017, Rustom et. al., 2004, Watkins et. al., 2005, Onfelt et. al., 2004, Kretschmer et. al., 2019). TNTs can be formed between heterocellular cocultures, as MCF7 and MDA cell lines derived from breast cancers, and SKOV3 and OVCAR3 cells derived from ovarian cancers, and non-malignant ECs (Endothelial cells) and MSCs, cancer cells and MSCs or ECs, prostate cancer cells and osteoblasts (Kretschmer et. al., 2019, Pasquier et. al., 2013). Although in some cases, TNTs are formed only in the homogenous population e.g. malignant mesothelioma cells or just normal mesothelioma cells but not between heterogeneous population consisting cocultures between malignant mesothelioma cells and the normal mesothelioma cells (Lou et. al., 2012). Detailed explanation of TNT connected cells is given in Table 1.

b) Molecules positive for TNT markers: Unfortunately, there are no molecular markers identified yet that are specific for TNTs. However, TNTs have shown to be positive for several proteins like transmembrane protein CD59, CD81 and CD86. TNTs are also shown to be positive for H-Ras protein (Transforming protein P21), and lectin (Arkwright et. al., 2010, Rainy et. al. 2013) as well as other molecules as MyosinX (MyoX), M-sec (also known as TNFaip2 (tumour necrosis factor- $\alpha$ -induced protein 2)) etc. (Gousset et. al., 2013, Ohno et. al., 2010) that were not exclusive markers. On the other hand, recent papers are looking at methods to identify by proteomics the specific TNT composition (Gousset et. al., 2019). Indeed, only by specific markers we will be able to identify TNTs both *in vitro* and most importantly *in vivo*.

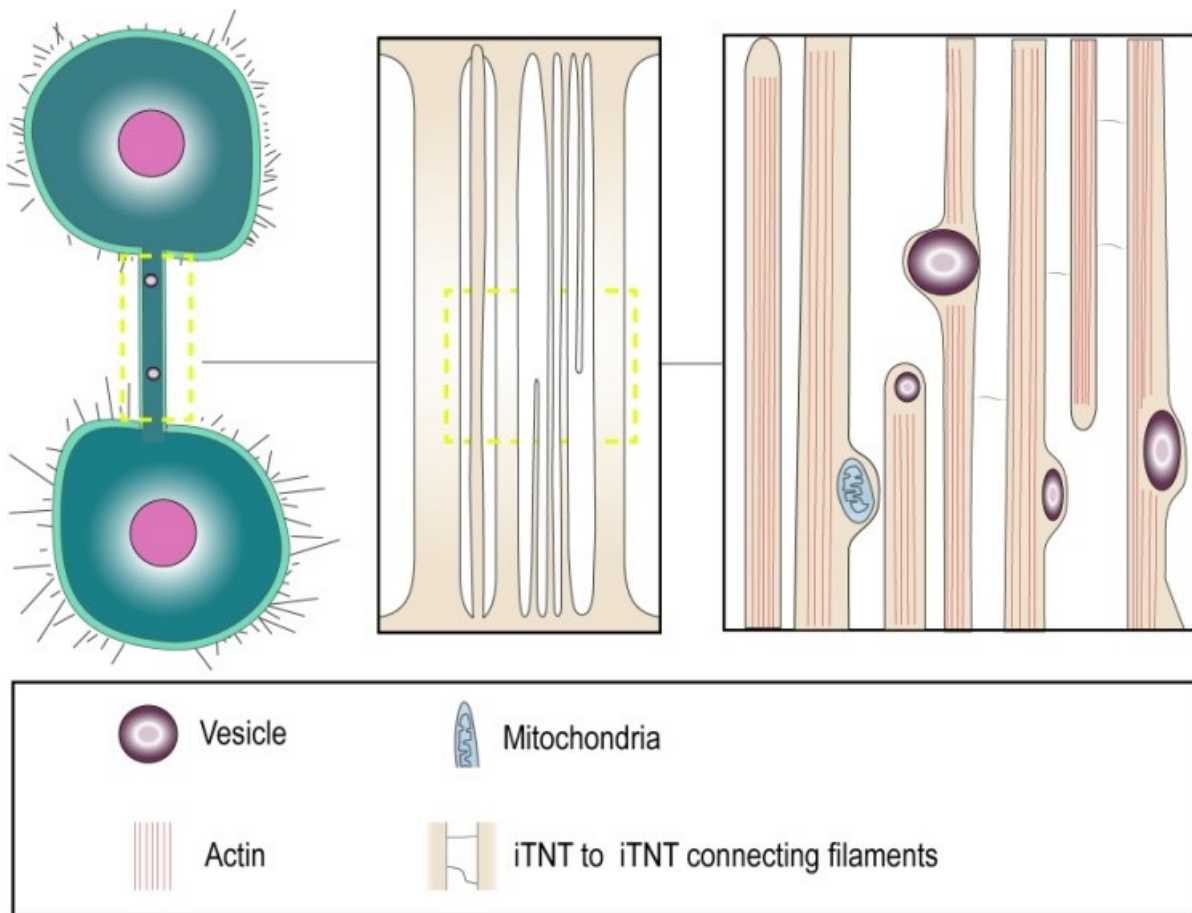
c) Tunneling nanotubes *in vivo*: Although major studies involving TNTs have been conducted *in vitro*, because of the lack of specific markers the identification of TNT *in vivo* is difficult, however, there have been several evidences of TNT like connections observed *in vivo* or under 3-D organoid culture conditions mainly based on their morphological aspect (Figure 4). During development, TNT like structures were shown in chick and sea urchin embryos (Miller et. al., 1995, Teddy et. al., 2004). Tunneling nanotubes were also formed between mesenchymal stromal cells and alveolar macrophages, *in vivo*, in mice (Jackson et. al., 2016). TNTs are observed between tumor cells derived from squamous cell carcinomas (SCC) cultured under 2-D and 3-D conditions and also in SCC tissues (Sáenz-de-Santa-María et. al., 2017). Actin based functional TNTs that transfer intracellular material are observed in spheroids made from human MSCs (Zhang et. al., 2018). In studies by Naphade et. al., in 2015 lysosomal defects have been corrected by the transport of vesicles from the diseased cells to the healthy cells by TNT like structures *in vivo*. This is done by transplantation of hematopoietic stem cells (HSCs) in the cystinotic kidneys. Detailed explanation is given in Table 2.



**Figure 4: Long flexible connections invivo:** Invivo description of long flexible extensions found in mouse neural crest cells. a) Scale bar 50um, b) Scale bar 10um (Cells are labeled with Venus Fluorescence reporter) (Pyrgaki et. al., 2010)

d) Structural organization of TNT: Since the majority of studies on TNTs have employed low-resolution tools (i.e. light /confocal microscopy) until date it has been very difficult to distinguish them from other membranous protrusions (e.g., filopodia). Recent data from our lab, using correlative Electron Microscopy show that TNTs are structurally different than other actin based cellular protrusions like filopodia. Specifically, by using for the first time correlative light- and cryo-electron microscopy approaches and Focused-Ion Beam Scanning Electron Microscopy (FIB-SEM), our lab has demonstrated that in neuronal cells TNTs are specific structures with a distinct ultrastructure, different from other cellular protrusions. They are comprised of a bundle of open-ended “individual Tunneling Nanotubes” (iTNTs), held together by N-cadherin based connections. Each iTNT is surrounded by a plasma membrane and contains highly organized parallel actin bundles that allow the intercellular transfer of membrane-bound compartments and organelles like mitochondria (Sartori-Rupp et. al., 2019) as shown in Figure 5.

Following this study, we proposed to call “TNT” only the membrane protrusion that fulfill the following criteria: 1. that connect distant cells, 2. that are open-ended; 3. that contain actin fibers and 4. that allows the transfer of cargoes. However, for simplification, I will continue to use the term TNTs while describing the current literature, as none of the current studies have looked at all the four criteria when studying TNTs.



**Figure 5:** Schematic diagram showing structure of TNTs. The TNTs that are connecting cells can either be single thick connections or a bundle of thin individual TNTs (iTNTs). iTNTs contain vesicles and mitochondria. Membranous compartments within iTNTs appear to be connected by thin filaments to actin on one side and to the inner side of the plasma membrane on the other. Thin membrane threads coil between and around several iTNTs. (Sartori-Rupp et. al., 2019).

Table 1 : Overview of TNTs.

Cell type	Cytoskeleton	Cargo	Ref.
PC12	F-actin, myosinVa	DID-labeled organelles, Membrane components (c-HA-Ras), Endosome/lysosome related organelles, Lipid anchored proteins (EGFP-f), EGFP-actin	(Bukoreshtliev et al., 2009, Rustom et al., 2004)
HEK293T	F-actin	Calcium signaling (IP3R, Electrical coupling through gap junction at the TNT end); Fluorescent prion protein construct (GFP-PrPwt)	(Smith et al., 2011)
RT4	F-actin	NF (Not found)	(Kabaso et al., 2011)
T24	F-actin	NF	(Kabaso et al., 2011)
MSTO-211H	F-actin	Vesicles, Proteins, Mitochondria	(Lou et al., 2012a, Lou et al., 2012b)
HUVEC	F-actin	Electrical signals	(Wang et al., 2010)
HeLa	F-actin	Ca <sup>2+</sup> , Vesicles	(Hase et al., 2009, Schiller et al., 2013)
Jurkat and primary T cells	F-actin	HIV protein Gag, Mitochondria, Membrane components (CD81, CD59, GPI-anchored GFP, TM-proteins (ICAM-I, HLA-Cw7), Cytosolic stain CFSE, Death signals (Fas ligand, caspase-3), Virus (GFP-Gag)	(Arkwright et al., 2010, Schiller et al., 2013)
ARPE-19	F-actin	Mitochondria, Ca <sup>2+</sup> , Small molecules such as Lucifer Yellow	(Wittig et al., 2012)
Normal rat kidney cells (NRK)	F-actin, Myosin Va	Endosome-related organelles, Depolarization signals	(Rustom et al., 2004, Wang et al., 2010)
RPTEC	F-actin	Endosomes, Lysosomes	(Domhan et al., 2011)
Cardiac myoblast H9c2 cell	F-actin, Microtubules	Mitochondria, Nanoparticles quantum dots (CdSe/ZnS)	(He et al., 2010)
Human lung carcinoma A549	F-actin, Microtubules	Wheat germ agglutinin	(Wang et al., 2012b)
Mouse Cath. a-differentiated (CAD) neuronal cells	F-actin	Exogenous and endogenous membrane GFP-PrP, Proteinaceous aggregates (PrPSc)	(Gousset et al., 2009)
MDM	F-actin, Microtubules	Golgi and Endoplasmic reticulum, Mitochondria, Endosome/lysosome related organelles, Lysosomes, Membrane components (MHC-I), Bacteria (Mycobacterium bovis BCG), HIV Env and Gag proteins	(Kadiu and Gendelman, 2011a, Kadiu and Gendelman, 2011b, Onfelt et al., 2006)
Raw264.7 macrophages	F-actin	Ca <sup>2+</sup>	(Hase et al., 2009)
Myeloid (dendritic) cells	ND	Ca <sup>2+</sup> , Surface receptors (HLA-A, B, C class I MHC), Small molecules such as Lucifer Yellow	(Watkins and Salter, 2005)



THP-1 monocytes	F-actin	Calcium fluxes (Ca <sup>2+</sup> , Fura-2), Lucifer yellow, Surface receptors (HLA-A, B, C class I MHC)	(Watkins and Salter, 2005)
NK cells	F-actin, Microtubules	Membrane components (MHC-I), Death signals	(Chauveau et al., 2010)
Bacteria	ND	Proteins, Genetic materials	(Dubey and Ben-Yehuda, 2011)

Table 2: TNTs between different cell types

Cell type	Cytoskeleton	Cargo	Ref.
HEK293T and COS-7 in co-culture	NF	Membrane receptors GPCRs	(Guescini et al., 2012)
EPC and HUVEC in co-culture	F-actin	Lysosomes	(Yasuda et al., 2011)
EBV-transformed human B cell line (721.221) and human peripheral blood NK cells in co-culture	NF	GPI-GFP (HLA-Cw6)	(Onfelt et al., 2004)
MMSC and RTC in co-culture	NF	Mitochondria, Cytoplasmic components (Calcein)	(Plotnikov et al., 2010)
Primary neurons and astrocytes in co-culture	F-actin, Microtubules	Depolarization signals	(Wang et al., 2012a, Victoria et al., 2016)
Dendritic cells and cerebellar granule neurons	NF	PrPSc	(Langevin et al., 2010)
Dendritic cells and THP-1 monocytes in co-culture	NF	Calcium fluxes (Ca <sup>2+</sup> , Fura-2)	(Watkins and Salter, 2005)
CMs and FBs in co-culture	F-actin, Microtubules	Mitochondria, Endosomal vesicles, Ca <sup>2+</sup> , Cytosolic GFP	(He et al., 2011)
HSCs and macrophages in co-culture	NF	Vesicular exchange (endosome/lysosome system)	(Naphade et al., 2015)
MSCs and CMs in co-culture	NF	Mitochondria	(Cselenyak et al., 2010)
Vascular smooth muscle cells and MSCs in co-culture	NF	Mitochondria	(Vallabhaneni et al., 2012)
EPC and CMs in co-culture	NF	Mitochondria, Soluble proteins (GFP)	(Koyanagi et al., 2005)
Human MSCs and adult CMs in co-culture	F-actin, Microtubules	Mitochondria	(Acquistapace et al., 2011)
DaMSCs and MEF in co-culture	F-actin, Microtubules	4-Oct	(Rolf et al., 2012)

## 2) Cellular mechanisms involved in the formation of TNTs:

### a) Role of Stress and inflammation on TNT formation:

One of the major questions in the TNT field is how TNTs are formed and specifically what are the stimuli that trigger TNT formation. Until date this issue is still not very clear. However, one intriguing finding is that various stress and inflammatory cues and molecules are shown to regulate TNT formation (A Rustom, 2016).

Superoxide produced by NOX2 (NADPH oxidase 2) induces TNT formation between bone marrow stromal cells (BMSCs) and AML cells (Marlein et. al., 2017). Rotenone induced oxidative stress leads to formation of TNTs between MSCs and corneal epithelial cells (CECs). TNTs are formed by filopodial outgrowth, this is due to stress induced inflammatory response of NF $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells)/TNF $\alpha$ IP (Jiang et. al., 2016). In CAD (mouse catecholaminergic neuronal cell line) cells, exposure of cells to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) also showed an increase in levels of reactive oxygen species (ROS). This result in an increase in the number of TNT connected cells (Abounit et. al., 2016a). Addition of inflammatory mediators from the conditioned medium of macrophages, to MCF7 cells increased formation of TNTs (Patheja and Sahu, 2017). Blocking androgen receptors in prostate cancer cells stressed them which leads to increase TNT formation by phosphatidylinositol 3-kinase (PI3K)/AKT (Protein Kinase B) and Eps8 (epidermal growth factor receptor kinase) dependent manner (Kretschmer et. al., 2019). Exposure of PC12 cells with UV (Ultraviolet) light shows an increase in TNTs (Wang and Gerdes, 2015). In rat astrocytes p53 (tumor protein p53; is involved in regulation of cell cycle and apoptosis) activation leads to TNT formation through AKT/mTOR (Target of Rapamycin), PI3K and epidermal growth factor-receptor (EGF-R) dependent pathway (Wang et. al., 2011). Fas/CD95 cell death signalling cascade is important for the formation of TNTs in Jurkat and primary T cells. This pathway is dependent on the Rho GTPases, as its inhibitor, toxin B of *Clostridium difficile*, or inhibitor of Cdc42 (a member of the Rho GTPase family) secramine reduced the Fas dependent TNT formation (Hanna et. al., 2017b).

The next open question is what are the pathways that are activated in response to the original trigger in order to form TNTs. It is clear that when a cell is extending a membrane protrusion like in the case of filopodia and cilia, there are two major pathways that contribute to the extension of the protrusion; the membrane trafficking that provides protein and lipids for the actual growth of the protrusion and the cytoskeleton which provides

either the force to generate the protrusion and/or gives it the structural stability. Consistent with this, lipids, small GTPases regulating membrane trafficking and actin cytoskeleton have been the major actors in TNT formation.

b) Role of Lipids on TNT formation: Lipids have a role in TNT formation, however studies of the specific membrane composition of TNTs are still scarce. In mesothelioma cells TNTs are shown to be enriched in lipid rafts (Thayanithy et. al., 2014a). Reduction in sphingolipid or cholesterol leads to decrease in TNTs. These TNTs are positive for gangliosides in human Daudi B cell lines, thus the lipid composition of the nanotubes may have an influence over the elasticity fluidity of membrane. This may indirectly influence the nanotube growth (Toth et. al., 2017).

### c) Role of Small GTPases on TNT formation:

Small GTPases have been shown to regulate different membrane pathways (see below) and there is substantial evidence from several studies that TNT formation is regulated by several different small GTPases.

In RAW 264.7 cells, M-sec protein (expressed in myeloid cells and homologous to Sec 6; part of the exocyst complex) regulates TNTs through RalA GTPase (Hase et. al., 2009). In urothelial carcinoma-derived 5637 cancer cells, RalGPS2 (Ras-independent Guanine Nucleotide Exchange Factor (GEF) for RalA) interacts with LST-1 (leukocyte specific transcript), RalA and Sec5 (component of exocyst complex) to regulate TNT formation (D'Aloia et. al., 2018). In HeLa cells, overexpression of LST1 protein increased TNT formation and transfer of MHC-I (Major Histocompatibility Complex-class I) protein through TNTs (Schiller et. al., 2013b). LST1 recruits RalA protein, filamin, forming a multimolecular complex with myosin, myoferlin and M-sec, and regulates TNTs (Schiller et. al., 2013a).

TNTs can form between B and T cells, through plasma membrane patches enriched with H-Ras (a small GTPase), and H-Ras freely diffuses through these TNTs (Rainy et. al., 2013).

Viral protein of HTLV (human T-cell leukemia virus type 1) protein P8 shows an increase in TNT connections in the T cells and monocytes (Omsland et. al., 2018). In bronchial cell carcinoma, protein RASSF1A (Ras association domain family 1 isoform A) shows an induction in TNT formation through Rab11 (Dubois et. al., 2018). Similarly, Rab8a and Rab11a also have been reported to form TNTs. This acts through the cascade mechanism through VAMP3 and positively regulates TNT formation (described in this thesis) (Zhu et. al., 2016, Zhu et. al., 2018).

mTOR pathway inhibition by drug everolimus resulted in negative effect on TNTs (Lou et. al., 2012). In bladder cancer cells changes in the AKT/mTOR pathways resulted in an increase in the number of TNTs (Lu et. al., 2017).

#### d) Role of Cytoskeleton associated Proteins on TNT formation:

Since TNTs are primarily made of actin and microtubules, several proteins which modify cytoskeleton also show an effect on TNT formation.

Focal Adhesion Kinase (FAK) interacts with matrix metalloprotease (MMP-2) in SCCs and forms TNTs (Sáenz-de-Santa-María et. al., 2017). In B cells, interaction between integrin and fibronectin or laminin results in TNT formation (Osteikoetxea-Molnar, 2016). Fascin, a protein mainly involved in filopodia formation, localised at the base of the TNTs in the mesothelioma cells and regulates TNT formation (Lou et. al., 2012). However, in neuronal cells fascin increases filopodia and not TNTs (Gousset et. al., 2013). This again suggests that TNTs observed in different cells and context might be different structures and/or that TNT involve different cell specific pathways in different cells.

Although overexpression of N-cadherin had no effect on TNT formation (Gousset et. al., 2013), endogenous N-cadherin is present between iTNTs (Sartori-Rupp et. al., 2019). The U53 cells connected by TNTs showed enrichment at formation sites with proteins like  $\alpha$ -catenin and E-cadherins (Jansens et. al., 2017). Nef protein (accessory protein to HIV virus) is transferred through TNTs and regulates the formation of TNTs through MyoX (Uhl et. al., 2019). Overexpression of MyoX increases TNT formation in CADs and in osteoclasts (Tasca et. al., 2017, Gousset et. al., 2013).

In the macrophage cells, actin regulating Rho GTPases like Rac and Cdc42 acts through Wiskott-Aldrich syndrome protein (WASP), WASP family verprolin-homologous 2 (WAVE-2) and Arp2/3 (Actin Related Protein 2/3), on TNT biogenesis (Hanna et. al., 2017b). But in neuronal CAD cells, the proteins positively regulating filopodia like IRSp53 (Insulin receptor substrate p53) and VASP (Vasodilator-stimulated phosphoprotein) shows a decrease in TNT formation while the proteins negatively regulating the filopodia formation i.e. Eps8 shows an increase in TNT formation, this indicates that filopodia and TNT are regulated in opposite manner (Delage et. al., 2016). Similarly, TNTs are decreased by addition of Arp2/3 inhibitor compound, CK-666 in CAD cells (Sartori-Rupp et. al., 2019). Non-muscle myosin 2 protein acts as an inhibitor of TNTs in the B cells (Osteikoetxea-Molnar, 2016).



### 3) Organelle transfer by TNTs:

The major function of TNTs is to exchange different cargoes between cells. Several cargoes and different cellular organelles are transported through TNTs as summarized in Table 1. In the next section I will summarize the studies highlighting the potential role that TNT mediate organelle transfer could have in different physio-pathological conditions.

#### a) Effect of transfer of cellular organelles under various conditions:

Several studies have shown that mitochondria transfer occurs through TNTs. Transport of mitochondria through TNTs helps to alleviate various stress conditions as described below.

Upon exposure to UV rays, PC12 cells induce TNT formation, also undergo apoptosis. These TNTs transport mitochondria from the healthy cells to the apoptotic cells in co-culture, reversing the effects of apoptosis (Wang and Gerdes, 2015). Stress induction by  $H_2O_2$ , results in an increased number of TNT connected cells (Abounit et. al., 2016a). In human MSCs, on coculturing stressed (treated with  $H_2O_2$ ) and the non-stressed cells, mitochondria transfer from the non-stressed cells through TNTs. This results in downregulation of expression of S616-phosphorylated dynamin-related protein 1 (regulates fragmentation/fission of mitochondria) (Lu et. al., 2017). Rotenone induced oxidative stress caused the transfer of mitochondria from MSCs to corneal epithelial cells (CECs). In the alkali injured cornea eye model of the mouse, upon transplantation of the healthy MSCs, there seemed to be a rescue effect in the corneal epithelium by the transfer of mitochondria through TNTs (Jiang et. al., 2016).

Several molecular proteins were also involved in TNT formation and mitochondria transfer. p53 activation by the stressed cells plays a major role in the formation of TNTs in rat astrocytes. This is dependent on AKT/ mTOR and PI3K pathway; usually stressed cells initiate the formation of TNTs to unhealthy cells and transfer mitochondria (Wang et. al., 2011). Miro1 (mitochondrial Rho-GTPase) helps in the movement of mitochondria on microtubules. It facilitates transfer of intercellular mitochondria from MSCs, resulting in rescuing effects of the damaged (treated with proinflammatory supernatant of macrophages) epithelial cells (Ahmad et. al., 2014). Mitochondria transfers from BMSCs to AML cells through TNTs, due to superoxide produced by NOX2. Blocking of NOX2 in BMSCs results in impaired mitochondria transfer, and results in apoptotic death of AML cells (Marlein et. al., 2017). From the above examples it is shown that TNTs are formed

between various kinds of cells, and typically under stressful conditions, transfer of healthy mitochondria results in reversing the stressful effect.

The phenomenon of mitochondria transfer through TNTs is also seen in the cells upon infection by pathogenic organisms. This transfer is usually beneficial to the infected acceptor cells, as in the case of *E. coli* infection, the alveolar macrophages, upon receiving healthy mitochondria resulted in enhancing the phagocytic ability and bacterial clearance (Jackson et. al., 2016). Mitochondria transfer through TNTs from uninfected cells rescued cells from apoptosis/necrosis in early stages of infection in cells infected with porcine reproductive and respiratory syndrome virus (PRRSV) (Guo et. al., 2018).

Similarly, mitochondria transfer through TNTs occurs in several different disease conditions such as ischemia (results from shortage of oxygen for cellular metabolism), Acute Respiratory Distress Syndrome (ARDS; Respiratory failure resulting from rapid inflammation), sepsis, asthmatic inflammation (onset of acute inflammation in asthma conditions) etc. Transport of mitochondria by Miro1 from MSCs compensates mitochondrial impairment in ischemic differentiated cells (Babenko et. al., 2018, Jiang et. al., 2016). In ARDS and sepsis, mesenchymal stromal cells transfer healthy mitochondria through TNTs that results in increased immune functions of macrophages, both *invitro* and *in vivo* (Jackson et. al., 2016). Recovery of simulated ischemia/reperfusion in bone marrow derived MSCs and cardiomyocytes i.e. H9C2 cells, was shown upon mitochondria transfer through TNTs (Han et. al., 2016). Similarly, iPSC-MSCs (human induced pluripotent stem cell (iPSC)-derived mesenchymal stem cells) to epithelial cells mitochondria transfer through TNTs alleviate asthmatic inflammation both *invitro* and *in vivo* (Yao et. al., 2018).

Lysosomes are also shown to be transferred through TNTs (Abounit et. al., 2016a, Osteikoetxea-Molnar, 2016). Lysosomes are found to travel unidirectionally inside TNTs in PC12 cells (Rustom et. al., 2004). TNTs are shown to form between different cell types such as epithelial or mesenchymal cells and transport lysosomes through them. There have been several effects of lysosomal transfer, for example in SCCs the cells deficient in FAKs transfer autophagosomes/lysosomes to the FAK proficient cells, to adapt to stress conditions (Sáenz-de-Santa-María et. al., 2017). TNTs transport lysosomes, alpha-synuclein between astrocytes, CAD cells and primary neurons (Rostami et. al., 2017, Abounit et. al., 2016a).

In addition to mitochondria and lysosome transfer through TNTs, that have been observed more frequently, TNTs have also been shown to transport variety of other organelles and

various molecules. They include exosomes, micro-RNA (Thayanithy et. al., 2014a, Thayanithy et. al., 2014b), ER, golgi, endosomes (Wang et. al., 2011, Smith et. al., 2011), microvesicles (Giusceni et. al., 2012), Dil or DiO labelled organelles (Rustom et. al., 2004), GM1/ GM3 (Ganglioside enriched) vesicles (Osteikoetxea-Molnar, 2016). It also transports different proteins and receptors such as MHC-II (Major Histocompatibility Complex-class II), B7-2 (CD86) (Osteikoetxea-Molnar, 2016), IP3 (inositol triphosphate) (Smith et. al., 2011), synaptophysin (an early endosomal marker), myosinVa (motor protein) (Rustom et. al., 2004), GPCRs (G-protein coupled receptors) (Guescini et. al., 2012), CD133 (stem cell marker) (Reichert et. al., 2016), MHC-I, GPI (glycosylphosphatidylinositol) anchored proteins (Onfelt et. al., 2004),  $\text{Ca}^{+2}$  and LPS (Lipopolysaccharide) signals (Smith et. al., 2011, Osteikoetxea-Molnar, 2016). Our lab has shown that TNTs mediate the intercellular transfer of different amyloid proteins involved in neurodegenerative diseases (prions, alpha synuclein, huntingtin and tau) and proposed that TNTs facilitate the spreading of pathology and progression of the diseases (Victoria and Zurzolo, 2017, Abounit et. al., 2016a, Abounit et. al., 2016b, Gousset et. al., 2009, Costanzo et. al., 2013). Thus, in this respect TNTs may represent a novel therapeutic target to halt the progression of these diseases.

### b) Effect of transfer of cellular organelles in cancer:

Cancer is a group of diseases that is characterized by uncontrollable cell division, migration, and invasion of cells to different parts of body, from origin site, leading to the general perturbation of equilibrium in the normal cells and body functions. Progression of carcinogenesis occurs following different mechanisms of cell to cell signalling in the tumour micro environment such as paracrine mediated mechanisms and exosome mediated cellular content transfer between cells (Kaminska et. al., 2015, Quail and Joyce, 2013, Roma-Rodrigues et. al., 2014). Since cell-cell interactions are shown to play such a vital role in cancer progression, the possible role of TNTs in cancer is a topic of many current studies

Emil Lou et. al., in 2012, showed TNTs are present in mesothelioma cell lines (MSTO 211H), human mesothelioma cells, malignant pleural mesothelioma and lung adenocarcinoma tissue specimens. TNTs indeed play a role in the bidirectional transfer of cellular contents such as proteins, mitochondria and golgi vesicles in MSTO 211H cells. This transfer of mitochondria may be implicated in phenotypical changes and aggressivity of cancer cells.

Pasquier et. al., in 2013 showed that functional TNTs transporting mitochondria were formed between the endothelial cells and cancer cells. As mentioned before TNTs are known to transport micro-RNAs, and this seems to lead to an increase in oncogenesis of the cells. TNTs have been shown to play role in regulating the invasiveness of cancer cells, especially in pancreatic carcinoma. The number of TNTs increases after the treatment of cells with chemotherapeutic agent doxorubicin, thus they may play a role in the drug resistance in cancer (Desir et. al., 2018). Tunneling nanotubes are involved in the transfer of mitochondria in heterogonous mixture of bladder cancer cells in co-culture, which results in increased invasiveness of these cancer cells (Lu et. al., 2017).

### c) Effect of transfer of cellular organelles in neurodegeneration:

Neurodegenerative diseases are adult onset diseases which are characterized by progressive destruction of neurons leading to a loss of variety of homeostatic functions in the body. There are several neurodegenerative diseases that are identified and described till date such as Alzheimer's, Huntington, Parkinson's, Amyotrophic lateral sclerosis etc. Often the causes of neurodegenerative diseases are the proteins that are involved in the normal and developmental functions of neurons such as alpha-synuclein, huntingtin, tau, presenilin-1 etc. (Winner et. al., 2011). Most of the neurodegenerative diseases can be familial or sporadic in nature. The familial form of the disease is associated with the genetic mutations of the protein causing the disease but the sporadic form of the disease is attributed to both genetic and environmental factors (Rietdijk et. al., 2017). Upon onset of the disease, the proteins misfold, aggregate and accumulate in the cells leading to neurotoxicity (Walsh and Selkoe, 2016). Thus, the intercellular transfer of misfolded protein aggregates to the target cells, which further leads to aggregation or misfolding in the target cells causing neurodegenerative disorders, is most widely recognized explanation of disease progression. In 2003, Braak and his colleagues showed a staging system for the spread of disease in mice, in which it develops in a particular region of the brain and then spreads to the different connected regions. This study provided the evidence that spread of neurodegenerative diseases throughout the brain is due to the spread of misfolded proteins between different parts of the brain.

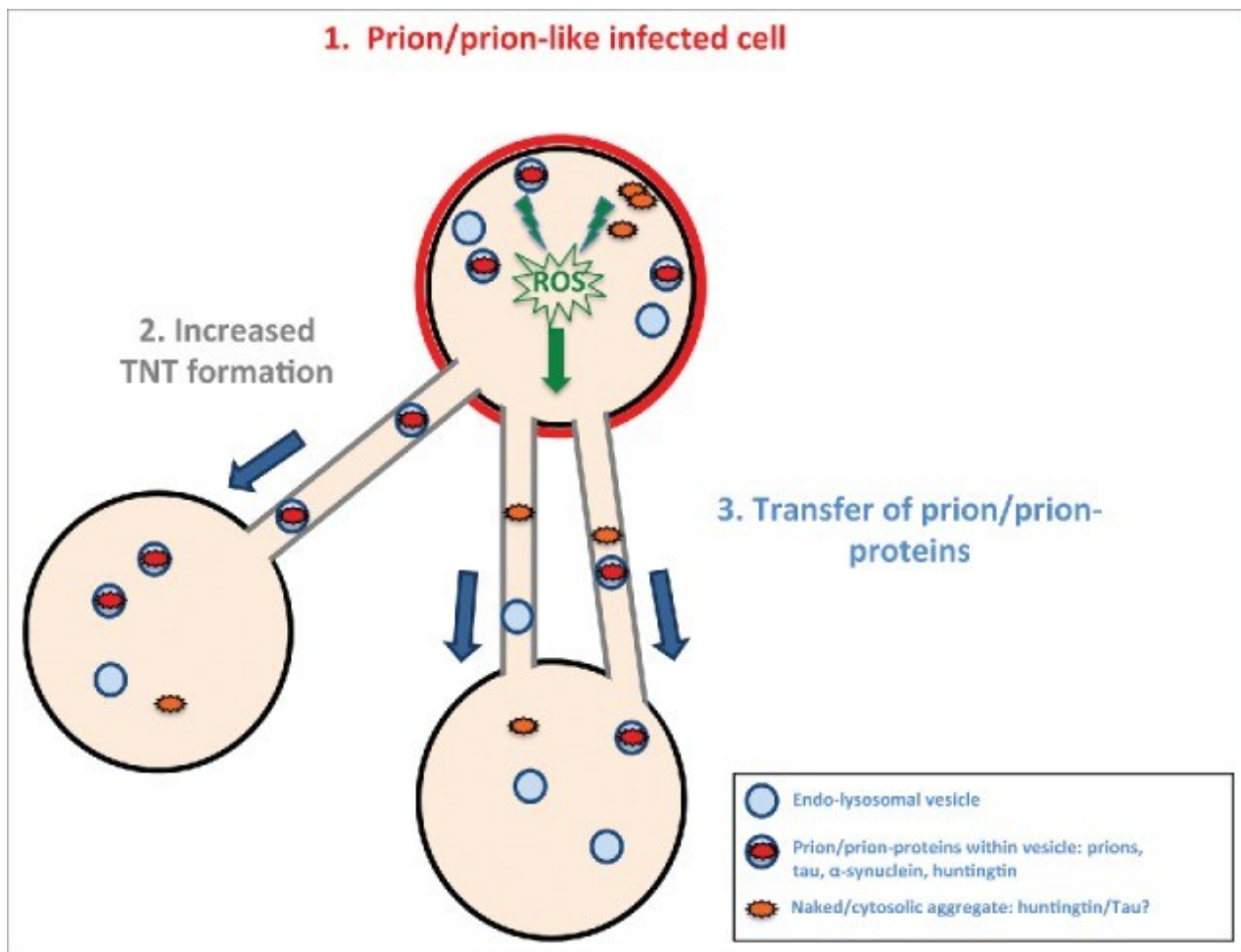
Alzheimer's disease is characterised by the presence of A $\beta$  peptides (beta amyloid) which is made by beta and gamma secretases by amyloid precursor protein (APP) mainly accumulating outside the cells (Udayar et. al., 2013), and of tau aggregates accumulating inside the neurons in tangles and neurofibrils (Goedert and Spillantini, 2017). A $\beta$  peptides were transferred through TNTs and cause cytotoxicity in the neurons (Wang et. al., 2011). Presence of tau shows an increase in TNTs in the neurons, and they propagate through TNTs (Abounit et. al. 2016b, Tardivel et. al., 2016) shown in Figure 6.

Presence and spread of misfolded alpha synuclein is one of the major characteristics of Parkinson's disease (Brundin et. al., 2010, Hansen et. al., 2011). TNTs are formed between the human neuronal cell line SH-SY5Y and primary human pericytes and

have been shown to transfer alpha synuclein (Dieriks et. al., 2017). Alpha synuclein is also transported through TNTs in CADs and neurons. As mentioned above this transfer occurs usually through lysosomes inside the TNTs from the diseased to healthy cells (Abounit et.

al., 2016a). Interestingly when alpha synuclein is transported through TNTs in astrocytes, mitochondria are transported in the opposite direction from the healthy astrocytes to the diseased cells enabling a rescue mechanism (Rostami et. al., 2017). Our lab has also shown that transfer of alpha synuclein is cell type specific. Alpha synuclein transfer occurs between different cell types such as neurons to astrocytes, or astrocytes to astrocytes, but less efficiently between astrocytes to neurons. Furthermore, we also showed that astrocytes are much more efficient in degrading alpha synuclein than neurons (Loria et. al., 2017).

Rhes (striatal-specific protein) protein is also transported between the TNTs and is responsible for the transport of the mutant huntingtin between cells, interestingly these TNTs carry Rab5a positive vesicles inside them (Sharma and Subramaniam, 2019).



**Figure 6:** Model of prion/prion-like protein induced formation of TNTs and spreading. Prions or prion-like proteins such as tau fibrils after “infecting” a cell (in red) (1) induce via an unknown (possible common) mechanism (for example, induced via radical oxygen species (ROS) from oxidative stress) an increase in TNT number (2). The prion/prion-like aggregates would then be propagated via TNT-mediated intercellular trafficking from infected cells to naïve cells (Abounit et. al., 2016b).

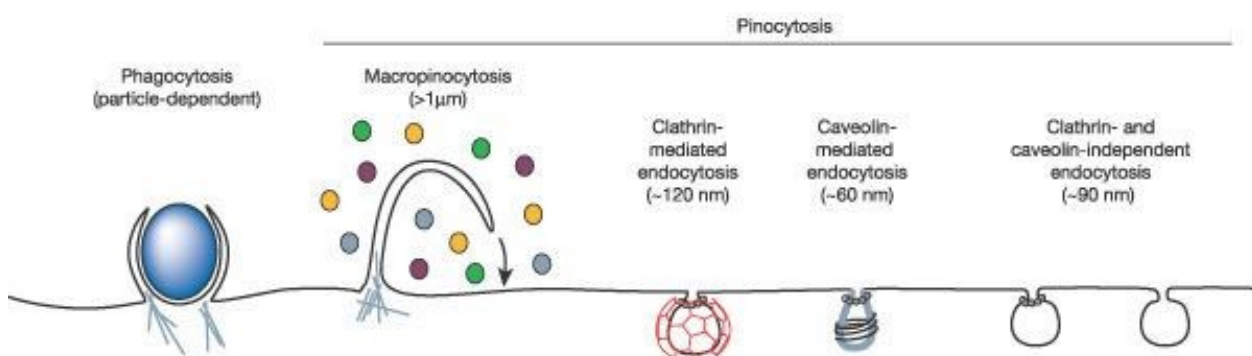
## **Chapter 2: Overview of vesicle trafficking and the role of Rab proteins in cellular functions**

### **1) Vesicle trafficking in cells**

Intracellular vesicle trafficking can be divided into two main pathways i.e. endocytosis and exocytosis.

Exocytosis is mainly used to expel cellular substances, while endocytosis is involved the uptake of molecules/solutes by the cells. Exocytosis involves fusion of vesicular membranes with the plasma membrane.

Endocytosis internalizes lipids and proteins of plasma membranes in the cells (Doherty and McMahon, 2009). Endocytosis can be divided into phagocytosis (uptake of large particles) and pinocytosis (uptake of fluid and solutes) (Conner and Schmid, 2003). Endocytosis is important for nutrient uptake, cell adhesion and migration, signaling, pathogen entry, synaptic transmission, receptor down regulation, antigen presentation, cell polarity, mitosis, growth and differentiation, and drug delivery (Doherty and McMahon, 2009). The different pathways of endocytosis are summarized in Figure 7.



**Figure 7: Vesicle trafficking in cells:** Representation of different types of endocytic pathways depending upon the cargo internalized and the proteins associated with the internalization process. (Conner and Schmid, 2003)



### a) Endocytic Pathways:

*Phagocytosis* occurs in specialized cells such as macrophages, monocytes and neutrophils. These cells are able to rearrange actin cytoskeleton which could lead to internalization of molecules and pathogens, such as bacteria (Conner and Schmid, 2003, Aderem and Underhill, 1999, Dramsi and Cossart, 1998). Among the proteins that are involved in this process, Rho GTPases play an important role (Hall and Nobes, 2000).

*Pinocytosis* is widespread in a variety of cells. It is divided into four main processes - macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (Connor and Schmid, 2003).

*Macropinocytosis*: is the uptake of cellular substances especially, solutes. This process occurs usually in dendritic cells since they have to sense the cellular environment during immune surveillance (Steele-Mortimer et. al., 2000).

*Clathrin mediated endocytosis (CME)*: Plays an important role in immune response, neurotransmission, intercellular communication, signal transduction, cellular and systemic homeostasis (Conner and Schmid, 2003). In CME, cytoplasmic domains of the plasma membrane proteins are recognized by the adaptor proteins (AP2) and then packaged into clathrin coated vesicles and internalized in the cells (A Sorkin, 2004). For example, iron bound transferrin receptors (TFRs) and the low-density lipoprotein receptors (LDLRs) undergo CME.

*Caveolae mediated endocytosis*: In caveolae mediated endocytosis, caveolin forms loops in the inner leaflet of the plasma membrane and forms membrane invaginations (Conner and Schmid, 2003). It acts through actin and dynamin. Caveolin regulates endocytosis, transcytosis (the uptake of macromolecules from one end and its expulsion from the other end of the cell) and cell signaling (Pelkmans and Helenius, 2002). Caveolar endocytosis involves caveolin coat and is usually involved in the internalization of glycosphingolipids and viruses (Mayor and Pagano, 2007, Sandvig et. al., 2008).

*Clathrin independent endocytosis*: The clathrin independent endocytosis involves internalization of the receptors such as beta integrins, MHC-I, GPI and CD59. ARF6 mediates clathrin independent endocytosis in different cell types including HeLa cells, COS (CV-1 in Origin with SV40 genes) cells, MCF7 cells, B cells, cardiomyocytes, hippocampal neurons, and dendritic cells (Grant and Donaldson, 2009, Naslavsky et. al., 2004b).

### b) Recycling of Endosomes (recycling pathway):

The cargoes that are internalized by endocytosis undergo either recycling or degradation (Bhuin and Roy, 2014). Endocytic recycling involves the return of membrane and receptors back to the plasma membrane, and this process involves vesicles to be transported back to the plasma membrane (Cai et. al., 2013). The recycling pathway is regulated by Rabs, ARF and polarity proteins (Radhakrishna and Donaldson, 1997, Balasubramanian et. al., 2007). They belong to family of small GTPases (Takai et. al., 2001). They are involved in various steps of vesicle trafficking in cells. There are around 60 Rab GTPases in mammals (Kiral et. al., 2018). I will be talking about the Rabs and ARF proteins in detail, in part 2 of this chapter and chapter 3 respectively.

The recycling pathway involves the internalization of cargo by clathrin coated pits or clathrin independent endocytosis at the membrane which are eventually pinched off from the plasma membrane to form early endosomes. These early endosomes are associated with specific Rab molecules which allow vesicles to fuse and form the multivesicular body (MVB). Afterwards, they are recycled back to the membrane (which usually occurs for plasma membrane receptors) (Katzmann et. al., 2002) or are fused with lysosomes for degradation of vesicle content (Cullen and Steinberg, 2018). This process is summarised in Figure 8.

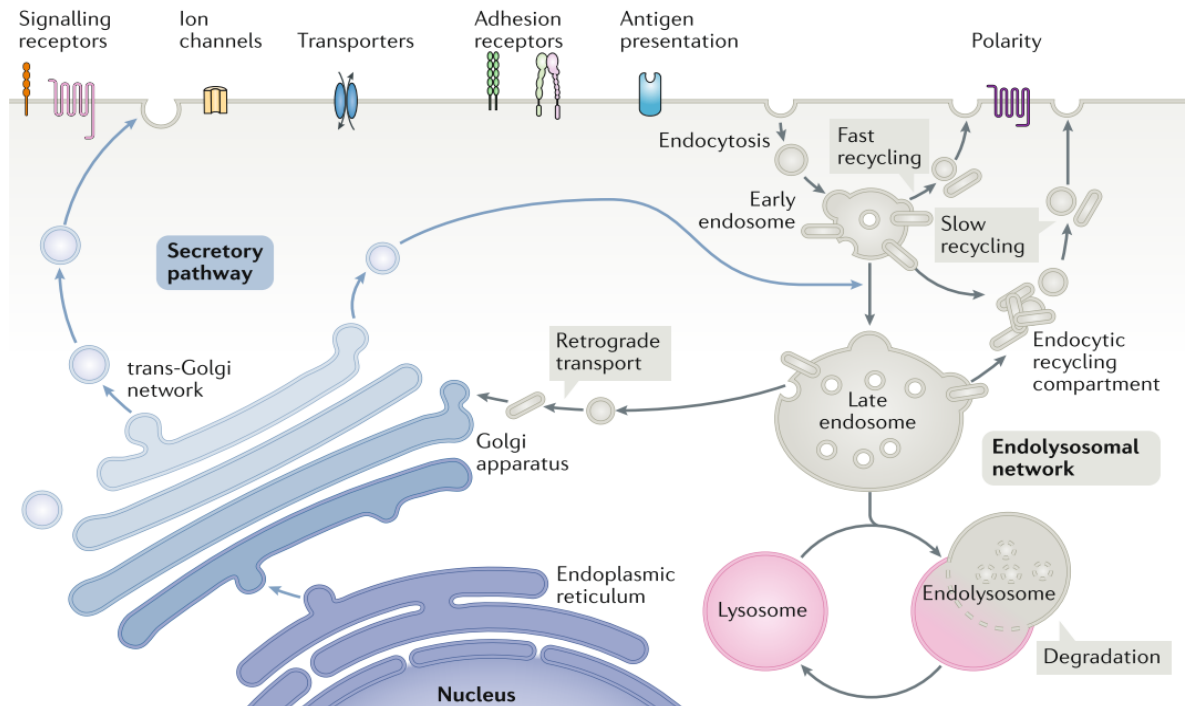
There is a balance between uptake of contents by endocytosis and their recycling back to the plasma membrane. These two pathways control various cell processes such as cell migration, cytokinesis, maintenance of cell polarity, cell adhesion and junction formation (Grant and Donaldson, 2009). Recycling of membrane is quite frequent; for instance, in mouse macrophages plasma membrane recycling occurs around 1-5 times per hour (Steinman et. al., 1983). The cargoes meant for recycling are usually first targeted to the endocytic recycling compartment (ERC) which is localised near Microtubule organizing center (MTOC) at the perinuclear region of the cell (Grant and Donaldson, 2009). I will discuss ERC and give more information on recycling pathway in part 2 of this chapter.

Recycling pathway is involved in several different functions:

a) In myoblast fusion: Rac is enriched at the cell-to-cell contact sites and is involved in cell fusion by remodeling the actin cytoskeleton. Rac is also involved in the migration of cells by ARF6 mediated recycling (Palamidessi et. al., 2008). Rac localization and activation is affected by the activation of ARF6 (Chen et. al., 2003).

b) In learning and memory: Learning and memory, usually involves two receptors i.e. AMPAR (Alpha-Amino-3-Hydroxy-5-Methyl-4-Isoxazole Propionic Acid-Receptor) and NMDA (N-methyl-D-Aspartate) receptor. Receptor recycling is essential for formation of long term memory or neuronal structures like dendritic spines. Several Rab proteins are involved in this process for e.g. Rab11, Rab10 etc. (Glodowski et. al., 2007, Wang et. al., 2008).

c) In cytokinesis: Recycling endosomes are involved in the late step of cytokinesis, by associating with the cytokinetic bridges (Ai and Skop, 2009).



**Figure 8: The endolysosomal network:** The integration between the endolysosomal network and the secretory pathway helps to establish, maintain, and remodel the cell surface proteome. Following their endocytosis, internalized integral membrane proteins enter the early endosome, where most sorting is initiated. Selected cargo can be recycled back to the cell surface by means of tubulo-vesicular transport carriers either directly, termed ‘fast recycling’, or by transit through the endocytic recycling compartment, termed ‘slow recycling’. Recycling back to the cell surface can occur by passage through the trans-Golgi network and entry into the secretory pathway (termed ‘retrograde transport’). Other cargoes are targeted for degradation within the lysosome. This is principally achieved through cargo sorting into intraluminal vesicles (ILVs). Iterative rounds of cargo sorting and ILV biogenesis, coupled with maturation of the early endosome, result in the formation of the late endosome (also known as the multivesicular endosome or multivesicular body). The late endosome is able to fuse with lysosome to form an endolysosome within which ILVs and their accompanying cargoes are degraded (Cullen and Steinberg, 2018)

c) Role of Cytoskeleton in vesicle trafficking: Inside the cell, vesicles are usually transported on microtubule or actin tracks (Grant and Donaldson, 2009, Radhakrishna and Donaldson, 1997, Balasubramanian et. al., 2007). Several vesicle trafficking processes as endocytosis, exocytosis and vesicle recycling involves and/or affects actin modifying proteins. Vesicle trafficking is important for cell migration, formation of cellular protrusions, lamellopodia, pseudopods etc. Actin regulating proteins such as Rho A, Rho B, Rho D, Rac and Cdc42 are also involved in membrane trafficking in cells. Rac promotes the formation of membrane ruffles and lamellipodia while Cdc42 affects the formation of finger like protrusions like filopodia and microspikes. Both of these proteins act by inducing actin polymerization and branching (Lamarche et. al., 1996). Endocytosis of various receptors has an effect on several cytoskeletal based cellular functions like cell migration or formation of cellular protrusions like pseudopods (which is a filament like extension from the plasma membrane) (Powelka et. al., 2004). Rac is also involved in TFR endocytosis and apico-basal polarization of cells (Lamaze et. al., 1996, Jou et. al., 2000). PDGF (Platelet Derived Growth Factor) binds to its receptor to induce a variety of cellular functions such as cell migration, formation of dorsal ruffles. PDGF induces macropinocytosis and involves actin regulating proteins such as Rac and P21 activated kinases (A Ridley, 2001).

Vesicle recycling also regulates formation of cellular protrusions. Rac induced membrane ruffles and lamellopodia are formed due to actin polymerization. Recycling vesicular membrane gives rise to the new membranes necessary for the formation of these structures (Bretscher and Aguado-Velasco, 1998). Rac and Cdc42 also stimulate the adherens junction assembly in the epithelial cells. E-cadherin is recycled through the endosomal pathway and is involved in the formation of adherens junctions (A Ridley, 2001, Le et. al., 1999). Rab8 recycles transferrin positive vesicles back to the plasma membranes. These vesicles participate in the formation of cellular protrusions (Hattula et. al., 2006).

Exocytosis also involves several actin modifying proteins. Cdc42 is affected by Rab8a. Knocking down Rab8a and Rab11a, leads to reduced activation of Cdc42. This may explain the correlation between actin and vesicle trafficking in the cells. This pathway also regulates the apical exocytosis mediated by Rab11 (Bryant et. al., 2010). In infections, as by *Shigella* and *Listeria*, the bacteria move through the cells by forming actin comets. They are secreted outside the cells with the activation of Cdc42, N-WASp, Arp2/3 complex inducing long protrusions by the cells (Frischknecht and Way, 2001). Rac and/or Cdc42

directly activates phospholipaseC $\gamma$  which can generate IP3 and induce secretion of vesicles. Rac induces actin polymerization associated with secretory granules through WAVEs/SCARs and Arp2/3 complex (Kumari and Mayor, 2008).

We became interested in Rab proteins as Rabs are involved in the regulation of the plasma membrane by the maintenance of the lipid content in the cells (Dambournet et. al., 2011). Rabs also regulate the recycling of the vesicles back to the membrane which may be involved in the membrane dynamics and lead to the formation of TNTs (described in this thesis).

## 2) Overview of Rab proteins:

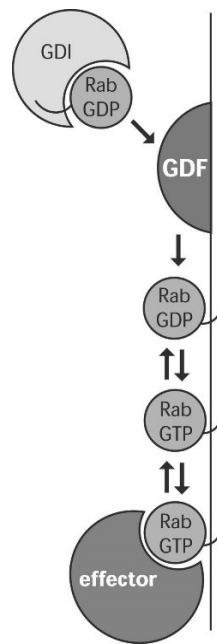
Small GTPases are divided into Ras, Rho, Rab, Sar1/Arf, and Ran family (Takai et. al., 2001). Their molecular weight ranges from 21-25 kDa. There are around 60 Rab GTPases in mammals, 31 in *Drosophila* and 11 in yeast (Kiral et. al., 2018, Zhang et al., 2007). They were first identified in 1980s as proteins isolated from yeast and then in rat brains (Rab-like protein from rat brain). They are numbered according to sequence of discovery as Rab1, Rab2 etc. (Das and Guo, 2011).

Rab proteins are master regulators of cellular functions, as shown in Figure 10. They are involved in vesicle trafficking, migration of cells and formation of cellular protrusions like filopodia, lamellipodia (Das and Guo, 2011).

a) Regulation of Rab proteins: Rabs function through GTP (Guanosine 5'-triphosphate) bound (active) –GDP (Guanosine 5'-diphosphate) bound (inactive) cycle. They are regulated by GEFs (Guanine nucleotide Exchange Factors), GAPs (GTPase Activating Proteins) and GDIs (Guanine Dissociation Inhibitors).

As shown in the Figure 9, Rabs are regulated by several different proteins. Rabs can bind to REP (Rab escort protein) which presents it to geranylgeranyl transferase to be geranylated (Pfeffer and Aizavan, 2004, S Pfeffer, 2017). The geranylation of Rabs allows it to bind the membranes and is usually targeted to their respective membranes with the help of REP (Kinsella and Maltese, 1992). GDF (GDI Displacement Factor) dissociates Rab-GDI complexes. Once the Rabs are dissociated from the GDI

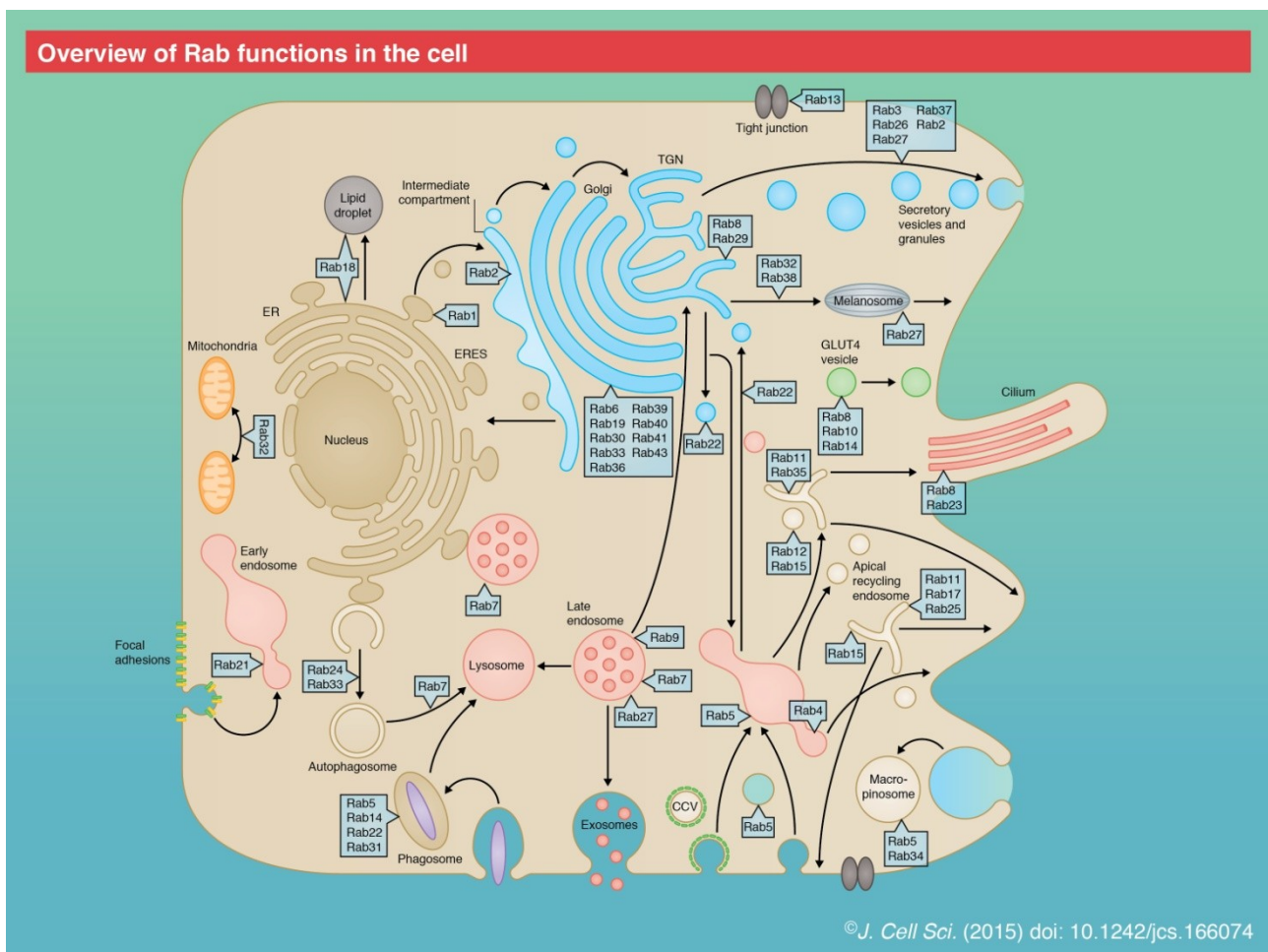
they are acted on by the GEFs which convert Rab-GDP to Rab-GTP and this conversion allows the Rabs to bind their effectors (Grosshans et. al., 2006, Dirac-Svejstrup et. al., 1997). Most of the Rab proteins contain two switch elements in their structure, switch I and switch II which change their conformation upon binding to  $\gamma$  phosphate of GTP. The switch regions are unfolded in their GDP bound state and adopt distinct conformations on binding to GTP (Lee et. al., 2009). GAPs are necessary for the cycling of GTP-Rab proteins to their GDP bound forms (Dumas et. al., 1999, Seabra and Wasmeier, 2004). After converting to their GDP bound forms, Rabs are extracted from the membrane and sent back to the cytosol by the GDI (Grosshans et. al., 2006, Heo et. al., 2006, Gavriljuk et. al., 2013).



**Figure 9: Overview of Rab cycle:** Rabs in their GDP-bound conformations are present in cytosol bound to GDI. GDI presents the Rab to the membrane; GEF displaces the GDI and allows the Rab to bind to the membrane. Rabs are then activated by a specific GEF, converting the Rab into its GTP-bound form. The GTP-bound Rab is then able to bind effectors. If a Rab is not activated, it can be re-extracted by GDI for redelivery to another membrane (S Pfeffer, 2005).



**b) Functions of Rab proteins:** The Rab proteins accumulate at their target membranes thus they are used as the marker of organelles. Rab proteins are involved in tethering and docking of the vesicles to its membrane in the cells and this leads to fusion of respective vesicle with the membrane. Each of the Rab protein controls a specific pathway of membrane trafficking (Bhuin and Roy, 2014). They are involved in different processes as summarized in Table 3 and shown in the Figure 10 below.



**Figure 10:** Overview of Rab functions in the cell (Zhen and Stenmark, 2015)

Table 3: Overview of Rab proteins

Rab	Intracellular Localization	Function	Effector
Rab1 A, B	ER–Golgi intermediate	ER–Golgi trafficking, Intra golgi	p115/Uso1, GM130, giantin, golgin-84, GCC185, MICAL-1, MICAL COOH-terminal like, JRAB/MICAL-L2, OCRL1, INPP5B, Cog6, GBF1 (Arf1 GEF), Iporin, Chlamydia pneumoniae Inc protein Cpn 0585, Trypanosoma brucei golgin Tbg63
Rab2A, B	ER, ER–Golgi intermediate, Golgi	ER–Golgi trafficking	INPP5B, golgin-45, RIC-19 ( <i>Caenorhabditis elegans</i> ortholog of ICA69), PKC $\iota/\lambda$ , GM130, GAPDH, <i>Drosophila melanogaster</i> germ cell-less homolog 1, GARI, Fam71f2, Fam71b
Rab3A-C	TGN-apico-lateral membranes, Secretory vesicles, Plasma membrane	Exocytosis of secretory granules and vesicles from TGN to apico-lateral membranes, Neurotransmitter release	rabin3, RIM1 $\alpha$ , RIM2 $\alpha$ , granuphilin, Noc2, Munc18-1, rabphilin, INPP5B, SNAP-29, synapsin, polymeric IgA receptor (Rab3b), Gas8 (Rab3b), Zwint-1 (Rab3c), OCRL1; Warburg Micro/Martsolf syndromes (Rab3GAP)
Rab3D (Rab16)	Secretory vesicles, plasma membrane	Exocytosis, regulated in nonneuronal cell	rabin3, RIM1 $\alpha$ , RIM2 $\alpha$ , granuphilin, Noc2, Munc18-1, rabphilin, INPP5B, SNAP-29, synapsin, polymeric IgA receptor (Rab3b), Gas8 (Rab3b), Zwint-1 (Rab3c), OCRL1; Warburg Micro/Martsolf syndromes (Rab3GAP)
Rab 4A, B	Early and recycling endosomes	Endocytic recycling to plasma membrane	Rabaptin-4, $\beta$ CD2AP, D-AKAP2, Rabip4, Rabip4=, Rabaptin-5 $\alpha$ , Rabaptin-5, Syntaxin 4, Dynein LIC-1, Rab coupling protein (RCP), Rabenosyn-5
Rab5, A, B, C	Clathrin coated vesicles and early endosomes, PM	Endocytic internalization and early endosome fusion	Vac1, EEA1 (early endosome antigen 1), Rabenosyn-5, CORVET (Class C core vacuole/endosome tethering), Rabaptin-5/5 $\beta$ , Rabex-5, INPP5B, OCRL1, PI3 kinases (hVPS34-p150, p110 $\beta$ -p85 $\alpha$ ), Rabankyrin-5, APPL1, APPL2, Huntingtin-HAP40, caveolin-1, angiotensin II type 1A receptor, Rabi6p4'

Rab6 A, A'	Golgi	Endosome to Golgi, intra-Golgi transport, Golgi to ER	Rab6 interacting protein 1/2A/2B, Cog6, kinesin Rab6-KIFL, GCC185, giantin, OCRL1, ELKS, INPP5B, golgin SCYL1BP1, golgin-97, golgin-245, hVps52 (GARP/VFT complex), dynein light chain DYNLRB1, p150 (Glued) subunit of dynein/dynactin complex, mint3 adaptor protein, Bicaudal-D1/2, VFT complex, golgin Sgm1 (TMF/ARA160); Geroderma osteodysplastica (golgin SCYL1BP1) Rabkinesin6, GAPCenA
Rab6B	Golgi	Intra-Golgi transport, preferentially expressed in neuronal cells	Rab6 interacting protein 1/2A/2B, Cog6, kinesin Rab6-KIFL, GCC185, giantin, OCRL1, ELKS, INPP5B, golgin SCYL1BP1, golgin-97, golgin-245, hVps52 (GARP/VFT complex), dynein light chain DYNLRB1, p150 (Glued) subunit of dynein/dynactin complex, mint3 adaptor protein, Bicaudal-D1/2, VFT complex, golgin Sgm1 (TMF/ARA160); Geroderma osteodysplastica (golgin SCYL1BP1)
Rab7A	Late endosomes, lysosomes/vacuole, melanosomes, phagosomes	Late endosome to lysosome	HOPS (Homotypic fusion and protein sorting) complex, Rabring7, proteasome alpha-subunit PSMA7, Vps34/p150 PI3-kinase complex, oxysterol binding protein related protein 1, RILP
Rab7B	Lysosomes	Late endosome to lysosome	HOPS (Homotypic fusion and protein sorting) complex, Rabring7, proteasome alpha-subunit PSMA7, Vps34/p150 PI3-kinase complex, oxysterol binding protein related protein 1, RILP
Rab8 A, B	Median Golgi and TGN, Cell membrane, Vesicles, Primary cilia	Basolateral protein transport from median Golgi and TGN, Exocytosis	Rab8IP (IP-interacting protein), Rabphilin, MICAL-1, MICAL COOH-terminal like, MICAL-L1, JRAB/MICAL-L2, TRIP8b (Rab8b), FIP-2, optineurin, otoferlin, RIM1, RIM2, Noc2, OCRL1, Sro7 (Sec4), cenexin3
Rab9 A	Late endosomes	Transport from late endosomes to <i>trans</i> -Golgi	p40, TIP47 (TIP-Tail interacting protein), TIP47, INPP5B, GCC185, PI3P PIKfyve kinase associated protein p40, Ndel, 14-3-3 protein theta, HPS4
Rab9B	Late endosomes, golgi	Transport from late endosomes to <i>trans</i> -Golgi	p40, TIP47 (TIP-Tail interacting protein), TIP47, INPP5B, GCC185, PI3P PIKfyve kinase associated protein

			p40, Ndel, 14-3-3 protein theta, HPS4
Rab10	Median Golgi and TGN, Sorting endosomes, GLUT4 vesicles	Basolateral protein transport from median Golgi and TGN, Exocytosis	Rim1, MICAL-1, MICAL COOH-terminal like, MICAL-L1, JRAB/MICAL-L2, <i>Chlamydia pneumoniae</i> Inc protein Cpn 0585
Rab11 A, B	TGN/post-Golgi vesicles and recycling endosomes	Transport from the Golgi, and apical and basolateral endocytic recycling	Rabphilin11, Rab11BP (BP-Binding protein), FIP2, FIP3, FIP4 (FIFP-Family interacting protein), Sec15, RIP11(RIP-Rab interacting protein), Sec15, Rab11-FIP1 to FIP5 [FIP3 3 eferin/arfophilin, FIP5 3 Rip11, FIP1c 3 Rab coupling protein (RCP)], D. melanogaster nuclear fallout, arfophilin-2, myosin Vb, PI4-kinase $\beta$ , rabphilin-11, Rab6 interacting protein 1, Rabin3, <i>Chlamydia pneumoniae</i> Inc protein Cpn 0585, Sec2 (Ypt31/32), Gyp1 (Ypt32); Huntington's disease
Rab12	Peripheral region of cell to perinuclear centrosomes, secretory vesicles, Golgi	Transport from peripheral region of cell to perinuclear centrosomes, Exocytosis	Rab interacting lysosomal protein-like 1 (RILP-L1)
Rab13	Cell/tight junctions, TGN, RE	TGN/RE to plasma membrane	Delta-PDE, MICAL-1, MICAL COOH-terminal like, MICAL-L1, JRAB/MICAL-L2, protein kinase A, INPP5B, OCRL1
Rab14	Early endosomes and Golgi, GLUT4 vesicles	Transport between early endosomes and Golgi, Apical membrane trafficking	FIP2, RCP, Rip11, D-AKAP2
Rab15	Early and recycling endosomes	Sorting endosome/RE to plasma membrane	REP15 (REP-Rab escort protein), MICAL-1, MICAL COOH-terminal like, MICAL-L1, JRAB/MICAL-L2, Rab15 effector protein
Rab17	Epithelial specific; apical recycling endosome	Transport through apical recycling endosomes, Transcytosis	
Rab18	ER-Golgi intermediate, Lipid Droplets	ER-Golgi trafficking, Lipid Droplet formation	
Rab19	Golgi	Unknown	D-AKAP2, ddGCC88, dGolgin97, Wdr38, oxidative stress-induced growth inhibitor family member 2 ( <i>D. melanogaster</i> GRIP domain proteins)

Rab20	In apical dense tubules, endocytic structures, Endosome, Golgi	In apical endocytosis/recycling	INPP5E
Rab21	Early endosomes	Endosomal transport	$\alpha$ -Integrin subunit
Rab22 A	Early endosomes and TGN	Endosomal transport, protein recycling to plasma membrane	Rabex-5, EEA1, rabenosyn-5, RAD51, INPP5B, OCRL1, rKIAA1055
Rab23	Plasma membranes and early endocytic vesicles	Transport between plasma membranes and early endocytic vesicles	
Rab24	ER/ <i>cis</i> -Golgi region and on late endosomal structures	Autophagy-related processes	COOH-terminal binding protein 1
Rab25	Epithelial specific; apical recycling endosome	Transport through apical recycling endosomes	Integrin $\beta$ -1 subunit, FIP2, Rip11; epithelial cancers
Rab26	TGN-apico-lateral membranes, Secretory Granules	Exocytosis of secretory granules and vesicles from TGN to apico-lateral membranes	RIM1
Rab27 A, B	Melanosomes	Exocytosis	Melanophilin, Rabphilin-3, Noc2, Granuphilin (SLP4), Slp1-5, Slac2-a (melanophilin), Slac2-b, granuphilin, MyRIP (Slac2-c), Rim2, Rabphilin, Noc2, Munc13-4, Golga4/p230;
Rab30	ER, Golgi	Unknown	Cog4, Golga4/p230, dGCC88, dGolgin97, dGolgin245 ( <i>D. melanogaster</i> GRIP domain proteins)
Rab31(Rab22B)	Early endosomes and TGN	Transport between TGN and early endosomes and vice versa, M6P receptor transport to endosome	OCRL1
Rab32	Mitochondria, melanosomes	TGN to melanosome, mitochondrial fission	Varp/Ankrd27, PKA
Rab33 A	Golgi, dense-core vesicles	Autophagosome formation	ATG16L, GM130, rabaptin-5, rabex-5
Rab33B	Golgi	Autophagosome formation	Rab33b-BP
Rab34	Golgi, macropinosomes	Intra-Golgi transport, peri-Golgi positioning of	RILP, Hmunc13, RILP-L1

		lysosome	
Rab35	Recycling endosomes, PM	Apical endocytic recycling, Actin assembly	MICAL COOH-terminal like, MICAL-L1, MICAL-1, OCRL1, fascin, Centaurin $\beta$ 2
Rab36	Golgi	Unknown	MICAL-1, MICAL-L1, RILP, RILP-L1, GAPCenA, Leprecan
Rab37	TGN-apico-lateral membranes, Secretory Granules	Exocytosis of secretory granules and vesicles from TGN to apico-lateral membranes	RIM1
Rab38	Melanosomes	TGN to melanosome	Varp/Ankrd27
Rab39	Golgi	Unknown	Caspase-1
Rab40 A, B, C	Golgi, RE	Endosome/intracellular transport	Elongin B/C, Cullin5, D-AKAP2, RILP-L1, RME-8
Rab41	Golgi	Unknown	Cog6, Golga4/p230, D-AKAP2
Rab42	Unknown	Unknown	Unknown
Rab43	ER, Golgi	ER to Golgi, Shiga toxin transport	Unknown
Rab44	Unknown	Unknown	Unknown
Rab45	Perinuclear region	Unknown	Unknown

Modified from (Hutagalung and Novick, 2011, Bhuin and Roy, 2014)

- Vesicle budding :

Rab proteins have been shown to regulate vesicle budding from different organelles. Rab1 controls budding from the ER (Allan et. al., 2000). Rab9 regulates membrane budding from the Trans-Golgi network (TGN). Rab5 regulates the half-life formation of clathrin coated pits, while Rab7 regulates the budding of the vesicles from the golgi complex (Lombardi et. al., 1993, Diaz and Pfeffer, 1998).

- Vesicle transport :

Rabs are involved in the transport of vesicles; Rab6 is involved in the microtubule-based transport from the golgi, while Rab5 has been implicated in the attachment and movement of early endosomes along the microtubules (Zerial and McBride, 2001). Rabs are involved in early and late endosomes trafficking and fusion, specifically Rab5 localises to early endosomes, while late endosomes require Rab7 localization (Rink et. al. 2005). The switch between Rab5 and Rab7 is essential to identify these organelles and to progress in the endocytic pathway. This is due to interaction of Rab5 with the HOPS/VPS (homotypic fusion and protein-sorting/class C vacuole protein-sorting) complex (GEF of Rab7). As the endosomes migrate from the cell periphery to the center of the cell Rab5 is replaced by Rab7 (Rink et. al., 2005).

- Vesicle fusion :

The exocyst is a complex of proteins that only functions in exocytosis in the exocytosis by acting as a tether between the vesicles and the plasma membrane (TerBush et. al., 1996). This complex has been identified in yeast, where it was shown to regulate the fusion of Rab loaded vesicles to the plasma membrane. Rab5 regulates the transport of the clathrin coated vesicles from the plasma membrane to the early endosomes and its fusion. EEA1 (Early Endosome Antigen 1) is the effector of Rab5 and allows the docking of the Rab5 bearing membranes (generally early endosomes) together (McBride et. al., 1999). Different Rab proteins regulate the effect of SNARES involved in fusion. For example, in our studies Rab8 regulates VAMP3 (involved in vesicle fusion) while Rab35 regulates the vesicle scission protein i.e. EHD1 and these proteins have been indicated in TNT formation (described in this thesis). This indicates that molecules affecting membrane fusion and scission regulates cell protrusions.

- Rabs and endosome recycling :

Several Rab proteins are involved in the recycling pathway. Rab11/EHD1 presence on the tubules is the indication ERC (Grant and Donaldson, 2009). Early endosomes lose Rab5 and acquires Rab11, and this result in the formation of the tubules by the early endosomes and the main body of the early endosomes gives rise to the multivesicular body. The ERC is usually situated near the golgi or MTOC (microtubule organizing centre) (Maxfield and McGraw, 2004). Recycling of endosomes involves rapid and slow recycling pathways. The rapid recycling occurs in endosomes that are Rab 4/5 positive, while slow recycling occurs in endosomes positive for Rab 4/11 (Hao et. al., 2000, Mayor et. al., 1993, Sonnichsen et. al., 2000).

The rapid recycling occurs by movement of the cargo from early endosomes directly to the plasma membrane. This pathway usually involves Rab4 and Rab35 and recycles transferrin, glycosphingolipids (Maxfield and McGraw, 2004, Kouranti et. al., 2006) MHC-receptors (Walseng et. al., 2008).

In slow recycling, cargo travels from early endosomes to the ERC, and then back to the plasma membrane. In mammals this pathway involves adaptor protein (AP2) protein and ARF6, usually this is involved in recycling of MHC-I (Lau and Chou, 2008). ARF6 also regulates the uptake and recycling of lipid rafts back to the plasma membrane (Balasubramanian et. al., 2007).

Several proteins and receptors are recycled with the help of Rab proteins. MHC-I is recycled by Rab11, Rab22a, EHD1 in association with ARF6 (Hattula et. al. 2006, Weigert et. al., 2004, Powelka et. al., 2004, Magadan et. al., 2006).  $\beta$ -integrins are recycled by Rab11, EHD1 (Powelka et. al. 2004, Jovic et. al., 2007), TFRs are recycled by Rab8, Rab22a (Hattula et. al. 2006, Weigert et. al., 2004, Magadan et. al., 2006), AMPA receptors by Rab 10 (Glodowski et. al., 2007).

The active form of Rab22a induces formation of large recycling tubules and accumulated vesicles. This stopped MHC-I recycling. Rab11a may co-ordinate different steps of MHC-I recycling (Weigert et. al., 2004). EHD1 regulates integrin recycling back to the plasma membrane. EHD1 is localized in the vesicles and maybe involved in cell spreading and migration (Jovic et. al., 2007). Similarly, EHD2 regulates GLUT4 receptor recycling back to the plasma membrane (Park et. al., 2004). EHD1 is also involved in recycling of the MHC-I receptors back to the plasma membrane in association with ARF6, EHD1 is tubule



inducing factor in this case, SNX-4 (Sorting nexin) proteins in the yeast whose absence allows the degradation of TFR (Traer et. al., 2007)

The recycling of T cell receptors depends upon the lipid rafts, protein tyrosine kinase activity and clathrin coated pits in cells. This process depends on the recycling of these complexes and its interaction with the ligands (Monjas et. al., 2004, Liu et. al., 2000).

- Rabs and Cilia formation:

Cilia are cellular projections that interpret and transmit a variety of extracellular signals. Cilia are structures made up of microtubules; their cell surface is made of molecules like smoothened, GPCRs, somatostatin and rhodopsin. Cilia formation involves both the polarized membrane trafficking and microtubule organization (S Sorokin, 1962). Cilia forms in a stepwise manner where the centrioles form the basal bodies, and they dock on the cell cortex to induce the formation of cilium. Assembly of cilia involves protein trafficking from the cytoplasm to the base of the cilium (Ishikawa and Marshall, 2011). Ciliary defects are associated with a wide variety of disorders such as retinal degeneration, Biedel Bardet syndrome etc. (Das and Guo, 2011).

Rab29 is localized at the base of cilia and regulates its formation by recruiting smoothened and interacting with Rab8, Rab11 and IFT20 (Intraflagellar Transport protein 20 homolog) and promoting ciliary growth (Onnis et. al., 2015, Vivar et. al., 2016). Rab8a regulates ciliogenesis in several different species such as mice, *C. elegans*, Zebrafish etc. Rab8a is usually localized to the base of the ciliary membrane (Westlake et. al., 2011). Rab8a works with Rab10 in a redundant manner. Rab8a interacts with Rab11 in a cascade mechanism involving Rabin8 to regulate cilia formation (Westlake et. al., 2011). Rab11 is regulated by ODF2/Cenexin protein and PI3K which generates phosphatidylinositol 3-phosphate (PI3P) (Hehnly et. al., 2012) and PI3P activates Rab11 (Franco et. al., 2014). Rab23 regulates cilia length by acting as inhibitor of cilia specific Shh (Sonic Hedgehog) signaling (Huangfu et. al., 2003, Yoshimura et. al., 2007, Lim and Tang, 2015). Rab28 is also involved in cilia formation and is localized to the ciliary body and at the root of the rat photoreceptor cells and regulates intraflagellar transport (Estrada-Cuzcano et. al., 2012, Jensen et. al., 2016).

- Rab and immunological synapse formation:

Immune synapse is the interface between T cells and antigen presenting cells, where the molecular interactions take place. It involves several receptors one of them is T cell receptor (M Dustin, 2014). T cell receptors (TCRs) are continuously taken up and recycled back to the plasma membrane. Upon encountering antigen presenting cells the T cells polarize towards the antigen presenting cell, and the recycling endosomes are localized towards the immunological synapse and exocytic t-SNAREs (soluble NSF attachment protein receptors) are accumulated at this site (Das et. al., 2004). Rab proteins are important for this function (Gorska et. al., 2009). In Jurkat T cells, EPI64C acts as GAP for Rab35 and regulates the TCRs and transferrin recycling (Patino–Lopez, 2008). Rab29 interacts with Rab8a, Rab11a and IFT20 for assembly of immune synapse and TCR trafficking. Rab11 recruits FIP3 (Rab11 Family of interacting proteins-3) to interact with Rac which induces actin remodeling for formation of immune synapse (Bouchet et. al., 2016). Rab8 and Rab11 interact with the SNARE protein VAMP3 (Vesicle associated membrane protein) for TCR trafficking to the immunological synapse, and formation of immune synapse (Finetti et. al., 2015). Transmembrane adaptor LAT (Linker for activation of T cells) is internalized by clathrin independent pathway, mediated by ARF6 via Rab27a and Rab37 vesicles (Soares et. al., 2013). The cytotoxic T lymphocytes need VAMP8 / Rab11 mediated vesicle fusion to fuse cytolytic granules with the membrane (Parker et. al., 2018). Immune synapse formation is regulated by Rab11 and myosinVb motor protein which further interact actin cytoskeleton (Gorska et. al., 2009).

- Rabs and disease :

Membrane trafficking is involved in many processes of the neurons, alteration in membrane trafficking has been implicated in neurodegeneration and cancer (Kiral et. al. 2018).

Neurodegenerative diseases: Alpha synuclein associated with lewy bodies are hallmark of Parkinson disease (Gitler et. al., 2008). Alpha synuclein is involved in vesicular trafficking and shown to interact with several different Rab proteins. Mutant alpha synuclein interacts with Rab5, Rab8 and Rab3a (Dalfo et. al., 2004). Alpha synuclein impairs ER-Golgi traffic and overexpression of Rab1 in these neurons rescues this effect and protects, loss of dopaminergic neurons (Cooper et. al., 2006). Further in dopaminergic neurons it was shown that Rab3a and Rab8a rescue the effect of alpha synuclein in mice (Gitler et. al.,

2008). Rab11 is involved in regulating the levels of A $\beta$  by controlling the recycling of beta secretase (Udayar et. al., 2013).

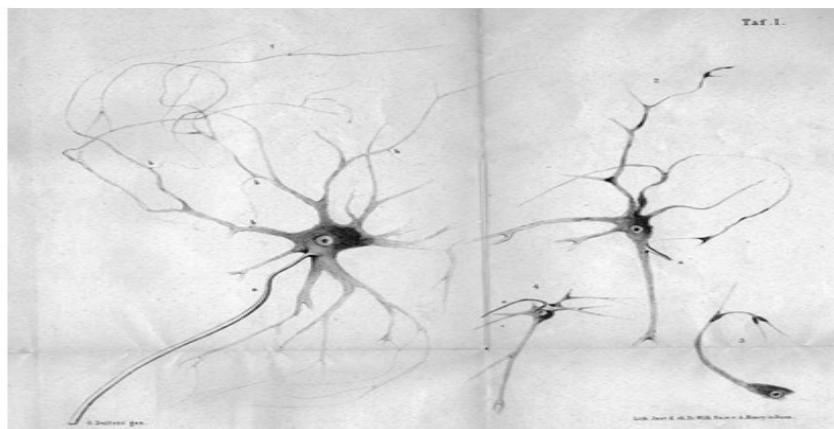
Huntington is autosomal recessive genetic disorder which is caused by expansion of the trinucleotide repeat of the huntingtin gene (htt). Huntingtin prevents the trafficking of Rab8 and disrupts clathrin mediated trafficking to the lysosomes (Sahlender et. al., 2005, del Toro et. al., 2009). Huntingtin also interacts with the Rab11. Overexpression of dominant negative form of Rab11 disrupted the motor functions in mice (Li et. al., 2008, Li et. al., 2009a, Li et. al., 2009b).

Mutations in Rab7 causes Charcot marie tooth syndrome 2b, which results in overexpression tenfold of Rab7 (Cherry et. al., 2013). In rare human disease Griscelli syndrome, mutations in Rab27a and myosinVa cause the accumulation of melanosomes due to inability of granule movement (Zerial and McBride, 2001).

Cancer: Membrane trafficking also plays an important role in cancer due to loss of cell polarity and involved in enabling metastasis of cells (Mosesson et. al., 2008). Rab25 is upregulated in breast cancer and ovarian cancer (Cheng et. al., 2004).

b) Role of Rabs in polarization: The case of neuronal polarization:

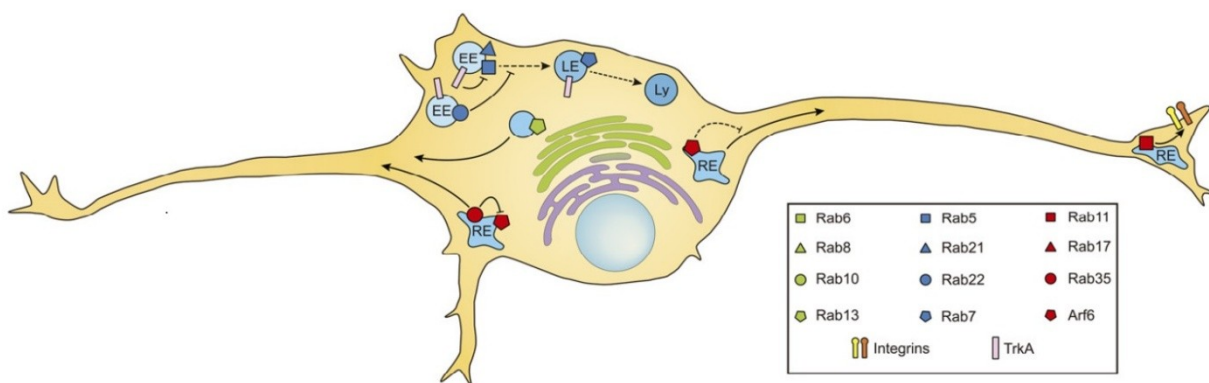
Otto Friedrich Karl Deiters (1834–1863) gave the first description of neurons as “The cell body continues without interruption into a more or less large number of processes that run in long courses, often branching repeatedly and extending finally into immeasurably fine processes called protoplasmic processes (the dendrites). These are immediately distinguishable from an outstanding single process that arises from the cell body or from the root of one of the larger protoplasmic processes (the axons).” This is referred in Figure 11. More detailed description of the neuron was given by Cajal in 1899, as “Every neuron, then, possesses a receptor apparatus, the body and the dendritic prolongations, an apparatus of emission, the axon, and an apparatus of distribution, the terminal arborization of the nerve fiber.”



**Figure 11: Neurons:** The drawings in are from the spinal cord gray matter at 300–400 times magnification (Otto Friedrich Karl Dieters)

The neurons contain two different structures as the axons and the dendrites. Cultured hippocampal neurons undergo different stages of development from stage 1 to stage 5, from bearing immature neurites to forming fully functional neurons bearing axons and dendrites (Craig and Banker, 1994, Dotti et. al., 1988).

Neurons are polarised cells. They contain polarized structures as multiple dendrites and a single axon; they need specific proteins to maintain their polarization. These proteins are synthesized in the somatodendritic regions and are trafficked along the intracellular trafficking machinery to maintain neuronal polarity (Bentley and Banker, 2016). This indicates the importance of vesicle trafficking in regulating the neuronal polarization.



**Figure 12: Membrane trafficking contribution to neurite elongation: in neuroblastoma.** Rab GTPases from TGN derived vesicles (green), early/late endosomes (blue) and recycling endosomes (red) are represented in a neuroblastoma cell line. The identity of several Rabs involved in membrane trafficking is illustrated by triangles, squares, circles, and pentagons. The vesicle routes promoting neurite outgrowth are represented by continued lines, whereas inhibitory activities are shown as dashed lines (Villaroel-Campos, 2014).

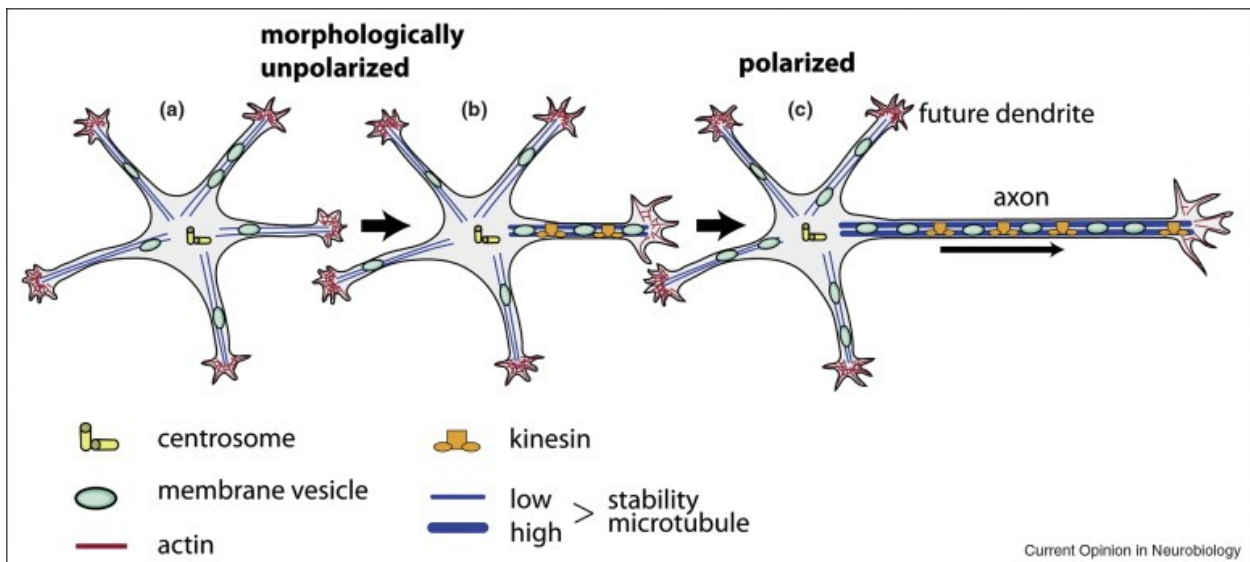
Neuronal cells and epithelial cells may share a similar mechanism of polarization. This was first proposed by Dotti and Simons in 1990, in MDCK cells, the vesicular stomatitis virus (VSV) G protein, is delivered to the basolateral surface, whereas the influenza fowl plague virus (FPV) hemagglutinin (HA) is delivered apically similarly these proteins are segregated in mature hippocampal neurons where (VSV) G protein was sorted somatodendritically while the (FPV) HA protein was directed to the axons.

Neuronal polarity and the formation of axons and the dendrites require recycling endosomes (Horton and Ehlers, 2003) Figure 12. There are two models which describes recycling pathways involved in neurons, the first model proposes sorting of the vesicles from the TGN-RE (Trans-Golgi network-Recycling endosome) compartment either to axons or dendrites. Usually TFRs are targeted in a selective manner to the dendrites, while the axonal protein NgCAM ((neuron—glia CAM)/L1) is transported to both the axons and dendrites (Burack et. al., 2000). Microtubule polarity may be responsible for the transport of vesicles towards specific compartments (Baas, 1998). Thus, both the recycling pathway and the cytoskeleton are involved in maintaining the polarity of neurons.

The second model proposes a specific fusion event in which the molecules or the receptors can be transported both to the axons and the dendrites but they can fuse only in the axon, as the protein NgCAM (Burack et. al., 2000, Sampo et. al., 2003).

Along with directing proteins for maintaining axons and dendrites, vesicle trafficking is also involved in formation of neuronal structures such as neurite outgrowth as shown in Figure 13, formation of dendritic spines etc. Several proteins involved in vesicle trafficking are also involved in the formation of neuronal processes. For example, exocyst complex is one of the major components of the recycling endosomes, especially Sec 6/8 is localized at the growth cones and the neurites (Hazuka et. al., 1999). Rab11 and TFRs are present at the base of dendritic spines under normal conditions in neurons (Schmidt and Haucke, 2004). Rab11 is regulating localisation of N-cadherin in neurons in turn regulating polarisation of the neurons (Barnat et. al., 2017). It is also reported that Rab5 acts upstream of Rab11 in regulating the migration of neurons, while the Rab7 regulates the late stage of migration of neurons (Kawauchi et. al., 2010). In the post mitotic cultured neurons, downstream of Ras protein PI3K action regulates the levels of PIP3 in cells. Overexpression of active subunit of PI3K leads to the formation of axons. PTEN (phosphatase and tensin homolog deleted on chromosome 10) is known to act on the neuronal polarisation and its effect is opposite of PI3K since it converts PIP3 to PIP2 and increases its levels. Over expression of PTEN in neurons leads to the decrease in

formation of axons. Recycling pathway is important for maintaining and formation of the dendritic spines (Park et. al., 2006).



**Figure 13: Mechanisms of neuronal polarization.** Initially, all neurites are assumed to be equal. a) Cytoskeleton is changes occur before polarization. b) These cytoskeletal changes are present in the axons and kinesin one shows more preference to the future axons. c) The cytoskeleton is more dynamic and giving rise to the growth cone in one of the neurite, the microtubule motor proteins are trafficked along axon (Witte and Bradke, 2008).

### **Chapter 3: Overview of the Rabs and downstream pathways involved in TNT regulation**

#### **1) Rab8a:**

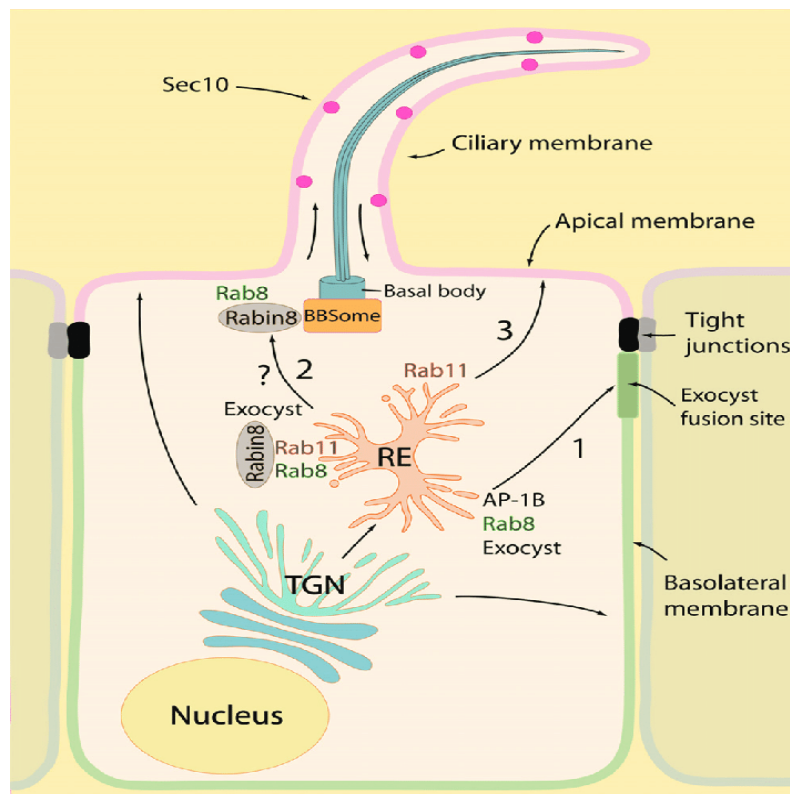
Rab8a is localized at the basolateral membrane, golgi and tubulovesicular structures. It is involved in recycling of endosomes and basolateral transport of membrane proteins (Lee et. al., 2003). Rab8a colocalises with several different proteins as ARF6, Rab11 (Hattula et. al., 2006)  $\beta$  integrins under polarized conditions (Peranen et. al., 1996) and IFT20 (Finetti et. al., 2015). Rab8a is involved in vesicle trafficking and its effects, such as exocytosis (Huber et. al., 1993, Hattula et. al., 2006), membrane recycling and polarisation of cells, see Figure 14. For example, overexpression of active form of Rab8a shows increase in formation of tubular and reticular structures in cells. Rab8a interacts with EHBP1L1 (EH domain-binding protein 1-like 1), Bin 1 and dynamin complex to regulate apical membrane transport in rat intestinal cells (Huber et. al., 1993). Rab8a is involved in the recycling of T cell receptors in immune cells (NIH3T3 cells) (Finetti et. al., 2015). It colocalizes with and is involved in the transfer of membrane protein perforin to the immunological synapse (Lesteberg et. al., 2017).

Rab8a is involved in both membrane recycling and formation of cellular structures. Rab8a positive tubules are involved in membrane formation at membrane ruffling and the formation of protrusions, by recycling vesicles back to the membrane. Knockdown of Rab8a results in formation of stress fibers (Hattula et. al., 2006).

Formation of Rab8a positive intracellular tubules depends on actin and microtubules in the cells (Huber et. al., 1993). Rab8a has been shown to have an effect on both microtubules and actin reorganization and induced the formation of cellular protrusions in BHK (Baby hamster kidney) cells similar to lamellipodia and filopodia (Peranen et. al., 1996). Overexpression of dominant negative form of RhoA in cells increased the presence of tubular structures positive for Rab8a inside the cellular protrusions. Importantly, these Rab8a positive vesicles were shown to fuse with the plasma membrane protrusions giving rise to filopodia (Hattula et. al., 2006). Under polarized conditions active Rab8a is recycled back slowly. This allows it to form complexes with its effectors at the plasma membrane and enables its interaction with actin. This results in formation of tubulovesicular structures into cellular protrusions (Peranen et. al., 1996). Over expression of inactive form of Rab8a shows an increase in the stress fibers but the active form of Rab8a shows redistribution of actin filaments in the cells. It was also shown that Rab8a localises with actin in lamellopodia like structures formed in the cells overexpressing Rab8a. However, the



mechanisms responsible for the inter-regulation of Rab8a and actin is unknown (Peranen et. al., 1996).



**Figure 14: Role of Rab8 and Rab11 in cell:** The sorting of biosynthetic cargoes to different plasma membrane domains takes place either at the TGN or in recycling endosomes in polarized epithelial cells. Vesicular trafficking to the cilium may originate in recycling endosomes where Rab8 or Rab11 or both may regulate the organization of distinct subdomains. The exocyst has been shown to play a role in vesicular trafficking from the recycling endosomes (RE) to the basolateral membrane (arrow 1) as well as in trafficking to the cilium in association with Rab8, Rabin8, and Rab11 (arrow 2). Vesicles may be directed to the base of the cilium by the interaction of Rabin8 and BBS1. Rab11 also has a known role in sorting to the apical membrane (arrow3) (Kang and Folsch, 2009).

## 2) Rab11a:

Rab11 is involved in transport of recycling endosomes back to the plasma membrane (Das and Guo, 2011). Rab11a is enriched at the base of the cilia and is involved in the maintenance of the primary cilia (Knodler et. al., 2010), as shown in Figure 14.

Rab11a-Rab8a with the help of Rabin8 and Cdc42 regulates epithelial lumenogenesis (Bryant et. al., 2010). GTP bound Rab11 colocalises with and recycles transferrin positive endosomes in CHO, BHK, and COS1 cells (Ullrich et. al., 1999, Ren et. al., 1998, Naughtin et. al., 2010). Rab11 is associated with MVBs and is involved in fusion of the autophagosomes to MVBs (Fader et. al., 2009).

Importantly Rab8 and Rab11 are involved in cilia formation with the help of Rabin8 (GEF of Rab8a). Rabin8 is shown to be the link between Rab11 dependent activation of Rab8a and the two Rabs act in a cascade that leads to the formation of cilia (Knodler et. al., 2010). In my thesis work (together with another student) we found a role for Rab11a and Rab8a in TNT formation (see below and results). However, Rabin8 does not have an effect on the formation of the tunneling nanotubes in CAD cells (Zhu et. al., 2018). GRAB (GRAB/Rab3IL1 (guanine nucleotide exchange factor for Rab3A; Rab3A interacting protein (rabin3)-like 1), which acts in a similar manner to Rabin8. It acts as a downstream effector of Rab11a and Rab8a and is involved in axon outgrowth (Furusawa et. al., 2017). Rab11 interacts with GRAB and overexpression of Rab11 causes GRAB to shift to the membranes (Horgan et. al., 2013).

In my thesis work we showed that GRAB, is involved in the formation of the TNTs. However, it was not involved in the cascade mechanism for the regulation of TNTs (Zhu et. al., 2018).

### 3) VAMP3:

The targeting, docking and fusion of vesicles with the acceptor membranes require interaction with actin and microtubule cytoskeleton, recruitment of cytosolic docking complexes and specific recognition between integral membrane proteins known as v- and t-SNARES. SNARE proteins are divided into distinct 'v' and 't' snares on the basis of their cellular localization. v-SNAREs are vesicle localized while the t-SNAREs are localized to the target vesicles. But this nomenclature is not specific since v-SNAREs are also localized at the target vesicles while the t-SNAREs are localized to the vesicles (Ungermann and Langosch, 2005). SNARE proteins are involved in phagocytosis, exocytosis, cell spreading, and integrin recycling (Hackam et. al., 1998, Skalski et. al., 2005). VAMP3 (Vesicle associated membrane protein 3) belongs to the family of SNARE proteins (Banerjee et. al., 2017).

The major role of VAMP3 is in regulation of membrane recycling from endosome to the plasma membrane, as I explain in the examples cited below. VAMP3 regulates the recycling and intracellular distribution of  $\alpha 5\beta 1$  integrins and therefore affects cell migration and lamellopodia formation in CHO-K (Chinese Hamster Ovary) cells (Tayeb et. al., 2005). In platelet cells, megakaryocytes, VAMP3 is involved in fibrinogen recycling (Banerjee et. al., 2017). In dendritic cells, MHC-I is concentrated to Rab11a, VAMP3/Cellulobrevin positive endosomes at the ERC (Nair-Gupta et. al., 2014). In NK cells recycling endosomes are involved in the secretion of granules, thus upon inactivation of Rab11 or VAMP3, cells are unable to recycle the cytokines back to the plasma membrane. VAMP3 also regulates the fusion of the tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) from the recycling endosomes to the plasma membrane (Murray et. al., 2005). VAMP3 is localized near phagocytic cups and helps in engulfment of organism to be phagocytosed (Bajno et. al., 2000). Rab8a, Rab11a and IFT20 (Intraflagellar Transport protein; involved in the formation and maintenance of primary cilium) and act in concert for the recycling of T cell receptors. The IFT20 and the Rab8 positive vesicles colocalise with each other but VAMP3 is necessary for vesicles to fuse with plasma membrane and then undergo recycling. Furthermore, Rab8 was shown to be involved in the recruitment of VAMP3 (Finnetti et. al., 2015). VAMP3 is also involved in trafficking of the integrins from Rab11 positive recycling endosomes (Skalski et. al., 2005).

VAMP3 is also shown to interact with the smoothened (transmembrane protein found in the cilia, necessary for signal transduction) and Rab8a in ciliary growth (Finnetti et. al.,

2015). Cilia usually forms in a stepwise manner where the centrioles form the basal bodies, and they dock on the cell cortex to induce the formation of cilium. Assembly of cilia involves protein trafficking from the cytoplasm to the base of the cilium (Ishikawa and Marshall, 2011).

#### 4) Rab35 and its interactors:

Rab35 was first cloned from the human skeletal muscle and is ubiquitously expressed. It was described as 201 amino acid containing protein, that exhibits homology to Rab1A, Rab1B, and YPT1 at its N terminus and was initially named as H-ray protein (Zhu et. al., 1994). Due to its similarity to Rab1 protein, it was renamed to Rab1C, but eventually it came to be known as Rab35 (Klinkert and Echard, 2016). Rab35 is the only Rab protein that localizes both to the plasma membrane and the endosome (Klinkert and Echard, 2016). It is involved in cytokinesis and fast endocytic recycling in cells. Rab35 regulates the recycling of various receptors such as MHC-I, MHC-II, TCRs, N-Cadherin, TFRs (Dikshit et. al., 2014).

Rab35 has several effector proteins such as OCRL (Oculo-Cerebro-Renal syndrome of Lowe), ACAP2 ( $\beta$ -centaurin), and MICAL-L1 (molecules interacting with CasL-Like 1) (Dambournet et. al., 2011). Rab35 in its GTP bound state binds to lipids, phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) at the plasma membrane (Heo et. al., 2006). Rab35 is necessary for recycling of the synaptic vesicle proteins through ESCRT (endosomal sorting complexes required for transport) machinery, which enables the degradation of synaptic vesicle proteins (Sheehan and Waites, 2019). The GTP bound form of Rab35 directly binds to the enzyme OCRL. OCRL hydrolyzes PIP2 to PI4P (Phosphatidylinositol-4-phosphate). Mutation in OCRL causes the disease known as OCRL syndrome (Dambournet et. al., 2011). Depletion of either OCRL or Rab35 leads to delays in trafficking of the internalized mannose-6-phosphate receptor to the TGN. Rab35 in its GTP bound state binds to PIP2 and PIP3 at the plasma membrane (Heo et. al., 2006).

The presence of large vacuoles at the cytokinetic bridge suggests the role of endocytic recycling in cytokinesis in drosophila S2 cells and HeLa cells. Rab35 is involved in fast endocytic recycling and its downregulation resulted in the accumulation of the endocytic markers in the various vacuoles throughout the cells leading to failed cytokinesis. It was subsequently shown that Rab35 is involved in localisation of PIP2 and Septin to the cytokinetic bridge (Kouranti et. al., 2006). ARF6 negatively regulates Rab35 activation and its role in cytokinesis through its effector EPI64B (Chesneau et. al., 2012). Kobayashi and Fukuda, in 2013, showed that NGF (Nerve Growth Factor) activation induces neurite outgrowth due to Rab35 association to the ARF6 positive endosomes. Rab35 then recruits ACAP2 and MICAL-L1 to form a complex. MICAL-L1 directly acts as a binding site for

EHD1. While ACAP2 indirectly regulates the recruitment of EHD1 to the membrane by inactivating ARF6 and thereby influencing the levels of PI4P on the membrane of the vesicles which allows for the binding of the EHD1 to the vesicle membrane, and EHD1 then acts to regulate the neurite outgrowth.

#### a) MICAL-L1:

MICAL (molecules interacting with CasL) proteins cause F-actin depolymerisation which is NADPH dependent. This action due to N-terminal FAD-containing domain catalyzes a strong NADPH oxidase activity in free state. MICAL-L1 (MICAL Like proteins) proteins are homologous to these proteins but are encoded by the different genes. They interact with actin cytoskeleton, but they lack the NADPH oxidizing activity. MICAL-like proteins are commonly involved in membrane recycling (M. Vanoni, 2017). MICAL-L1 proteins have a calponin homology (CH), LIM, proline rich and coiled-coil domains, and are associated with late endosomes and tubular recycling endosomes (Abou-Zeid et. al., 2011, Bahl et. al., 2016).

MICAL-like proteins have the binding capacity for a broad range of proteins including Rab8a/b, Rab10, Rab13 and Rab15. MICAL-L1 interacts with Rab-EHD complex of proteins (M Vanoni, 2017).

Loss of MICAL-L1 leads to impairment in recycling of transferrin and integrin receptors back to the plasma membrane (Sharma et. al., 2010). MICAL-L1 links Rab8a and EHD1 and regulates the recruitment of EHD1 to the membrane tubules (Sharma et. al., 2009). MICAL-L1 interacts with EHD1 and GRAF1 (GTPase Regulator Associated with Focal Adhesion Kinase) at TREs (tubular recycling endosomes) in cells (Cai et. al., 2014). It is involved in the formation of elongated tubular endosomal network in the human dendritic cells (Compeer and Boes, 2014). MICAL-L1 also recruits EHD1 molecules to the recycling endosomes. MICAL-L1 along with another BAR (Bin, Amphiphysin and Rvs) domain protein syndapin-2 (also known as PACSIN-2) binds to phosphatidic acid, and this leads to the stabilization of the two proteins and helps in the nucleation of the recycling endosomes by the syndapin-2 (Giridharan et. al., 2013). Loss of MICAL-L1 has a role in trafficking of the EGF- receptor back to the plasma membrane, though it has no effect on late endosomes (Abou-Zeid et. al., 2011).

Rab35 regulates the localisation of MICAL-L1 and Rab8 to the ERC membranes by ARF6 and MICAL-L1. MICAL-L1 binds to its partner CRMP2 (Collapsin Response Mediator Protein-2; CRMP2 helps the vesicles bind to the microtubule motor protein dynein and

kinesin) and directs the vesicles to membranes (Giridharan et. al., 2012, Rahajeng et. al., 2010). MICAL-L1 interacts with GTP bound form of Rab35 and Rab10 through its coiled-coil region (Rahajeng et. al., 2012). Rab35 has a role in the interaction with MICAL-L1 and this recruits Rab8a on the tubular endosomes by MICAL-L1. MICAL-L1 is regulated by ARF6. Overexpression of activated ARF6 in the cells resulted in the recruitment of MICAL-L1 in the tubular endosomes (Rahajeng et. al., 2012).

Loss of MICAL-L1 leads to loss of focal adhesion turnover, cell migration and cell spreading. MICAL-L1 also recruits EHD1 to Src positive endosomes and is involved in the recycling of the Src protein (non-receptor tyrosine kinase) back to the plasma membrane (Reinecke et. al., 2014). Impaired recycling endosome is responsible for production of binucleated cells during the cytokinetic process, and MICAL-L1 and EHD1 are two components whose depletion leads to this phenomenon, along with the loss of Rab11 on these intercellular bridges and they have formed abnormally large intercellular bridges. Loss of MICAL-L1 leads to the elongation of spindle fibers during the cytokinetic process in the cells (Reinecke et. al., 2015).

#### b) ACAP2:

ACAP2 (Arf GAP with coiled coil, ANK repeat and PH domain) or Centaurin  $\beta$ 2 is found in a wide variety of multicellular organisms ranging from *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana* to mammals including humans and mouse. It is expressed in different tissues in humans such as amygdala, retina, and testis. It is usually localized at the cell periphery (Jackson et. al., 2000).

It has been shown that the activity of ACAP2 is dependent on PIP2 and phosphatidic acid levels in cells (Jackson et. al., 2000). Rab35 is involved in early stages of phagocytosis, specifically in the pseudopod extension. In *C. elegans*, a homolog to mammalian ACAP1 and ACAP2 i.e. CNT1 (Centaurin 1) is recruited by Rab10 in the intestinal epithelium and is necessary for regulating the PIP2 levels in the recycling endosomes (Shi et. al., 2012).

Rab35 downregulates the effects of ARF6 through ACAP2 and its recycling-based effects on the  $\beta$  integrins and EGF receptors and this has an effect on cell adhesion and migration (Allaire et. al., 2013). ACAP2 is also shown to mediate the transcytosis of the apical marker of polarized cell i.e. podocalyxin in 3D culture model of MDCK cells (Mrozowska and Fukuda, 2016).

### c) ARF6:

ARF GTPases are small proteins of 21kDa. They are divided into three classes, class-I includes ARFs 1–3, class-II includes ARFs 4 and 5, and class-III ARF includes ARF6 (Jackson and Casanova, 2000). ARF6 was discovered in the 1980s in rabbit liver membranes as a protein that localises with and acts as a cofactor of Cholera toxin B by activating adenylate cyclase activity (Kahn et. al., 1984).

ARF6 GEFs belongs to ARNO/cytohesin and EFA6 (exchange factor for ARF6) families (Jackson and Casanova, 2000) or Arf-GEP100 (Guanine Nucleotide Exchange Protein for ADP-ribosylation Factor 6) (Someya et. al., 2001). ARF6 GAPs includes Git1, ACAP2 etc. (Jackson et. al., 2000).

ARF6 is involved in different steps of vesicle trafficking. Clathrin coat formation is dependent on the elevated levels of PIP2 in the cells. Levels of PIP2 is dependent on the activation of PIP5 kinase by ARF6 (Krauss et. al., 2003, Honda et. al., 1999). When the cells overexpress the active form of ARF6 i.e. GTP bound form of ARF6, this leads to formation of PIP2 positive actin coated vesicles. In turn, this impairs the recycling of beta integrins, plakoglobins, MHC-I back to the plasma membrane as they are trapped in these vesicles. Thus, cycling of ARF6 between active and inactive forms is important for the recycling of vesicles back to the plasma membrane (Brown et. al., 2001). Phospholipase D (PLD) interacts with phosphatidylinositol-4-phosphate 5 (PI4P) kinase to convert PI4P to PIP2. This PIP2 in turn is necessary for the activity of PLD. ARF6 mutants which have inability to activate PLD show defects in the recycling and failure to form protrusions by cells (Jovanovic et. al., 2006). ARF6 is involved in non clathrin dependent endocytosis and recycling back to the plasma membrane. It is involved in recycling of actin regulating proteins such as Cdc42 and Rac and PAR6 complex (Donaldson and Jackson, 2011). ARF6 is involved in the internalization of the integral membrane proteins independent of AP2 proteins, such as IL-2 receptor alpha subunit Tac (Radhakrishna and Donaldson, 1997, Peters et. al., 1995, Cavenagh et. al., 1996). Fc  $\gamma$  receptor mediated endocytosis depends on ARF6 (Zhang et. al., 1998, Niedergang et. al., 2003).

ARF6 is involved in regulating the cytoskeleton and its activity. As consequence, ARF6 has been shown to be involved in cell spreading (Song et. al., 1998). Activated form of ARF6 has a role in forming protrusive membrane structures that are not particularly rich in F-actin (Brown et. al., 2001). ARF6 colocalizes with Rac1 at the plasma membrane. The over expression of both ARF6 and Rac1 induces formation of dorsal ruffles and actin



mediated protrusions. ARF6 plays a role in Rac1 mediated formation of lamellipodia by affecting the recycling Rac1 to the plasma membrane. In addition, ARF6 also has an independent capability of altering the cortical actin cytoskeleton upon activation, independent of Rac1 (Radhakrishna et. al., 1999).

Protein kinase C acts through ARNO, which is a GEF for ARF6 and regulates reorganization of actin and the formation of membrane protrusions (Frank et. al., 1998). ARF6 activates PIP5 kinase, and this activation requires phosphatidic acid that is the product of phospholipase D, at the ruffling membranes (Honda et. al., 1999, Brown et. al., 2001, Cockcroft et. al., 1994). ARF6 forms lamellopodia through ARNO and also affects cellular motility (Santy et. al., 2001). It is involved in dendritic branching and neurite outgrowth, through ARNO (Hernandez-Deviez et. al., 2002). It is also involved in cell polarity, and maintenance of adherens junctions in cells (Palacios and D'souza-Schorey, 2003, Palacios et. al., 2002).

#### d) EHD1:

Endocytic recycling involves the return of the membranes and receptors back to the plasma membrane, and this process involves the tubular and vesicular vesicles carriers in the cells where they are easily transported back to the plasma membrane through the microtubules. EHD family of proteins was found to be important in endocytic recycling and they are predominantly associated to the ERC (Cai et. al., 2013). EHD family of proteins is homolog to Eps15 protein. They contain the EH domain (Eps15 homology domain) that is used to interact with the phosphatidylinositols (Posey et. al., 2014). They have a molecular weight of 60 kDa.

Rabenosyn 5 interacts with EHD1 to recycle TFR or MHC-I back to the plasma membrane (Naslavsky et. al., 2004a). EHD proteins are involved in the tubule formation in muscle cells and regulates the fusion of myoblasts. Loss of EHD1 results in mislocalisation and defective recycling of key muscle proteins. EHD1 regulates BIN1 mediated tubule formation (Posey et. al., 2014). EHD1 is involved in spermatogenesis and, lens development in mice. Its deletion in lens tissue resulted in decreased epithelial proliferation, reduced expression of E-cadherin and adherens junction proteins (Arya et. al., 2015). EHD1 and Rab11 act together in recycling of the AMPAR to the membrane. EHD1 has also has a role in the movement of NgCAM from the somatodendritic region to the axon (Moore and Baleja, 2012).

It was shown that EHD1 directly colocalised with smoothened (as mentioned before smoothened is involved in signal transduction and is primarily situated at the base of the cilia) and trafficked along cilium (Bhattacharyya et. al., 2016). EHD1 is necessary for the formation of proper spindle fibers during the early stages of cytokinesis (Reinecke et. al., 2015). EHD1 and EHD4 are involved in the vesiculation and recycling of the membranes decorated with MICAL-L1, while EHD3 is a membrane tubulating protein (Cai et. al., 2013). Similarly, EHD1 and EHD3 interacts with Rab11 and Rab8 cascade and regulating the initiation of cilia formation, along with EHD1, SNARE protein namely SNAP29 is needed for the formation of the vesicles assembled at the base of the cilia which eventually results in the growth of cilia (Lu et. al., 2015).

EHD1 has been shown to be involved in several diseases. EHD1 is upregulated in the spinal cord as well as neurons, microglia and astrocytes, in rat model of Spinal cord Injury. EHD1 induced the up regulation of Trk A and the recycling of TrkA, which induces the uptake of NGF in the neurons which is important for neurite outgrowth (Wu et. al., 2016).

Table 4: General overview of Rab proteins and their effectors.

Protein	Function	Binding/ Interacting partners	Reference
Rab8a	Exocytosis, membrane recycling, polarisation of cells	ARF6, Rab11, IFT20	Lee et. al., 2003, Hattula et. al., 2006, Finetti et. al., 2015
Rab11	Vesicle recycling, cilia formation	Rab8a, Rabin8, Cdc 42, GRAB	Das and Guo, 2011, Knodler et. al., 2010 Bryant et. al., 2010, Furusawa et. al., 2017
VAMP3	Cilia formation, Integrin receptor recycling	Rab8a, smoothened, Rab11	Finetti et. al., 2015, Skalski et. al., 2005
Rab35	Fast endocytic recycling, cytokinesis	OCRL, ACAP2 ( $\beta$ -centaurin), and MICAL-L1	Dambournet et. al., 2011, Klinkert and Echard, 2016
MICAL-L1	Actin cytoskeleton interaction, membrane recycling	Rab8a/b, Rab10, Rab13, Rab35, Rab15, EHD1	Rahajeng et. al., 2012, M. Vanoni, 2017, Abou-Zeid et. al., 2011, Bahl et. al., 2016).
ACAP2	Vesicle recycling, cell adhesion and cell migration	Rab35, ARF6	Allaire et. al., 2013, Jackson et. al., 2000
ARF6	Non clathrin dependent endocytosis, vesicle recycling	ARNO/cytohesin, Arf-GEP100, EFA6, Git1, ACAP2	Someya et. al., 2001, Jackson et. al., 2000, Donaldson and Jackson, 2011
EHD1	endocytic recycling	Rabenosyn 5, MICAL-L1, Rab11, smoothened	Naslavsky et. al., 2004a Moore and Baleja, 2012, Bhattacharyya et. al., 2016

## ***Section 2***

# **Aims and Objectives**

## **Aims and Objectives:**

Tunneling nanotubes (TNTs) are F-actin based intercellular membrane connections, non-adherent to substrate, which are able to transport cytoplasmic contents and cellular organelles between two connected cells. Their diameter ranges from 50-150 nm and their length can extend up to several hundred micrometers (Rustom et. al., 2004, Sartori-Rupp et. al., 2019). They transport a variety of different organelles and molecules like lysosomes, mitochondria, golgi, cellular vesicles, calcium, RNA etc. (Rustom et. al., 2004, Abounit et. al., 2016a, Lu et. al., 2017, Wang et. al., 2012).

TNTs are also involved in several disease conditions. They transport different pathogens like HIV (Eugenin et. al., 2008), bacteria (Sisakhtnezhad and Khosravi, 2015) and were shown to spread pathogenic protein aggregates causing neurodegeneration like prions, alpha synuclein, huntingtin, tau,  $\alpha\beta$  peptides etc. (Zhu et. al., 2015, Sharma and Subramanian, 2019, Wang et. al., 2011, Dieriks et. al., 2017, Dilsizoglu Senol et. al., 2019, Tardivel et. al., 2016, Costanzo et. al., 2013). More recently TNTs have been described to have an important role in intercellular communication between cancer cells and stroma and were proposed to be involved in promoting invasiveness of cancer cells (Lou et. al., 2012, Pasquier et. al., 2013).

TNTs can be formed by two different mechanisms 1) Cell protrusion mediated mechanism in which the cells form a filopodia like protrusion which eventually connects the two cells giving rise to a TNT (Gousset et. al., 2013, Rustom et. al., 2004) 2) Cell dislodgement mechanism: Two cells come into contact with each other and then form a functional TNT after moving apart (Onfelt et. al., 2004).

TNT like structures have been shown in a wide variety of cells like e.g. NK cells, macrophages, HEK cells, CAD, PC12, SH-SY5Y cells etc. (Yin et. al., 2017, Rustom et. al., 2004, Watkins et. al., 2005, Onfelt et. al., 2005, Gousset et. al., 2009, Gousset et. al., 2013, Sartori-Rupp et. al., 2019). Furthermore, TNTs can be formed between homocellular or heterocellular cocultures (Kretschmer et. al., 2019).

TNTs are regulated by several different proteins, several stress factors, actin modifying proteins or small GTPases. Stress conditions or signals like serum deprivation, exposure to UV light, increases in the levels of ROS in cells lead to an increase in TNT formation (Wang et. al., 2011, Abounit et. al., 2016, Wang et. al., 2015). Several proteins like M-sec, RalA, and LST1 are shown to regulate TNT formation (Hase et. al., 2009, D'Aloia et. al.,

2018, Schiller et. al., 2013). In addition, different actin modifying proteins have been shown to regulate TNTs like Focal adhesion kinases (FAK) and matrix metalloproteases (Sáenz-de-Santa-María et. al., 2017), Fascin (Lou et. al., 2012), MyosinX (Uhl et. al., 2019, Gousset et. al., 2013), Eps8 (Delage et. al., 2016), Rac and Cdc42 (Hanna et. al., 2017a). However, the mechanism of TNT formation is not completely understood. Several pathways could lead to TNT formation and that this could also be cell type specific (Abounit et. al., 2012, Marzo et. al., 2013, Gousset et. al., 2013).

### **Role of Rabs in TNT formation and vesicle transfer:**

Endocytic recycling involves the return of receptors back to the plasma membrane (Cai et. al., 2013). The membrane recycling process regulates the plasma membrane composition by maintaining the balance between endocytosis and exocytosis of lipids and protein components, this in turn contributes to several cellular processes such as cytokinesis, cell migration, polarisation and signal transduction (Grant and Donaldson, 2009).

Rab proteins are the master regulators of cellular functions. They are involved in functions such as vesicle trafficking, migration of cells and formation of protrusions like filopodia, lamellipodia. Rabs function through GTP bound (active) – GDP bound (inactive) cycle (Das and Guo, 2011). In my thesis I have contributed to show that Rab8a and Rab11a regulate TNT formation (Zhu et. al., 2018).

Rab8a is a major regulator of vesicle trafficking (Huber et. al., 1993, Hattula et. al., 2006). Rab8a positive tubules are involved in membrane formation at membrane ruffling and the formation of protrusions, by recycling back to the membrane. Rab8a positive tubules are also positive for ARF6 and Rab11 (Hattula et. al., 2006). In addition, Rab8a is also involved in the formation of different cellular structures such as immune synapse, cilia etc. (Lesteborg et. al., 2017, Lu et. al., 2015). Rab8a is also involved in the formation of cellular protrusions like filopodia and lamellopodia and cilia formation, by affecting actin and microtubule organization (Peranen et. al., 1996). Interestingly, knockdown of Rab8a results in formation of stress fibers (Hattula et. al., 2006).

Rab11 is involved in transport of recycling endosomes back to the plasma membrane (Das and Guo, 2011). Rab11a is enriched at the base of the cilia and is involved in the maintenance of the primary cilia (Knodler et. al., 2010). Rab8 and Rab11 are involved in cilia formation with the help of Rabin8 (which is a GEF of Rab8a) (Knodler et. al., 2010). GRAB (GRAB/Rab3IL1 (guanine nucleotide exchange factor for Rab3A; Rab3A interacting protein (rabin3)-like 1) is acting as a downstream effector of Rab11a and GEF of Rab8a,

was shown to be involved in axon outgrowth (Furusawa et. al., 2017). Overexpression of Rab11 causes GRAB to shift to the membranes (Horgan et. al., 2013). SNARES (soluble NSF attachment protein receptors) are involved in phagocytosis, exocytosis, cell spreading, and integrin recycling in cells (Hackam et. al., 1998, Skalski et. al., 2005). VAMP3 (Vesicle associated membrane protein 3) belongs to the family of SNARE proteins (Banerjee et. al., 2017). Rab8a, Rab11a and IFT20 act with each other for the recycling of T cell receptors. The IFT20 and the Rab8 positive vesicles colocalise with each other but VAMP3 is necessary for vesicles to fuse with plasma membrane and then undergo recycling. Rab8 is important in the recruitment of VAMP3 (Finnetti et. al., 2015). VAMP3 is also involved in trafficking of the integrins from Rab11 positive recycling endosomes (Skalski et. al., 2005). VAMP3 is shown to interact with the smoothened and Rab8a in ciliary growth (Finnetti et. al., 2015). Another Rab protein shown to have a central role in vesicle recycling is Rab35. Rab35 is involved in fast endocytic recycling. It plays a key role in TFR recycling and cytokinesis (Kouranti et. al., 2006, Klinkert and Echard, 2016, Dikshit et. al., 2014). ARF6 (ADP Ribosylation Factor) negatively regulates Rab35 in cytokinesis (Chesneau et. al., 2012). EHD family of proteins have an EH domain (Eps15 homology domain) are homolog to the Eps15 protein. EHD family of proteins is associated to the ERC, and EHD1 and EHD4 are involved in the vesiculation and recycling of the membranes decorated with MICAL-L1 (Cai et. al., 2013). Loss of MICAL-L1 leads to the elongation of spindle fibers during the cytokinetic process in the cells (Reinecke et. al., 2015). Reduction of PI4P either by activation of PIP5KI-gamma expression or by Sac1-induced dephosphorylation of PtdIns4P to PtdIns, caused a reduction in EHD1 associated tubular membranes (Jovic et. al., 2007).

In an interesting study by Kobayashi and Fukuda, 2013 it was shown that NGF activation positively regulates neurite outgrowth due to the association of Rab35 to the ARF6 positive endosomes. Rab35 recruits ACAP2 and MICAL-L1 to form a tripartite complex. MICAL-L1 directly acts as a binding site for EHD1 on the PM, which then has a positive effect on the neurite outgrowth. Indeed, EHD1 facilitates pinching off the vesicles and therefore their recycling to the PM, thus providing the lipids and proteins necessary for the neurite outgrowth. In addition, this process is also facilitated by ACAP2 which indirectly regulates the recruitment of EHD1 to the membrane by inactivating ARF6. ARF6 inactivation leads to an increase of the levels of PI4P on the membrane of the vesicles, which allows for the binding of the EHD1 to the membrane and favors its action to promote neurite outgrowth. In the second part of my thesis I was interested in exploring whether this pathway could also have a role in TNT formation.

## **Objectives**

Following a screen made by a previous PhD student in the lab, who screened 41 Rabs for a possible role of these small GTPases in TNT formation, the objectives of my thesis are two-fold

- To characterize the specific Rabs involved in TNT formation.
- To uncover the downstream effectors of Rabs involved in TNT formation

Specifically, in the first part of my thesis I helped the characterization of the downstream pathway involved in the Rab11a, Rab8a cascade that regulates TNT formation, while in the second part I characterized the role of Rab 35 that was another positive hit for TNTs in the Rab screen.



## ***Section 3***

# **Results**

## **Article 1**

## **Title: Rab11a–Rab8a cascade regulates the formation of tunneling nanotubes through vesicle recycling.**

### **Summary of results and discussion:**

It has been shown that Rab proteins are involved in several cellular processes such as cytokinesis, formation of cilia, immune synapse etc. They are shown to be master regulators of cellular functions (Das and Guo, 2011). Thus, we screened several different Rab proteins to identify the ones involved in TNT formation. In this study Seng Zhu (previous PhD student in the lab) set up a screen for TNTs, based on the ability of overexpressed Rab proteins to increase or decrease TNT mediated vesicle transfer between a donor and acceptor cell population. Out of the 41 different Rab proteins screened, Rab8a, Rab11a and Rab35 were strong positive hits. Rab8a is involved in the formation of different cellular structures such as immune synapse and cilia formation (Lesteborg et. al., 2017, Lu et. al., 2015). Rab8a is also involved in the formation of cellular protrusions like filopodia and lamellopodia in the cells, by affecting actin and microtubule organization (Peranen et. al., 1996). Rab8 and Rab11 are involved in cilia formation with the help of Rabin8 (GEF of Rab8a) (Knodler et. al., 2010). Upon overexpression of each of these proteins it was observed that GTP bound forms of Rab8a and Rab11a were increasing TNTs. We also showed that they regulate TNTs through a cascade mechanism. However, while Rabin8 increased filopodia formation, we demonstrated that this was not the case for TNTs as Rabin8 overexpression did not affect TNTs. Another GEF of Rab8a, i.e. GRAB was shown before to be involved in axon outgrowth through the cascade mechanism by Rab11a-Rab8a (Furusawa et. al., 2017). Thus, we looked at the role of GRAB with respect to TNTs. We found that overexpression of GRAB leads to an increase in TNT connected cells, but this seems to be independent of Rab8-Rab11 cascade. On the other hand, we found that VAMP3 which is known to interact with Rab8 and to be responsible for the final fusion of vesicles back to the membrane (Finetti et. al., 2015) was involved. VAMP3 was also shown to interact with Rab8 at the base of the cilium and regulates ciliary outgrowth (Patrussi and Baldari, 2016).

The data presented here provide new insights into the factors involved in TNT formation and intercellular vesicle transfer in neuron-like CAD cells. Our results show that Rab11a, Rab8a and VAMP3 increase both the number of TNT-connected cells and the vesicle transfer through TNTs in CAD cells. We demonstrate that Rab11a and Rab8a work in the

same pathway and employ VAMP3 as an effector to induce functional TNTs but not filopodia. Of interest, this Rab11a-Rab8a signaling cascade does not require Rabin8, which on the other hand is essential to mediate Rab8a-activated filopodia formation. Thus, in addition to providing mechanistic details about the role of Rab proteins in TNT formation, these data also strengthen our hypothesis that filopodia and TNTs are different structures, which rely on different mechanisms of formation

Personal contribution:

I showed the role of GRAB proteins in the TNT formation. In addition, I performed all the experiments that the reviewers had asked to sustain the initial findings during the review process of the paper. I wrote the revised results and answered the reviewers.

## RESEARCH ARTICLE

# Rab11a–Rab8a cascade regulates the formation of tunneling nanotubes through vesicle recycling

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## ABSTRACT

Tunneling nanotubes (TNTs) are actin-enriched membranous channels enabling cells to communicate over long distances. TNT-like structures form between various cell types and mediate the exchange of different cargos, such as ions, vesicles, organelles and pathogens; thus, they may play a role in physiological conditions and diseases (e.g. cancer and infection). TNTs also allow the intercellular passage of protein aggregates related to neurodegenerative diseases, thus propagating protein misfolding. Understanding the mechanism of TNT formation is mandatory in order to reveal the mechanism of disease propagation and to uncover their physiological function. Vesicular transport controlled by the small GTPases Rab11a and Rab8a can promote the formation of different plasma membrane protrusions (filopodia, cilia and neurites). Here, we report that inhibiting membrane recycling reduces the number of TNT-connected cells and that overexpression of Rab11a and Rab8a increases the number of TNT-connected cells and the propagation of vesicles between cells in co-culture. We demonstrate that these two Rab GTPases act in a cascade in which Rab11a activation of Rab8a is independent of Rabin8. We also show that VAMP3 acts downstream of Rab8a to regulate TNT formation.

**KEY WORDS:** Rab GTPase, Tunneling nanotube, Vesicle recycling, Vesicle transfer

## INTRODUCTION

Tunneling nanotubes (TNTs) are thin membranous structures that connect distant cells. Observed in different cell types, TNTs are normally observed as straight membrane protrusions hovering above the substrate between two cells (Rustom et al., 2004). TNTs act as conduits between cells that allow the exchange of both cell-surface molecules and cytoplasmic content such as endosomes, mitochondria, endoplasmic reticulum and calcium ions (Abounit and Zurzolo, 2012; Marzo et al., 2012). TNTs can also be hijacked by different pathogens, including prions (Gousset et al., 2009; Zhu et al., 2017), bacteria (Onfelt et al., 2006) and viruses (Kadiu and Gendelman, 2011; Sherer and Mothes, 2008; Sowinski et al., 2008), thus participating in the propagation of a wide range of diseases. Interestingly, several misfolded proteins associated with neurodegenerative diseases, such as  $\beta$ -amyloid (A $\beta$ ), tau,  $\alpha$ -synuclein ( $\alpha$ -syn) and huntingtin polyglutamine aggregates, have been found

inside TNTs, supporting the hypothesis that TNTs are a preferential highway for the spreading of proteinaceous aggregates (Abounit et al., 2016a,b; Marzo et al., 2012; Victoria and Zurzolo, 2017; Wang et al., 2011).

TNTs principally comprise continuous actin filaments that are enclosed in a lipid bilayer (Austefjord et al., 2014). In some cells (e.g. macrophages), TNTs have been shown to also contain microtubules organized in bundles parallel to the major axis (Sanchez et al., 2017). The formation of TNTs can be impaired by the use of F-actin-depolymerizing drugs, such as latrunculin and cytochalasin D (Bukoreshtliev et al., 2009; Gousset et al., 2009; Rustom et al., 2004), suggesting that actin polymerization plays an important role in TNT formation. However, the mechanism underlying TNT formation is not completely understood and it is possible that different mechanisms are involved in different cell types (Abounit and Zurzolo, 2012; Gousset et al., 2013).

Time-lapse imaging studies have previously suggested two possible mechanisms of TNT formation: (1) actin-driven protrusion outgrowth (Abounit and Zurzolo, 2012; Reichert et al., 2016; Rustom et al., 2004) and (2) cell dislodgement (Davis and Sowinski, 2008; Rustom et al., 2004; Sowinski et al., 2008). Based on our current understanding, an intercellular bridge is established by the outgrowth of a filopodia-like protrusion containing F-actin from either one or both cells. After extension, the tip of the filopodia-like protrusion contacts the target cell directly (or through adhesion molecules) and could (or not) fuse with the receiving cell (Rustom et al., 2004). On the other hand, it has been proposed that when two cells come into physical contact with each other, they could either form an immune synapse (Dustin et al., 2010; Reichert et al., 2016) or fuse temporarily. After plasma membrane fusion, cells continue to migrate in opposing directions, stretching out the plasma membranes to form TNTs that could be formed by either one or two cells. Although the first mechanism is more common in immobile cells such as neurons, the latter is mainly found in motile cells such as macrophages and other cells of the immune system (Abounit and Zurzolo, 2012).

Several proteins controlling actin polymerization and depolymerization have been shown to play a role in filopodia formation. Among these proteins, the cell division control protein 42 homolog (CDC42), a small GTPase of the Ras superfamily, regulates actin polymerization through direct binding to the neural Wiskott–Aldrich syndrome protein (N-WASP), which subsequently activates Arp2/3, a protein complex that promotes actin branching (Higgs and Pollard, 1999). Treatment of Jurkat cells with the CDC42-specific inhibitor secramine A blocks TNT formation (Arkwright et al., 2010). Similarly, inhibition of CDC42 in HeLa cells results in the decreased formation of TNTs induced by the overexpression of M-Sec, whereas Rac1 inhibition shows no effect (Hase et al., 2009). On the other hand, in macrophages, both the CDC42 and Rac1 pathways are involved in TNT formation through WASP-dependent actin remodeling (Hanna et al., 2017). In

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neurons, the actin regulator proteins CDC42, insulin receptor substrate of 53kDa (IRS $\beta$ 53) and vasodilator-stimulated phosphoprotein (VASP) work as a network to increase filopodia formation (Disanza et al., 2013). Interestingly, we showed that the same network inhibits TNT formation in neuronal cells while increasing filopodia formation. These and other data suggest that the same actin modifiers might have opposite actions on filopodia and TNT formation, indicating that these two structures, although similar, are distinct organelles with different functions (Delage et al., 2016; Gousset et al., 2013).

In addition to actin polymerization, formation of cellular protrusions (TNT, filopodia, cilia, etc.) also requires vesicular traffic. Rab GTPases are considered to be master regulators of intracellular membrane trafficking (Fukuda, 2008; Stenmark, 2009). Rab GTPases are localized in different membrane compartments to control the specificity and directionality of membrane trafficking pathways, mostly related to the trafficking of vesicles. Of particular interest, Rab GTPases are also involved in the regulation of cytoskeleton dynamics, including the formation of cell protrusions (Diekmann et al., 2011; Klöpper et al., 2012; Villarroel-Campos et al., 2016). Rab8 localizes to the primary cilium membrane during ciliogenesis, and cilia formation is inhibited or promoted when a Rab8 dominant-negative or positive form is expressed, respectively (Follit et al., 2010; Nachury et al., 2007). This suggests that the activity of Rab8 is crucial for the biogenesis of cilia. Rab8 is functionally linked to ADP-ribosylation factor 6 (Arf6). When the active form of Arf6 is present, cell protrusions induced by Rab8 can be inhibited (Hattula et al., 2006), indicating that inhibition may be related to membrane trafficking via endosome recycling rather than through the trans-Golgi network (TGN) (Hattula et al., 2006; Villarroel-Campos et al., 2016). On the other hand, Rab11 has been shown to promote neuritogenesis, both in hippocampal neurons in culture and in PC12 cells differentiated with nerve growth factor (NGF) through its interaction with protrudin (Shirane and Nakayama, 2006). Rab11 also increases axon outgrowth, a process that requires remodeling of the actin cytoskeleton. In hippocampal neurons, depletion of Rab11 reduces axonal length, which is also controlled by cyclin-dependent protein kinase5 (Cdk5) via an inhibitory phosphorylation of lemur kinase 1 (LMTK1), which in turn can activate Rab11 (Takano et al., 2012).

We decided to investigate the role of Rab GTPases in TNT formation and therefore performed a screen by overexpressing 41 GFP–Rab GTPases in our neuronal model CAD cells, (Matsui and Fukuda, 2011; Tsuboi and Fukuda, 2006). Based on this screening, Rab8a and Rab11a seemed promising candidates so we chose to investigate their specific involvement in TNT formation. Although two recent independent observations indicated that Rab8a and/or Rab11a are involved in TNT formation, the specific mechanism was not investigated (Burtey et al., 2015; Zhu et al., 2016). Our results showed that Rab8a and Rab11a positively regulate TNT formation and function in vesicle transfer between connected cells, via their active GTP-bound form. We demonstrated that Rab11a and Rab8a act through a cascade pathway to regulate downstream v-SNARE VAMP3-mediated fusion, suggesting that they increase vesicle recycling to the cell surface in order to form TNTs. Importantly, we demonstrated that this Rab cascade also regulates filopodia formation in our cell model, but through different effectors. Thus, in addition to improving our knowledge about TNT formation, our data demonstrate that TNTs are specific novel structures that are different from filopodia and other cellular protrusions.

## RESULTS

### High-content screening reveals that Rab8a and Rab11a promote DiD-labeled vesicle transfer between cells

To determine whether and which Rab GTPases affect TNT formation, we set up a screen whereby 41 GFP–Rab GTPases were overexpressed in our CAD cell line model. The transfer of cargos between cells, which is the main function of TNTs, was used to perform a preliminary screen and subsequently to study the formation of TNTs. To perform the transfer experiments, donor cells were transfected with 41 different GFP–Rab GTPases and incubated with the nonspecific membrane dye DiD (Vybrant™ DiD), which is quickly internalized and labels all internal vesicular compartments. Acceptor cells were transfected with H2B–mCherry and co-cultured with donor cells in a 1:1 ratio (Fig. 1A) (Delage et al., 2016). After 16 h of co-culture in 96-well plates, cells were fixed and images acquired to analyze the percentage of acceptor cells containing DiD-labeled vesicles transferred from donor cells (Fig. 1A). Images were analyzed with Cellprofiler (Fig. 1B). The percentage of acceptor cells containing DiD-labeled vesicles from each condition was normalized to control condition (cells transfected with an empty vector tagged with GFP) (Table S1). GFP–myosin10 and GFP–VASP were employed as positive and negative controls, as they respectively increase and decrease TNT formation and vesicle transfer (Delage et al., 2016; Gousset et al., 2013). We used three additional conditions as controls for cell-to-cell contact-mediated transfer: (i) mixture, where cells were just mixed and not incubated in co-culture, which gives the background noise; (ii) filter, where cells in co-culture were separated by a Transwell filter that impairs cell-to-cell contact; and (iii) supernatant, where the supernatant of donor cells was challenged with acceptor cells cultured separately, which measures transfer through secretion (Fig. 1C–F). To call hits from 41 GFP–Rab GTPases, two threshold values (105% and 95%) were set to score all conditions. When the relative percentage was higher than 105%, a hit was scored as ‘1’; when a hit was lower than 95%, it was scored as ‘–1’; and when a hit was between 95% and 105%, it was scored as ‘0’ (Table S1). The score values obtained from the average of triplicates from four experiments were summed, and a comparative analysis of the 41 GFP–Rab GTPases tested was performed. Among others, cells overexpressing Rab8a and Rab11a increased vesicle transfer between cells. These results were intriguing, because both these Rabs are involved in formation of cilia and other cell protrusions (Cox et al., 2000; Eva et al., 2010; Hattula et al., 2006; Peränen, 2011). Furthermore, two independent reports suggest that Rab8 or both Rab8 and Rab11 might be involved in regulating TNT formation, although the mechanism was not explored (Burtey et al., 2015; Zhu et al., 2016).

### Rab11a positively regulates TNT formation and function via the active GTP-bound form

Rab11 localizes at the endocytic recycling compartment/recycling endosome (ERC/RE), the TGN and post-Golgi vesicles. It influences several cellular processes, including exocytic and recycling processes (controlling both secretion and composition of plasma membrane), cytokinesis, phagocytosis, cell migration, immunological synapse and primary cilia formation (Campa and Hirsch, 2017). To understand whether the increase in vesicle transfer is related to an increase in TNTs, we investigated the effect of Rab11a on TNTs by calculating the percentage of TNT-connected cells. Because no specific marker of TNTs is currently available, we quantified TNTs between cells (labeled with wheat germ agglutinin; WGA) (Delage et al., 2016). TNTs were identified in culture according to the current definition of TNTs as

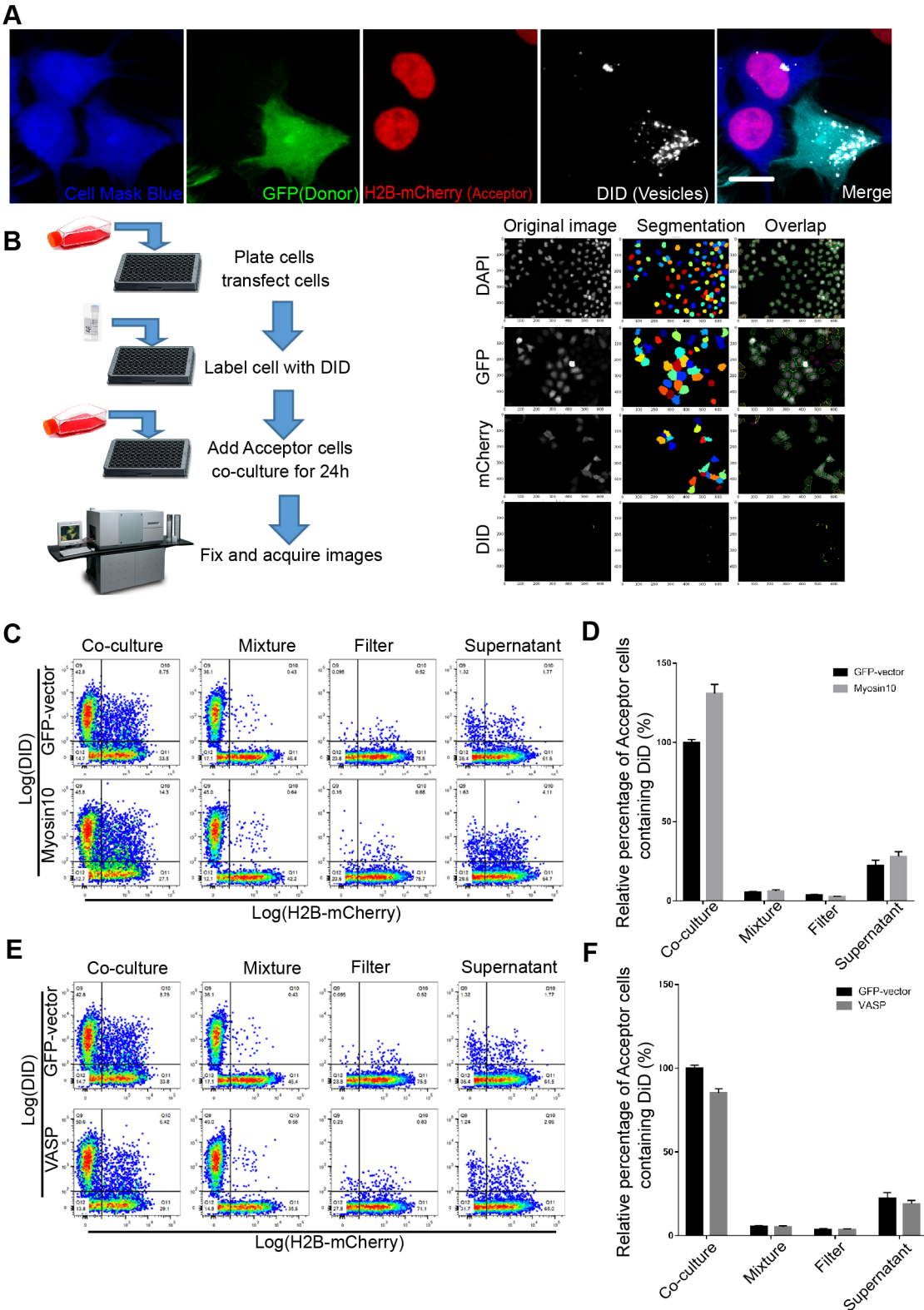


Fig. 1. See next page for legend.

membranous stretches between cells that do not contact the substrate (Fig. S1A) (Rustom et al., 2004). To rule out possible effects resulting from the distance between cells on TNT formation, we seeded different concentrations of cells (Fig. S1B) and determined the range of cell concentrations (Fig. S1C,D) for which the number

of TNT-connected cells was not affected (Fig. S1E). This measured the tolerance of the system and assured the reproducibility of our data. For all subsequent experiments, cells were plated at the same concentration within the determined tolerance regime. By using this method, we overexpressed wild-type (WT) GFP-Rab11a and



**Fig. 1. Co-culture system for intercellular transfer of DiD-labeled vesicles and high-content screening assay.** (A) An example of the co-culture system setup. Donor cells were transfected with GFP–vector or GFP–Rab plasmids and labeled with DiD; acceptor cells were transfected with H2B–mCherry. Cells were fixed after co-culture and labeled with HCS CellMask™. DiD-labeled vesicles transferred to acceptor cells were detected as white spots in cells with red nuclei. (B) Left panel shows high-content screening setup and workflow. Cells were co-cultured as described in A and fixed after co-culture. Right panel shows a representative image of process of segmentation of cells and vesicles. (C) Representative flow cytometry raw data (dot plots) of the co-culture of donor cells expressing GFP–vector or GFP–myosin10 and labeled with DiD and acceptor cells expressing H2B–mCherry. Acceptor cells containing DiD-labeled vesicles from the entire population of acceptor cells after co-culture were quantified. The various conditions were donor and acceptor cells co-cultured, cultured separately and mixed just before sorting (mixture); cultured separated by a filter (filter); and acceptor cells challenged with donor cells supernatant (supernatant). (D) Bar graphs showing the relative percentage of acceptor cells from the experiment described in C (GFP–vector=100±1.8, GFP–myosin10=130.9±5.6). (E,F) As C,D, except that GFP–vector or GFP–VASP was expressed in donor cells (GFP–vector=100.0±1.8, GFP–VASP=85.2±2.4). Data show mean±s.e.m. from three independent experiments. Scale bar: 10 µm.

observed a significant increase in the percentage of TNT-connected cells (Fig. 2A,B and Table S2).

To confirm that the intercellular connections induced by Rab11a were functional TNTs, we performed transfer assays measuring the transfer of labeled vesicles between cells in co-culture (Abounit et al., 2015). Two populations of cells, donor cells (transfected with GFP–Rab11a-WT) with internal vesicles labeled with a fluorescent membrane dye (Vybrant™ DiD) and acceptor cells (transfected with H2B–mCherry) were mixed at a 1:1 ratio and co-cultured for 16 h (Delage et al., 2016). Overexpression of GFP–Rab11a-WT significantly increased the transfer of DiD-labeled vesicles, as measured by two different methods, quantitative fluorescent image analysis (Fig. 2C) and flow cytometry (Fig. S2A). Again, we used two controls to exclude the effect of vesicle transfer by secretion: (1) cells were co-cultured using a Transwell filter to separate the two populations, and/or (2) the conditioned medium from donor cells (cultured in a separate dish) was applied to acceptor cells. In both conditions, transfer of vesicles was very low and not affected by the overexpression of GFP–Rab11a-WT, showing that vesicle transfer between cells relied on cell-to-cell contact, and not on secretion (Fig. S2A). We also found that Rab11a-WT protein was present in acceptor cells, which indicates that Rab11a-positive vesicles could transfer between cells through TNTs (Fig. S2B). These combined results show that the increase in TNTs induced by Rab11a corresponds to an increase in cargo transfer between cells, suggesting that the TNTs induced by Rab11a are functional.

Rab GTPases switch between two conformations, an inactive form bound to guanosine diphosphate (GDP) and an active form bound to guanosine triphosphate (GTP), to regulate different cellular processes. Thus, cells were transfected with GFP–Rab11a-Q70L (constitutively active mutant) and GFP–Rab11a-S25N (dominant-negative mutant) for 40 h. For GFP–Rab11a-Q70L, the increase in the percentage of TNT-connected cells was similar to that observed with GFP–Rab11a-WT (Fig. 2A,B and Table S2). Similarly, we observed an increase in vesicle transfer between cells (Fig. 2C). By contrast, GFP–Rab11a-S25N overexpression showed no increase in the number of TNT-connected cells (Fig. 2A,B and Table S2) nor an increase in vesicle transfer (Fig. 2C). Importantly, when the expression time of GFP–Rab11a-S25N was extended to 52 h, the percentage of TNT-connected cells was significantly decreased (Fig. S3A), indicating that the effectiveness of the

dominant-negative proteins depends on the time elapsed after transfection. We speculate that the levels of expression of dominant-negative Rabs at 40 h after transfection are insufficient to show dominant-negative effects (e.g. trapping their activator's GEFs) (Ramalho et al., 2002).

To further confirm the effect of Rab11a on TNTs, Rab11a was knocked down in an acute manner (more than 90%) using short hairpin RNA (shRNA; Fig. 2D). In this condition, both the number of TNT-connected cells (Fig. 2E,F and Table S2) and vesicle transfer between the two cell populations (Fig. 2G) were substantially decreased. Rescue experiments in which GFP–Rab11a-WT was overexpressed in Rab11a knockdown cells showed that both the number of TNT-connected cells (Fig. 2E,F and Table S2) and vesicle transfer were rescued (Fig. 2G). Together, these results suggest that Rab11a plays a role in the formation of functionally active TNTs and that the active GTP-bound form of Rab11a is needed.

### Rab8a positively regulates TNT formation and function via the active GTP-bound form

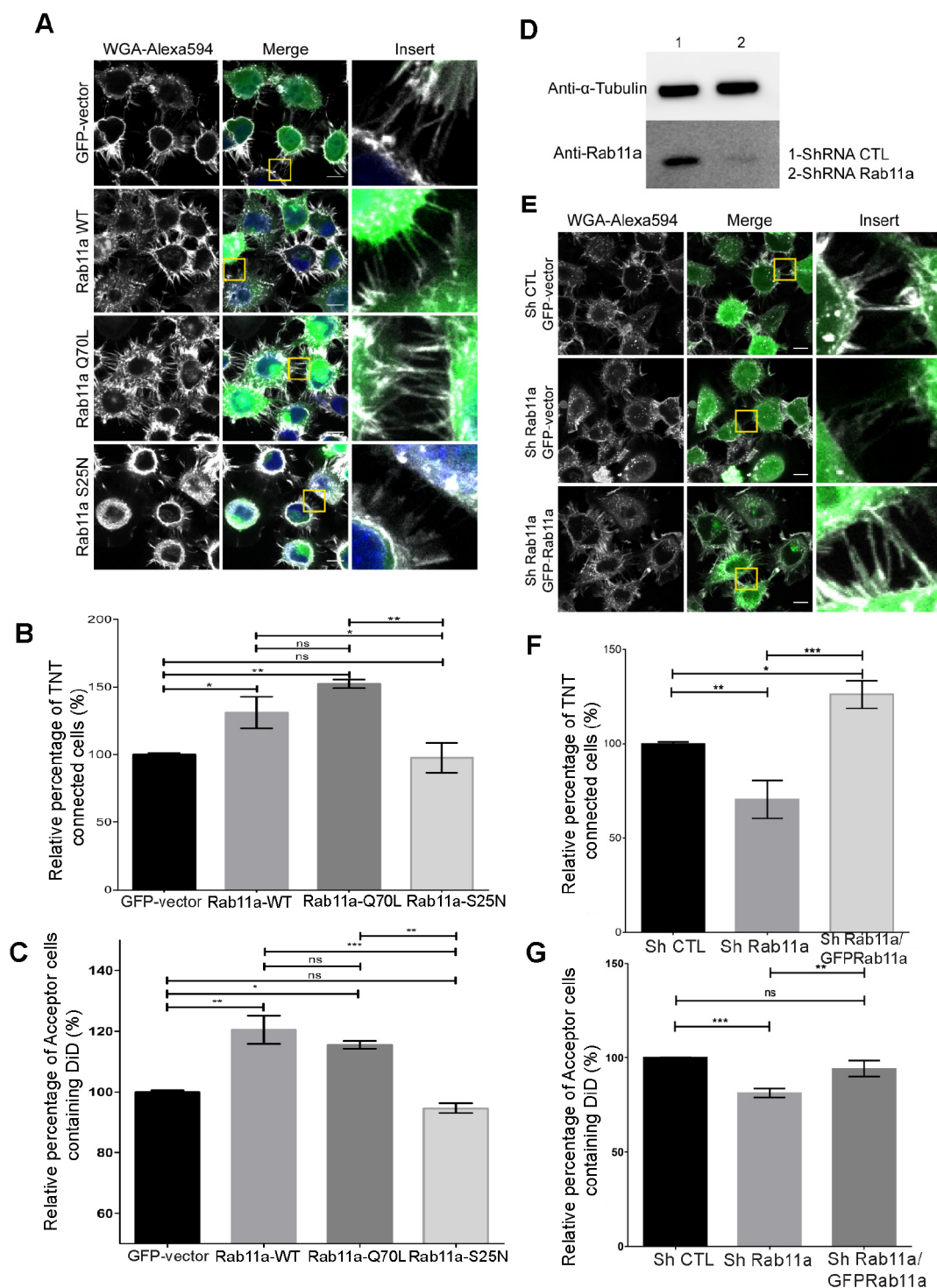
Rab8 localizes in the TGN, where it recycles endosomes, vesicular and tubular structures in the cytosol, membrane protrusions and the plasma membrane. Rab8 is reported to be involved in several transport pathways, and it induces actin polymerization and cell surface protrusion formation (Hattula et al., 2002, 2006). We overexpressed GFP–Rab8a-WT, GFP–Rab8a-Q67L (constitutively active mutant) and GFP–Rab8a-T22N (dominant-negative mutant) for 40 h by transient transfection and found that GFP–Rab8a-WT and GFP–Rab8a-Q67L significantly increased the number of TNT-connected cells (Fig. 3A,B and Table S2). On the other hand, expression of GFP–Rab8a-T22N had no significant effect on TNT-connected cells (Fig. 3A,B and Table S2) unless its expression time was extended to 52 h. In the latter case, the percentage of TNT-connected cells was significantly decreased (Fig. S3A).

Consistent with a positive role of Rab8a in TNT formation, the transfer of vesicles increased in cells overexpressing GFP–Rab8a-WT and GFP–Rab8a-Q67L (Fig. 3C and Fig. S2C). On the other hand, expression of GFP–Rab8a-T22N resulted in a decrease in vesicle transfer between cells (Fig. 3C). By comparison with Transwell plate co-culture or conditioned medium, the effect of secretion on vesicle transfer in this condition was negligible, indicating that the transfer of vesicles between cells was mediated through cell–cell contact and not secretion (Fig. S2C). Similar to Rab11a, Rab8a protein could also transfer between cells through cell–cell contact and not secretion (Fig. S2D).

To further confirm the effect of Rab8a on TNTs, we acutely depleted Rab8a (more than 90%) with a specific shRNA (Fig. 3D). Downregulating the expression of Rab8a decreased the number of TNT-connected cells (Fig. 3E,F and Table S2) as well as DiD-labeled vesicle transfer between cells (Fig. 3G). Of importance, when overexpressing GFP–Rab8a-WT in Rab8a knockdown cells, both the number of TNT-connected cells (Fig. 3E,F and Table S2) and vesicles transferred (Fig. 3G) were rescued to control levels. All these results suggest that Rab8a is a positive regulator of TNTs through its GTP-active form.

An isoform of Rab8a (Rab8b) has been shown to have an effect on apical transport (Sato et al., 2014). Thus, we also checked the role of Rab8b in TNT formation. We found that overexpression of GFP–Rab8b-WT in CAD cells had no effect on either the number of TNT-connected cells (Fig. S3B) or the number of acceptor cells containing vesicles following co-culture (Fig. S3C). Overall, the





**Fig. 2.** See next page for legend.

data indicate that only the Rab8-specific isoform Rab8a has a positive effect on TNT formation.

### Rab11a-Rab8a cascade in TNT formation

Of interest, Rab11 and Rab8 have been shown to work in a cascade pathway in several cellular processes, such as cyst lumen formation in MDCK cells (Roland et al., 2011), primary cilium generation (Westlake et al., 2011) and axon outgrowth (Furusawa et al., 2017). To assess whether Rab8a and Rab11a GTPases work in the same

pathway to modulate TNTs, we depleted one of the two Rabs and overexpressed the other one and quantified the number of TNT-connected cells and vesicle transfer between cells. We found that overexpressing Rab8a in shRNA-depleted Rab11a cells still led to an increase in the number of TNT-connected cells (Fig. 4A,B and Table S2) and rescued vesicle transfer between cells to control levels (Fig. 4C). By contrast, in cells depleted of Rab8a using shRNA, Rab11a overexpression had no effect on the number of TNT-connected cells (Fig. 4D,E and Table S2) or the number of

**Fig. 2. Rab11a positively regulates functional TNTs via the active GTP-bound form.**

(A) Representative confocal images of TNT-connected cells after transfection of GFP–vector, GFP–Rab11a-WT, GFP–Rab11a-Q70L and GFP–Rab11aS25N. Inserts are enlargements of boxed regions in the merge panel. (B) Bar graph showing the relative percentage of TNT-connected cells from experiment described in A (GFP–vector=100±0.5, GFP–Rab11aWT=131.0±4.7, GFP–Rab11a-Q70L=152.4±2.2, GFP–Rab11aS25N=97.6±11.0). (C) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with GFP–vector, GFP–Rab11a-WT, GFP–Rab11a-Q70L or GFP–Rab11a-S25N and labeled with DiD (GFP–vector=100±0.0, GFP–Rab11aWT=120.4±4.6, GFP–Rab11aQ70L=115.5±1.2, GFP–Rab11aS25N=94.6±1.6). (D) Western blot analysis of cells transfected with shRNA non-targeting (ShCTL) or targeting Rab11a (ShRab11a), showing the expression of Rab11a and  $\alpha$ -tubulin as loading control. (E) Representative confocal images of TNT-connected cells after transfection with ShCTL/GFP–vector, ShRab11a/GFP–vector or ShRab11a/GFP–Rab11a. Inserts are enlargements of framed regions in merge panel. (F) Bar graph representing relative percentage of TNT-connected cells from experiment described in E (ShCTL/GFP–vector=100±0.5, ShRab11a/GFP–vector=70.5±4.5, ShRab11a/GFP–Rab11a=126.1±5.1). (G) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with ShCTL/GFP–vector, ShRab11a/GFP–vector or ShRab11a/GFP–Rab11a and labeled with DiD (ShCTL/GFP–vector=100±0.2, ShRab11a/GFP–vector=81.3±2.4, ShRab11a/GFP–Rab11a=94.2±4.2). All graphs show mean±s.e.m. from three independent experiments (ns, not significant; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001; by one-way ANOVA with Tukey's multiple comparison post test). Scale bars: 10  $\mu$ m.

vesicles transferred between cells (Fig. 4F). These experiments showed that the effect of Rab8a on TNTs could be independent of Rab11a activation, whereas the effect of Rab11a on TNTs was dependent on the presence of Rab8a, indicating that Rab8a acts downstream of Rab11a in the regulation of TNT formation. Interestingly, when co-transfected in CAD cells, GFP–Rab8a and strawberry-Rab11a appeared to locate on the end of TNTs. This suggests that the two proteins cooperate on the same endosome at the base of the TNT for its formation (Fig. S4A).

A similar Rab activation cascade has been implicated in the *de novo* generation of the primary cilium (Westlake et al., 2011). It was demonstrated that Rab11 regulates Rab8 function by activating Rabin8 (Westlake et al., 2011), which is a major guanine nucleotide exchange factor (GEF) for Rab8 and an essential component for cell protrusion formation, such as ciliogenesis and neurite outgrowth (Wang et al., 2015). However, overexpression of GFP–Rabin8 did not affect the number of TNT-connected cells compared with the GFP control (Fig. S5A,B) nor affect the transfer of DiD-labeled vesicles between cells (Fig. S5C,D). Similarly, knockdown of Rabin8 by shRNA (Fig. S5E) had no effect on either the number of TNT-connected cells (Fig. S5F) or the number of vesicles transferred (Fig. S5G,H). This suggests that Rabin8 is not involved in the Rab11a–Rab8a cascade leading to TNT increase.

It has been reported that MICAL-L1 binds to GTP-bound Rab8a and stabilizes it on tubular membranes (Sharma et al., 2009; Rahajeng et al., 2012). Rab35 is known to interact with MICAL-L1 and to regulate neurite outgrowth (Kobayashi et al., 2014). Additionally, Rab35 also activates MICAL1 (Fremont et al., 2017; Deng et al., 2016). Because Rab35 was one of the positive hits in the screening (Table S1), we overexpressed MICAL-L1 and MICAL1 and checked their effect on TNT formation (Fig. S4B) and vesicle transfer (Fig. S4C). However, no significant effect on either vesicle transfer or TNT formation was observed, thereby indicating that Rab8 and Rab11 act independently of MICAL1 and MICAL-L1 proteins.

**GRAB regulates TNT formation independently of Rab8a**

It has been reported that GRAB (a guanine nucleotide exchange factor for Rab8a) mediates the Rab11a–Rab8a cascade mechanism and facilitates axonal growth (Furusawa et al., 2017). Therefore, we tested the effect of GRAB on TNT formation. We overexpressed wild-type GRAB in CAD cells and found that there was an increase in the number of TNT-connected cells (Fig. 5A,B and Table S2) and in vesicle transfer between cells in co-culture (Fig. 5C,D). To validate whether GRAB acts through the same pathway as Rab11a and Rab8a, we knocked down Rab8a by shRNA and overexpressed wild-type GFP–GRAB. We could still see an increase in TNT formation between cells (Fig. 5E,F and Table S2) and also an increase in vesicle transfer (Fig. 5G,H). This is an indication that GRAB does not act upstream of Rab8a and that it might act through an independent pathway that does not involve Rab8a.

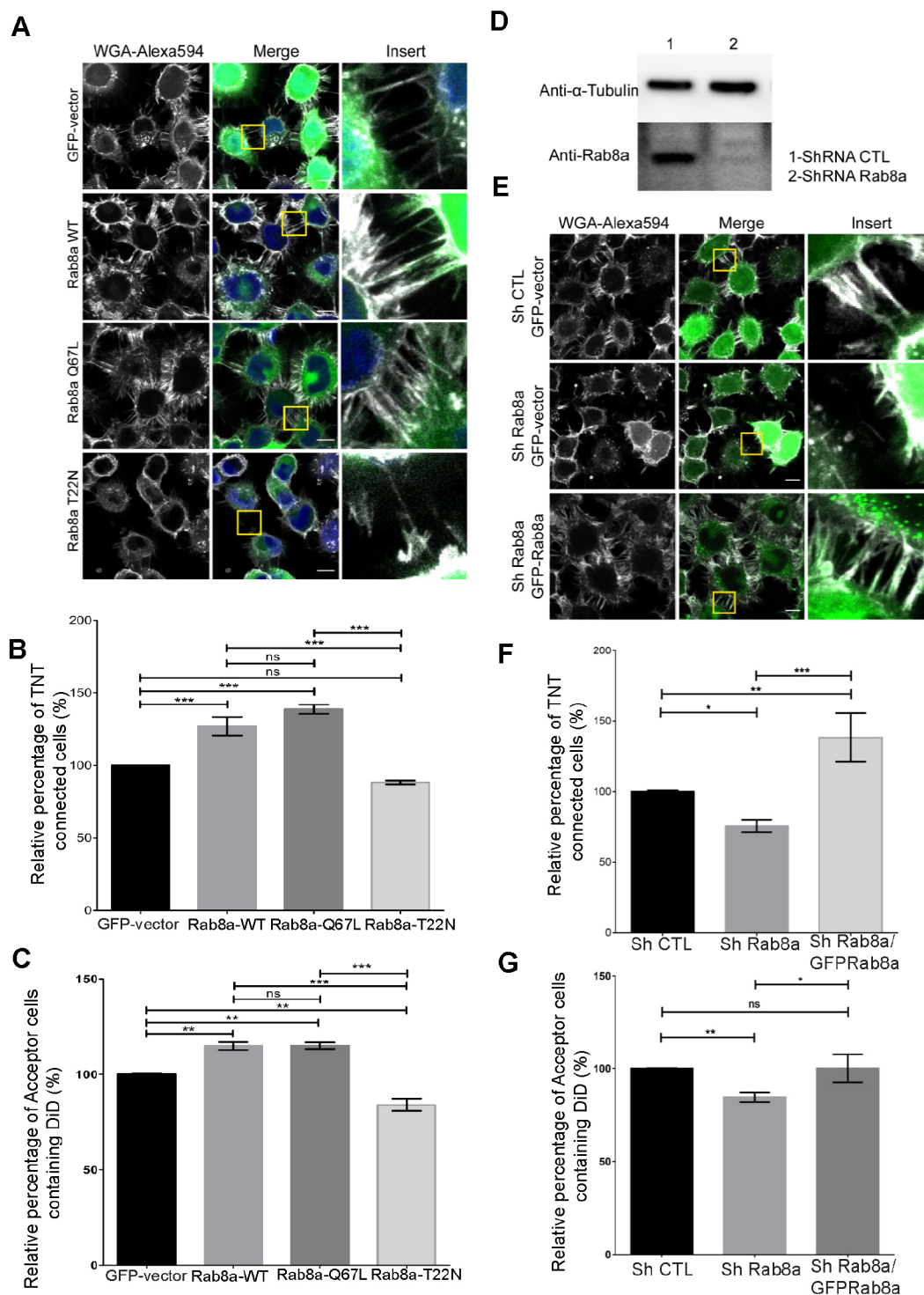
**VAMP3 acts downstream of Rab8a in regulating TNT formation**

Rab8 and Rab11 have been shown to be crucial in vesicle recycling from the plasma membrane (Chen et al., 1998; Zhang et al., 2005). Blocking of either Rab8 or Rab11 pathways leads to an inhibition of the TGN-to-plasma membrane transport of recycling endosomes, as well as inhibition of plasma membrane recycling (Rowe et al., 2008); thus, one possibility is that TNT formation could be affected by this process. Primaquine (PMQ) interferes with membrane recycling from endosomes to the plasma membrane through direct interaction with endosomes (van Weert et al., 2000). We used this drug to inhibit transferrin recycling to the plasma membrane (Fig. 6A) and quantified the number of TNT-connected cells after 4 h of PMQ treatment. The results showed a significant decrease in the number of TNT-connected cells (Fig. 6B,C and Table S2), suggesting that vesicle recycling to the plasma membrane is involved in TNT formation.

The cascade of Rab11 activation couples cargo transport from the TGN and recycling endosomes to vesicle docking and fusion at the plasma membrane. By interacting with the v-SNARE VAMP3, Rab8 is responsible for the final docking/fusion step in T cell receptor recycling to the immune synapse (Finetti et al., 2015). Rab8 also interacts with VAMP3 at the base of the cilium, where VAMP3 regulates ciliary growth and targeting of Smoothed to the plasma membrane (Patrussi and Baldari, 2016). To test whether the Rab cascade affects TNTs through an increase in vesicle recycling, as mediated by VAMP3, we overexpressed GFP–VAMP3 and quantified the number of TNT-connected cells and vesicle transfer between cells. We found that there was an increase in both the percentage of TNT-connected cells (Fig. 6D,E and Table S2) and the number of DiD-labeled vesicles transferred (Fig. 6F and Fig. S6A). Comparison between the total transfer of vesicles and the supernatant-dependent transfer showed that the transfer of vesicles was mainly mediated by cell-to-cell contact (Fig. S6A). Interestingly, we also found that VAMP3-positive vesicles could transfer between cells in a cell–cell contact-dependent manner (Fig. S6B).

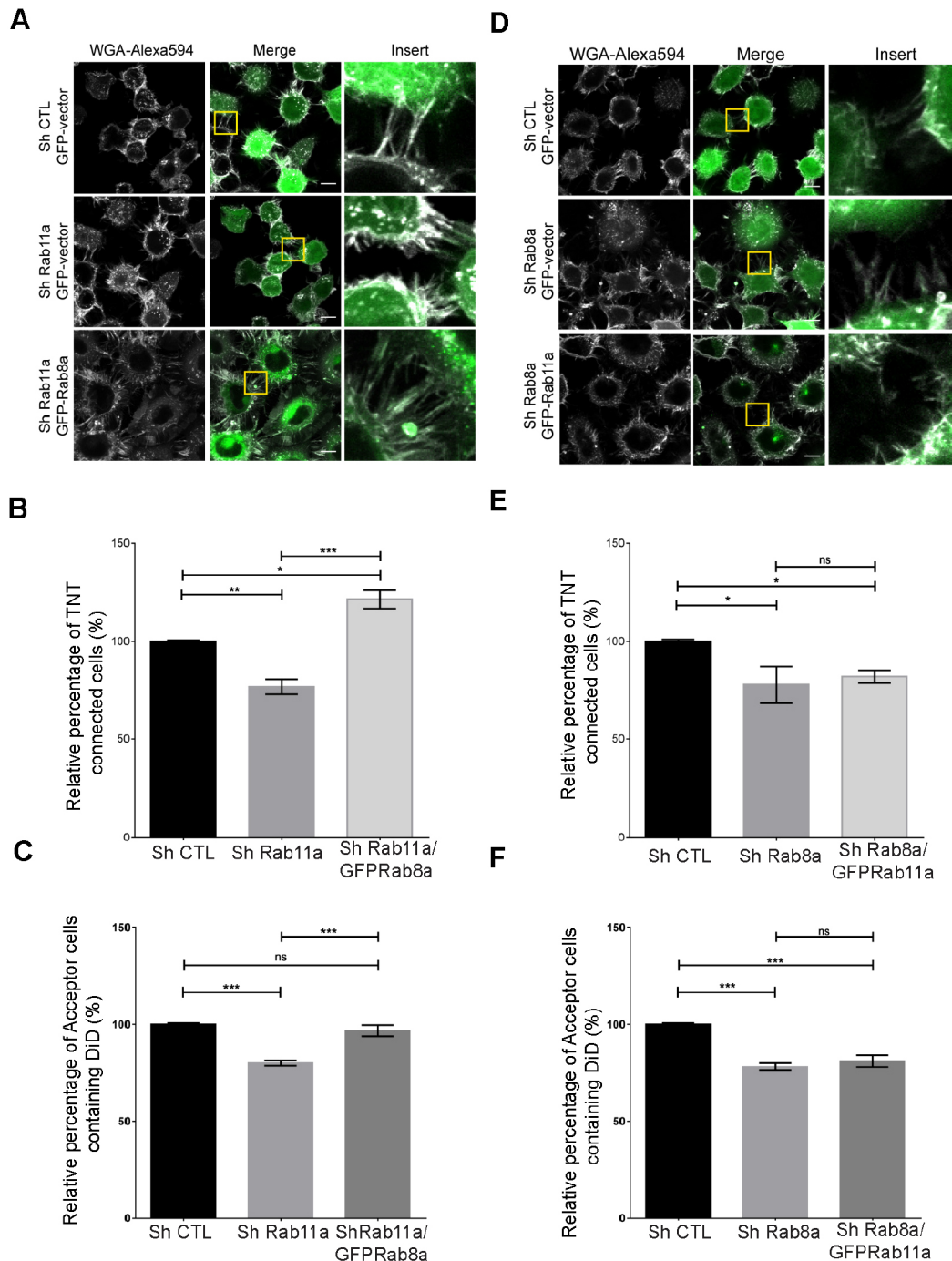
To confirm the function of VAMP3 in regulating TNT formation, knockdown of VAMP3 by shRNA (Fig. 6G) decreased the number of TNT-connected cells (Fig. 6H,I and Table S2) and decreased the transfer of vesicles between cells (Fig. 6J). On the other hand, overexpressing VAMP3 in VAMP3 knockdown cells restored both the number of TNT-connected cells (Fig. 6H,I and Table S2) and vesicle transfer between cells (Fig. 6J). These results suggest that VAMP3 is involved in the regulation of TNT formation.

To confirm whether VAMP3 is a downstream effector of Rab8a in regulating TNT formation, GFP–Rab8a-WT was overexpressed



**Fig. 3. Rab8a positively regulates functional TNTs via the active GTP-bound form.** (A) Representative confocal images of TNT-connected cells after transfection with GFP-vector, GFP-Rab8a-WT, GFP-Rab8a-Q67L or GFP-Rab8a-T22N. Inserts are enlargements of boxed regions in merge panel. (B) Bar graph representing relative percentage of TNT-connected cells from experiment described in A (GFP-vector=100, GFP-Rab8a-WT=124.2 $\pm$ 3.1, GFP-Rab8a-Q67L=138.8 $\pm$ 2.1, GFP-Rab8a-T22N=88.3 $\pm$ 0.9). (C) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with GFP-vector, GFP-Rab8a-WT, GFP-Rab8a-Q67L or GFP-Rab8a-T22N and labeled with DiD (GFP-vector=100.0 $\pm$ 0.57, GFP-Rab8a-WT=114.9 $\pm$ 2.004, GFP-Rab8a-Q67L=115 $\pm$ 1.729, GFP-Rab8a-T22N=84.02 $\pm$ 3.145). (D) Western blot of cells transfected with shRNA non-targeting (ShCTL) or targeting Rab8a (ShRab8a) and  $\alpha$ -tubulin as loading control. (E) Representative confocal images of TNT-connected cells after transfection with ShCTL/GFP-vector, ShRab8a/GFP-vector, and ShRab8a/GFP-Rab8a. Inserts are enlargements of the framed region in merge panel. (F) Bar graph representing the relative percentage of TNT-connected cells from experiment described in E (ShCTL/GFP-vector=100 $\pm$ 0.57, ShRab8a/GFP-vector=75.67 $\pm$ 1.8, ShRab8a/GFP-Rab8a=138.3 $\pm$ 17.3). (G) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with ShCTL/GFP-vector, ShRab8a/GFP-vector or ShRab8a/GFP-Rab8a and labeled with DiD (ShCTL/GFP-vector=100 $\pm$ 0.2, ShRab8a/GFP-vector=84.5 $\pm$ 2.4, ShRab8a/GFP-Rab8a=100.1 $\pm$ 7.5). All graphs show mean $\pm$ s.e.m. from three independent experiments (ns, not significant; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001; by one-way ANOVA with Tukey's multiple comparison post test). Scale bars: 10  $\mu$ m.

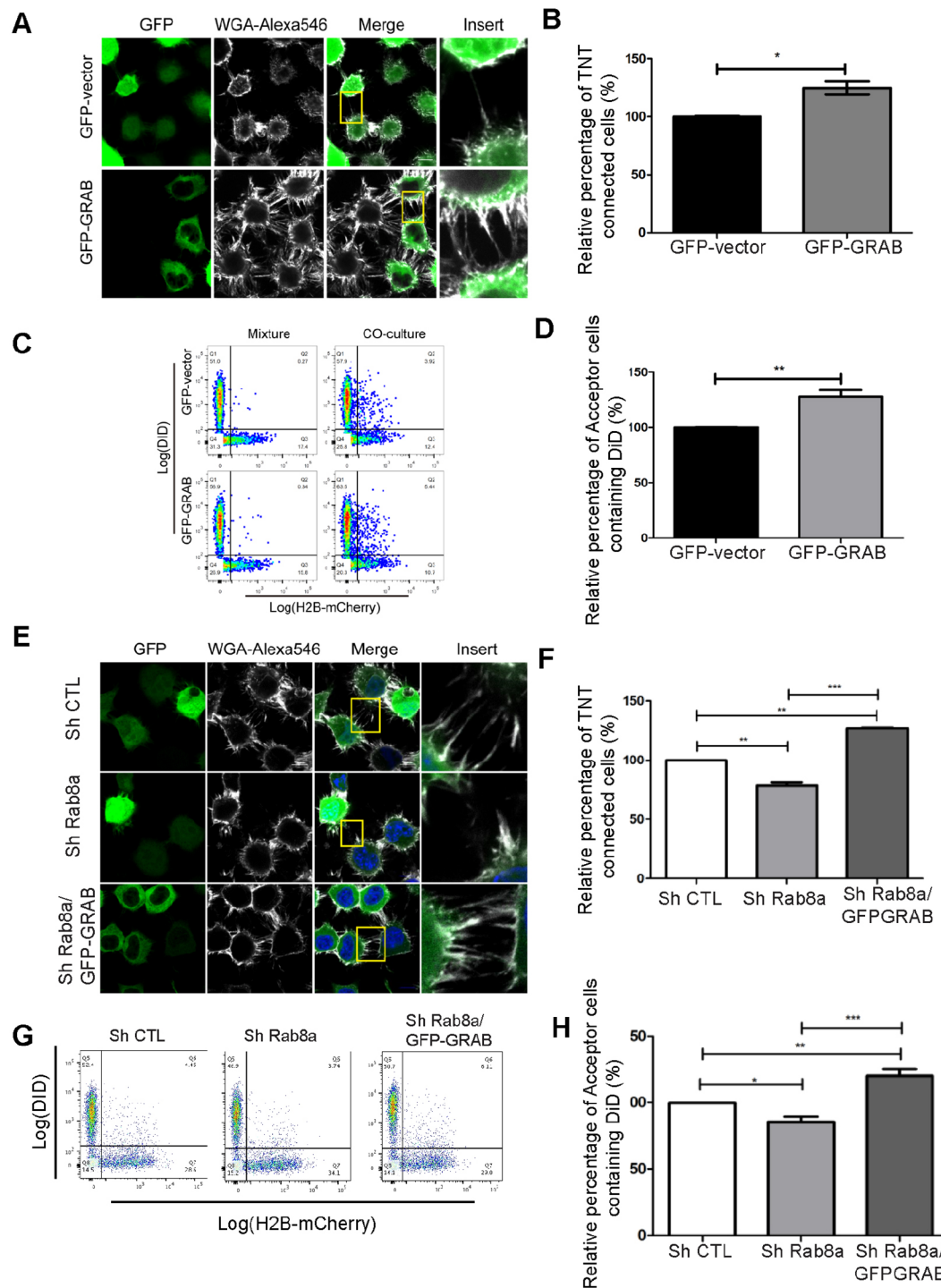




**Fig. 4. Rab11a-Rab8a cascade in TNT formation.** (A) Representative confocal images of TNT-connected cells after transfection with ShCTL/GFP-vector, ShRab11a/GFP-vector or ShRab11a/GFP-Rab8a. Inserts are enlargements of framed regions in merge panel. (B) Bar graph showing relative percentage of TNT-connected cells from experiment described in A (ShCTL/GFP-vector=100±0.57, ShRab11a/GFP-vector=76.74±3.9, ShRab11a/GFP-Rab8a=121.7±4.74). (C) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with ShCTL/GFP-vector, ShRab11a/GFP-vector or ShRab11a/GFP-Rab8a and labeled with DiD (ShCTL/GFP-vector=100±0.69, ShRab11a/GFP-vector=79.91±1.3, ShRab11a/GFP-Rab8a=96.7±2.94). (D) Representative confocal images of TNT-connected cells after transfection with ShCTL/GFP-vector, ShRab8a/GFP-vector or ShRab8a/GFP-Rab11a. Inserts are enlargements of the framed regions in merge panel. (E) Bar graph showing the relative percentage of TNT-connected cells from the experiment described in D (ShCTL/GFP-vector=100±0.57, ShRab8a/GFP-vector=77.97±5.51, ShRab8a/GFP-Rab11a=81.95±3.15). (F) Bar graph representing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with ShCTL/GFP-vector, ShRab8a/GFP-vector or ShRab8a/GFP-Rab11a and labeled with DiD (ShCTL/GFP-vector=100±0.6, ShRab8a/GFP-vector=78.05±1.9, ShRab8a/GFP-Rab11a=80.91±2.9). All graphs are from three independent experiments and show mean±s.e.m. (ns, not significant; \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001; by one-way ANOVA with Tukey's multiple comparison post test). Scale bars: 10 μm.

in VAMP3 knockdown cells. Under these conditions, neither the number of TNT-connected cells (Fig. 7A,B and Table S2) nor the vesicles transferred between cells (Fig. 7C) were affected.

Furthermore, overexpressing GFP-Rab11a-WT in VAMP3 knockdown cells had no effect on the number of TNT-connected cells (Fig. 7A,B and Table S2) nor vesicle transfer between cells



**Fig. 5. GRAB regulates formation of TNT independent of Rab8a.** (A) Representative confocal images of TNT-connected cells after transfection with GFP-vector or GFP-GRAB. Inserts are enlargements of the framed region in merge panel. (B) Bar graph representing relative percentage of TNT-connected cells from the experiment described in A (GFP-vector=100±0.5, GFP-GRAB=125±5.4). (C) Raw data (dot plots) of flow cytometry from a representative experiment showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with either GFP-vector or GFP-GRAB and labeled with DiD. (D) Bar graph representing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures of the experiment described in C (GFP-vector=100.0±0.5, GFP-GRAB=128.0±5.8). All graphs show mean±s.e.m. from three independent experiments (\* $P < 0.05$ , \*\* $P < 0.01$ ; by unpaired Student's *t*-test). (E) Representative confocal images of TNT-connected cells after transfection with ShCTL/GFP-vector, ShRab8a/GFP-vector or ShRab8a/GFP-GRAB. Inserts are enlargements of the framed regions in merge panel. (F) Bar graph showing relative percentage of TNT-connected cells from experiment described in E (ShCTL/GFP-vector=100, ShRab8a/GFP-vector=78.2±3.4, ShRab8a/GFP-GRAB=126.9±0.6). (G) Raw data (dot plots) of flow cytometry from a representative experiment showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, in which the donor cells were transfected with ShCTL/GFP-vector, ShRab8a/GFP-vector or ShRab8a/GFP-GRAB and labeled with DiD. (H) Bar graph representing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures of the experiment described in G (ShCTL/GFP-vector=99.5±0.5, ShRab8a/GFP-vector=85.1±10.2, ShRab8a/GFPGRAB=120.0±12.1). All graphs show means±s.e.m. (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; by one-way ANOVA with Tukey's multiple comparison post test) from two independent experiments. Scale bars: 10  $\mu$ m.

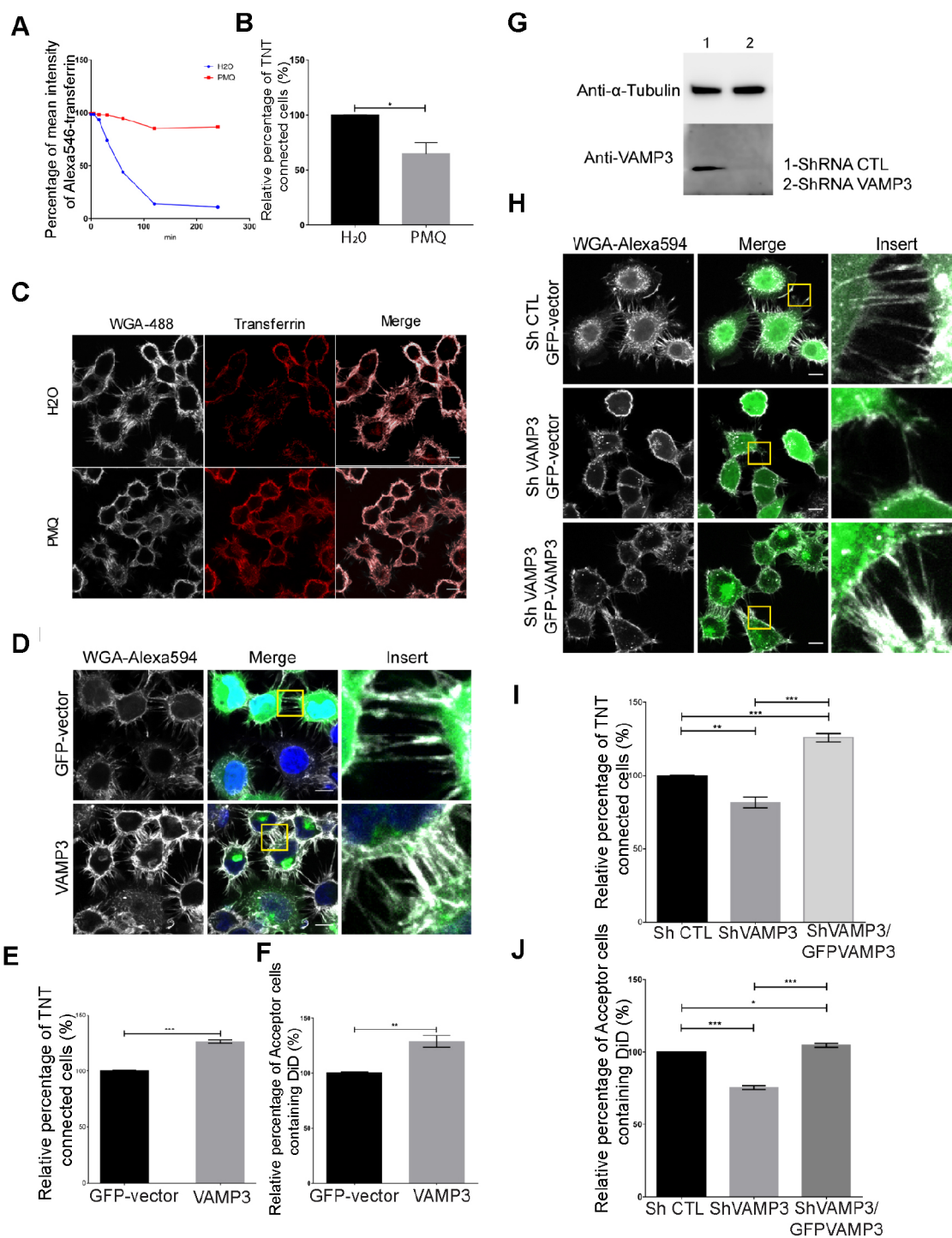


Fig. 6. See next page for legend.

(Fig. 7C). These results indicate that VAMP3 is involved in the Rab11a–Rab8a cascade, regulating TNT formation by acting downstream of Rab8a.

#### Rab8a and Rab11a promote both TNTs and filopodia formation, with different mechanisms

Both Rab11 and Rab8 have been shown to be involved in filopodia formation in different contexts (Cox et al., 2000; Eva et al., 2010; Hattula et al., 2006; Peränen, 2011). Although we have shown that

in CAD cells filopodia and TNTs are formed by different mechanisms leading to actin remodeling (Delage et al., 2016), the question arises whether, in our cell model, the same Rab cascade also leads to an increase in filopodia formation. In contrast to TNTs, attached filopodia exhibit vinculin-positive focal adhesions at the tip (Bohil et al., 2006; Schafer et al., 2010). By automatically detecting vinculin-positive peripheral cellular protrusions, we observed that overexpression of Rab8a or Rab11a also increased the number of vinculin-positive protrusions (Fig. S7A,B). Inversely,



**Fig. 6. VAMP3 positively regulates functional TNTs.** (A) Relative mean intensity of Alexa Fluor 546-transferrin bound to cells after treatment with Primaquine (PMQ) or H<sub>2</sub>O for 0 min (H<sub>2</sub>O=98.6, PMQ=99.2), 5 min (H<sub>2</sub>O=98.6, PMQ=99.3), 15 min (H<sub>2</sub>O=93.6, PMQ=98.4), 30 min (H<sub>2</sub>O=74.1, PMQ=98.1), 60 min (H<sub>2</sub>O=44.0, PMQ=94.6), 120 min (H<sub>2</sub>O=13.8, PMQ=85.4) or 240 min (H<sub>2</sub>O=11.0, PMQ=86.7) analyzed by flow cytometry. (B) Bar graphs showing relative percentage of TNT-connected cells after treatment with PMQ or H<sub>2</sub>O for 4 h (H<sub>2</sub>O=100±0.57, PMQ=64.92±10.07). The graph shows mean±s.e.m. from three independent experiments (\**P*<0.05; by unpaired Student's *t*-test). (C) Representative confocal images of TNT-connected cells after treatment with PMQ or H<sub>2</sub>O for 4 h. (D) Representative confocal images of TNT-connected cells after transfection with GFP–vector or GFP–VAMP3. Inserts are enlargements of the framed regions in merge panel. (E) Bar graph representing relative percentage of TNT-connected cells from experiment described in D (GFP–vector=100.0±0.5, GFP–VAMP3=126.4±1.7). (F) Bar graph representing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with either GFP–vector or GFP–VAMP3 and labeled with DiD (GFP–vector=100.2±0.73, GFP–VAMP3=129.0±5.3). The graphs show mean±s.e.m. from three independent experiments (\*\**P*<0.01, \*\*\**P*<0.001; by unpaired Student's *t*-test). (G) Western blot of cells transfected with shRNA non-targeting (ShCTL) or targeting VAMP3 (ShVAMP3), showing the expression of VAMP3 and  $\alpha$ -tubulin as loading control. (H) Representative confocal images of TNT-connected cells after transfection with ShCTL/GFP–vector, ShVAMP3/GFP–vector or ShVAMP3/GFP–VAMP3. Inserts are enlargements of the framed region in merge panel. (I) Bar graph representing relative percentage of TNT-connected cells from experiment described in H (ShCTL/GFP–vector=100±0.4, ShVAMP3/GFP–vector=81.6±3.6, ShVAMP3/GFP–VAMP3=126±2.9). (J) Bar graph representing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with ShCTL/GFP–vector, ShVAMP3/GFP–vector or ShVAMP3/GFP–VAMP3 and labeled with DiD (ShCTL/GFP–vector=100, ShVAMP3/GFP–vector=75.3±1.2, ShVAMP3/GFP–VAMP3=104.5±1.2). The above graphs show mean ±s.e.m. from three independent experiments (\**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001; by one-way ANOVA with Tukey's multiple comparison post test). Scale bars: 10  $\mu$ m.

knocking down Rab8a or Rab11a was associated with a decrease in vinculin-positive protrusions (Fig. S7C,D). This result indicates that Rab8a and Rab11a increased both the number of TNTs and the number of attached filopodia. Interestingly, we found that overexpression of Rabin8 increased the number of vinculin-positive protrusions (Fig. S7E,F), whereas it had no effect on TNT formation and vesicle transfer between the cells (Fig. S5). Thus, Rabin8 could be the effector of Rab11a, and GEF of Rab8a, in promoting attached filopodia formation, but not for TNTs. On the other hand, overexpression of VAMP3, which increased TNTs (Fig. 6), decreased the number of attached filopodia (Fig. S7E,F). Together, these results suggest that VAMP3 could act as an effector of Rab8a in regulating TNT formation, but not the formation of attached filopodia.

## DISCUSSION

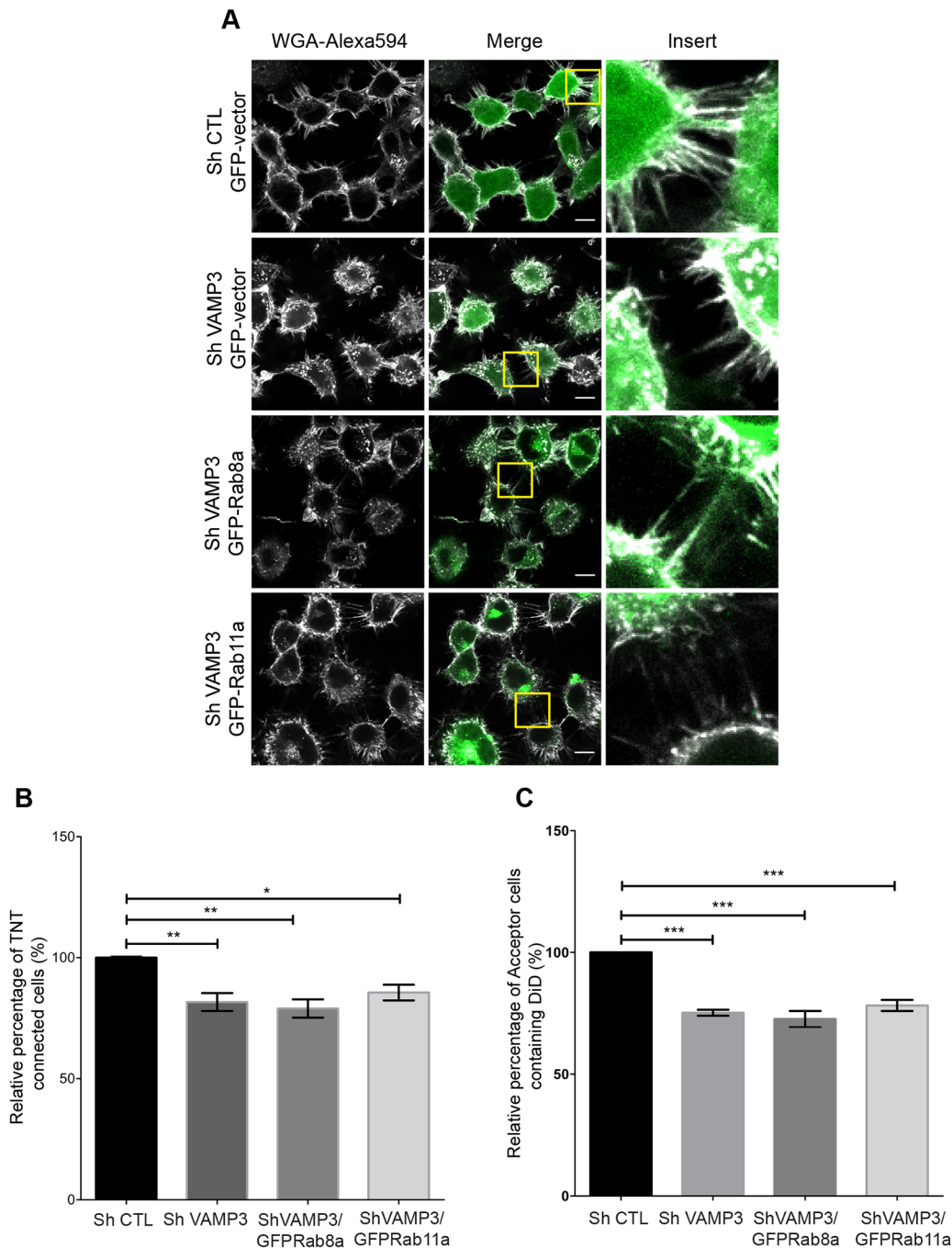
The data presented here provide new insights into the factors involved in TNT formation and intercellular vesicle transfer in neuron-like CAD cells. Our results show that Rab11a, Rab8a and VAMP3 increase both the number of TNT-connected cells and vesicle transfer through TNTs in CAD cells. We have demonstrated that Rab11a and Rab8a work in the same pathway and employ VAMP3 as an effector to induce functional TNTs but not filopodia. Of interest, this Rab11a–Rab8a signaling cascade does not require Rabin8, which is essential in mediating Rab8a-activated filopodia formation. Thus, in addition to providing mechanistic details about the role of Rab proteins in TNT formation, the data also strengthen our hypothesis that filopodia and TNTs are different structures, which rely on different mechanisms of formation.

The fact that Rab effectors are highly diverse illustrates that Rab GTPases control multiple biochemical events. The functions of Rab GTPases and their effectors are mostly related to the vesicular traffic between donor and recipient compartments. Some distinct Rab effectors are involved in the sorting of cargo into vesicles by acting on budding, uncoating and motility along actin or microtubule filaments (Stenmark, 2009). Through their activation, Rab GTPases control the maturation of vesicles, as well as vesicle shuttling between different membrane compartments (Zhen and Stenmark, 2015). The function of activated Rab GTPases is to recruit effectors such as coat proteins (Carroll et al., 2001), cytoskeletal motors (Roland et al., 2007; Wu et al., 2002), kinases and phosphatases (Shin et al., 2005) and membrane tethering/fusion proteins (Nielsen et al., 2000; Simonsen et al., 1998). Because TNTs are novel cellular structures consisting of membranous channels containing actin that bridge distant cells and allow the exchange of different materials/cargos (Abounit and Zurzolo, 2012), it is likely that specific Rab proteins are involved in TNT formation. To assess this, we set up a screen where all 41 Rab subfamilies were tested for possible roles in TNT-mediated vesicle transfer in CAD cells. Although Rab8a, Rab11a and Rab35 could increase vesicle transfer between cells, we found that Rab39 and Rab40 had negative effects (Table S1).

We decided to focus on Rab8 and Rab11, for which there is evidence supporting their role in TNT formation in other cell systems, although the mechanism was unexplored (Burtey et al., 2015; Zhu et al., 2016). Rab11 and Rab8 could work independently from each other or in the same pathway (Hattula et al., 2006). In addition, because TNTs are membrane protrusions containing actin filaments, these two Rab GTPases could affect TNT formation by playing a role either in modulating actin dynamics and/or by regulating the supply of the membrane-specific lipids and proteins required for TNT development.

Both Rab8 and Rab11 can be found associated with the trans-Golgi network and recycling endosomes, and have been shown to be essential in the trafficking of proteins from the Golgi to the plasma membrane (Rowe et al., 2008). Intriguingly, Rab8 and Rab11 could also modulate actin dynamics (Castillo-Romero et al., 2010; Hattula et al., 2002; Ramel et al., 2013). Rab11 is known to be involved in the recycling of endocytosed proteins. However, Rab11 also regulates actin dynamics during formation of membrane protrusions and is required for the spatial control of Rac1 activity through the control of cell–cell communication during collective cell migration by inducing cell protrusions through the regulation of moesin activation (Ramel et al., 2013). On the other hand, Rab8 has been shown to drive cytoskeletal reorganization in HeLa cells, either through RhoA GTPase, calpain or MT1-MMP activation (Bravo-Cordero et al., 2016). However, knocking down RhoA GTPase in DNA damage-induced senescent cells did not induce a significant reduction in mCherry transfer between cells, suggesting that Rab8 could regulate TNT formation not only through actin polymerization (Biran et al., 2015).

Of interest, Rab8 has been reported to target vesicles to the cilium to promote ciliary membrane elongation (Nachury et al., 2007). Endogenous Rab8 localizes to the primary cilium and to the BBsome, which associates with the ciliary membrane. VAMP3, which is involved in the docking and/or fusion of synaptic vesicles with the plasma membrane, interacts with Rab8 at the base of the cilium to regulate ciliary growth and transport of specific receptors, such as Smoothened and fibrocystin to the cilium (Boehlke et al., 2010; Folliot et al., 2010). By interacting with VAMP3, Rab8 is also responsible for the final docking/fusion step



**Fig. 7. VAMP3 acts downstream of Rab8a in regulating TNTs.** (A) Representative confocal images of TNT-connected cells after transfection with ShCTL/GFP-vector, ShVAMP3/GFP-vector, ShVAMP3/GFP-Rab8a or ShVAMP3/GFP-Rab11a. Inserts are enlargements of the framed region in merge panel. (B) Bar graph showing relative percentage of TNT-connected cells from the experiment described in A (ShCTL/GFP-vector=100±0.4, ShVAMP3/GFP-vector=81.6±3.6, ShVAMP3/GFP-Rab8a=79.0±3.7, ShVAMP3/GFP-Rab11a=85.6±3.2). (C) Bar graph representing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with ShCTL/GFP-vector, ShVAMP3/GFP-vector, ShVAMP3/GFP-Rab8a or ShVAMP3/GFP-Rab11a and labeled with DiD (ShCTL/GFP-vector=100, ShVAMP3/GFP-vector=75.3±1.2, ShVAMP3/GFP-Rab8a=72.7±3.3, ShVAMP3/GFP-Rab11a=78.3±1.4). The graphs show mean±s.e.m. from three independent experiments (ns, not significant; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001; by one-way ANOVA with Tukey's multiple comparison post test). Scale bars: 10  $\mu$ m.

in T cell receptor recycling to the immune synapse (Patrussi and Baldari, 2016).

Our results clearly show that VAMP3 promotes TNT formation by acting downstream of Rab8a in CAD cells. It is therefore possible that Rab8a uses a similar mechanism to facilitate TNT formation by transporting vesicles and/or specific proteins or receptors to the

bases of TNTs. This could be initiated by the fusion of a contractile vacuole with the cell membrane to form negative and positive curvature at the TNT tip and base, respectively. Subsequently, the extension of TNTs could be supported by membrane intake through motor proteins and exocytosis involving myosin10 (Bishai et al., 2013; Gousset et al., 2013) and Rab8 (Wang et al., 2015). Our



results show an impairment of membrane recycling from endosomes to the plasma membrane with PMQ, leading to a decrease in the number of TNT-connected cells, which is in perfect accordance with this model.

There is evidence supporting a model in which, during membrane flow from one organelle to another, the compartment transition from an upstream Rab to a downstream Rab is regulated by the recruitment of effectors (Hutagalung and Novick, 2011). The Rab11–Rab8 cascade has been shown to be involved in several cellular processes, including transferrin receptor recycling, primary cilium formation and axon outgrowth. Regarding TNTs, our results show that Rab8a depletion inhibits TNT formation induced by Rab11a, whereas Rab11a depletion does not affect the formation of TNTs induced by Rab8a. This indicates that Rab8a acts as a downstream effector of Rab11a to regulate TNT formation. How this occurs is still unclear. However, when overexpressed together, Rab11a and Rab8a appear to colocalize at the base of the TNT, which suggests that the two proteins cooperate on the same endosome at the base of the TNT for its formation. On the other hand, from this qualitative analysis, the two proteins do not appear to colocalize inside TNTs.

Even though we have shown that the downregulation of Rab11a and Rab8a by shRNA is close to 90%, we did not see a similar decrease in 90% of the TNT-connected cells. This is expected, as other effectors have been described to increase TNT formation, such as M-sec (Hase et al., 2009) and myosin-10 (Gousset et al., 2013). On the other hand, the data suggest that these proteins might act in an independent (or parallel) pathway to the Rab11a–Rab8a cascade described here.

M-sec is known to be a component of the exocyst complex. It interacts with RalA (Ras-like small GTPase) to regulate the formation of TNTs (Kimura et al., 2016). RalA interacts with CDC42 through Ral1 binding protein for the formation of filopodia (Ikeda et al., 1998). Additionally, CDC42 has been shown to regulate the formation of TNTs in Raw264.7 cells (Hanna et al., 2017). However, in the case of CAD cells, CDC42 negatively regulates the formation of TNTs (Delage et al., 2016). Thus, we believe that this pathway is not responsible for TNT formation in our cell model.

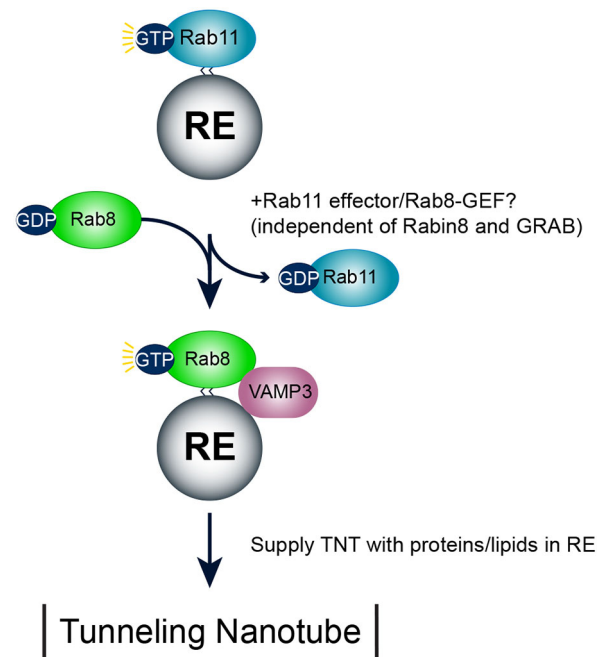
However, some proteins of the exocyst complex (e.g. Sec15 and Exo70) have been shown to interact with Rab11 (Takahashi et al., 2012; Wu et al., 2005). From preliminary experiments, overexpression of Sec15 in CAD cells results in only a slight decrease in vesicle transfer between cells in co-culture; consequently, we did not pursue this further. However, Exo70 could be an interesting candidate because it is known to induce membrane curvature and actin-free filopodia (Zhao et al., 2013) and it also interacts with Rab11 in the exocytosis process (Takahashi et al., 2012). Further research is necessary to explore this possibility.

Guanine nucleotide exchange factor for Rab3A (GRAB), a GEF of Rab8, has been shown to regulate axon outgrowth (Furusawa et al., 2017). These results indicated that GRAB regulates axonal outgrowth via activation and recruitment of Rab8A to Rab11A-positive endosomes in a Cdk5-dependent manner. GRAB might act as a GEF for regulating the formation of TNTs and the transfer of vesicles. But, from our results, we conclude that GRAB acts on TNT formation irrespective of Rab8a and is not involved in a Rab8a–Rab11a pathway in regulating the formation of TNT. Because GFP–GRAB promoted vesicle transfer in Rab8a-KD cells, GRAB might activate unknown Rabs other than Rab8a to increase vesicle transfer. Rabin8, a close homolog of GRAB, can activate both Rab8 and Rab10 to promote neurite outgrowth of

PC12 cells. Overexpression of Rab10 (WT/QL) had no effect on neurite outgrowth, whereas its knockdown decreased neurite outgrowth (Homma and Fukuda, 2016). Thus, Rab10 is a likely candidate for an alternative GRAB target in regulating the formation of TNTs between the cells. However, since Rab10 did not come up as a possible positive regulator in our initial screening of 41 Rabs, we believe that further studies are needed to explore the mechanism of Rab10 and GRAB in regulating the formation of TNTs.

In other circumstances, for example in *de novo* generation of the primary cilium, Rab11 regulates Rab8 function by activating Rabin8, a GEF of Rab8 (Westlake et al., 2011). However, in our cell system, overexpression of Rabin8 did not affect TNT number, strongly suggesting that Rabin8 is not the effector between Rab8 and Rab11 in the regulatory pathway of TNTs. Furthermore, we showed that, in our cell system, the Rab11a–Rab8a cascade induces filopodia formation through Rabin8, whereas VAMP3 had no effect on filopodia. This indicates that filopodia and TNTs, although naively similar in appearance, are distinct structures with different mechanisms of formation and different functions. We propose that Rab11a and Rab8a facilitate the trafficking and fusion of vesicles, containing specific proteins and lipids necessary for TNT formation and function, with the plasma membrane (by interacting with the v-SNARE protein VAMP3). On the other hand, this cellular process could also regulate TNT formation simply by facilitating membrane accumulation at the site of TNT formation (see schematic in Fig. 8).

In summary, our results represent the first molecular evidence of a mechanism whereby Rab GTPases regulate TNT formation in neuron-like CAD cells. Our study further confirms that TNTs are regulated differently from other cell protrusions, including filopodia and primary cilia. Future studies are required to provide further



**Fig. 8. Schematic of Rab11a–Rab8a–VAMP3 cascade in regulating TNTs.** GTP-bound Rab11a acts upstream of Rab8a, while VAMP3 acts downstream of Rab8a in regulating TNT formation. Rab11a-induced activation of Rab8a is independent of Rabin8 and GRAB. One possibility is that Rab11a is released from the recycling endosome (RE) when Rab8a is activated. GTP-bound Rab8a interacts with VAMP3, which functions as a Rab8a effector and facilitates the fusion of RE-derived vesicle to supply lipids and specific proteins needed for TNT formation.

insights into the involvement of these and other Rab proteins (as from our screen) in vesicular trafficking and actin remodeling in TNT formation to better characterize the differences and similarities between TNT and other membrane protrusions.

## MATERIALS AND METHODS

### Cell lines, plasmids and transfection procedures

The mouse catecholaminergic neuronal CAD cell line (mouse catecholaminergic neuronal cell line, Cath.aDifferentiated) (Gousset et al., 2013) was grown in Gibco OptiMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. pEGFP-C1-Rab plasmids library, pEGFP-C1-Rab8a(Q67L), pEGFP-C1-Rab8a(T22N), pEGFP-C1-Rab11a(Q70L), pEGFP-C1-Rab11a(S25N) and pEGFP-C1-Rabin8, and pEGFP-C1-GRAB were prepared as described previously (Furusawa et al., 2017; Ishida et al., 2012; Matsui and Fukuda, 2011; Tsuboi and Fukuda, 2006). GFP-VAMP3 was a kind gift from Thierry Galli (Center of Psychiatry and Neuroscience, INSERM U894, Paris France). GFP MICAL1 and GFP MICAL-LI were a kind gift from Arnaud Echard (Institut Pasteur, Paris). The shRNA non-target control (SHC016-1EA), shRNA Rab8a (TRCN0000100422) and shRNA Rab11a (TRCN0000100344) were from Sigma Aldrich. ShRNA Rabin8 was prepared as described previously (Homma and Fukuda, 2016). Anti-Rabin8 was purified as described (Homma and Fukuda, 2016). Anti-Rab8a antibody (ab188574), Anti-Rab11a antibody (ab128913) and anti-VAMP3 antibody (ab2102) were from Abcam. GFP-vector and H2B-mCherry-vector were from AddGene. CAD cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### High-content screening of Rab-GTPases

Confluent CAD cells were mechanically detached and counted, and 5000 cells were plated for 6 h in 96-well plates (Greiner Bio-One). The 41 GFP-Rab GTPase plasmids were mixed with 10  $\mu$ l Opti-MEM, and 0.2  $\mu$ l Lipofectamine was mixed with 10  $\mu$ l Opti-MEM. After mixing these two compartments for 15 min, the transfection mixture was added to cells and 80  $\mu$ l additional complete medium was added to cells. After 16 h of transfection, cells were labeled with lipophilic tracer Vybrant<sup>TM</sup> DiD (long-chain dialkylcarbocyanine) in complete medium for 30 min at 37°C and rinsed with PBS. 5000 CAD cells that had been transfected with H2B-mCherry were added to each well and co-cultured for 24 h. Cells were fixed with 4% PFA for 20 min at room temperature and labeled with HCS CellMask<sup>TM</sup> Blue. Images were acquired with a 20 $\times$  objective from Opera Phenix High Content Screening System (PerkinElmer). Fifty images of different fields from each well were acquired and analyzed using Cellprofiler (<http://cellprofiler.org/>).

### Quantification of TNT-connected cells

Confluent CAD cells were mechanically detached and counted, and 300,000 cells were plated for 6 h in six-well plates. Cells were transfected with the appropriate plasmids. At 24 h post-transfection, cells were detached and counted, and 220,000 cells were plated for 16 h on Ibidi  $\mu$ -dishes (Biovalley, France). At 16 h post-seeding, cells were fixed with fixative solution 1 (2% PFA, 0.05% glutaraldehyde and 0.2 M HEPES in PBS) for 20 min at 37°C, followed by a second 20 min fixation with fixative solution 2 (4% PFA and 0.2 M HEPES in PBS) at 37°C. The cells were gently washed with PBS and labeled with WGA-Alexa Fluor 594 (Sigma) (1:300 in PBS) for 20 min at room temperature, washed and sealed with Aqua-Polymount (Polysciences, Inc.). Cells were labeled with WGA-Alexa Fluor 594 for the plasma membrane and DAPI for the nucleus in all the experiments.

Image stacks (0.3  $\mu$ m) covering the whole cellular volume were acquired using a confocal microscope (Zeiss LSM 700) controlled by ZEN software. To evaluate the number of TNT-connected cells, manual analysis was performed for transfected cells with TNTs. The criterion for identifying TNTs was that a continuous connection could be found when moving along the stacks after removing the bottom three slices. The two cells connected by at least one continuous connection were marked as TNT-connected cells. Each experiment was performed at least in triplicate. Image analyses and

displays of raw data, such as Z-projections, were obtained using ICY software (Gousset et al., 2013).

### Fluorescence image analysis to quantify the transfer of DiD-labeled vesicles

Confluent CAD cells were mechanically detached and counted, and 800,000 cells were plated for 6 h in T25 flasks. The cells were transfected with the appropriate GFP-tagged constructs for donor cells and H2B-mCherry for acceptor cells for 24 h in complete medium. The donor cells were labeled using a 333 nM solution of Vybrant<sup>TM</sup> DiD in complete medium for 30 min at 37°C. Cells were then washed with PBS and 0.01% trypsin, resuspended in complete medium and counted. The labeled donor cells were mixed in a 1:1 ratio with H2B-transfected acceptor cells and plated at subconfluence (220,000 cells) on Ibidi  $\mu$ -dishes (Biovalley, France) for 16 h at 37°C. Cells were fixed with 4% PFA. Cells were washed and sequentially stained for 30 min with a 1  $\mu$ g ml<sup>-1</sup> solution of HCS CellMask<sup>TM</sup> Blue, which stains the entire cell volume (i.e. cytoplasm and nucleus). Samples were washed and sealed with Aqua-PolyMount (Polysciences, Inc.). The cells were imaged with an inverted confocal microscope (Zeiss LSM700) controlled by ZEN software. Quantification was carried out using ICY software (<http://icy.bioimageanalysis.org/>).

### Flow cytometry to analyze the transfer of DiD-labeled vesicles

Confluent CAD cells were mechanically detached and counted, and 800,000 cells were plated for 6 h in T25 flasks. Cells were transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions, with the appropriate GFP-tagged constructs for donor cells and with H2B-mCherry for acceptor cells, for 24 h in complete medium. Donor cells were detached, counted and labeled with a 333 nM solution of Vybrant<sup>TM</sup> DiD in complete medium for 30 min at 37°C. Cells were then washed with PBS and 0.01% trypsin, resuspended in complete medium and counted.

The labeled donor cells were mixed in a 1:1 ratio with H2B-mCherry-transfected acceptor cells and plated at subconfluence (120,000 cells per well) on 24-well plates for 16 h at 37°C. Each independent co-culture was performed in triplicate. To verify that the transfer of vesicles between cells is cell-cell dependent and not through secretion, two populations of cells were co-cultured with a Transwell insert (i.e. a filter; 0.4  $\mu$ m) that could separate two populations of cells but share the medium. Alternatively, the supernatant from donor cells after overnight culture was taken and added to acceptor cells for another 16 h of culture. Cells were then washed with PBS, mechanically detached from the dish by pipetting up and down with 500  $\mu$ l PBS and then passed through sterile 40-mm nylon cell strainers (BD Falcon<sup>TM</sup>) in order to obtain single-cell suspensions. Cell suspensions were fixed with 500  $\mu$ l of 4% PFA (2% final solution). Flow cytometry data were acquired using an LSR Fortessa flow cytometer (BD Biosciences). GFP fluorescence was analyzed at 488 nm excitation wavelength, RFP and mCherry fluorescence were analyzed at 561 nm excitation wavelength and DiD fluorescence was analyzed at 640 nm excitation wavelength. Samples were analyzed at a high flow rate, corresponding to 200–400 events per second, and 10,000 events were acquired for each condition. Data were analyzed using FlowJo analysis software.

### Quantification of vinculin-positive peripheral focal adhesion

For indirect immunofluorescence labeling of vinculin, 90,000 cells were plated for 16 h on Ibidi  $\mu$ -dishes and then fixed in 4% PFA in PBS for 15 min at 37°C. Cell samples were quenched with 50 mM NH<sub>4</sub>Cl for 15 min, then permeabilized with 0.01% saponin in PBS containing 2% BSA (w/v) for 20 min at 37°C. After a first 1-h incubation with mouse anti-vinculin antibody (V9264, Sigma) diluted 1:500 in PBS containing 0.01% saponin and 2% BSA (w/v), cells were thoroughly washed and incubated for 40 min with goat anti-mouse Alexa Fluor 488 (Invitrogen) diluted 1:500 in PBS containing 0.01% saponin and 2% BSA (w/v). Cells were washed and sequentially stained for 20 min with a 3.3  $\mu$ g  $\mu$ l<sup>-1</sup> solution of WGA Alexa Fluor 647 nm conjugate, for 30 min with 1  $\mu$ g ml<sup>-1</sup> solution of HCS CellMask<sup>TM</sup> Blue, which stains the entire cell volume (i.e. cytoplasm and nucleus), and for 5 min with a 0.2  $\mu$ g  $\mu$ l<sup>-1</sup> solution of DAPI. Samples were

washed and sealed with Aqua-Poly/Mount (Polysciences, Inc.). The bottom of the cell (in contact with the plastic dish) was imaged with an inverted confocal microscope (Zeiss LSM700) controlled by ZEN software. Displayed images corresponded to stack projections. Only linear corrections were applied, using the software ImageJ. Vinculin-positive peripheral focal adhesion was automatically detected and counted using ICY software (<http://icy.bioimageanalysis.org/>).

### Western blot

Cells transfected with shRNA were lysed in NP-40 lysis buffer (25 mM Tris, pH 7–8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100). Protein concentration in the cell lysate was quantified using a Bradford protein assay (Bio-Rad). Protein samples were incubated at 100°C for 5 min and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes (GE Healthcare Life Sciences). Membranes were blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (Sigma) (TBS-T) for 1 h. Membranes were then incubated at 4°C with a primary antibody, rabbit anti-Rab8a, anti-Rab11a, anti-VAMP3 and anti-Rabin8 and mouse anti- $\alpha$ -tubulin (Sigma) or anti-rabbit GAPDH (Cell Signaling Technology) diluted in 5% nonfat milk overnight (1:500 and 1:10,000, respectively) then washed several times with TBS-T. After 1 h incubation with horseradish peroxidase conjugated with the respective IgG secondary antibody (1:10,000) (GE Healthcare Life Sciences), membranes were washed with TBS-T and protein bands on the membrane were detected using an ECL-Plus immunoblotting chemiluminescence system (GE Healthcare Life Sciences). Membranes were imaged using ImageQuant LAS 500TM camera (GE Healthcare Life Sciences).

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: S.Z., C.Z.; Methodology: S.Z., Y.K., C.Z.; Software: S.Z.; Validation: S.Z., S.B.; Formal analysis: S.Z., S.B.; Investigation: S.Z., S.B., S.S., Y.K., M.F.; Resources: S.Z.; Data curation: S.Z.; Writing - original draft: S.Z., S.B., M.F., C.Z.; Writing - review & editing: S.Z., S.B., M.F., C.Z.; Visualization: S.Z., C.Z.; Supervision: M.F., C.Z.; Project administration: C.Z.; Funding acquisition: C.Z.

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### Supplementary information

Supplementary information available online at <http://jcs.biologists.org/lookup/doi/10.1242/jcs.215889.supplemental>

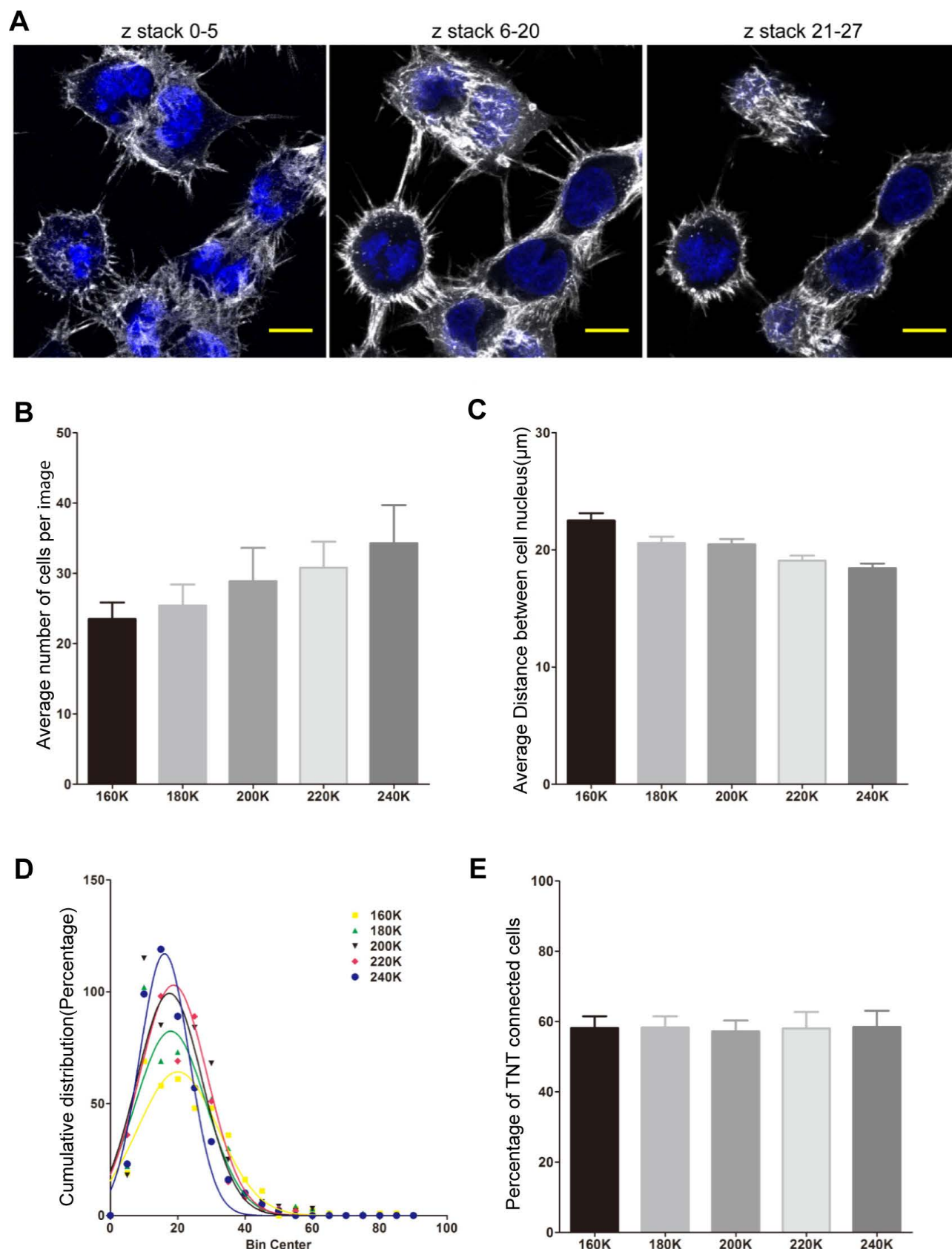
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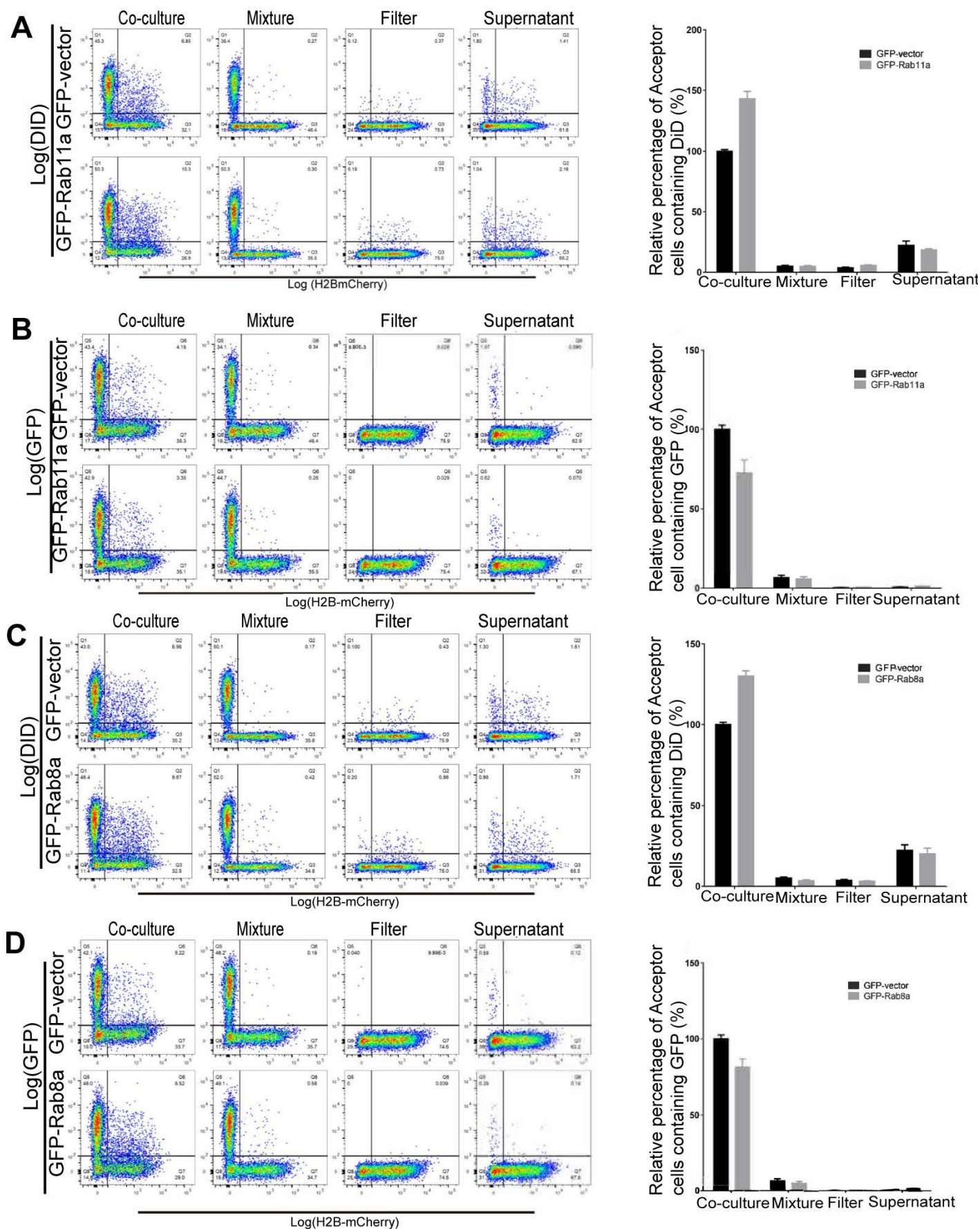
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**Fig. S1. The percentage of TNT connected cells does not change within a range of cell distance.** A. Representative confocal images of TNT formation between CAD cells. Images were acquired in 27 stacks, TNTs were present from stack 6 to stack 20. WGA-Alexa594 (white) was used to label cell plasma membrane and DAPI (blue) was used to label the nucleus. (Scale bar=10μm). B. Average number of CADs cells per images when 160K to 240K cells were plated (160K=23.77±1.39,180K=25.70±1.77,200K=29.21±2.769,220K=31.16±2.163,240K=34.70±3.149). C. Average distance between two cell nuclei from conditions as in (B) in mm(160K=22.51±0.62,180K=20.60±0.53,200K=20.49±0.43,220K=19.10±0.42,240K=18.44±0.40). D. Cumulative distribution of cell nuclear distance from conditions in (B). E. Bar graph representing average percentage of TNT connected cells from experiment described in (B) (160K=58.62±1.9 ,180K=58.75±1.91,200K=57.62±1.87,220K=58.49±2.72,240K=58.91±2.72)

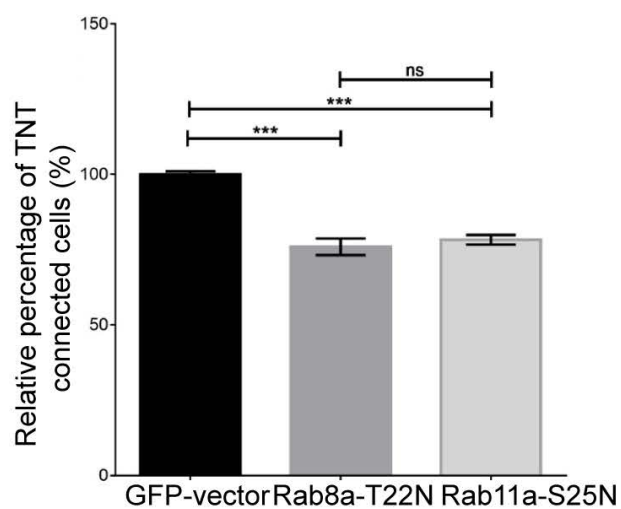




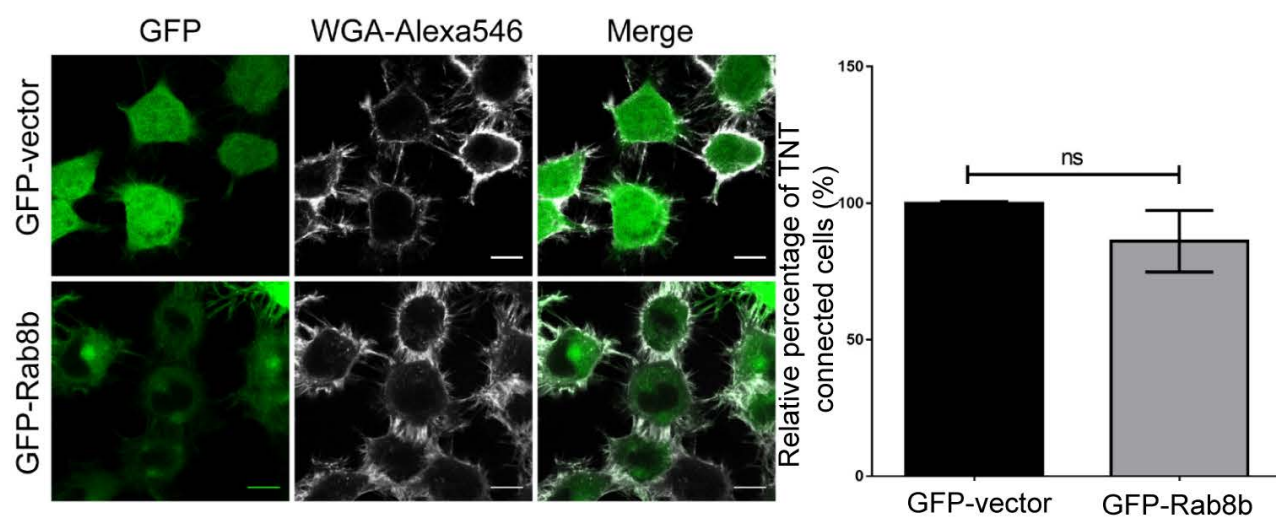
**Fig. S2. DiD-labeled vesicles transfer between cells overexpressing Rab11a or Rab8a is mediated by cell-to-cell contact.** A. Left panel, Raw data (dot plots) from a representative experiment showing the transfer of DiD-labeled vesicles to the acceptor cells population (H2B-mCherry) from mixture, co-culture with filter GFP-vector or GFP-Rab11a, coculture with/ without filter and supernatant between donor cells transfected with GFP-vector or GFP-Rab11a and acceptor cells. Right panel, Bar graph representing relative percentage of DiD positive acceptor cells by flow cytometry from conditions indicated in left panel mixture GFP-vector=5.03±0.67 or GFP-Rab11a=4.186±0.76, co-culture with filter GFP-vector=3.66±0.58, GFP-Rab11a=5.58±0.57 coculture without filter GFP-vector=100.0±1.4 or GFP-Rab11a=143.1±6.02 and supernatant GFP-vector=22.2±3.14 or GFP-Rab11a= 18.46±0.95. B. Left panel, Raw data (dot plots) from a representative experiment showing the transfer of GFP protein to the acceptor cells population (H2B-mCherry) from mixture, co-culture without filter with filter, and supernatant between donor cells transfected with GFP-vector or GFP-Rab11a and acceptor cells. Right panel shows bar graph representing relative percentage of GFP protein positive acceptor cells by flow cytometry from conditions indicated in left panel for mixture (GFP-vector=6.5±2.25 GFP-Rab11a=5.37±1.38), co-culture with filter (GFP-vector=0.35 ±0.17, GFP-Rab11a=0.44±0.08), coculture without filter (GFP-vector=100±3.84, GFP-Rab11a =72.64± 8.26) and supernatant (GFP-vector=0.85±0.21, GFP-Rab11a=1.13 ±0.12). C. Left panel, Raw data (dot plots) from a representative experiment showing the transfer of DiD-labeled vesicles to the acceptor cells population (H2BmCherry) from mixture, co-culture (with/without filter) and supernatant between donor cells transfected with GFP-vector or GFP-Rab8a and acceptor cells. Right panel, Bar graph representing relative percentage of DiD positive acceptor cells by flow cytometry from conditions indicated in left panel for mixture GFP-vector=5.03±0.67 or GFP-Rab8a=3.46±0.6, co-culture with filter GFP-vector=3.6±0.58, GFP-Rab8a =3.193 coculture without filter GFP-vector=100.0±1.4 or GFP-Rab8a=130.1±3.23 and supernatant (GFP-vector=22.2±3.14 or GFP-Rab8a= 20.04±3.6). D. Left panel, Raw data (dot plots) from a representative experiment showing the transfer of GFP protein to the acceptor cells population (H2B-mCherry) from mixture coculture with filter coculture without filter and supernatant between donor cells transfected with GFP-vector or GFP-Rab8a and acceptor cells. Right panel, Bar graph representing relative percentage of GFP protein positive acceptor cells by flow cytometry from conditions indicated in left panel for mixture (GFP-vector=9.09± 1.25, GFP-Rab8a=4.95±1.2), co-culture without filter (GFP-vector=100± 3.764 GFP-Rab8a= 81.64±5.40) with filter (GFP-vector=0.32±0.18, GFP-Rab8a=0.54±0.11), and supernatant (GFP-vector=0.57±0.29, GFP-Rab8a=0.54 ±0.11). Data represent the mean ± s.e.m of at least 3 independent experiments in triplicate setting.



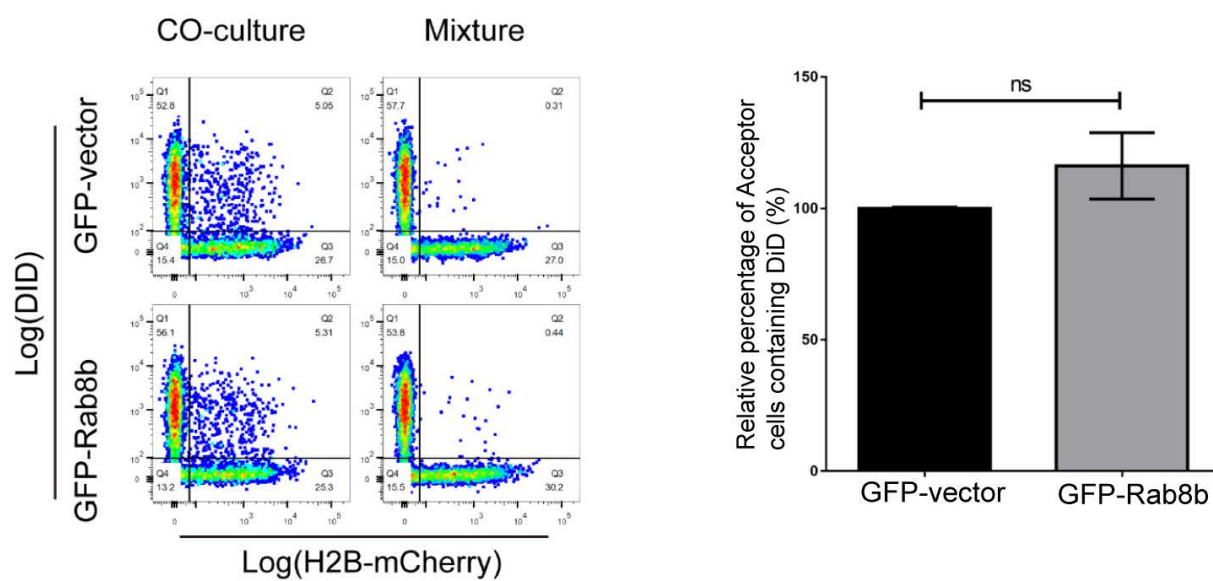
**A**



**B**

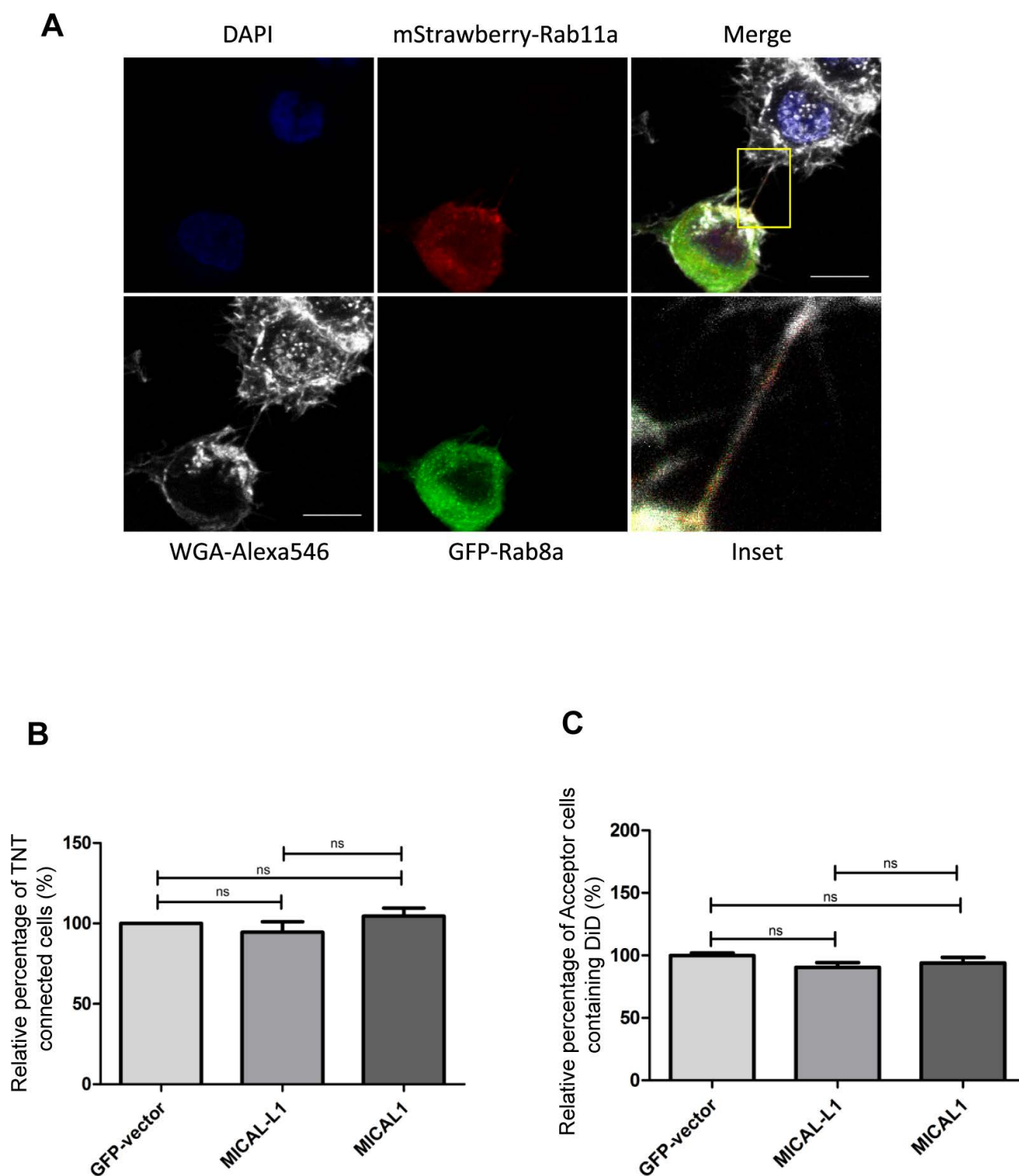


**C**

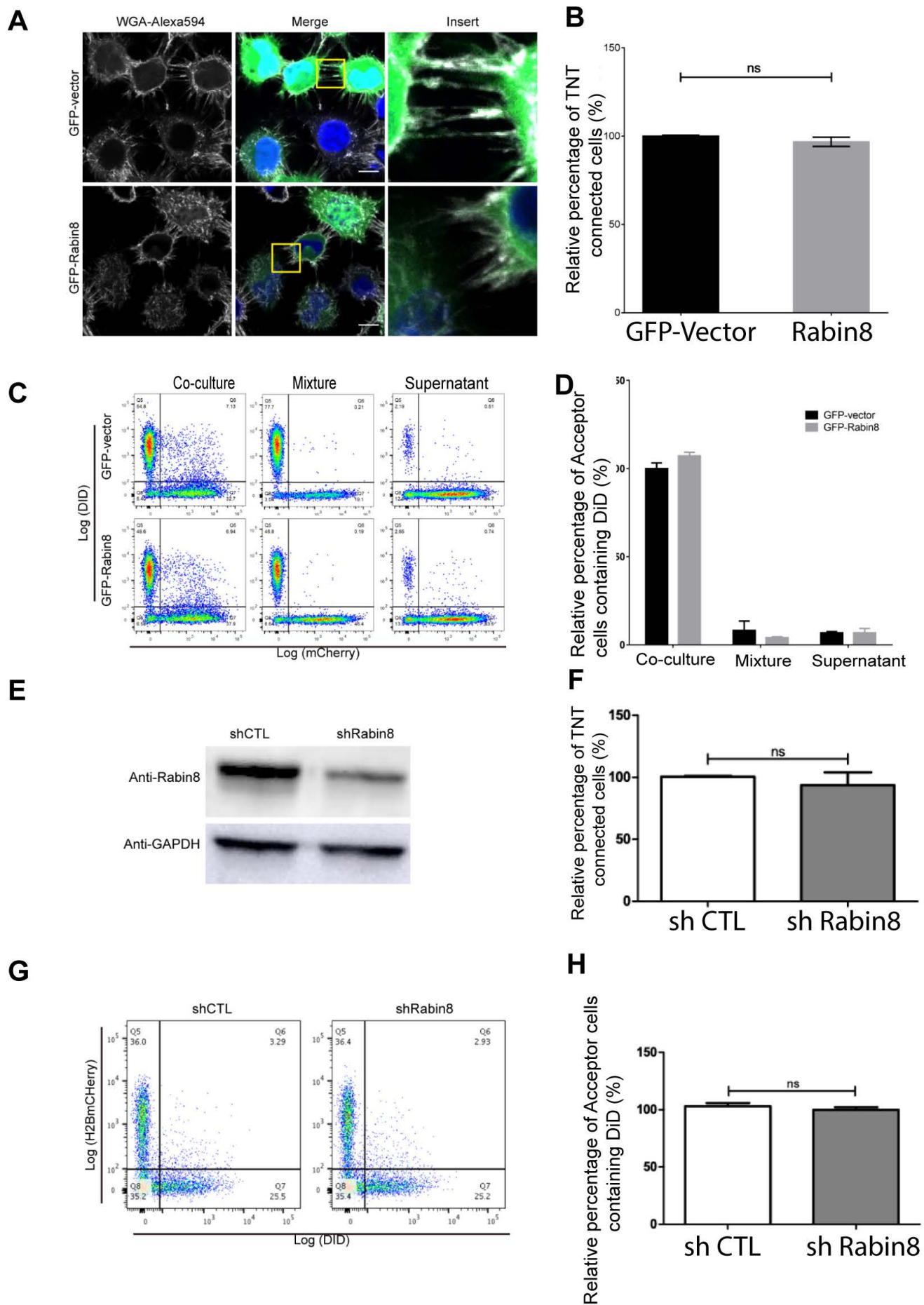


**Fig.S3. Overexpression of inactive mutants of Rab11a and Rab8a for 52 hours significantly decreased the number of TNT-connected cells and Rab8b has no role in the TNT formation.**

A. Bar graph showing relative percentage of TNT connected cells after transfection with either GFP-vector, GFP-Rab8a-T22N and Rab11a-S25N for 52hr (GFP-vector=100± 0.577, GFP-Rab8a-T22N=75.94±1.60 and Rab11a-S25N=78.29±0.92). The data represents the mean ± s.e.m, of at least 3 independent experiments (ns, no significant; \*\*\*,  $p < 0.001$  by One-way ANOVA with Tukey's multiple comparison post test). B. Left panel show representative confocal images of TNT connected cells after transfection with GFP-vector and GFP-Rab8b. Right panel is showing bar graph of relative percentage of the TNT-connected cells from the experiment described in the left panel (GFP-vector = 100.0 ± 0.5774 ,GFP-Rab8b= 86.05 ± 11.27), the graph shows mean ± s.e.m from three independent experiments (ns, no significant by Unpaired t test) C. Left panel shows the dot plot of the flow cytometry analysis of DiD transfer between donor cells overexpressing GFP-vector or GFP-Rab8b and acceptor cells expressing H2Bmcherry(GFP-vector= 100.0 ± 0.5774,GFP-Rab8b= 116.2 ± 12.67). Right panel shows bar graph of the relative percentage of the acceptor cells with DiD. Data shows mean ± s.e.m from three independent experiments (ns, no significant by unpaired student t test).



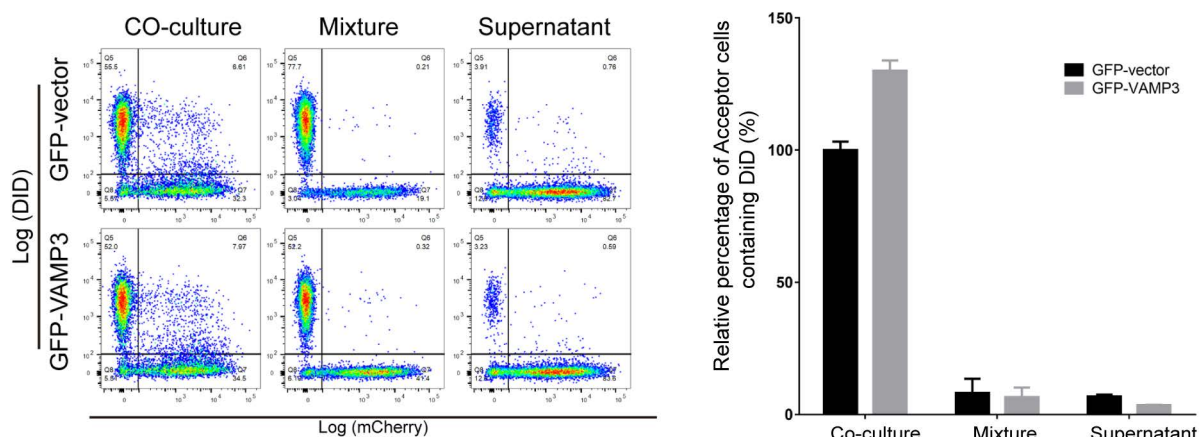
**Fig.S4 Rabs do not colocalise on TNT and MICAL1 and MICAL-L1 has no effect on TNT formation:** A Images show cells cotransfected with GFP-Rab8a(green) and mStrawberry-Rab11a(red). The DAPI (blue) and WGA-Alexa 594(white) was used to label the nucleus and cell membrane respectively. Inset shows the magnification of the region marked with yellow box from merge. (Scale bar =10μm) B. Bar graph representing the relative percentage of TNT-connected cells transfected with GFP-Vector, GFP-MICAL-L1, GFP-MICAL1. (GFP-Vector= 100±1.90, GFP-MICAL-L1= 90.46±3.75, GFP-MICAL1= 93.86±4.57) C. Bar graph showing the relative percentage of acceptor cells containing DiD labeled vesicles from the co-cultures, in which the donor cells were transfected with either GFP-Vector, GFP-MICAL-L1, GFP-MICAL1 and labeled with DiD. All the above bar graphs show mean ± s.e.m from three independent experiments (GFP-Vector= 100±0.00, GFP-MICAL-L1= 94.51±6.49, GFP-MICAL1= 104.5±4.98) (ns, no significant by One-way ANOVA with Tukey's multiple comparison post test).



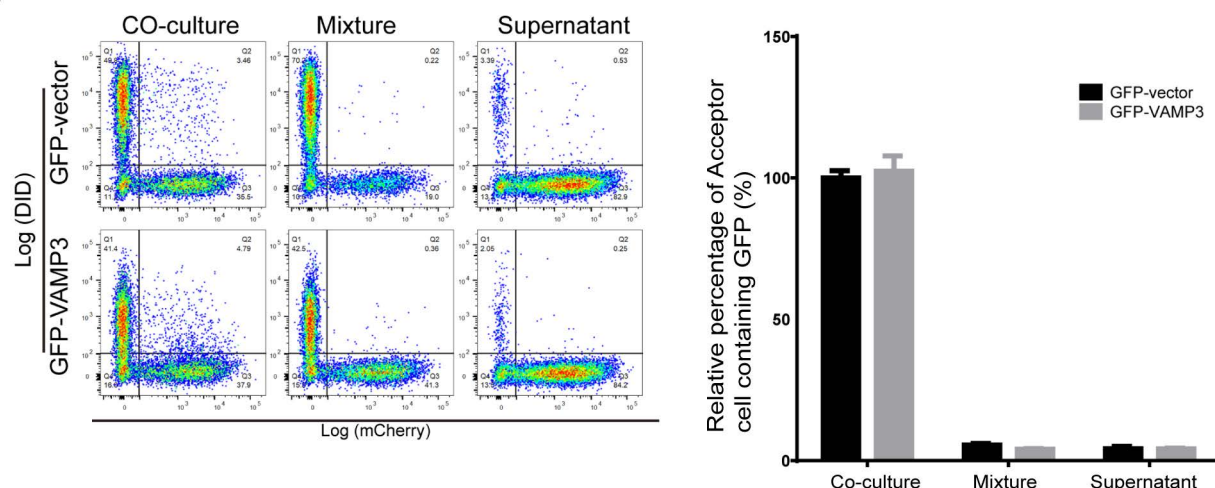
**Fig.S5. Rabin8 has no effect on the number of TNT-connected cells and vesicle transfer between cells.** A. Representative confocal images of TNT connected cells transfected with GFP vector or GFP-Rabin8. Inserts are enlargements of the framed region in merge panel. WGAAlexa594 (white) was used to label cell plasma membrane to show TNT between cells. (Scale bar=10µm). B. Bar graph showing relative percentage of TNT connected cells transfected with GFP-vector or GFP-Rabin8(GFP-vector=100 ±0.57, GFP-Rabin8=96.80 ±2.60). Data shows mean ± s.e.m from three independent experiments (ns, no significant; by unpaired t test). C. Raw data (dot plots) from a representative experiment showing the transfer of DiD labeled vesicles to the acceptor cells population (H2B-mCherry) from co-culture, mixture and supernatant between donor cells transfected with GFP-vector or GFP-Rabin8 and acceptor cells by flow cytometry. D. Bar graph showing relative percentage of DiD positive acceptor cells from the experiment described in (C) (GFP-vector or GFP-Rabin8). Data represent the mean ± s.e.m of three independent experiments in triplicate setting (ns, no significant; by unpaired student t test). E. Western blot analysis of extracts from cells transfected for 48h with ShRNA non-targeting (CTL) or targeting Rabin8 and GAPDH as loading control. F. Bar graph showing quantification of relative percentage of TNT connected cells transfected with ShCTL+GFP-vector or ShRabin8+GFP (ShCTL+GFP-vector= 100.5 ± 0.5, ShRabin8+GFP= 93.74 ± 10.37). Data shows mean ± s.e.m from two independent experiment (ns, no significant; by unpaired student t test). G. Raw data (dot plots) from a representative experiment showing the transfer of DiD labeled vesicles to the acceptor cells population (H2B-mCherry) from co-culture between ShCTL+GFPvector and shRabin8+GFP-vector transfected donor cells and acceptor cells. H. Bar graph showing relative percentage DiD positive acceptor cells from the experiment described in (G) (ShCTL+GFPvector= 100.0 ± 0.57, shRabin8+GFP-vector= 100.2 ± 2.09). Data shows mean ± s.e.m (ns, no significant; by unpaired t test) from one experiment performed in triplicate.)



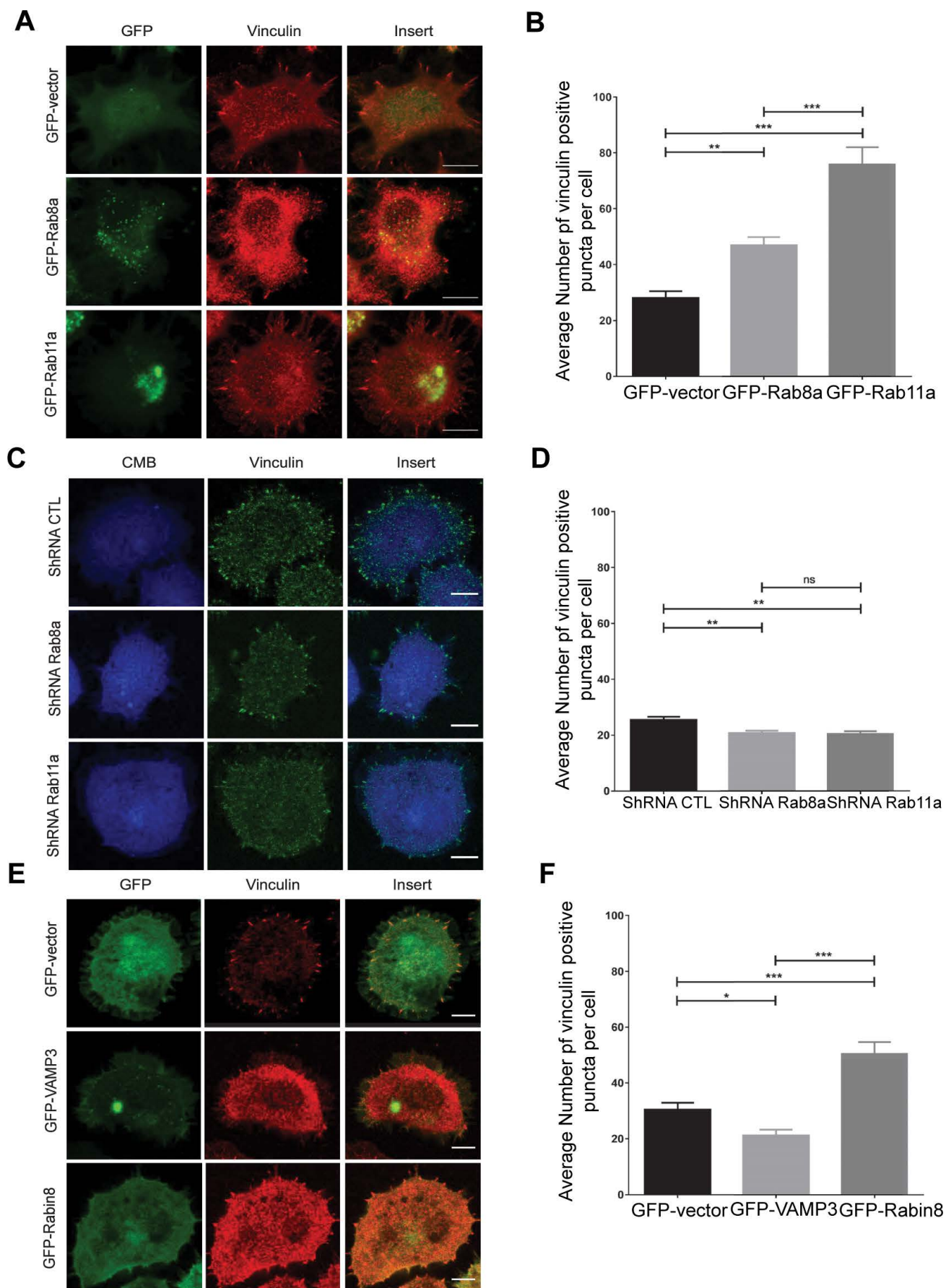
A



B



**Fig. S6. DiD labeled vesicles transfer between cells affected by VAMP3 overexpression was mediated by cell-to-cell contact.** A. Left panel, Raw data (dot plots) from a representative experiment showing the transfer of DiD labeled vesicles to the acceptor cells population (H2BmCherry) from co-culture, mixture, and supernatant between donor cells transfected with GFPvector or GFP-VAMP3 and acceptor cells, was analyzed by flow cytometry. Right panel, bar graph showing the relative percentage of DiD positive acceptor cells from the experiment described in the left panel (coculture GFPvector=100.0±1.5 or GFP- VAMP3=127.8±3.19 mixture GFPvector=4.5±1.38 GFP- VAMP3=4.15±1.18 supernatant GFPvector=3.18±0.01 GFP- VAMP3=3.7±0.74) Data represent the mean ± s.e.m of at least 3 independent experiments in triplicates. B. Left panel, Raw data (dot plots) from a representative experiment showing the transfer of GFP-Vector or GFP-VAMP3 protein to the acceptor cells population (H2B-mCherry) from mixture, co-culture (with/without filter) and supernatant between GFP-vector or GFP-VAMP3 transfected donor cells and acceptor cells and analyzed by flow cytometry. Right panel, Bar graph showing relative percentage of DiD positive acceptor cells from the experiment described in the left panel. Data represent the mean ± s.e.m, of at least 3 independent experiments in triplicates.



**Fig. S7. Rab8a and Rab11a promote attached filopodia formation.**

A. Representative confocal images of cells transfected with either GFP- vector, GFP-Rab8a or GFP-Rab11a. Cells were immunostained with anti-vinculin antibody (red). B. The bar graph represents the average number of vinculin positive puncta per cell from the experiment described in (A) (GFP- vector=  $29.26 \pm 1.91$ , GFP-Rab8a=  $40.84 \pm 2.43$  or GFP-Rab11a=  $67.59 \pm 5.87$ ) C. Representative confocal images of cells transfected with either ShCTL, ShRab8a or ShRab11a. Cells were immunostained with anti-vinculin antibody (Green) and stained with HCS CellMaskTM. Quantification of average number of vinculin positive puncta per cell of the experiment described in (C) (ShCTL=  $28.11 \pm 1.25$ , ShRab8a=  $20.78 \pm 0.84$ , ShRab11a=  $23.11 \pm 0.87$ ). E. Representative confocal images of cells transfected with either GFP-vector, GFPVAMP3 or GFP-Rabin8. Cells were immunostained with anti-vinculin antibody (Red). Representation of the average number of vinculin positive puncta per cell from the experiment described in (E) (GFP-vector=  $30.52 \pm 2.40$ , GFPVAMP3=  $21.15 \pm 2.12$ , GFP-Rabin8=  $50.49 \pm 4.21$ ). All the above graphs show mean  $\pm$  s.e.m from three independent experiments (ns, no significant \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  by one-way ANOVA with Tukey's multiple comparison post test). Scale bar=10 $\mu$ m



**Table S1. Data of High content subfamily-wide screening of 41 Rab GTPases overexpressed in CAD cells.** Relative percentage of acceptor cells containing DID labeled vesicles that transferred from donor cells. Data were normalized to control conditions. Values of score when applying two thresholds (95% and 105%) to data and sum of 4 experiments.

	Exp1	Exp2	Exp3	Exp4		Exp1	Exp2	Exp3	Exp4		Sum
MyoX	107.93%	109.37%	112.50%	110.39%	MyoX	1	1	1	1		4
GFP-v	100.00%	100.00%	100.00%	100.00%	GFP-v	0	0	0	0		0
VASP	92.13%	88.02%	81.51%	93.09%	VASP	-1	-1	-1	-1		-4
1A	109.17%	94.51%	84.88%	55.33%	1A	1	-1	-1	-1		-2
2A	110.05%	86.38%	80.39%	103.71%	2A	1	-1	-1	0		-1
3A	112.08%	80.95%	95.25%	101.51%	3A	1	-1	0	0		0
4A	115.66%	91.82%	95.29%	98.63%	4A	1	-1	0	0		0
5A	104.66%	76.50%	87.56%	106.94%	5A	0	-1	-1	1		-1
6A	106.59%	80.34%	86.77%	100.27%	6A	1	-1	-1	0		-1
7	101.78%	84.04%	90.57%	111.21%	7	0	-1	-1	1		-1
8A	107.01%	108.08%	105.63%	128.07%	8A	1	1	1	1		4
9A	115.70%	100.75%	89.89%	97.69%	9A	1	0	-1	0		0
10	105.29%	86.96%	85.37%	92.61%	10	1	-1	-1	-1		-2
11A	117.84%	109.95%	113.80%	113.33%	11A	1	1	1	1		4
12	109.42%	77.45%	87.59%	106.15%	12	1	-1	-1	1		0
13	111.35%	87.43%	89.02%	103.90%	13	1	-1	-1	0		-1
14	110.73%	93.12%	95.37%	95.85%	14	1	-1	0	0		0
15	97.54%	92.79%	98.66%	107.64%	15	0	-1	0	1		0
17	96.41%	96.10%	94.23%	109.72%	17	0	0	-1	1		0
18	86.38%	104.31%	98.22%	105.74%	18	-1	0	0	1		0
19	98.54%	100.14%	96.50%	107.10%	19	0	0	0	1		1
20	98.16%	92.85%	98.85%	95.93%	20	0	-1	0	0		-1
21	100.56%	104.45%	93.08%	99.04%	21	0	0	-1	0		-1
22A	96.99%	102.68%	88.51%	105.15%	22A	0	0	-1	1		0
23	91.60%	103.93%	99.42%	105.98%	23	-1	0	0	1		0
24	103.02%	102.75%	94.73%	95.79%	24	0	0	-1	0		-1
25	107.50%	96.46%	96.04%	91.11%	25	1	0	0	-1		0
26	108.86%	98.59%	79.29%	94.74%	26	1	0	-1	-1		-1
27A	109.41%	99.98%	94.92%	104.40%	27A	1	0	-1	0		0
28	112.98%	96.42%	95.79%	110.77%	28	1	0	0	1		2
29/7L1	108.33%	107.84%	88.99%	111.77%	29/7L1	1	1	-1	1		2
30	95.64%	104.62%	104.67%	95.82%	30	0	0	0	0		0
32	99.32%	98.44%	92.22%	96.71%	32	0	0	-1	0		-1
33A	100.09%	107.99%	117.41%	94.90%	33A	0	1	1	-1		1
34	102.04%	106.63%	80.69%	96.67%	34	0	1	-1	0		0
35	116.37%	106.11%	116.65%	108.16%	35	1	1	1	1		4
36	89.69%	105.77%	91.37%	97.60%	36	-1	1	-1	0		-1
37	96.08%	96.22%	90.52%	99.32%	37	0	0	-1	0		-1
38	88.35%	101.39%	110.89%	100.96%	38	-1	0	1	0		0
39A	91.44%	91.82%	88.58%	92.65%	39A	-1	-1	-1	-1		-4
40A	79.21%	92.13%	90.86%	92.26%	40A	-1	-1	-1	-1		-4
41/43	95.75%	107.68%	74.22%	102.27%	41/43	0	1	-1	0		0
42/7B	106.34%	100.20%	71.50%	95.93%	42/7B	1	0	-1	0		0
43/42	86.40%	105.30%	91.29%	100.40%	43/42	-1	1	-1	0		-1

**Table S2. Data showing average percentage of TNT connected cells.** Cells transfected with the plasmids as mentioned and the corresponding average of percentage of TNT connected cells showing  $\pm$  s.e.m.

Plasmids	Average Percentage of TNT connected cells
GFP-vector	57.44 $\pm$ 2.50
Rab11a-WT	76.68 $\pm$ 4.13
Rab11a-Q70L	92.25 $\pm$ 3.01
Rab11a-S25N	65.70 $\pm$ 3.95
Sh CTL	56.71 $\pm$ 3.41
Sh Rab11a	40.81 $\pm$ 0.99
Sh Rab11a/GFP-Rab11a	59.34 $\pm$ 3.30
GFP-vector	51.32 $\pm$ 1.9
Rab8a-WT	76.4 $\pm$ 2.06
Rab8a-Q67L	79.17 $\pm$ 5.07
Rab8a-T22N	53.47 $\pm$ 1.94
Sh CTL	56.71 $\pm$ 3.41
Sh Rab8a	46.15 $\pm$ 4.238
Sh Rab8a/GFP-Rab8a	69.07 $\pm$ 13.14
Sh CTL	52.35 $\pm$ 3.40
Sh Rab11a	39.94 $\pm$ 1.32
Sh Rab11a/GFP-Rab8a	61.43 $\pm$ 1.228
Sh CTL	52.35 $\pm$ 3.40
Sh Rab8a	40.98 $\pm$ 4.86
Sh Rab8a/GFP-Rab11a	42.78 $\pm$ 2.403
GFP-Vector	44.47 $\pm$ 6.79
GFP-GRAB	56.18 $\pm$ 10.64
ShCTL/GFP	38.86 $\pm$ 1.71
shRab8/GFP	30.38 $\pm$ 0.38
shRab8/GRAB	49.36 $\pm$ 2.36
H2O	68.92 $\pm$ 2.876
PMQ	44.17 $\pm$ 5.077
GFP-vector	56.56 $\pm$ 4.94
VAMP3	71.45 $\pm$ 6.07
Sh CTL	55.04 $\pm$ 1.050
Sh VAMP3	44.97 $\pm$ 1.463
Sh VAMP3/GFP-VAMP3	69.84 $\pm$ 1.017
Sh CTL	56.65 $\pm$ 2.04
Sh VAMP3	46.18 $\pm$ 2.078
Sh VAMP3/GFP-Rab8a	51.45 $\pm$ 4.92
Sh CTL	56.65 $\pm$ 2.04
Sh VAMP3	46.18 $\pm$ 2.07
Sh VAMP3/GFP-Rab11a	49.46 $\pm$ 2.22

## **Article 2**

## **Title: Role of Rab 35 and its effectors on Tunneling nanotubes**

Bhat S, Ljubojevic N, Zhu S, Echard A, Fukuda M, Zurzolo C.

**Abstract:** Tunneling nanotubes (TNTs) are F-actin rich structures that connect distant cells, allowing the transport of many cellular components, including vesicles, organelles, and different kind of molecules. TNTs are implicated in key cellular processes, such as development, immunity, and tissue regeneration, but also in the transmission of various pathogens. Since Rab GTPases are the major regulators of vesicle trafficking and also participate in actin cytoskeleton regulation, we examined the role of this protein family in TNT formation. First, we performed a screening of several different Rab proteins for its effect on TNT-dependent vesicle transfer. Rab35 was one of the Rabs to have a positive effect on vesicle transfer. The analysis of Rab35, a protein involved in endocytic recycling, cytokinesis, and neurite outgrowth, showed that the GTP-Rab35 bound form also increases TNT formation. Neurite outgrowth is an essential process in order to establish neural connectivity and vesicle recycling plays a crucial role in this process. Rab35 interacts with several proteins, that are involved in vesicle trafficking such as such as ACAP2 (acts as GAP of ARF6), MICAL-L1 (molecule interacting with CasL-like 1, which plays a role in vesicle recycling) EHD1 (a molecular scissor that has a role in vesicle scission). At the ARF6 positive endosomes, Rab35 recruits ACAP2 and MICAL-L1, and forms a complex that binds to EHD1 to regulate neurite outgrowth. Our data strongly suggest that these effectors may also be involved in the formation of TNTs. Individually, ACAP2, EHD1 and ARF6-GDP regulate TNT formation in a positive manner. But MICAL-L1 overexpression in cells shows no effect on TNTs. Also, preliminary data, indicates that Rab35 and EHD1 acts in a cascade mechanism to regulate TNT formation. This indicates that TNT formation and neurite outgrowth may act in a similar, but not exact pathway. The molecules identified here that have a role in TNT formation, constitute potential molecular targets for therapies aiming to block the spreading of pathogens that transfer through TNTs. This study proves that proteins that have a role in vesicle trafficking and neurite outgrowth, such as Rab proteins, are also involved in TNT formation.

## **Introduction:**

Tunneling nanotubes (TNTs) are F-actin based intercellular membrane connections, non-adherent to substrate and transport cytoplasmic contents and cellular organelles. Their diameter ranges from 50-150 nm and their length can extend upto several hundred micrometers (Rustom et. al., 2004, Sartori-Rupp et. al., 2019). TNTs transport lysosomes (Rustom et. al., 2004, Abounit et. al., 2016a), mitochondria (Lu et. al, 2017, Wang and Gerdes, 2015), RNA and calcium (Antanaviciute et. al., 2014, Wang et. al., 2012).

TNTs have been shown to be involved in several pathogenic conditions, as they are hijacked by different pathogens; by viruses like HIV (Eugenin et. al., 2008) and bacteria (Sisakhtnezhad and Khosravi, 2015). In several cases of neurodegenerative diseases, they have been involved in the spreading of proteins like prions, alpha synuclein, huntingtin etc. to the healthy neighboring cells (Zhu et. al., 2015, Sharma and Subramaniam, 2019, Wang et. al., 2011, Dieriks et. al., 2017, Dilsizoglu Senol et. al., 2019, Tardivel et. al., 2016). TNTs have also been found in several types of cancer, where their presence was shown to result a more aggressive phenotype (Lou et. al., 2012, Pasquier et. al., 2013). Furthermore, TNT like structures plays a role in development, as shown in chick and sea urchin embryos (Miller et. al., 1995, Teddy et. al., 2004).

TNTs are predominantly made of F-actin. F-actin affecting agents such as cytochalasin B, and latrunculin A or telytoxin (a cyanobacterial nucleoside which affects F-actin polymerization) show a reduction in TNTs in AML cells, neurons, astrocytes, PC12, bone marrow derived MSCs, SW13, SH-SY5Y cells and CAD cells. On the other hand, microtubule affecting agents as nocodazole (which disrupts the microtubule), and paclitaxel (microtubule stabilizing agent) had no effect on TNTs in the majority of the cells (e.g. CAD, SH-SY5Y, RAW 264.7, PC12, PC3 cell lines) (Marlein et. al., 2017, Wang et. al., 2011, Rustom et. al., 2004, Han et. al., 2016, Dilsizoglu Senol et. al., 2019, Zhang et. al., 2018, Hanna et. al., 2017b, Kretschmer et. al., 2019, Abounit et. al., 2016a). There are two major mechanisms by which TNTs are formed: 1) They can be formed after two cells come in contact followed by their dislodgement or 2) they can be formed by actin protrusions dependent mechanism in which one cell sends a filopodia like protrusions that becomes a TNT when it fuses with the opposite cell (Onfelt et. al., 2005, Abounit et. al. 2012, Marzo et. al., 2012, Reichert et. al., 2016).

We have shown that in neuronal cells which are immobile the actin driven protrusion mechanism is the most active (Gousset et. al., 2009, Gousset et. al., 2013). Indeed,

different actin modifying proteins have been shown to regulate TNTs like focal adhesion kinases (FAK) and matrix metalloproteases (Sáenz-de-Santa-María et. al., 2017), fascin (Lou et. al., 2012), MyosinX (Uhl et. al., 2019, Gousset et. al., 2013), Rac and Cdc42 (Hanna et. al., 2017a). Interestingly we have shown that in CAD cells the CDC42/IRSp53/VASP cascade that increases filopodia formation decreased TNTs, while Eps8 acts in decreasing filopodia and increasing TNTs (Delage et. al., 2016). We also found the Arp2/3 inhibition, increased TNTs. We therefore concluded that in neuronal cells the regulation of TNTs and filopodia seems to employ the same molecules which act in an opposite manner (Delage et. al., 2016, Sartori-Rupp et. al., 2019).

In addition to actin polymerization the formation of a membrane protrusion also requires the support of lipids and proteins at the plasma membrane site where the extension occurs. Endocytic recycling mediates the return of receptors back to the plasma membrane (Cai et. al., 2013). The membrane recycling process is regulating the plasma membrane composition by maintaining the balance between uptake and recycling, this in turn contributes to several cellular processes such as cytokinesis, cell migration and polarisation and signal transduction (Grant and Donaldson, 2009).

Rab proteins are known to be master regulators of cellular functions. They are involved in processes such as vesicle trafficking, migration of cells and formation of protrusion like filopodia, lamellipodia. Rabs function through GTP bound (active) – GDP bound (inactive) cycle (Das and Guo, 2011). 41 Rabs were screened in our lab to identify the specific Rabs regulating TNTs; Rab8, Rab11 and Rab35 was shown to regulate TNTs positively (Zhu et. al., 2018). We have previously characterized the mechanism by which Rab8 and Rab11 regulate TNT formation (Zhu et. al., 2018).

Rab35 is involved in fast endocytic recycling and is known to recycle TFRs and plays a key role in cytokinesis (Kouranti et. al., 2006, Klinkert and Echard, 2016, Dikshit et. al., 2014). ARF6 (ADP Ribosylation Factor) negatively regulates Rab35 in cytokinesis (Chesneau et. al., 2012). EHD family of proteins have an EH domain (Eps15 homology domain) are homolog to the Eps15 protein. EHD family of proteins is associated with ERC, and EHD1 and EHD4 are involved in the recycling of the membranes decorated with MICAL-L1 (Cai et. al., 2013). Also, loss of MICAL-L1 leads to the elongation of spindle fibers during the cytokinetic process in the cells (Reinecke et. al., 2015). Reduction of PI4P either by activation of PIP5KI-gamma expression or by Sac1, a phosphatase that specifically induces dephosphorylation of PtdIns4P to PtdIns, caused a reduction in EHD1 associated tubular membranes (Jovic et. al., 2007).

Interestingly, association of Rab35 to the ARF6 positive endosomes was shown to induce neurite outgrowth following NGF activation (Kobayashi and Fukuda, 2013). The authors demonstrated that Rab35 recruits both ACAP2 and MICAL-L1 to form a tripartite complex. MICAL-L1 directly acts as a binding site for EHD1, which then has a positive effect on the neurite outgrowth. On the other hand, ACAP2 indirectly regulates the recruitment of EHD1 to the membrane by inactivating ARF6 and may thereby influence the levels of PI4P on the membrane of the vesicles, which in turn allows the binding of EHD1 to the membrane and EHD1 function in regulating neurite outgrowth. Based on these findings, here we analysed a possible role of Rab 35 in TNT formation.

Neurite outgrowth involves both recycling of membrane and the cytoskeletal assembly. Since we had already shown the need of cytoskeleton modification and the involvement of membrane recycling for TNT formation (Delage et. al., 2016, Zhu et. al., 2018) in neuronal cells, we postulated that Rab35 may act through a similar pathway as neurite outgrowth, in regulating TNT formation. By overexpressing/dowregulation of Rab35 and different downstream effectors, we found that Rab35 positively regulates TNT formation and vesicle transfer between connected cells, via its active GTP-bound form. We demonstrated that Rab35 acts through a cascade pathway to regulate downstream EHD1 mediated vesicle recycling. Importantly, we show that Rab35 acts through ARF6 and ACAP2 but does not require MICAL-L1. This suggests that this pathway is specific for TNT and is different from the one shown to be activated in the case of neurite elongation, where MICAL-L1 is required. Thus, our data concur to demonstrate that activation of vesicle recycling through Rab35 is necessary for TNTs formation and that TNTs are specific novel structures that differ from other neurite protrusions.

## **Results**

### **Rab35-GTP shows an increase in TNTs**

Rab35 has been implicated in a variety of cellular processes such as neurite outgrowth, cilia formation and cytokinesis (Kouranti et. al., 2006, Kobayashi and Fukuda, 2013, Kuhns et. al., 2019). In our previous screening, based on the effect of Rab protein overexpression to affect TNT-mediated vesicle transfer in CAD cells in 96 well plate, one positive hit was Rab35 (Zhu et. al., 2018). Thus, we decided to validate this result by overexpressing Rab35 and its active and inactive forms of CAD cells grown in 35cm cell plate. Specifically, we transfected the wild type (WT), active (Q67L) and inactive (S22N) form of Rab35–GFP in CAD cells. After transfection, cells were cultured for 16 hours and then fixed to check eventual effect on the number of TNT connected cells. Because no specific marker of TNTs is currently available, we quantified TNTs between cells (labeled with wheat germ agglutinin; WGA) (Delage et. al., 2016). TNTs were identified in culture according to the current definition of TNTs as membranous stretches between cells that do not contact the substrate (Fig. 1a) (Rustom et al., 2004, Abounit et. al., 2012). Overexpression of Rab35-WT GFP and Rab35-Q67L GFP shows an increase in number of TNT connected cells (Figure 1 a, b). While overexpression of inactive form of Rab35-S22N GFP shows a decrease the number of TNT (Fig 1 a, b). In order to be sure that the intercellular connections induced by Rab35 identified by morphological criteria were functional, next we performed transfer assays measuring the transfer of labeled vesicles between cells in co-culture as previously described (Abounit et. al., 2015). Two populations of cells, donor cells (transfected with GFP–Rab35-WT, active (Q67L) and inactive (S22N)) with internal vesicles labeled with a fluorescent membrane dye (Vybrant™ DiD) and acceptor cells (transfected with H2B–mCherry) were mixed at a 1:1 ratio and co-cultured for 16 hours (Delage et. al., 2016). Overexpression of GFP–Rab35-WT and Q67L significantly increased the transfer of DiD-labeled vesicles, as measured by two different methods, quantitative fluorescent image analysis (Figure 1 c) and flow cytometry (Figure S1 a, b). On the other hand, overexpression of the inactive form of Rab35-S22N GFP shows a decrease in vesicle transfer between the cells as compared to the control (Figure 1 c). In control conditions the cells were transfected with just empty vector CMV-GFP.

We used two controls to exclude the effect of vesicle transfer by secretion: (1) the conditioned medium from donor cells (cultured in a separate dish) was applied to acceptor cells (Supernatant) (2) Both the donor and acceptor populations were mixed after fixation.



(Mixture). In both conditions, transfer of vesicles was very low and not affected by the overexpression of GFP–Rab35-WT, showing that vesicle transfer between cells relied on cell-to-cell contact, and not on secretion (Fig. S1 a, b). Furthermore, downregulating Rab35 by using CRISPR shows decrease in endogenous Rab35 (Figure S2 b) and decreases in the number of TNT connected cells (Figure S2 a, d) and vesicle transfer between cells (Figure S2 d). Overexpression of Rab35-GFP in the cells depleted with endogenous Rab35 shows rescue effect on TNTs and vesicle transfer (Figure S2 a, d, e). Thus, all combined these data support that Rab35 increase in TNTs corresponds to an increase in cargo transfer between cells, suggesting that the TNTs induced by Rab35 are functional.

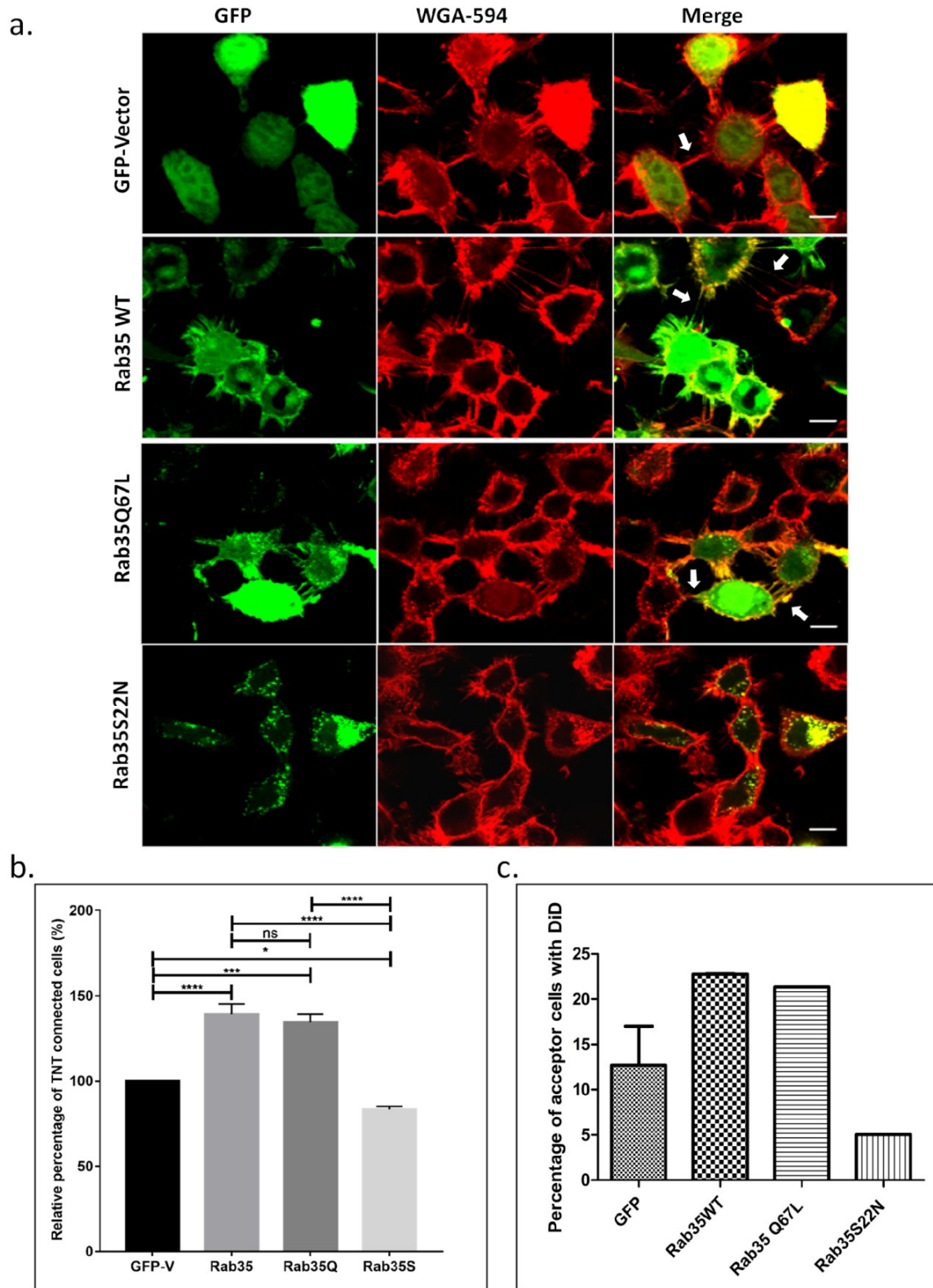


Figure 1

**Figure 1: Rab35 positively regulates TNT:** a) Confocal images of cells transfected with CMV-GFP, GFP-Rab35-WT, GFP-Rab35-Q67L, GFP-Rab35-S22N. b) Bar graphs representing the relative percentage of TNT connected cells described in (a) (CMV-GFP=100%, GFP-Rab35-WT=139±9.862%, GFP-Rab35-Q67L=134.4±8.664, GFP-Rab35-S22N=83.30±4.214%). c) Bar graph showing the percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with CMV-GFP, GFP-Rab35-WT, GFP-Rab35-Q67L, GFP-Rab35-S22N and labeled with DiD (CMV-GFP=12.69±4.301%, GFP-Rab35-WT=22.73±0.133%, GFP-Rab35-Q67L=21.35%, GFP-Rab35-S22N=5.054%). Graph (a) from three independent experiments and show mean±s.e.m. (ns, not significant; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001; by one-way ANOVA with Tukey's multiple comparison post test). Graph (b) from one experiment. Scale bars: 10 µm

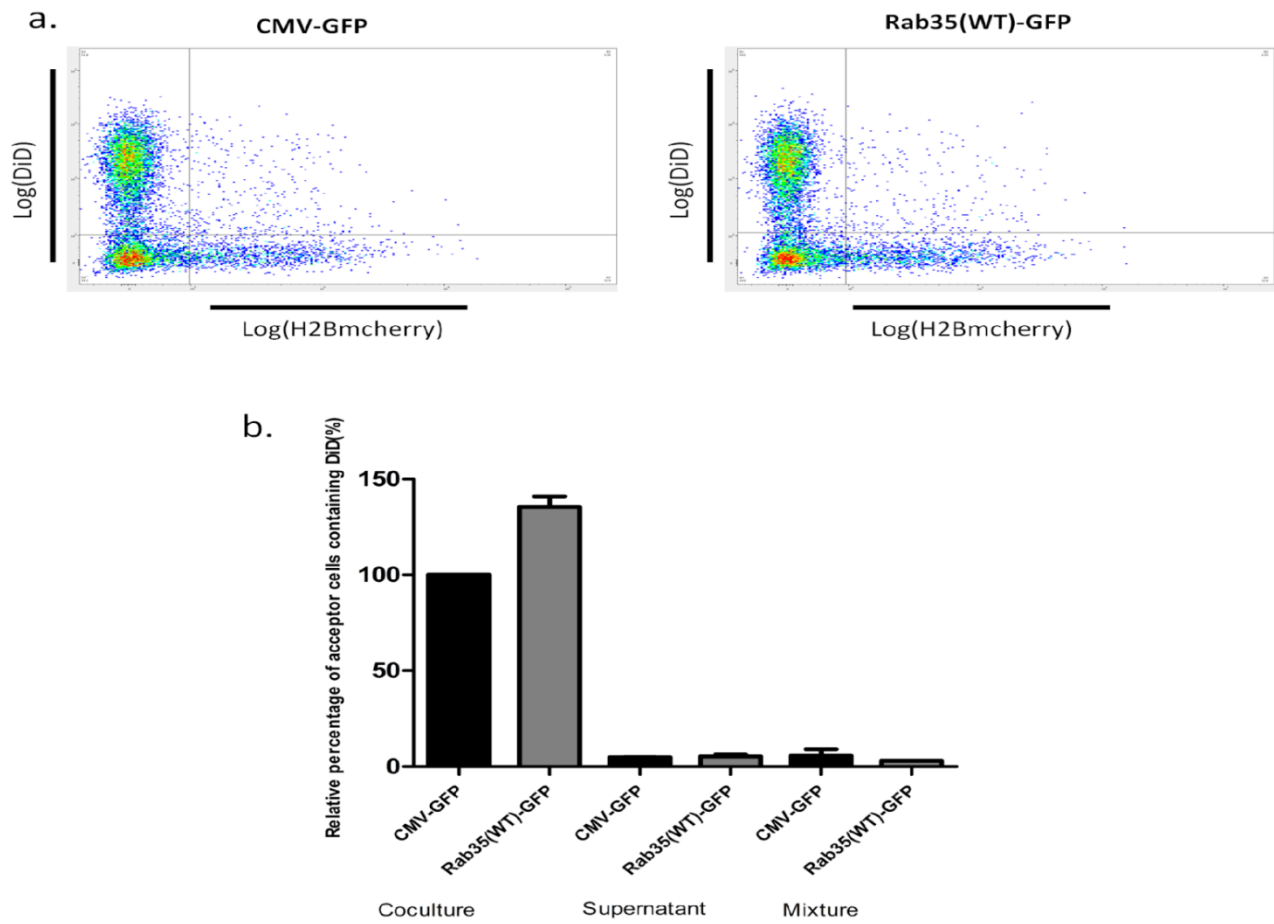


Figure S1

**Figure S1:**Rab35 positively regulates Vesicle transfer: a) Raw data (dot plots) of flow cytometry from a representative experiment showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with CMV-GFP, Rab35(WT)-GFP and labeled with DiD. b) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, supernatant and mixture where donor cells were transfected with CMV-GFP and Rab35(WT)-GFP (coculture-CMV-GFP=100%, Rab35(WT)-GFP= 135.5±5.47%, **supernatant-** CMV-GFP=4.75±0.15%, Rab35(WT)-GFP= 5.25±0.95%, **mixture-** CMV-GFP= 5.7±3.3%, Rab35(WT)-GFP=3%) Graphs from two independent experiments and show mean±s.e.m

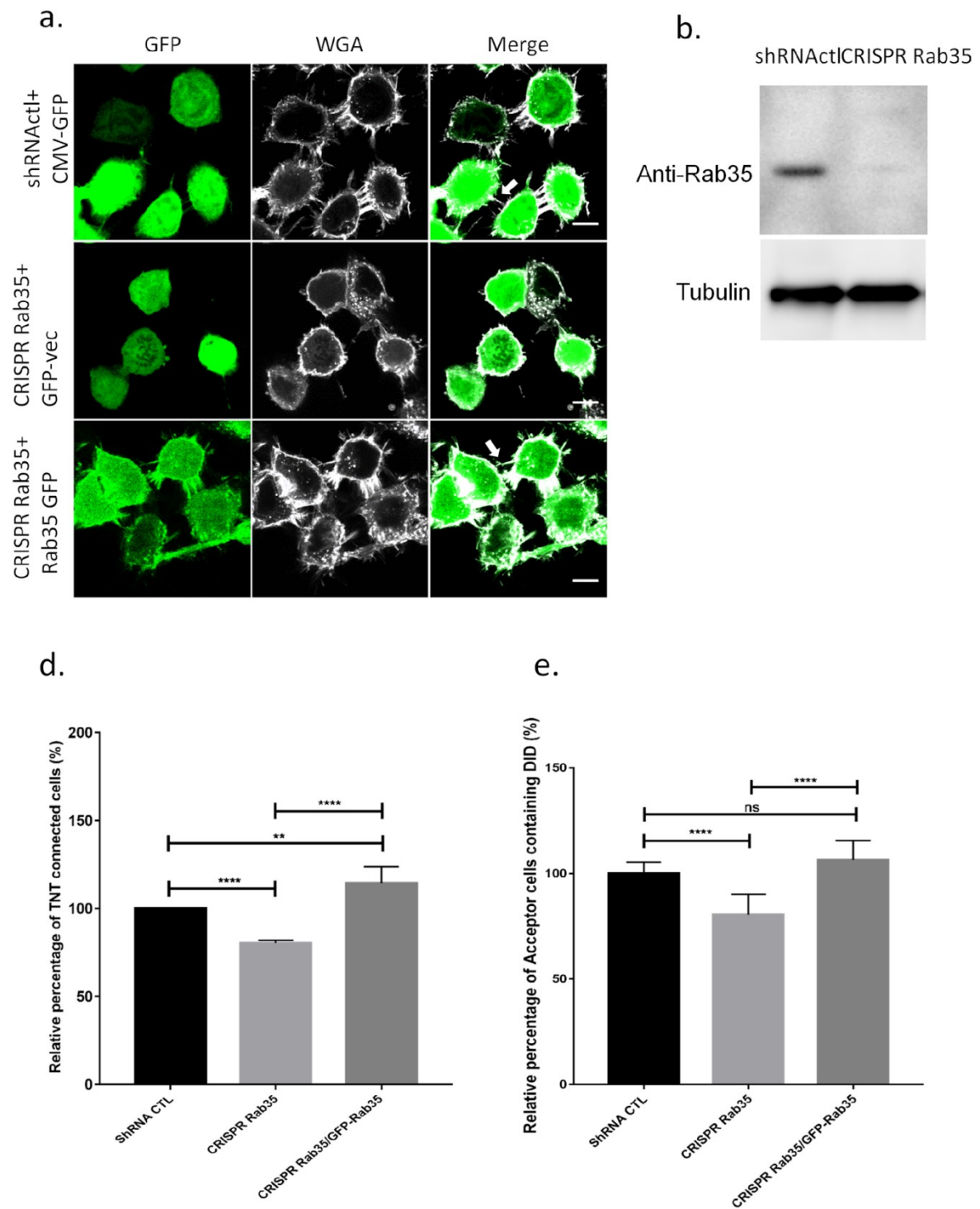


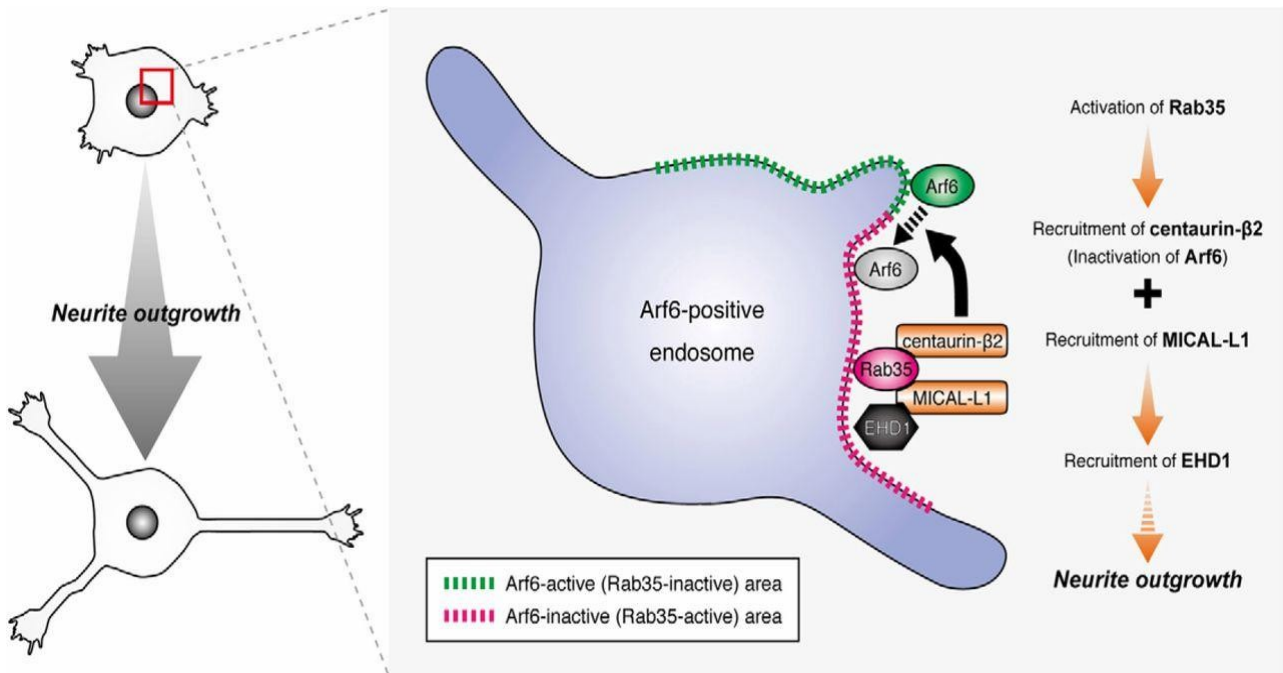
Figure S2

**Figure S2: Downregulation of Rab35 shows a decrease in TNT formation:** a) Confocal images of cells transfected with shCTL, CRISPR Rab35, CRISPR Rab35-GFP. b) Western blot of cells transfected with non-targeting (ShCTL) or targeting Rab35 (CRISPR Rab35), showing the expression of Rab35 and  $\alpha$ -tubulin as loading control. c) Bar graphs representing the relative percentage of TNT connected cells described in (a) (shCTL=100%, CRISPR Rab35=80.36 $\pm$ 10.03%, CRISPR Rab35-GFP=114.3 $\pm$ 5.172%) d) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with shCTL, CRISPR Rab35, CRISPR Rab35-GFP. (shCTL=100%, CRISPR Rab35=80.53 $\pm$ 8.35%, CRISPR Rab35-GFP=106.5 $\pm$ 2.38%) Graphs from three independent experiments and show mean $\pm$ s.e.m. (ns, not significant; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001; by one-way ANOVA with Tukey's multiple comparison post test). Scale bars: 10  $\mu$ m.

### ACAP2 shows an increase in TNTs

In order to understand by which pathway Rab35 was affecting TNTs we decided to look at downstream effectors and in particular we started with assessing the role of ACAP2 /  $\beta$  centaurin. ACAP2 indeed was shown to inhibit the formation of ARF6 dependent cellular protrusions by acting as GAP of ARF6 (Jackson et. al., 2000).

Kobayashi and Fukuda, in 2013 demonstrated that Rab35 recruits both ACAP2 and MICAL-L1 to form a tripartite complex. MICAL-L1 directly acts as a binding site for EHD1, which then has a positive effect on the neurite outgrowth. On the other hand, ACAP2 indirectly regulates the recruitment of EHD1 to the membrane by inactivating ARF6 and may thereby influence the levels of PI4P on the membrane of the vesicles, which in turn allows the binding of the EHD1 to the membrane and EHD1 function in regulating neurite outgrowth. As shown in the Figure 15 below.



**Figure 15: Role of Rab35 in neurite outgrowth:** Rab35 is accumulated at the perinuclear compartment at ARF6 positive endosomes upon NGF stimulation, and then recruits two distinct effectors MICAL-L1 and ACAP2/centaurin $\beta$ 2, at the same compartment, MICAL-L1 and ACAP2/centaurin $\beta$ 2 acts as a scaffold to EHD1 and recruits it to the same compartment and inactivates ARF6, and then facilitate neurite outgrowth.

By using the same methods described before, CAD cells were transfected with CMV-GFP (control) and ACAP2-WT GFP (donors) for 24 hours and re-plated for 16 hours as single cell population to analyse the percentage of TNT connected cells or in coculture



transfected with H2B-mcherry acceptor cells to analyze vesicle transfer cells. We found that overexpression of ACAP2-GFP results in an increase in the number of TNT connected cells (Figure 2 a, c), and vesicle transfer between the cells (Figure 2 b, d).

MICAL-L1 (MICAL Like proteins) was shown to work along with to ACAP2 to increase neurite protrusions (Kobayashi and Fukuda, 2013). MICAL-L1 are mostly involved in the membrane recycling activity (M. Vanoni, 2017). We therefore checked if overexpression of MICAL-L1-GFP would affect TNT formation and function by using the same assays as described above. Overexpression of MICAL-L1-GFP in the cells showed no significant effect either the number of TNT connected cells (Figure S3 a, b) or vesicle transfer between the cells (Figure S3c). Thus, these data indicated that Rab35 may regulate the TNT formation between the cells only through ACAP2 and not through MICAL-L1.

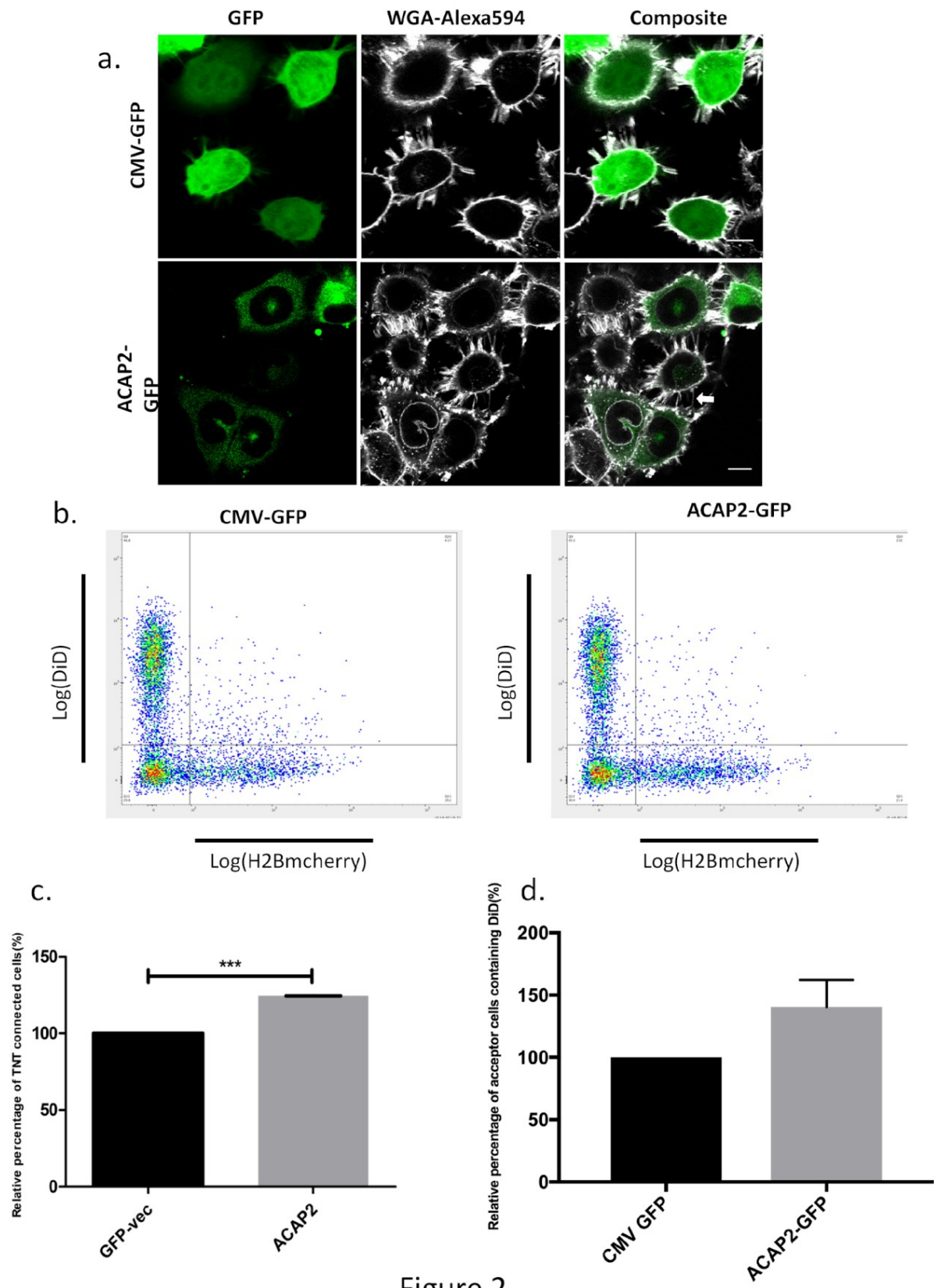


Figure 2

**Figure 2: ACAP2 positively regulates TNTs:** a) Confocal images of cells transfected with CMV-GFP, ACAP2-GFP. b) Raw data (dot plots) of flow cytometry from a representative experiment showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with CMV-GFP or ACAP2-GFP and labeled with DiD. c) Bar graphs representing the relative percentage of TNT connected cells described in (a) (CMV-GFP=100%, ACAP2-GFP=124.4±0.06%) c) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with CMV-GFP, ACAP2-GFP (CMV-GFP=100%, ACAP2-GFP=140.2±15.63%) Graph (a) from three independent experiments and show mean±s.e.m by unpaired students's t-test. (ns, not significant; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001; Graph (b) from two independent experiments. Scale bars: 10 µm.

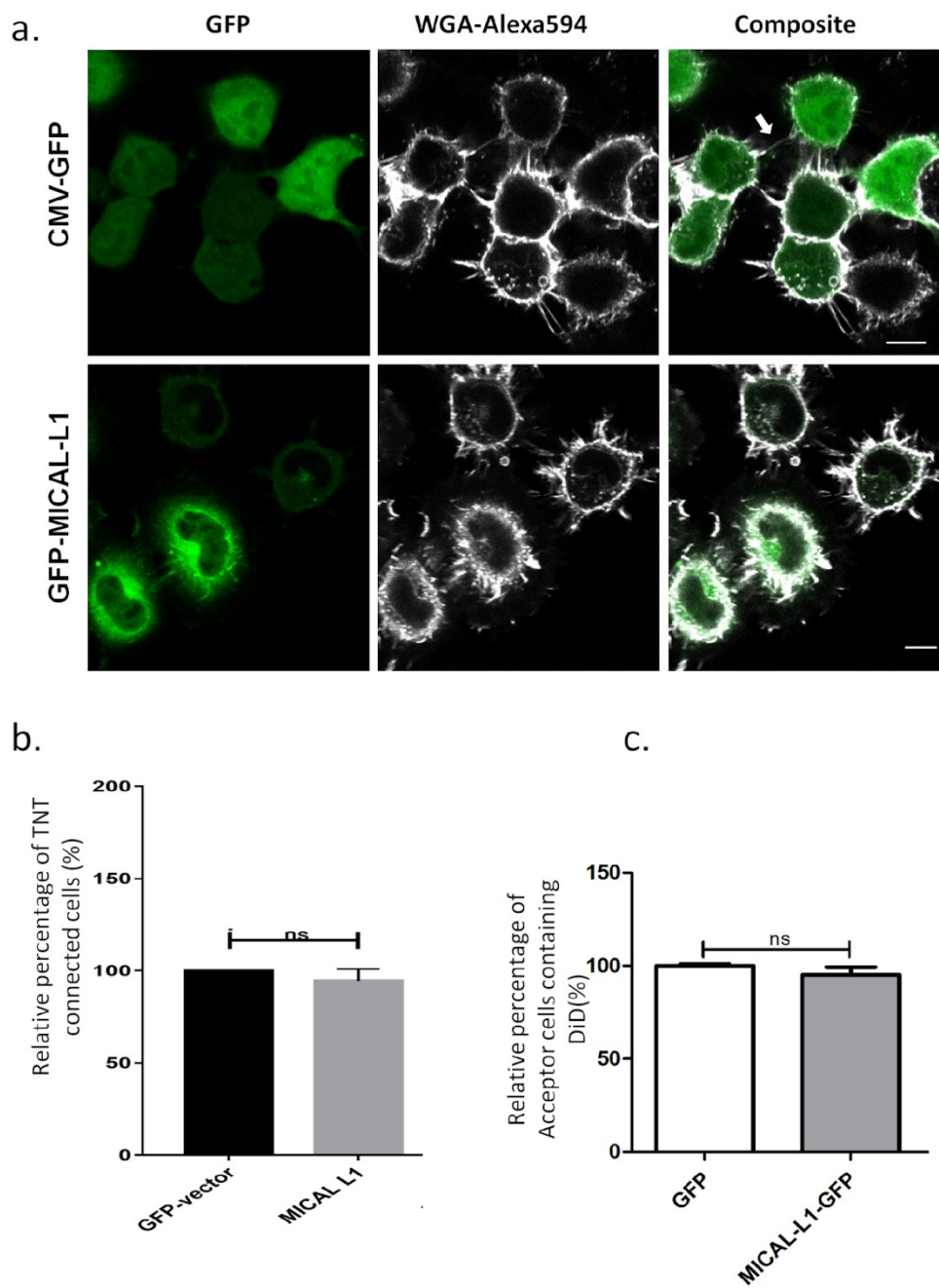


Figure S3

**Figure S3: MICAL-L1 has no effect on TNT formation:** a) Confocal images of cells transfected with CMV-GFP, MICAL-L1-GFP. b) Bar graphs representing the relative percentage of TNT connected cells described in (a) (CMV-GFP=100%, MICAL-L1-GFP=94.51±1.233%) c) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with CMV-GFP, MICAL-L1-GFP (CMV-GFP=100%, MICAL-L1-GFP=95.5±5.54%). Graphs from three independent experiments and show mean±s.e.m by unpaired students's t-test. (ns, not significant; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001) Arrows indicates TNTs. Scale bar = 10  $\mu$ m.

### ARF6-GDP shows an increase in TNTs

Next question we wanted to address was how ACAP2 through Rab35 would affect TNTs. ACAP2 is an ARF6 GAP, and inactivation of ARF6 by Rab35 through ACAP2 was shown to be required for successful neurite outgrowth of PC12 cells (Kobayashi and Fukuda, 2012). We hypothesized that a similar mechanism could be involved in TNT formation. Thus, cells were transfected with CMV-GFP (Control) ARF6-WT GFP (wild type), ARF6-Q67L GFP (active) and ARF6-T27N GFP (inactive) form in the cells and checked for their effect on TNTs as shown above. Overexpression of ARF6-WT-GFP showed a decrease in number of TNT connected cells (Figure 3 a, b) while vesicle transfer between the cells was not significantly affected (Figure 3 d, e). On the other hand, overexpression of ARF6-Q67L–GFP in the cells showed a decrease the number of TNT connected cells (Figure 3 a, b) and vesicle transfer between the cells (Figure 3 d, e). Furthermore ARF6-T27N-GFP overexpression in the cells shows an increase in the number of TNT connected cells (Figure 3 a, b) and increased vesicle transfer between cells (Figure 3 d, e). Overall these data indicate that inactive form of ARF6 plays a role on TNT formation and this inactivation of ARF6 may be due to its GAP activity on for ARF6.

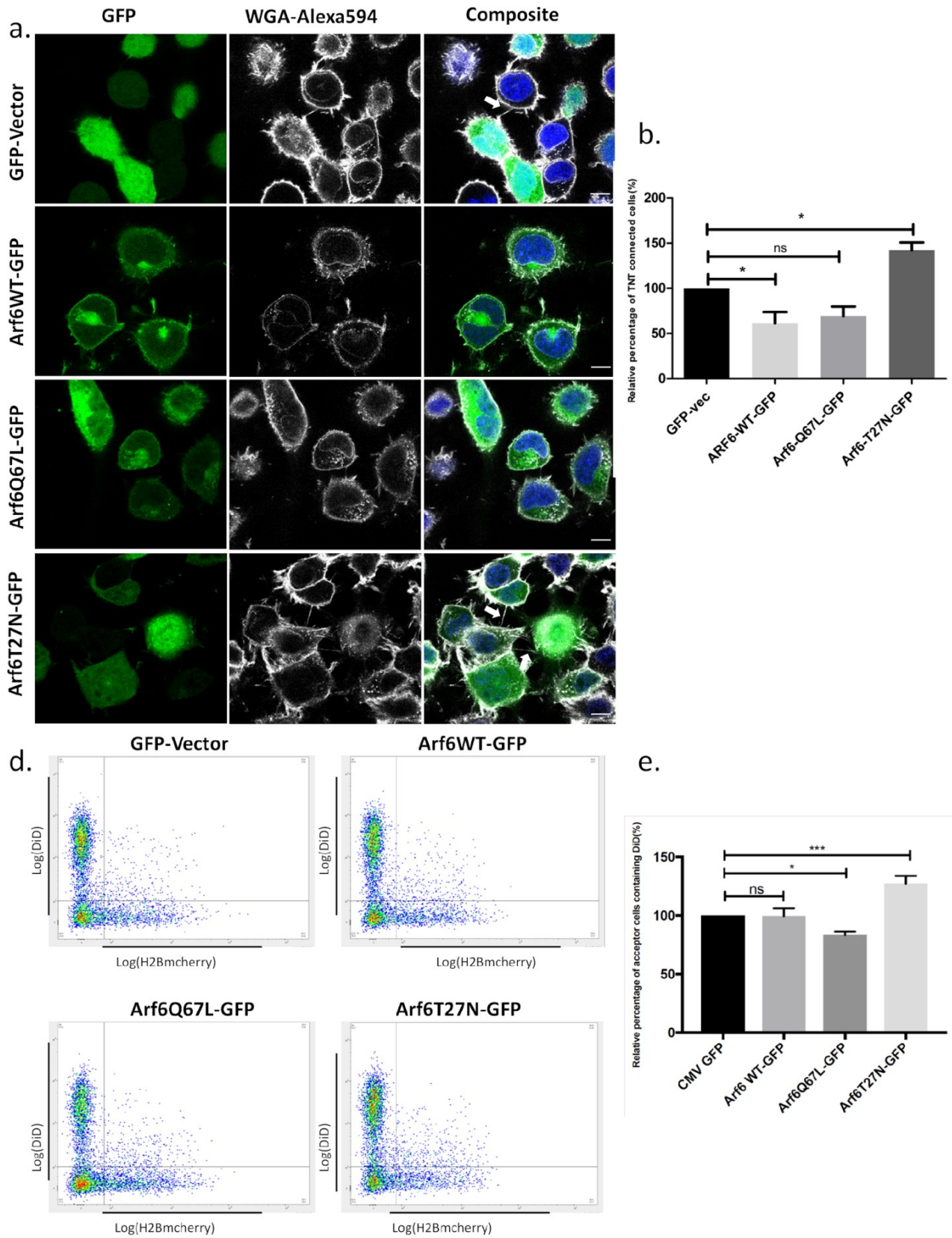


Figure 3

**Figure 3: ARF6-GDP positively regulates TNTs:** a) Confocal images of cells transfected with CMV-GFP, ARF6WT-GFP, ARF6Q67L-GFP, ARF6T27N-GFP. b) Raw data (dot plots) of flow cytometry from a representative experiment showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with CMV-GFP, ARF6WT-GFP, ARF6Q67L-GFP or ARF6T27N-GFP and labeled with DiD. c) Bar graphs representing the relative percentage of TNT connected cells described in (a) (CMV-GFP=100%, ARF6WT-GFP=69.61±4.651%, ARF6Q67L-GFP=69.23±3.694%, ARF6T27N-GFP=142.2±4.656%) d) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with CMV-GFP, ARF6WT-GFP, ARF6Q67L-GFP, ARF6T27N-GFP (CMV-GFP=100%, ARF6WT-GFP=99.53±0.471%, ARF6Q67L-GFP=83.54±16.46%, ARF6T27NGFP=127.3±27.32%) Graphs from three independent experiments and show mean±s.e.m. (ns, not significant; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001; by one-way ANOVA with Tukey's multiple comparison post test). Arrows indicate TNTs. Scale bars: 10 µm



### EHD1 shows an increase in TNTs.

Because active ARF6 converts PtdIns4P to phosphatidylinositol 4,5-bisphosphate (PtdIns (4,5) P<sub>2</sub>) by activation of PtdIns4P 5-kinase (PIP5K) (Brown et al., 2001; Jovic et. al., 2007), it was speculated that inactivation of ARF6 might be required to maintain PtdIns4P and to recruit EHD1. EHD1 in turn was shown to be essential in promoting neurite outgrowth following Rab35 activation (see schematic in Figure 15)

Thus, we next tested the role of EHD1. Overexpression of wild type form of EHD1 showed an increase in the number of TNT connected cells (Figure 4 a, c) and vesicle transfer between the cells (Figure 4 b, d).

By using shRNA against EHD1 we could see a decrease in the expression of endogenous EHD1 (Figure S4 b). These cells showed a decrease in the number of TNT connected cells (Figure S4 a, d) and vesicle transfer between the cells (Figure S4 c, e). In addition, by overexpressing EHD1, in cells depleted of endogenous EHD1, we could observe a rescue of the number of TNT connected cells (Figure S4 a, d) and the number of acceptor cells containing vesicles (Figure S4 c, e). Thus, altogether these data indicated that EHD1 regulates TNT formation between the cells.

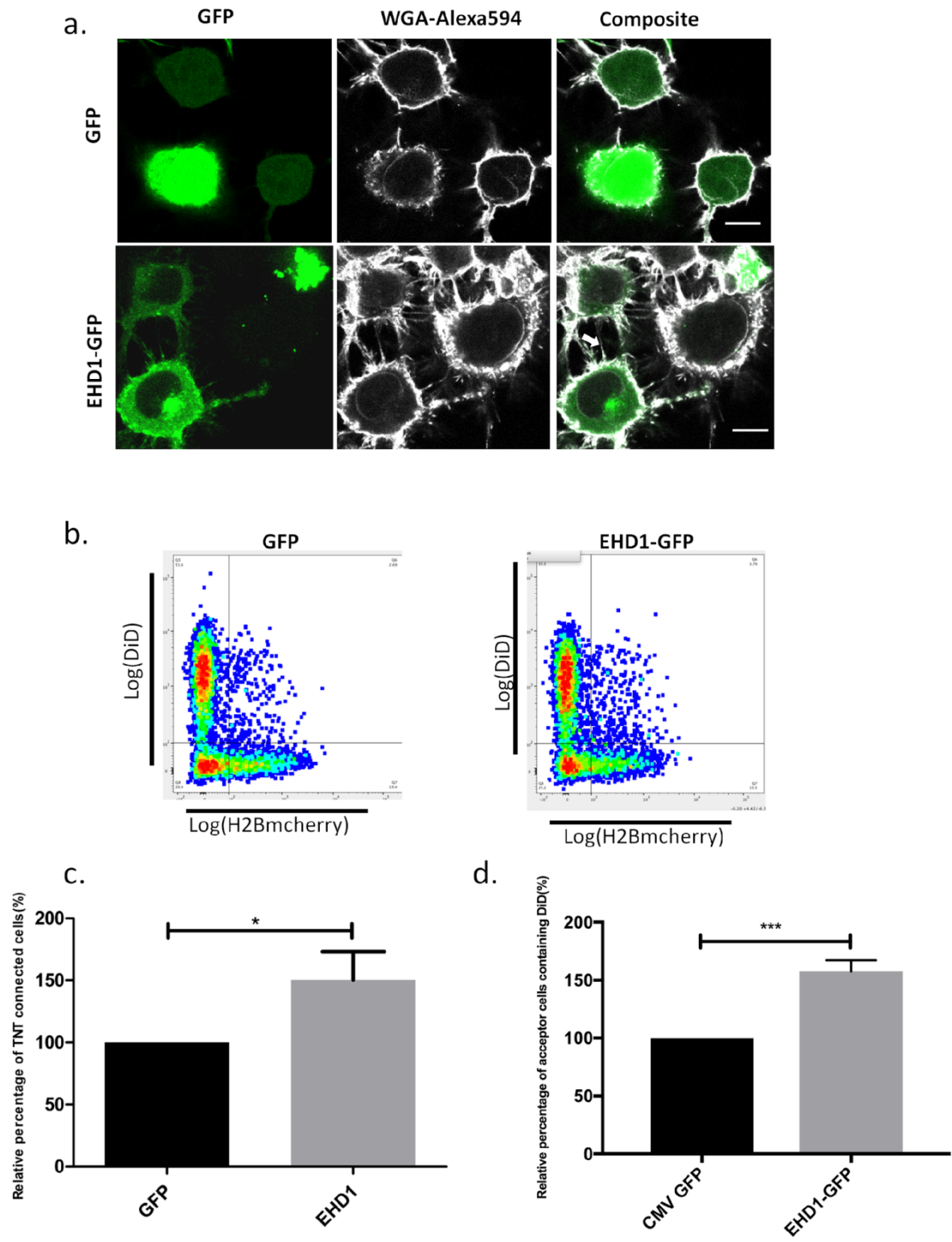


Figure 4

**Figure 4: EHD1 positively regulates TNT:** a) Confocal images of cells transfected with CMV-GFP, EHD1-GFP. b) Raw data (dot plots) of flow cytometry from a representative experiment showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with either GFP–vector or EHD1-GFP and labeled with DiD. c) Bar graphs representing the relative percentage of TNT connected cells described in (a) (CMV-GFP=100%, EHD1-GFP=150.5±23%) d) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with CMV-GFP, EHD1-GFP (CMV-GFP=100%,EHD1-GFP=157.6±5.549%); Graphs from three independent experiments and show mean±s.e.m by unpaired students's t-test (ns, not significant; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001; Scale bars: 10 µm.

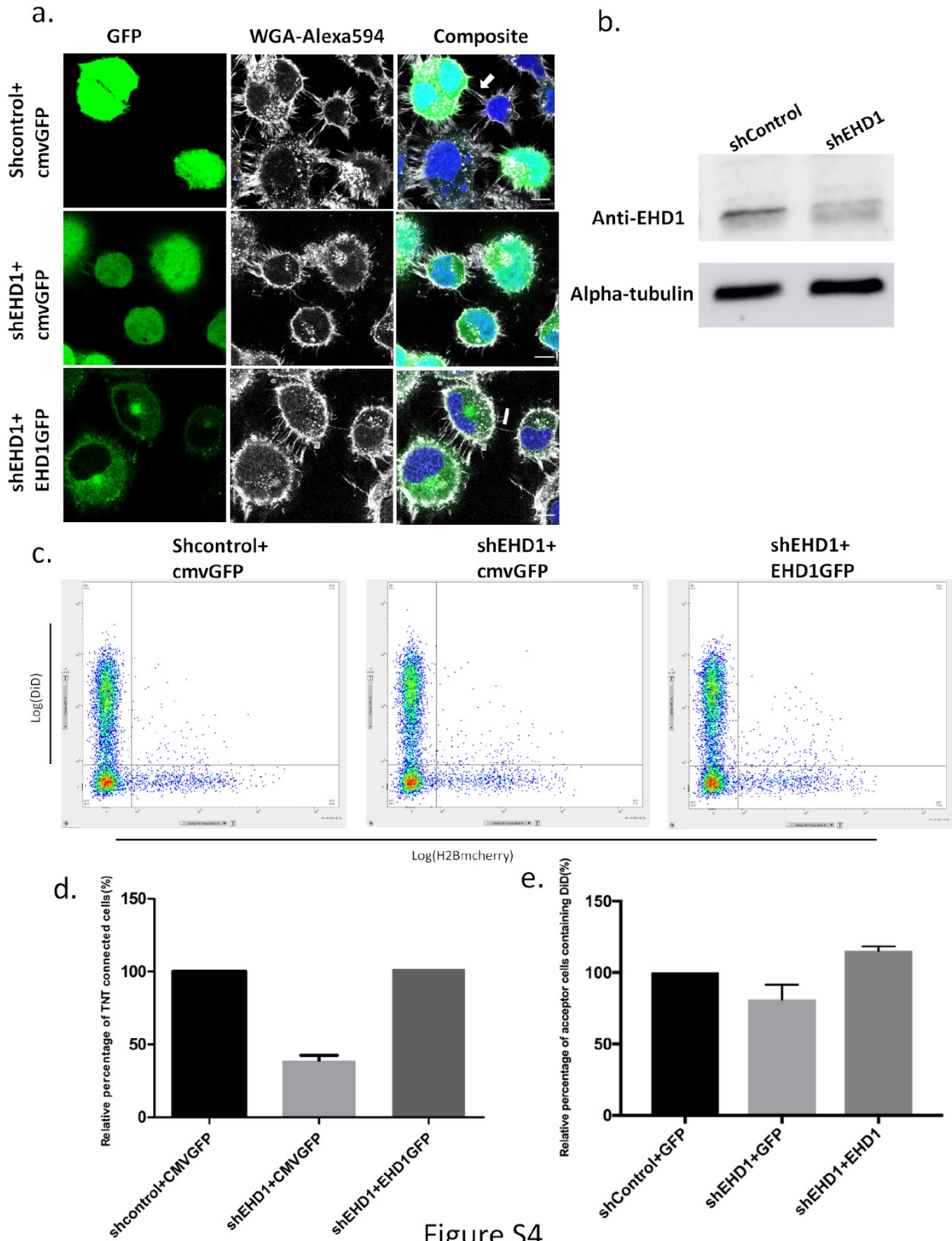


Figure S4

**Figure S4:** Down regulation of EHD1 shows a decrease in TNT formation: a) Confocal images of cells transfected with shControl+GFP, shEHD1+cmv-GFP, shEHD1+EHD1-GFP. b) Western blot of cells transfected with shRNA non-targeting (ShCTL) or targeting EHD1 (ShEHD1), showing the expression of EHD1 and  $\alpha$ -tubulin as loading control. c) Raw data (dot plots) of flow cytometry from a representative experiment showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with either shControl+GFP, shEHD1+cmv-GFP or shEHD1+EHD1-GFP and labeled with DiD. d) Bar graphs representing the relative percentage of TNT connected cells described in (a) (shControl+GFP=100%, shEHD1+cmv-GFP= 39.04 $\pm$ 5.029%, shEHD1+EHD1-GFP=102%). e) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with shControl+GFP, shEHD1+cmv-GFP, shEHD1+EHD1-GFP (shControl+GFP=100%, shEHD1+cmv-GFP=81.16 $\pm$ 7.295, shEHD1+EHD1-GFP=115.2 $\pm$ 2.201%) Graphs from two independent experiments and show mean $\pm$ s.e.m. Arrows indicate TNTs. Scale bars: 10  $\mu$ m

Rab35 acts upstream of EHD1 to regulate TNTs:

To check if Rab35 and EHD1 were working on the same pathway, we overexpressed Rab35-WT GFP in cells in which endogenous EHD1 has been depleted by shRNA and checked both the number of TNT connected cells and vesicle transfer. From the preliminary data, compared to the control we found a slight decrease in the number of TNT connected cells (Figure 5 a, c) and vesicle transfer between the cells (Figure 5 b, d).

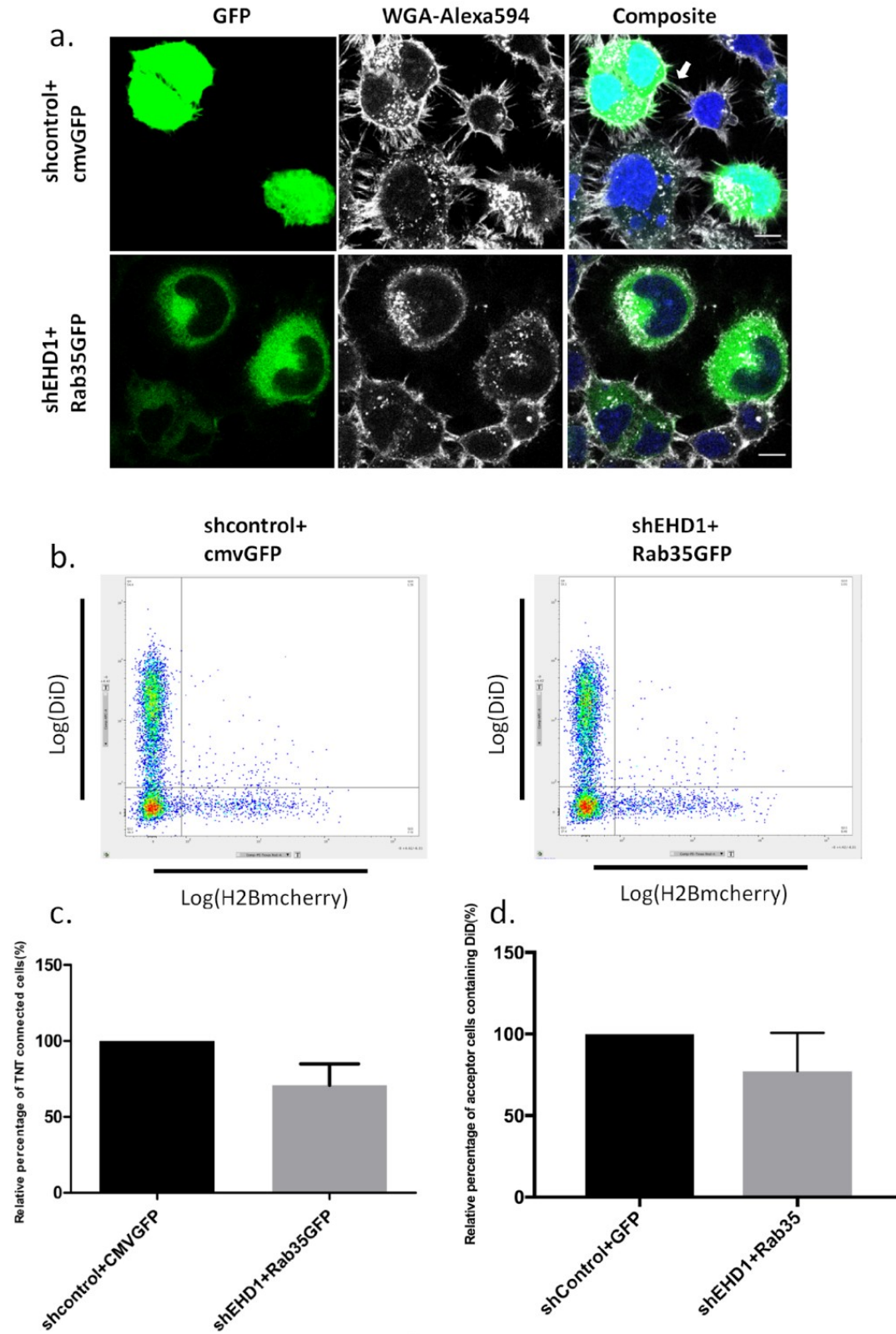


Figure 5

**Figure 5:** Rab35 acts upstream of EHD1 and regulates TNTs.: a) Confocal images of cells transfected with shControl+cmvGFP, shEHD1+Rab35GFP. b) Raw data (dot plots) of flow cytometry from a representative experiment showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with either shControl+cmvGFP or shEHD1+Rab35GFP and labeled with DiD. c) Bar graphs representing the relative percentage of TNT connected cells described in (a) (shControl+cmvGFP=100%, shEHD1+Rab35GFP=70.67±14.14%). d) Bar graph showing the relative percentage of acceptor cells containing DiD-labeled vesicles from the co-cultures, where donor cells were transfected with shControl+cmvGFP, shEHD1+Rab35GFP (shControl+cmvGFP=100%, shEHD1+Rab35GFP=77.31±16.88%). Graphs from two independent experiments and show mean±s.e.m; Arrows indicate TNTs. Scale bars: 10 µm.



These data indicate that there is a relation between the action of Rab35 and EHD1 and that EHD1 act downstream of Rab35 to regulate TNTs.

Overall, from the above data we can fairly speculate that Rab35, ACAP2, ARF6 and EHD1 are involved in the formation of TNTs (Figure 6).

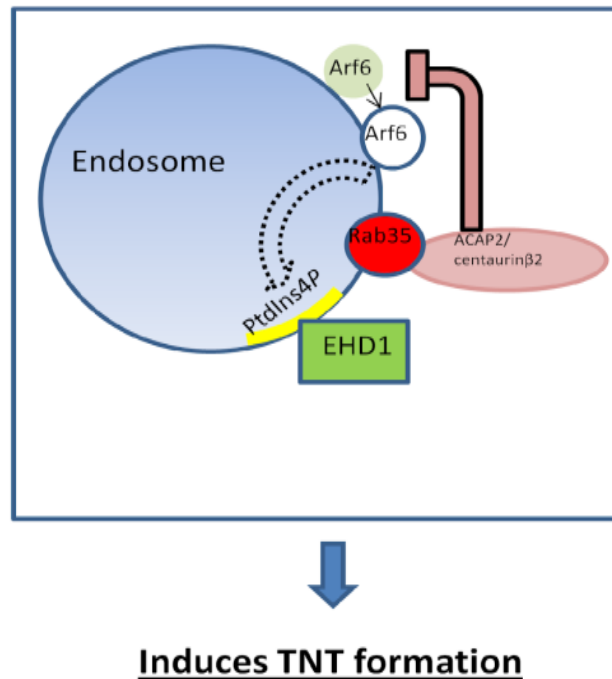


Figure 6

**Figure 6:** Schematic of Rab35 regulating TNTs: Rab35-GTP positively regulates TNT formation. Downstream effectors of Rab35 involving, ACAP2 also shows a positive regulation of TNTs. ACAP2 may act in maintaining the GDP bound form of ARF6 which in turn positively regulates TNT formation. This may influence the recruitment of EHD1 and positively regulate TNT formation. EHD1 acts downstream of Rab35 in regulating TNT formation.

## **Discussion:**

Tunneling nanotube are F actin rich structures. There are no molecular markers for detecting TNTs, thus we classify them as cell to cell connecting thin structures that are non-adherent to substrate, are F-actin positive and can transport various molecules, proteins and cellular organelles. Until date several molecular factors have been shown to regulate TNTs. Since majority of TNTs are made of F-actin, several actins regulating proteins have been studied with respect to TNTs like WASP, MyosinX, WAVE, Arp2/3, and shown to be positive regulators of TNTs in several cells (e.g. CADs, macrophages) (Gousset et. al., 2013, Hanna et. al., 2017b). But in neuronal CAD cells, the actin polymerising proteins like IRSp53, VASP, Arp2/3 have been shown to regulate TNTs negatively (Delage et. al., 2016, Sartori-Rupp et. al., 2019). In the neuronal cells, filopodia and TNTs are regulated in opposite manner, for e.g. Eps8 which is a bundling protein regulates filopodia number in a negative manner but it regulates the number of TNT connected cells in a positive manner; on the other hand, VASP increases filopodia number while decreasing the number of TNT connected cells (Delage et. al., 2016). This indicates that TNTs in macrophages and neuronal cells may be regulated by different mechanisms (Delage et. al., 2016, Sartori-Rupp et. al., 2019).

Thus, it is imperative to study the molecular effectors that are regulating TNTs. Previous studies in the lab have also shown that in addition to actin remodelling, membrane recycling plays an important part in TNT formation. Rab proteins have been shown to play an important role in membrane recycling (Grant and Donaldson, 2009). Rabs are also shown to be important in several different cellular functions like cytokinesis, vesicle trafficking and formation of cellular structures like cilia, lamellopodia and filopodia etc. (Das and Guo, 2011). There have been studies indicating the role of Rab8 and Rab11 on TNT formation (Zhu et. al., 2016). Previous study in the lab, has shown that upon screening 41 different Rabs, Rab8a and Rab11a are involved in TNT formation through the action of VAMP3, thus involving the fusion of recycling vesicles to the plasma membrane. All the three proteins are shown to regulate TNTs by acting through a cascade mechanism (Zhu et. al., 2018).

From this screening another candidate of Rab proteins i.e. Rab35 was also shown to positively regulate TNT formation. Thus, I decided to study the role of Rab35 and its effectors in the formation of TNTs.

Several different proteins have been involved in the neurite outgrowth in the cells. Neurites are the initial cellular protrusions which further gives rise to axons and dendrites in neuronal cells. Though it seems intuitive that the cytoskeleton may be one of the major influencers in the formation of the axons and dendrites, the formation of cellular protrusions is also influenced by vesicle trafficking (Horton and Ehlers, 2009). Majority of vesicle trafficking is guided by Rabs which regulates proteins that are directed to the neurite outgrowth (Schmidt and Haucke, 2000). There have been studies which indicate that Rabs and the recycling compartment are involved in TNT formation (Zhu et. al., 2016, Zhu et. al., 2018). This led us to take an educated guess that the neurite outgrowth and the formation of the TNTs may be related or they may form by a similar mechanism.

Rab35 and recycling endosomes are involved in neurite outgrowth of the cells (Kobayashi and Fukuda, 2013). During neurite outgrowth, Rab35 acts along with ACAP2 and MICAL-L1 to regulate ARF6 and EHD1 (Kobayashi and Fukuda, 2013). In our studies we have shown that GTP bound form of Rab35 shows an increase in TNT formation. But unlike neurite outgrowth, MICAL-L1 is not involved in TNT formation. However, we could show that several of the downstream effectors such as ACAP2, GDP-ARF6 and EHD1 are involved in TNT formation. ACAP2 has been shown to indirectly regulate neurite outgrowth, by acting as a GAP of ARF6 (Kobayashi and Fukuda, 2013). ARF6 activates PIP5kinase which converts PI4P to PIP2. Thus inactivation of ARF6 regulates the PI4P levels (Krauss et. al., 2003). EHD1 has been shown to bind to vesicles and membranes that are rich in PI4P (Jovic et. al., 2007). Thus, we can speculate that Rab35 indirectly regulates EHD1 through ACAP2 and ARF6 for TNT formation. Indeed, here we were able to demonstrate that Rab35 acts upstream of EHD1 in regulating the growth of TNTs. However, EHD1 was also shown to possess a binding site to MICAL-L1 (Sharma et. al., 2009) and this direct binding is involved in neurite outgrowth. It was shown that both the indirect regulation through ACAP2 and the direct regulation through MICAL-L1 of EHD1 is necessary in neurite outgrowth (Kobayashi and Fukuda, 2013). But in the case of TNT formation we demonstrated that MICAL-L1 is not involved. Thus, these data clearly show that even though they use similar pathways through the activation of Rab35 and the recruitment of EHD1, neurite outgrowth and TNT formation are regulated in different manner. On the other hand, the mechanism by which EHD1 works for both neurite outgrowth and TNT formation could be the same, that is through promoting endocytic recycling, and in particular the trafficking from recycling endosomes to the plasma membrane (Naslavsky and Caplan, 2011, Kobayashi and Fukuda, 2013). Endocytic recycling has been proposed to be crucial to supply membranes and/or proteins to neurite

tips and growing protrusions like cilia, filopodia and also TNTs to enable their extension (Sann et al., 2009, Zhu et. al., 2018, Lu et. al., 2015, Hattula et. al., 2006). This according with the current literature we propose that EHD1 may facilitate TNT growth by facilitating fission of vesicles targeted to the budding TNT from recycling endosomes in response to activation of Rab35.

However further studies are needed to validate this hypothesis and to demonstrate the role of all the effectors, and their interaction with each other to regulate TNT formation.

Specifically, to understand whether these proteins are acting along with each other or they act individually, it would be interesting to see if Rab35, ACAP2 and EHD1 act in the same or different pathway to regulate TNTs. For this we would perform a similar experiment described in the paper. We would downregulate the endogenous protein and over express the other protein and check its effect in the formation of TNTs. For example, to check whether Rab35 and ACAP2 are acting in the same or different pathway we plan to downregulate Rab35 and overexpress ACAP2. According to our hypothesis these two proteins should be acting in the same pathway and thus in this case we hypothesize to see an increase in TNT formation. On the other hand, if the activation of Rab35 recruits ACAP2 if we downregulate ACAP2 and overexpress Rab35 we should not see an effect on TNTs. Thus, we plan to perform these types of experiments for each protein that have been tested positive for TNT formation in my studies, for e.g. Rab35, ACAP2, ARF6 and EHD1 to check whether they form TNTs in a cascade mechanism.

MICAL-L1 has been shown to regulate neurite outgrowth by forming a tripartite complex with Rab35 and ACAP2 (Kobayashi and Fukuda, 2013), but over-expression of MICAL-L1 alone in CAD cells shows no effect on TNT formation. It would be interesting to investigate if a similar effect is shown by knocking down the endogenous MICAL-L1 to completely rule out the role of MICAL-L1 in TNT formation.

Finally, it would be interesting to study the interaction of Rab35 and its effectors by studying the colocalization of these proteins with respect to each other. Rab35 and its effectors were shown to interact with each other by co- immuno precipitation (Kobayashi and Fukuda, 2013), thus it would be also interesting to check if these proteins interact with each other by colocalization/ FRET studies or by co-immuno precipitation.

From all the above studies we plan to uncover the mechanism by which Rab35 and its effectors regulate TNTs. This study will provide a novel insight into the role of Rabs,

recycling endosomes and neurite outgrowth in TNT formation. This will provide us with a novel direction for the study of molecular effectors involved in TNT formation.

## **Materials and methods:**

### **Cell lines, plasmids and transfection procedures**

The mouse catecholaminergic neuronal CAD cell line (mouse catecholaminergic neuronal cell line, Cath.aDifferentiated) (Gousset et al., 2013) was grown in Gibco's OptiMEM supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. PEGFP-C1-Rab35 (WT), pEGFP-C1-Rab35 (Q67L), pEGFP-C1-Rab35 (S22N), centaurin- $\beta$ 2 (Cent $\beta$ 2-WT) and EHD1 WT and shRNA non-target control and shRNA against EHD1 were from Mitsunori Fukuda (Laboratory of Membrane Trafficking Mechanisms, Tohoku University, Japan). GFP MICAL-LI, GFP-ARF6 (WT), GFP-ARF6 (Q67L), GFP ARF6 (T27N), CRISPR Rab35 were from Arnaud Echard (Pasteur Institute, Paris, France). GFP-vector and mCherry-vector (AddGene). CAD cells were transiently transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions

### **Quantification of TNT-connected cells.**

Confluent CAD cells were mechanically detached and counted, and 300,000 cells were plated for 6 h in 6 well plate. Cells were transfected as described above, with the appropriate plasmids. At 24h post-transfection, cells were detached and counted, and 220,000 cells were plated for 16h on Ibidi  $\mu$ -dishes (Biovalley, France). After 16h post-seeding, cells were fixed with fixative solution 1 (2% PFA, 0.05% glutaraldehyde and 0.2 M HEPES in PBS) for 20 minutes at 37 °C followed by a second 20 minutes fixation with fixative solution 2 (4% PFA and 0.2 M HEPES in PBS) at 37 °C. The cells were gently washed with PBS and labeled with WGA-Alexa 594 (Sigma) (1:300 in PBS) for 20 minutes at room temperature, washed and sealed with Aqua-Polymount.

Image stacks (0.3 $\mu$ m) covering the whole cellular volume were acquired using a confocal microscope (Zeiss LSM 700) controlled by ZEN software. To evaluate the number of TNTs-connected cells, the manual analysis was performed for transfected cells, which possessed TNTs, were counted. The criteria to identify TNT are a continuous connection could be found when moving along the stacks after removing the bottom 3 slices. The two cells which were connected with at least one continuous connection were marked as TNT-connected cells. Each experiment was performed at least in triplicate. Image analyses and displays of raw data, such as Z-projections, were obtained using ICY software (Gousset et al., 2013).

### **Fluorescence image analysis to quantify the transfer of DiD-labeled vesicle.**

Confluent CAD cells were mechanically detached and counted, and 800,000 cells were plated for 6 h in T25 flasks. The cells were transfected with the appropriate GFP tagged constructs for donor cells and H2B-mCherry for acceptor cells for 24h in complete medium. The donor cells were labeled with a 333 nM solution of the lipophilic tracer Vybrant<sup>TM</sup> DiD (long-chain dialkylcarbocyanine) in complete medium for 30 min at 37 °C. Cells were then washed with PBS and 0.01% trypsin, subsequently resuspended in complete medium and counted. The labeled donor cells were mixed in a 1:1 ratio with H2B-transfected acceptor cells and plated at subconfluence (220,000 cells) on Ibidi  $\mu$ -dishes (Biovalley, France) for 16 h at 37 °C. Cells were fixed with 4% PFA. Cells were washed and sequentially stained for 30 min with a 1  $\mu\text{g. mL}^{-1}$  solution of HCS CellMask<sup>TM</sup> Blue, which stains the entire cell volume (i.e. cytoplasm and nucleus). Samples were washed and sealed with Aqua-Poly/Mount (Polysciences, Inc.). The cells were imaged with an inverted confocal microscope (Zeiss LSM700) controlled by ZEN software and quantification was done by using ICY software (<http://icy.bioimageanalysis.org/>).

### **Flow cytometry to analyze the transfer of DiD-labeled vesicle.**

Confluent CAD cells were mechanically detached and counted, and 800,000 cells were plated for 6 h in T25 flasks. Cells were transfected with the appropriate GFP tagged constructs for donor cells and with H2B-mCherry for acceptor cells, for 24h in complete medium. Donor cells were detached, counted and labeled with a 333 nM solution of the lipophilic tracer Vybrant<sup>TM</sup> DiD (long-chain dialkylcarbocyanine) in complete medium for 30 min at 37 °C. The donor cells were labeled with a 333 nM solution of the lipophilic tracer Vybrant<sup>TM</sup> DiD (long-chain dialkylcarbocyanine) in complete medium for 30 min at 37 °C. Cells were then washed with PBS and 0.01% trypsin, subsequently resuspended in complete medium and counted.

The labeled donor cells were mixed in a 1:1 ratio with H2B-mCherry transfected acceptor cells and plated at subconfluence (120,000 cells per well) on 24-well plates for 16 h at 37 °C. Each independent co-culture was performed in triplicate. To verify the transfer of vesicles between cells is cell-cell dependent not through secretion, two populations of cells were co-cultured with transwell insert which could separate two populations of cells but share the medium. Or the supernatant from donor cells after cultured for overnight was taken and add to acceptor cells to culture for another 16h. Cells were then washed with PBS, mechanically detached from the dish by pipetting up and down with 500  $\mu\text{L}$  PBS and

passed through sterile 40-mm nylon cell strainers (BD Falcon™) in order to obtain single-cell suspensions. Cell suspensions were fixed with 500 µl of 4% PFA (2% final solution). Flow cytometry data were acquired using an LSR Fortessa flow cytometer (BD Biosciences). GFP fluorescence was analyzed at 488 nm excitation wavelength, RFP and mCherry fluorescence were analyzed at 561 nm excitation wavelength, and DiD fluorescence was analyzed at 640 nm excitation wavelength. Samples were analyzed at a high flow rate, corresponding to 200–400 events per second and 10,000 events were acquired for each condition. The data was analyzed using FlowJo analysis software.

### **Western blot**

Cells transfected with ShRNA were lysed in NP-40 lysis buffer (25mM Tris, pH 7-8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100), and protein concentration in the cell lysate was quantified using a Bradford protein assay (Bio-Rad). Protein samples were incubated at 100 °C for 5 min and electrophoresed on 10% SDS-polyacrylamide gels. Proteins were transferred to PVDF membranes (GE Healthcare Life Sciences). Membranes were blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h. Membranes were then incubated at 4 °C with a primary antibody, rabbit anti-Rab35 (ab230838) anti-EHD1 (24657-1-AP), mouse anti- $\alpha$ -tubulin (Sigma) diluted in 5% nonfat milk overnight (1:500 and 1:10,000, respectively) then washed several times with TBS-T. After 1 h incubation with horseradish peroxidase-conjugated with respective IgG secondary antibody (1:10,000) (GE Healthcare Life Sciences), membranes were washed with TBS-T and protein bands on the membrane were detected using an ECL-Plus immunoblotting chemiluminescence system (GE Healthcare Life Sciences). Membranes were imaged using ImageQuant LAS 500™ camera (GE Healthcare Life Sciences).



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## ***Section 4***

# **Conclusion and Perspectives**

## **Conclusion and perspectives**

From screening 41 different Rab proteins, we found that Rab8a, Rab11a and Rab35 positively regulate contact dependent vesicle transfer between cells. Upon further investigation we demonstrated that individually Rab8a and Rab11a regulate the formation of TNTs. Since VAMP3 protein regulates the formation of cilia along with Rab8a and Rab11a (Finetti et. al., 2015), we investigated its role and found that VAMP3 positively regulates TNT formation. We further showed that all the three proteins Rab11a, Rab8a and VAMP3 act in cascade manner to regulate TNT formation. Rabin8 which interacts with Rab11a and acts as a GEF of Rab8a in cilia formation (Knodler et. al., 2010), shows no effect on TNTs. On the other hand, I found that GRAB a protein whose function is similar to Rabin8 (Furusawa et. al., 2017) upon over-expression in our neuronal cell model leads to an increase in TNT connected cells. However, I demonstrated that this TNT regulation is independent of the Rab11a-Rab8a cascade. This opens new questions for investigation such as to find the factors that act as a connecting link between Rab11a and Rab8a. Also, it would be intriguing to further investigate the role of GRAB and its interacting proteins that may regulate TNT formation.

Several Rab proteins are involved in the recycling pathway. The ERC is usually situated near the golgi or MTOC (microtubule organizing centre) (Maxfield and McGraw, 2004). Rab11/EHD1 presence on the tubules is the indication of ERC (Grant and Donaldson, 2009). Rac and Cdc42 stimulates adherens junction assembly in the epithelial cells by recycling of E-cadherin through the endosomal pathway (A Ridley, 2001, Le et. al., 1999). Rab8 recycles transferrin positive vesicles back to the plasma membranes. These vesicles participate in the formation of cellular protrusions (Hattula et. al., 2006). Thus, since the recycling of vesicles plays an important role in the formation of different cellular protrusions we propose that this is also important for the regulation of TNTs, however this needs to be further investigated.

Preliminary data (data not shown) has indicated that Rab8a and Rab11a are localized at the base of the TNTs. It would be interesting to study the role of Rab8 and Rab11 proteins in regulating TNT formation in live conditions, and whether the vesicles positive for these proteins are giving rise to TNTs. Primaquine which is a membrane recycling inhibitor and blocked the transferrin recycling in CAD cells (Zhu et. al., 2018) shows a negative effect on TNT connected cells. This shows that recycling of the membrane has an important effect on TNTs since it may supply lipids and proteins necessary for the outgrowth of TNTs.

During neurite outgrowth, Rab35 acts along with ACAP2 and MICAL-L1 to regulate ARF6 and EHD1 (Kobayashi and Fukuda, 2013). In our studies we have shown that GTP bound form of Rab35 leads to an increase in the number of TNT connected cells. But unlike neurite outgrowth, MICAL-L1 is not involved in TNT formation. However, we could show that several of the downstream effectors such as ACAP2, GDP-ARF6 and EHD1 are involved in TNT formation. ACAP2 has been shown to indirectly regulate neurite outgrowth, by acting as a GAP of ARF6. Active form of ARF6 activates PIP5kinase which converts PI4P to PIP2 (Krauss et. al., 2003). Thus, inactivation of ARF6 may regulate PI4P levels (Kobayashi and Fukuda, 2013). EHD1 binds to vesicles that are rich in PI4P (Jovic et. al., 2007). Therefore, we can speculate that Rab35 indirectly regulates EHD1 through ACAP2 and ARF6 for TNT formation. Indeed, here we have demonstrated that Rab35 acts upstream of EHD1 in regulating the growth of TNTs. However, EHD1 was also shown possess a binding site to MICAL-L1 (Sharma et. al., 2009). It was shown that both the indirect regulation through ACAP2 and the direct regulation through MICAL-L1 of EHD1 is necessary in neurite outgrowth (Kobayashi and Fukuda, 2013). But in the case of TNT formation we demonstrated that MICAL-L1 is not involved. Thus, these data clearly show that even though they use the similar pathway through the activation of Rab35, neurite outgrowth and TNT formation are regulated in different manner.

There are several different EHD proteins in humans (EHD 1-4). Some of them have a role in cilia formation and recycling pathway (Naslavsky et. al., 2011). It would also be interesting to see if these proteins have a role in TNT formation.

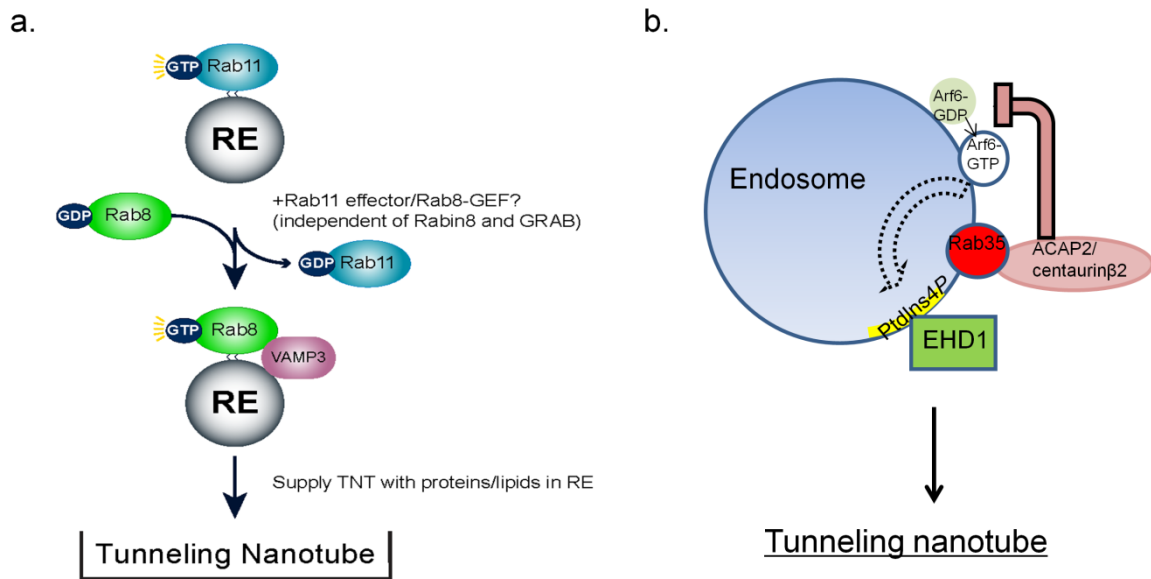
Along with directing proteins for neurite outgrowth and maintaining axons and dendrites, vesicle trafficking and recycling is also involved in the formation of different neuronal structures such as neurite outgrowth, formation of dendritic spines etc. Exocyst complex is one of the major components of the recycling endosomes, for e.g. Sec 6/8 is localized at the growth cones and the neurites (Hazuka et. al., 1999). Rab11 is regulating localisation of the N-cadherin in neurons and polarisation of the neurons (Barnat et. al., 2017). It is also reported that Rab5 acts upstream of Rab11 in regulating the migration of neurons, while the Rab7 regulates the late stage of migration in the neurons (Kawauchi et. al., 2010). Rac induced membrane ruffles and lamellopodia are formed due actin polymerization. Recycling membrane gives rise to newer membranes of these cellular structures (Bretscher and Aguado-Velasco, 1998). This indicates that the recycling pathway, neurite outgrowth and its effect on cytoskeletal structures are related.



Overall from our studies, Rab8a, Rab11a and Rab35, all three show a positive effect on contact dependent vesicle transfer by TNTs. Hence it might be an interesting pathway to study their effect with respect to each other, since all the three proteins Rab8a, Rab11a and Rab35 are also involved in the formation of cellular protrusions such as cilia (Onnis et. al., 2015, Kuhns et. al., 2019). Preliminary data suggests that downregulating Rab35 and overexpressing either Rab8 or Rab11 has no role on TNT formation (data not shown), they may work in another mechanism wherein Rab35 acts downstream of Rab8 and Rab11 and show an effect on TNTs. Further experiments are needed to validate this.

Previous studies done in our lab has shown that filopodia and TNTs, even though they are structures that are highly enriched in F-actin, are regulated by different manner. Especially in neuronal cells the proteins which are regulating filopodia in a positive manner are shown to negatively regulate TNTs e.g. IRSp53 and VASP, while Eps8 that negatively regulates filopodia, has a positive effect on TNTs (Delage et. al., 2016). MyosinX which has been shown to be a positive regulator for TNTs in many studies, regulates TNTs and filopodia by different domains on the protein (Gousset et. al., 2013).

Recent work in the lab suggests that actin structure in both the TNTs and filopodia are different (Sartori-rupp et. al., 2019). It would be interesting to study the role of Rab35, ACAP2, EHD1 in the ultrastructure of TNTs. From these studies we plan to establish the role of Rabs and its effect on TNT formation.



**Figure 16 : Working Model and Hypothesis :** a) Role of Rab11a-Rab8a-VAMP3 cascade regulating the formation of TNTs b) Role of Rab35 and its effectors involved in TNT formation.

## ***Section 5***

# **References**

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## **SYNTHÈSE EN FRANÇAIS**

(Texte traduit par Google translate)

La communication cellule-cellule est importante pour le développement et la fonction des organismes multicellulaires. Les interactions cellule-cellule se produisent à travers différents mécanismes entre des cellules adjacentes ou distantes. Des structures de connexion intercellulaire ont été montrées dans une grande variété d'organismes vivants comme les plasmodesmes dans les plantes, les cytonèmes chez la drosophile et les jonctions lacunaires chez les mammifères. Ces structures permettent le transport de molécules, d'ions, de métabolites et d'hormones.

Les nanotubes à effet tunnel sont principalement des protubérances membranaires intercellulaires à base de F-actine qui transportent le contenu cytoplasmique, les cargaisons et les organites. Ils étaient à l'origine décrits comme des ponts de connexion de cellule à cellule constitués de connexions membraneuses continues qui n'adhèrent pas au substrat. Leur diamètre varie de 50 à 150 nm. et leur longueur peut aller jusqu'à une centaine de micromètres (Rustom et. al., 2004, Sartori-Rupp et. al., 2019). Ils sont de nature extrêmement fragile, sensibles à la fixation lumineuse, chimique et mécanique. La composition cytosquelettique des TNT décrits est légèrement variée, une grande majorité contenant de la F-actine, mais une sous-population contenant également des microtubules (Antanaviciute et. Al., 2014). Les TNT se forment non seulement dans la population homogène mais aussi dans la population hétérogène de cellules (Lou et. Al., 2012, Kretschmer et. Al., 2019, Pasquier et. Al., 2013). Malheureusement, aucun marqueur moléculaire n'a encore été identifié, qui soit spécifique aux TNT. Bien que des études majeures impliquant des TNT aient été menées in vitro, en raison du manque de marqueurs spécifiques, l'identification des TNT in vivo est difficile, cependant, il y a eu plusieurs preuves de connexions de type TNT observées in vivo ou dans des conditions de culture organoïde 3D principalement basées sur leur aspect morphologique. Plus précisément, en utilisant pour la première fois des approches de microscopie corrélative à lumière et cryoélectron et la microscopie électronique à balayage par faisceau d'ions focalisés (FIB-SEM), notre laboratoire a démontré que dans les cellules neuronales, les TNT sont des structures spécifiques avec une ultrastructure distincte, différente de autres protubérances cellulaires. Ils se composent d'un ensemble de «nanotubes de tunnelage» (iTNT) ouverts, maintenus ensemble par des connexions basées sur la N-cadhérine. (Sartori-Rupp et. Al., 2019)

Les TNT peuvent être formés par deux mécanismes différents; a) Par l'extension de la protrusion cellulaire comme les filopodes dépendants de l'actine, pour former une connexion initiale qui se traduit par la formation de TNT (Rustom et. al., 2004, Yang et. al., 2016). b) Ou ils peuvent se former par contact de cellule à cellule, puis délogement des cellules, ce qui entraîne une connexion TNT fonctionnelle entre les cellules (figure 3) (Onfelt et. al., 2004, Reichert et. al., 2016, Pedicini et. al., 2018).

La formation de TNT est régulée par plusieurs facteurs et protéines différents. Il a été démontré que divers signaux et molécules de stress et inflammatoires régulent la formation de TNT (A Rustom, 2016). L'exposition des cellules à des produits chimiques comme le peroxyde d'hydrogène ( $H_2O_2$ ) ou à des conditions environnementales comme la lumière ultraviolette (UV) provoque une augmentation du nombre de cellules connectées au TNT. Des protéines qui régulent le stress dans les cellules comme le NF $\kappa$ B (facteur nucléaire kappa-activateur de chaîne légère des cellules B activées) / TNF $\alpha$ IP ou la protéine tumorale p53 sont impliquées dans la formation de TNT. Les lipides ont un rôle dans la formation de TNT, cependant les études de la composition membranaire spécifique des TNT sont encore rares. (Thayanithy et al., 2014). Il a été démontré que les petites GTPases régulent différentes voies membranaires et il existe des preuves substantielles de plusieurs études que la formation de TNT est régulée par plusieurs petites GTPases différentes, par exemple; RalGPS2 (facteur d'échange de nucléotides de guanine indépendant de Ras (GEF) pour RalA), RASSF1A (isoforme A de la famille 1 du domaine d'association Ras) régulent positivement les TNT. Comme les TNT sont principalement constitués d'actine et de microtubules, plusieurs protéines qui modifient le cytosquelette montrent également un effet sur la formation de TNT. Ces protéines incluent les protéines régulatrices des filopodes et des lamellipodes comme Rac et Cdc42, la protéine du syndrome de Wiskott-Aldrich (WASP), la famille WASP homologue à la verproline 2 (WAVE-2) et Arp2 / 3 (Actin Related Protein 2/3) sur les TNT dans différentes cellules. les types. Mais dans les cellules CAD neuronales, les protéines régulant positivement les filopodes comme IRSp53 (substrat récepteur de l'insuline p53) et VASP (phosphoprotéine stimulée par vasodilatateur) montrent une diminution de la formation de TNT tandis que les protéines régulant négativement la formation de filopodes, c'est-à-dire Eps8, montrent une augmentation de la formation de TNT, cela indique que les filopodes et le TNT sont réglementés de manière opposée (Delage et al., 2016).

La fonction principale des TNT est d'échanger différentes cargaisons entre les cellules. Il a été démontré qu'ils transportent plusieurs organites cellulaires et substances cellulaires

différents comme les récepteurs membranaires GPCR (récepteurs couplés aux protéines G), les lysosomes, les mitochondries, etc.

Plusieurs études mettent en évidence le rôle potentiel que pourrait avoir le transfert d'organelles médié par le TNT dans différentes conditions physiopathologiques.

Par exemple, dans les maladies neurodégénératives comme Alzheimer, Parkinson Huntington, etc., les TNT se sont avérés transporter plusieurs des protéines pathogènes responsables de la propagation de la maladie. Ce transport de protéines pathogènes vers les cellules saines conduit à la cytotoxicité dans les cellules saines. Par exemple; Les peptides  $\beta$  ont été transférés par les TNT et provoquent une cytotoxicité dans les neurones (Wang et al., 2011). L'alpha synucléine est également transportée à travers les TNT dans les CAD et les neurones. Ce transfert se produit généralement à travers les lysosomes à l'intérieur des TNT des cellules malades vers les cellules saines (Abounit et al., 2016a). Fait intéressant, lorsque l'alpha synucléine est transportée à travers les TNT dans les astrocytes, les mitochondries sont transportées dans la direction opposée des astrocytes sains aux cellules malades, ce qui permet un mécanisme de sauvetage (Rostami et al., 2017). Notre laboratoire a également montré que le transfert d'alpha-synucléine est spécifique au type cellulaire. Le transfert d'alpha-synucléine se produit entre différents types de cellules, comme les neurones aux astrocytes ou les astrocytes aux astrocytes, mais de manière moins efficace entre les astrocytes et les neurones. De plus, nous avons également montré que les astrocytes sont beaucoup plus efficaces pour dégrader l'alpha-synucléine que les neurones (Loria et al., 2017).

Les TNT étant essentiels dans plusieurs processus physiopathologiques, il était essentiel de les étudier plus en détail. Et comme il y avait plusieurs exemples mettant en évidence le rôle des protéines sur les TNT, j'ai choisi d'étudier le rôle des protéines Rab (petites GTPases) et son effet sur les TNT.

Les GTPases Rab sont l'une des principales familles de protéines impliquées dans le trafic de vésicules dans les cellules. Le trafic de vésicules est important pour l'absorption des nutriments, l'adhésion et la migration des cellules, la signalisation, l'entrée d'agents pathogènes, la transmission synaptique, la régulation des récepteurs, la présentation de l'antigène, la polarité cellulaire, la mitose, la croissance et la différenciation, et la délivrance de médicaments (Doherty et McMahon, 2009). Le trafic de vésicules intracellulaires peut être divisé en deux voies principales, à savoir l'endocytose et l'exocytose.



L'exocytose est principalement utilisée pour expulser les substances cellulaires, tandis que l'endocytose est impliquée dans l'absorption de molécules / solutés par les cellules. L'exocytose implique la fusion des membranes vésiculaires avec la membrane plasmique. L'endocytose intériorise les lipides et les protéines des membranes plasmiques dans les cellules (Doherty et McMohan, 2009).

L'un des processus du trafic de vésicules est la voie de recyclage des vésicules. Elle implique le processus de recyclage des vésicules vers la membrane plasmique. La voie de recyclage implique l'internalisation de la cargaison par des puits recouverts de clathrine ou une endocytose indépendante de la clathrine au niveau de la membrane qui est finalement pincée de la membrane plasmique pour former des endosomes précoces. Ces endosomes précoces sont associés à des molécules Rab spécifiques qui permettent aux vésicules de fusionner et de former le corps multivesiculaire (MVB). Ensuite, ils sont recyclés vers la membrane (ce qui se produit généralement pour les récepteurs de la membrane plasmique) (Katzmann et al., 2002) ou fusionnés avec des lysosomes pour la dégradation du contenu des vésicules (Cullen et Steinberg, 2018). Il existe un équilibre entre l'absorption du contenu par l'endocytose et leur recyclage vers la membrane plasmique. Ces deux voies contrôlent divers processus cellulaires tels que la migration cellulaire, la cytokinèse, le maintien de la polarité cellulaire, l'adhésion cellulaire et la formation de jonction (Grant et Donaldson, 2009).

Le trafic de vésicules est impliqué dans la formation de différentes structures cellulaires. À l'intérieur de la cellule, les vésicules sont généralement transportées sur des pistes de microtubules ou d'actine (Grant et Donaldson, 2009). Le recyclage des vésicules régule également la formation de protubérances cellulaires. Des volants de membrane et des lamellopodes induits par Rac se forment en raison de la polymérisation de l'actine. Le recyclage de la membrane vésiculaire donne naissance aux nouvelles membranes nécessaires à sa formation (Bretscher et Aguado-Velasco, 1998). Les cils sont des structures composées de microtubules; leur surface cellulaire est constituée de molécules comme le lissé, les GPCR, la somatostatine et la rhodopsine. La formation des cils implique à la fois le trafic des membranes polarisées et l'organisation des microtubules (S Sorokin, 1962). Le trafic membranaire est impliqué dans de nombreux processus des neurones, l'altération du trafic membranaire a été impliquée dans la neurodégénérescence et le cancer (Kiral et al. 2018). Les Rabs régulent également le recyclage des vésicules vers la membrane qui peuvent être impliquées dans la dynamique de la membrane et conduire à la formation de TNT (décrites dans cette thèse).

Les petites GTPases sont divisées en familles Ras, Rho, Rab, Sar1 / Arf et Ran (Takai et al., 2001). Il existe environ 60 GTPases Rab chez les mammifères, 31 chez la drosophile et 11 chez la levure (Kiral et al., 2018, Zhang et al., 2007). Ils ont été identifiés pour la première fois dans les années 1980. Les Rabs fonctionnent via le cycle lié au GTP (Guanosine 5'-triphosphate) (actif) –GDP (Guanosine 5'-diphosphate) (inactif). Ils sont régulés par les GEF (Guanine nucleotide Exchange Factors), les GAP (GTPase Activating Proteins) et les GDI (Guanine Dissociation Inhibitors).

Les protéines Rab s'accumulent au niveau de leurs membranes cibles et sont donc utilisées comme marqueur d'organites. Les protéines Rab sont impliquées dans l'attache et l'amarrage des vésicules à sa membrane dans les cellules, ce qui conduit à la fusion de la vésicule respective avec la membrane. Chacune des protéines Rab contrôle une voie spécifique de trafic membranaire (Bhuin et Roy, 2014). Il a été démontré que les protéines Rab régulent le bourgeonnement des vésicules à partir de différents organites. Les Rabs participent au transport des vésicules. Plusieurs protéines Rab sont impliquées dans la filière de recyclage. La présence de Rab11 / EHD1 sur les tubules est l'indication ERC (Endocytic Recycling Compartment) (Grant et Donaldson, 2009). Les endosomes précoces perdent Rab5 et acquièrent Rab11, ce qui entraîne la formation des tubules par les endosomes précoces et le corps principal des endosomes précoces donne naissance au corps multivesiculaire. L'ERC est généralement situé près du golgi ou MTOC (centre d'organisation des microtubules) (Maxfield et McGraw, 2004).

Voici les différentes protéines Rab et leurs effecteurs impliqués dans la formation de TNT.

Rab8a est localisé au niveau de la membrane basolatérale, des golgi et des structures tubulovésiculaires (Lee et al., 2003). Rab8a colocalise avec plusieurs protéines différentes comme ARF6, Rab11 (Hattula et al., 2006), les intégrines  $\beta$  en conditions polarisées (Peranen et al., 1996) et IFT20 (Finetti et al., 2015). Rab8a est impliquée dans le trafic de vésicules et ses effets, tels que l'exocytose (Huber et al., 1993, Hattula et al., 2006), le recyclage des membranes et la polarisation des cellules. Rab8a est impliquée dans la formation de la membrane au niveau du froissement de la membrane et la formation de protubérances comme les filopodes et les lamellipodes, et la formation de fibres de stress en recyclant les vésicules vers la membrane (Hattula et al., 2006). La surexpression de la forme inactive de Rab8a montre une augmentation des fibres de stress mais la forme active de Rab8a montre une redistribution des filaments d'actine dans les cellules. Il a également été montré que Rab8a se localise avec l'actine dans des structures semblables à des lamellopodes formées dans les cellules surexprimant Rab8a.

Cependant, les mécanismes responsables de l'inter-régulation de Rab8a et d'actine sont inconnus (Peranen et al., 1996).

Rab11 est impliqué dans le transport des endosomes recyclés vers la membrane plasmique (Das et Guo, 2011). Rab11a est enrichi à la base des cils et participe à l'entretien des cils primaires (Knodler et al., 2010). Rab11a-Rab8a à l'aide de Rabin8 et Cdc42 régule la luménogenèse épithéliale (Bryant et al., 2010). Rab8 et Rab11 sont impliqués dans la formation des cils avec l'aide de Rabin8 (FEM de Rab8a). Rabin8 se révèle être le lien entre l'activation dépendante de Rab11 de Rab8a et les deux Rabs agissent en cascade qui conduit à la formation de cils (Knodler et al., 2010). Dans mon travail de thèse (avec un autre étudiant), nous avons trouvé un rôle pour Rab11a et Rab8a dans la formation de TNT (voir ci-dessous et résultats). Cependant, Rabin8 n'a pas d'effet sur la formation des nanotubes à effet tunnel dans les cellules CAD (Zhu et al., 2018). GRAB (GRAB / Rab3IL1 (facteur d'échange de nucléotides guanine pour Rab3A; Rab3A interagissant protéine (rabin3) -like 1) agit de manière similaire à Rabin8. Il agit en tant qu'effecteur en aval de Rab11a et Rab8a et est impliqué dans la croissance des axones (Furusawa et al., 2017). Rab11 interagit avec GRAB et la surexpression de Rab11 provoque le déplacement de GRAB vers les membranes (Horgan et al., 2013). Dans mon travail de thèse, nous avons montré que GRAB, est impliqué dans la formation des TNT. Cependant, il n'était pas impliqué dans le mécanisme en cascade pour la régulation des TNT (Zhu et al., 2018).

Le ciblage, l'amarrage et la fusion des vésicules avec les membranes accepteurs nécessitent une interaction avec l'actine et le cytosquelette des microtubules, le recrutement de complexes d'accostage cytosolique et une reconnaissance spécifique entre les protéines membranaires intégrales connues sous le nom de SNARES (récepteurs solubles de la protéine de fixation NSF). VAMP3 (protéine membranaire associée aux vésicules 3) appartient à la famille des protéines SNARE (Banerjee et al., 2017). Il a été démontré que Rab8 était impliqué dans le recrutement de VAMP3 et aide au recyclage des vésicules. Il est également démontré que VAMP3 interagit avec le lissé (protéine transmembranaire trouvée dans les cils, nécessaire à la transduction du signal) et Rab8a dans la croissance ciliaire ((Finnetti et al., 2015). VAMP3 est également impliqué dans le trafic des intégrines de Rab11 positif le recyclage des endosomes (Skalski et al., 2005).

Rab35 a d'abord été cloné à partir du muscle squelettique humain et est exprimé de manière omniprésente. Il participe à la cytokinèse et au recyclage endocytaire rapide des cellules. Rab35 régule le recyclage de plusieurs récepteurs (Dikshit et al., 2014). Rab35

possède plusieurs protéines effectrices telles que l'OCRL (syndrome oculo-cérébro-rénal de Lowe), ACAP2 ( $\beta$ -centaurine) et MICAL-L1 (molécules interagissant avec CasL-Like 1) (Dambournet et al., 2011). Rab35 est impliqué dans le recyclage endocytaire rapide et sa régulation négative a entraîné l'accumulation de marqueurs endocytaires dans les diverses vacuoles à travers les cellules, entraînant un échec de la cytokinèse. Il a été démontré par la suite que Rab35 est impliqué dans la localisation de PIP2 et de Septin au pont cytokinétique (Kouranti et al., 2006). ARF6 régule négativement l'activation de Rab35 et son rôle dans la cytokinèse par son effecteur EPI64B (Chesneau et al., 2012). Kobayashi et Fukuda, en 2013, ont montré que l'activation du NGF (Nerve Growth Factor) induit la croissance des neurites en raison de l'association de Rab35 aux endosomes positifs ARF6. Rab35 recrute ensuite ACAP2 et MICAL-L1 pour former un complexe. MICAL-L1 agit directement comme un site de liaison pour EHD1. Alors que l'ACAP2 régule indirectement le recrutement d'EHD1 à la membrane en inactivant ARF6 et en influençant ainsi les niveaux de PI4P sur la membrane des vésicules, ce qui permet la liaison d'EHD1 à la membrane vésiculaire qui régule l'excroissance des neurites.

MICAL-L1 (molécules MICAL interagissant avec CasL - Like), interagit avec le cytosquelette d'actine et est couramment impliqué dans le recyclage des membranes (M. Vanoni, 2017). Les protéines de type MICAL ont la capacité de liaison pour une large gamme de protéines, y compris Rab8a / b, Rab10, Rab13 et Rab15. MICAL-L1 interagit avec le complexe de protéines Rab-EHD. Rab35 interagit avec MICAL-L1 et recrute Rab8a sur les endosomes tubulaires. MICAL-L1 est réglementé par ARF6. La surexpression d'ARF6 activé dans les cellules a entraîné le recrutement de MICAL-L1 dans les endosomes tubulaires (Rahajeng et al., 2012).

ACAP2 (Arf GAP avec bobine enroulée, répétition ANK et domaine PH) ou Centaurin  $\beta$ 2 se trouve dans une grande variété d'organismes multicellulaires allant de *Drosophila melanogaster*, *Caenorhabditis elegans* et *Arabidopsis thaliana* aux mammifères, y compris l'homme et la souris. Il a été démontré que l'activité de ACAP2 dépend des niveaux d'acide PIP2 et phosphatidiques dans la cellule de (Jackson et al. Al., 2000). Rab35 est impliqué dans les premiers stades de la phagocytose, en particulier dans l'extension des pseudopodes. Chez *C. elegans*, un homologue des mammifères ACAP1 et ACAP2, c'est-à-dire CNT1 (Centaurine 1) est recruté par Rab10 dans l'épithélium intestinal et est nécessaire pour réguler les niveaux de PIP2 dans les endosomes de recyclage (Shi et al., 2012).

Les GTPases ARF sont de petites protéines de 21 kDa. Ils sont divisés en trois classes, la classe I comprend les ARF 1 à 3, la classe II comprend les ARF 4 et 5, et la classe III ARF comprend ARF6 (Jackson et Casanova, 2000). La phospholipase D (PLD) interagit avec la phosphatidylinositol-4-phosphate 5 (PI4P) kinase pour convertir PI4P en PIP2. Ce PIP2 est à son tour nécessaire à l'activité de PLD. Les mutants ARF6 qui sont incapables d'activer la PLD présentent des défauts de recyclage et ne parviennent pas à former des protubérances par les cellules (Jovanovic et. Al., 2006). ARF6 est impliqué dans l'endocytose non dépendante de la clathrine et le recyclage vers la membrane plasmique. Il est impliqué dans le recyclage des protéines régulatrices d'actine telles que le complexe Cdc42 et Rac et PAR6 (Donaldson et Jackson, 2011)

Le recyclage endocytaire implique le retour des membranes et des récepteurs à la membrane plasmique, et ce processus implique les vésicules tubulaires et vésiculaires porteurs dans les cellules où ils sont facilement transportés vers la membrane plasmique à travers les microtubules. La famille de protéines EHD s'est avérée importante dans le recyclage endocytaire et elles sont principalement associées au CER (Cai et. Al., 2013). La famille de protéines EHD est homologue de la protéine Eps15. Il a été montré que l'EHD1 directement colocalisé avec lissé (comme mentionné précédemment lissé est impliqué dans la transduction du signal et est principalement situé à la base des cils) et trafiqué le long du cil (Bhattacharyya et. Al., 2016).

La première partie de la thèse décrit le rôle de Rab8a et Rab11a et de leurs effecteurs sur les TNT. Les résultats sont résumés comme suit. Il a été démontré que les protéines Rab sont impliquées dans plusieurs processus cellulaires tels que la cytokinèse, la formation de cils, la synapse immunitaire, etc. Elles se révèlent être des maîtres régulateurs des fonctions cellulaires (Das et Guo, 2011). Ainsi, nous avons criblé plusieurs protéines Rab différentes pour identifier celles impliquées dans la formation de TNT. Dans cette étude, le Dr Seng Zhu (ancien doctorant du laboratoire) a mis en place un dépistage des TNT, basé sur la capacité des protéines Rab surexprimées à augmenter ou diminuer le transfert de vésicules induit par le TNT entre un donneur et un accepteur de cellules. Sur les 41 protéines Rab différentes testées, Rab8a, Rab11a et Rab35 étaient de forts résultats positifs. Rab8a est impliquée dans la formation de différentes structures cellulaires telles que la synapse immunitaire et la formation de cils (Lesteborg et. Al., 2017, Lu et. Al., 2015). Rab8a est également impliquée dans la formation de protubérances cellulaires comme les filopodes et les lamellipodes dans les cellules, en affectant l'organisation de l'actine et des microtubules (Peranen et al., 1996). Rab8 et Rab11 sont impliqués dans la

formation des cils avec l'aide de Rabin8 (FEM de Rab8a) (Knodler et al., 2010). Lors de la surexpression de chacune de ces protéines, il a été observé que les formes liées au GTP de Rab8a et Rab11a augmentaient les TNT. Nous avons également montré qu'ils régulent les TNT via un mécanisme en cascade. Cependant, alors que Rabin8 augmentait la formation de filopodes, nous avons démontré que ce n'était pas le cas pour les TNT car la surexpression de Rabin8 n'affectait pas les TNT. Un autre FEM de Rab8a, c'est-à-dire GRAB, a été démontré auparavant comme étant impliqué dans la croissance des axones par le mécanisme en cascade par Rab11a-Rab8a (Furusawa et al., 2017). Ainsi, nous avons examiné le rôle du GRAB par rapport aux TNT. Nous avons constaté que la surexpression de GRAB conduit à une augmentation des cellules connectées au TNT, mais cela semble être indépendant de la cascade Rab8-Rab11. D'autre part, nous avons constaté que VAMP3 qui est connu pour interagir avec Rab8 et être responsable de la fusion finale des vésicules à la membrane (Finetti et al., 2015) était impliqué. Il a également été démontré que VAMP3 interagit avec Rab8 à la base du cil et régule l'excroissance ciliaire (Patrussi et Baldari, 2016).

Les données présentées ici fournissent de nouvelles informations sur les facteurs impliqués dans la formation de TNT et le transfert de vésicules intercellulaires dans les cellules CAD neuronales. Nos résultats montrent que Rab11a, Rab8a et VAMP3 augmentent à la fois le nombre de cellules connectées au TNT et le transfert de vésicules à travers les TNT dans les cellules CAD. Nous démontrons que Rab11a et Rab8a fonctionnent dans la même voie et utilisent VAMP3 comme effecteur pour induire des TNT fonctionnels mais pas des filopodes. Il est intéressant de noter que cette cascade de signalisation Rab11a-Rab8a ne nécessite pas Rabin8, qui d'autre part est essentiel pour assurer la médiation de la formation de filopodes activés par Rab8a. Ainsi, en plus de fournir des détails mécanistes sur le rôle des protéines Rab dans la formation de TNT, ces données renforcent également notre hypothèse selon laquelle les filopodes et les TNT sont des structures différentes, qui dépendent de différents mécanismes de formation.

Dans la deuxième partie de ma thèse, je me concentre sur l'étude du rôle de Rab35 et de ses effecteurs sur la formation de TNT. Les résultats sont résumés comme suit. Les nanotubes à effet tunnel (TNT) sont des structures riches en F-actine qui connectent des cellules distantes, permettant le transport de nombreux composants cellulaires, y compris les vésicules, les organites et différents types de molécules. Les TNT sont impliqués dans des processus cellulaires clés, tels que le développement, l'immunité et la régénération tissulaire, mais également dans la transmission de divers agents pathogènes. Étant donné

que les GTPases Rab sont les principaux régulateurs du trafic de vésicules et participent également à la régulation du cytosquelette d'actine, nous avons examiné le rôle de cette famille de protéines dans la formation de TNT. Tout d'abord, nous avons effectué un criblage de plusieurs protéines Rab différentes pour son effet sur le transfert de vésicules dépendant du TNT. Rab35 a été l'un des Rabs à avoir un effet positif sur le transfert des vésicules. L'analyse de Rab35, une protéine impliquée dans le recyclage endocytaire, la cytokinèse et la croissance des neurites, a montré que la forme liée au GTP-Rab35 augmente également la formation de TNT. La croissance des neurites est un processus essentiel pour établir la connectivité neuronale et le recyclage des vésicules joue un rôle crucial dans ce processus. Rab35 interagit avec plusieurs protéines impliquées dans le trafic de vésicules telles que ACAP2 (agit comme GAP de ARF6), MICAL-L1 (molécule interagissant avec CasL-like 1, qui joue un rôle dans le recyclage des vésicules) EHD1 (ciseau moléculaire qui a un rôle dans la scission des vésicules). Aux endosomes positifs ARF6, Rab35 recrute ACAP2 et MICAL-L1, et forme un complexe qui se lie à EHD1 pour réguler la croissance des neurites. Nos données suggèrent fortement que ces effecteurs peuvent également être impliqués dans la formation de TNT. Individuellement, ACAP2, EHD1 et ARF6-GDP régulent la formation de TNT de manière positive. Mais la surexpression de MICAL-L1 dans les cellules ne montre aucun effet sur les TNT. De plus, les données préliminaires indiquent que Rab35 et EHD1 agissent dans un mécanisme en cascade pour réguler la formation de TNT. Cela indique que la formation de TNT et l'excroissance des neurites peuvent agir dans une voie similaire, mais pas exacte. Les molécules identifiées ici qui ont un rôle dans la formation de TNT, constituent des cibles moléculaires potentielles pour des thérapies visant à bloquer la propagation des pathogènes qui se transmettent par les TNT. Cette étude prouve que les protéines qui ont un rôle dans le trafic de vésicules et la croissance des neurites, telles que les protéines Rab, sont également impliquées dans la formation de TNT .

De toutes les études menées au cours de ma thèse, nous avons tiré les conclusions suivantes. Du criblage de 41 protéines Rab différentes, nous avons constaté que Rab8a, Rab11a et Rab35 régulent positivement le transfert de vésicules dépendant du contact entre les cellules. Après une enquête plus approfondie, nous avons démontré que individuellement Rab8a et Rab11a régulent la formation de TNT. Puisque la protéine VAMP3 régule la formation de cils avec Rab8a et Rab11a (Finetti et al., 2015), nous avons étudié son rôle et constaté que VAMP3 régule positivement la formation de TNT. Nous avons en outre montré que les trois protéines Rab11a, Rab8a et VAMP3 agissent en cascade pour réguler la formation de TNT. Rabin8 qui interagit avec Rab11a et agit comme

un GEF de Rab8a dans la formation des cils (Knodler et al., 2010), ne montre aucun effet sur les TNT. D'un autre côté, j'ai trouvé que GRAB une protéine dont la fonction est similaire à Rabin8 (Furusawa et al., 2017) lors de la surexpression dans notre modèle de cellule neuronale conduit à une augmentation des cellules connectées au TNT. Cependant, j'ai démontré que cette régulation TNT est indépendante de la cascade Rab11a-Rab8a. Cela ouvre de nouvelles questions à rechercher, notamment pour trouver les facteurs qui agissent comme un lien entre Rab11a et Rab8a. En outre, il serait intéressant d'étudier plus en détail le rôle de GRAB et de ses protéines en interaction qui peuvent réguler la formation de TNT.

Plusieurs protéines Rab sont impliquées dans la filière de recyclage. L'ERC est généralement situé près du golgi ou MTOC (centre d'organisation des microtubules) (Maxfield et McGraw, 2004). La présence de Rab11 / EHD1 sur les tubules est l'indication de l'ERC (Grant et Donaldson, 2009). Rac et Cdc42 stimulent l'assemblage des jonctions adhérentes dans les cellules épithéliales en recyclant la E-cadhérine par la voie endosomale (A Ridley, 2001, Le et. Al., 1999). Rab8 recycle les vésicules positives pour la transferrine vers les membranes plasmiques. Ces vésicules participent à la formation de protubérances cellulaires (Hattula et. Al., 2006). Ainsi, puisque le recyclage des vésicules joue un rôle important dans la formation de différentes protubérances cellulaires, nous proposons que cela soit également important pour la régulation des TNT, mais cela doit être étudié plus avant.

Des données préliminaires (données non présentées) ont indiqué que Rab8a et Rab11a sont localisés à la base des TNT. Il serait intéressant d'étudier le rôle des protéines Rab8 et Rab11 dans la régulation de la formation de TNT dans des conditions vivantes, et si les vésicules positives pour ces protéines sont à l'origine de TNT. La primaquine qui est un inhibiteur du recyclage des membranes et a bloqué le recyclage de la transferrine dans les cellules CAD (Zhu et. Al., 2018) montre un effet négatif sur les cellules connectées au TNT. Cela montre que le recyclage de la membrane a un effet important sur les TNT car il peut fournir des lipides et des protéines nécessaires à la croissance des TNT.

Pendant la croissance des neurites, Rab35 agit avec ACAP2 et MICAL-L1 pour réguler ARF6 et EHD1 (Kobayashi et Fukuda, 2013). Dans nos études, nous avons montré que la forme liée au GTP de Rab35 entraîne une augmentation du nombre de cellules connectées au TNT. Mais contrairement à la croissance des neurites, MICAL-L1 n'est pas impliqué dans la formation de TNT. Cependant, nous pourrions montrer que plusieurs des effecteurs en aval tels que ACAP2, GDP-ARF6 et EHD1 sont impliqués dans la formation



de TNT. ACAP2 s'est avéré réguler indirectement la croissance des neurites, en agissant comme un GAP d'ARF6. La forme active d'ARF6 active la PIP5kinase qui convertit PI4P en PIP2 (Krauss et al., 2003). Ainsi, l'inactivation de l'ARF6 peut réguler les niveaux de PI4P (Kobayashi et Fukuda, 2013). L'EHD1 se lie aux vésicules riches en PI4P (Jovic et al., 2007). Par conséquent, nous pouvons spéculer que Rab35 régule indirectement EHD1 via ACAP2 et ARF6 pour la formation de TNT. En effet, nous avons ici démontré que Rab35 agit en amont de l'EHD1 en régulant la croissance des TNT. Cependant, EHD1 s'est également avéré posséder un site de liaison à MICAL-L1 (Sharma et al., 2009). Il a été démontré que la régulation indirecte via ACAP2 et la régulation directe via MICAL-L1 de EHD1 sont nécessaires dans la croissance des neurites (Kobayashi et Fukuda, 2013). Mais dans le cas de la formation de TNT, nous avons démontré que MICAL-L1 n'est pas impliqué. Ainsi, ces données montrent clairement que même si elles utilisent la voie similaire à travers l'activation de Rab35, l'excroissance des neurites et la formation de TNT sont régulées de manière différente.

Il existe plusieurs protéines EHD différentes chez l'homme (EHD 1-4). Certains d'entre eux jouent un rôle dans la formation des cils et la voie de recyclage (Naslavsky et al., 2011). Il serait également intéressant de voir si ces protéines ont un rôle dans la formation de TNT.

En plus de diriger les protéines pour la croissance des neurites et de maintenir les axones et les dendrites, le trafic et le recyclage des vésicules sont également impliqués dans la formation de différentes structures neuronales telles que la croissance des neurites, la formation d'épines dendritiques, etc. Le complexe d'exocystes est l'un des principaux composants du recyclage des endosomes, par exemple, Sec 6/8 est localisé au niveau des cônes de croissance et des neurites (Hazuka et al., 1999). Rab11 régule la localisation de la N-cadhérine dans les neurones et la polarisation des neurones (Barnat et al., 2017). Il est également rapporté que Rab5 agit en amont de Rab11 en régulant la migration des neurones, tandis que Rab7 régule le stade tardif de la migration dans les neurones (Kawauchi et al., 2010). Des volants de membrane et des lamellopodes induits par Rac se forment en raison de la polymérisation de l'actine. La membrane de recyclage donne naissance à de nouvelles membranes de ces structures cellulaires (Bretscher et Aguado-Velasco, 1998). Cela indique que la voie de recyclage, l'excroissance des neurites et son effet sur les structures cytosquelettiques sont liés.

Dans l'ensemble de nos études, Rab8a, Rab11a et Rab35, les trois montrent un effet positif sur le transfert de vésicules dépendantes du contact par les TNT. Par conséquent, cela pourrait être une voie intéressante pour étudier leurs effets les uns par rapport aux

autres, car les trois protéines Rab8a, Rab11a et Rab35 sont également impliquées dans la formation de protubérances cellulaires telles que les cils (Onnis et. Al., 2015, Kuhns et . al., 2019). Les données préliminaires suggèrent que la régulation négative de Rab35 et la surexpression de Rab8 ou Rab11 n'ont aucun rôle sur la formation de TNT (données non présentées), ils peuvent fonctionner dans un autre mécanisme dans lequel Rab35 agit en aval de Rab8 et Rab11 et montre un effet sur les TNT. D'autres expériences sont nécessaires pour valider cela.

Des études antérieures réalisées dans notre laboratoire ont montré que les filopodes et les TNT, même s'il s'agit de structures hautement enrichies en F-actine, sont régulés de différentes manières. Surtout dans les cellules neuronales, les protéines qui régulent les filopodes de manière positive se sont avérées réguler négativement les TNT, par exemple IRSp53 et VASP, tandis que l'Eps8 qui régule négativement les filopodes a un effet positif sur les TNT (Delage et al., 2016). MyosinX, qui s'est révélé être un régulateur positif pour les TNT dans de nombreuses études, régule les TNT et les filopodes par différents domaines sur la protéine (Gousset et. Al., 2013).

Des travaux récents en laboratoire suggèrent que la structure de l'actine dans les TNT et les filopodes est différente (Sartori-rupp et. Al., 2019). Il serait intéressant d'étudier le rôle de Rab35, ACAP2, EHD1 dans l'ultrastructure des TNT. À partir de ces études, nous prévoyons d'établir le rôle de Rabs et son effet sur la formation de TNT.

Ainsi, à partir des études menées au cours de mon travail de thèse, on peut conclure que les protéines Rab et certains effecteurs sont impliqués dans la formation du TNT. Les protéines qui peuvent être impliquées dans la croissance des neurites peuvent également être impliquées dans la formation de TNT.

**Titre :** Etude du rôle des protéines Rab et de leurs effecteurs sur les Tunneling nanotubes

**Mots clés:** Tunneling nanotubes, Rab8a, Rab11a, Rab35

**Résumé :** Les nanotubes de tunnellation (TNT) sont des structures riches en F-actine qui relient des cellules distantes, permettant ainsi le transport de nombreux composants cellulaires, notamment des vésicules, des organites et différents types de molécules. Les TNT sont impliqués dans des processus cellulaires clés, tels que le développement, l'immunité et la régénération des tissus, mais également dans la transmission de divers agents pathogènes.

Plusieurs facteurs moléculaires ont été identifiés pour participer à la régulation de la formation de TNT. Le complexe de l'exocyste est l'un des premiers facteurs moléculaires impliqués dans la formation de TNT. Ce complexe est également impliqué dans la fixation des vésicules sécrétoires pendant la sécrétion, ce qui suggère que les protéines qui régulent le trafic vésiculaire pourraient jouer un rôle dans la formation de TNT. Nous avons émis l'hypothèse que la formation de TNT est modulée par des protéines qui participent à la fois à la régulation du trafic vésiculaire et au remodelage du cytosquelette d'actine, et que ces deux processus sont essentiels pour la formation de ces structures.

Comme les GTPases de Rab sont les principaux régulateurs du trafic vésiculaire et participent également à la régulation du cytosquelette d'actine, nous avons examiné le rôle de cette famille de protéines dans la formation de TNT. Tout d'abord, nous avons effectué un criblage de plusieurs protéines de Rab différentes pour son effet sur le transfert de vésicule dépendant de TNT. Nous avons constaté que Rab8a, Rab11a et Rab35 ont un effet positif sur le transfert de vésicule. Des études supplémentaires ont démontré que la surexpression de Rab8a et Rab11a augmentait également le nombre de cellules connectées au TNT. Lors de la surexpression de VAMP3 (une autre protéine impliquée dans le trafic vésiculaire), nous avons également observé une augmentation du nombre de cellules connectées au TNT. Une analyse plus poussée a montré que les trois protéines, à savoir Rab11a, Rab8a et VAMP3, ont un effet sur la formation de TNT de manière cascade. Pour établir une relation entre Rab11a et Rab8a, nous avons vérifié le rôle de Rabin8 sur la formation de TNT (une protéine qui interagit avec Rab11 et qui active Rab8) et

nous avons constaté qu'elle n'avait aucun rôle dans la formation de TNT.

De plus, nous avons vérifié une autre protéine dont la fonction est similaire à Rabin8, à savoir GRAB (facteur d'échange de nucléotide de guanine pour Rab3A) et son rôle dans la formation de TNT. Les résultats montrent que la surexpression de GRAB augmente la formation de TNT, mais qu'elle agit de manière indépendante de Rab11 et Rab8a pour réguler la formation de TNT. L'analyse de Rab35, une protéine impliquée dans le recyclage des endocytos, la cytokinèse et la croissance des neurites, a montré que la forme liée au GTP-Rab35 augmente également la formation de TNT. La croissance des neurites est un processus essentiel pour établir la connectivité neuronale et le recyclage des vésicules joue un rôle crucial dans ce processus. Rab35 interagit avec plusieurs protéines impliquées dans le trafic vésiculaire, telles que ACAP2 (agit en tant que GAP de ARF6), MICAL-L1 (molécule interagissant avec CasL-like 1, qui joue un rôle dans le recyclage des vésicules) EHD1 (un ciseau moléculaire) qui joue un rôle dans la scission de la vésicule). Sur les endosomes positifs pour ARF6, Rab35 recrute ACAP2 et MICAL-L1 et forme un complexe qui se lie à EHD1 pour réguler la croissance des neurites. Nos données suggèrent fortement que ces effecteurs pourraient également être impliqués dans la formation de TNT. Individuellement, ACAP2, EHD1 et ARF6-GDP régulent la formation de TNT de manière positive. Mais la surexpression de MICAL-L1 dans les cellules ne montre aucun effet sur les TNT. En outre, des données préliminaires indiquent que Rab35 et EHD1 agissent dans un mécanisme en cascade pour réguler la formation de TNT. Ceci indique que la formation de TNT et la croissance des neurites peuvent agir de manière similaire, mais pas exactement. Les molécules identifiées ici qui jouent un rôle dans la formation de TNT constituent des cibles moléculaires potentielles pour les thérapies visant à bloquer la propagation d'agents pathogènes transférés à travers les TNT.

Cette étude prouve que les protéines jouant un rôle dans le trafic vésiculaire et la croissance des neurites, telles que les protéines de Rab, participent également à la formation de TNT.



**Title:** Study of the role of Rab proteins and their effectors on Tunneling nanotubes

**Keywords:** Tunneling nanotubes, Rab8a, Rab11a, Rab35.

**Abstract:** Tunneling nanotubes (TNTs) are F-actin rich structures that connect distant cells, allowing the transport of many cellular components, including vesicles, organelles, and different kind of molecules. TNTs are implicated in key cellular processes, such as development, immunity, and tissue regeneration, but also in the transmission of various pathogens.

Several molecular factors have been identified to participate in the regulation of TNT formation. One of the early molecular factors that is implicated in TNT formation is the exocyst complex. This complex is also involved in the tethering of secretory vesicles during secretion, which suggest that proteins that regulate vesicle trafficking could have a role in TNT formation. We have hypothesized that the formation of TNTs is modulated by proteins that participate in both, the regulation of vesicle trafficking and the remodelling of the actin cytoskeleton, and that these two processes are key for the formation of these structures.

Since Rab GTPases are the major regulators of vesicle trafficking and also participate in actin cytoskeleton regulation, we examined the role of this protein family in TNT formation. First, we performed a screening of several different Rab proteins for its effect on TNT-dependent vesicle transfer. We found that Rab8a, Rab11a and Rab35 have a positive effect on vesicle transfer. Additional studies demonstrated that Rab8a and Rab11a overexpression also increase the number of TNT connected cells. Upon overexpression of VAMP3 (another protein involved in vesicle trafficking), we also observed an increase in the number of TNT connected cells. Further analysis showed that all three proteins, i.e. Rab11a, Rab8a and VAMP3, show an effect on TNT formation in a cascade dependent manner. To establish a relationship between Rab11a and Rab8a, we checked the role on TNT formation of Rabin8 (a protein that interacts with Rab11a and activates Rab8a)

and we found that it has no role in TNT formation. Additionally, we checked another protein whose function is similar to Rabin8, i.e. GRAB (guanine nucleotide exchange factor for Rab3A) and its role in TNT formation. The results show that GRAB overexpression increases TNT formation, but it acts in a pathway independent of Rab11 and Rab8a to regulate TNT formation. The analysis of Rab35, a protein involved in endocytic recycling, cytokinesis, and neurite outgrowth, showed that the GTP-Rab35 bound form also increases TNT formation. Neurite outgrowth is an essential process in order to establish neural connectivity and vesicle recycling plays a crucial role in this process. Rab35 interacts with several proteins, that are involved in vesicle trafficking such as such as ACAP2 (acts as GAP of ARF6), MICAL-L1 (molecule interacting with CasL-like 1, which plays a role in vesicle recycling) EHD1 (a molecular scissor that has a role in vesicle scission). At the ARF6 positive endosomes, Rab35 recruits ACAP2 and MICAL-L1, and forms a complex that binds to EHD1 to regulate neurite outgrowth. Our data strongly suggest that these effectors may also be involved in the formation of TNTs. Individually, ACAP2, EHD1 and ARF6-GDP regulate TNT formation in a positive manner. But MICAL-L1 overexpression in cells shows no effect on TNTs. Also, preliminary data, indicates that Rab35 and EHD1 acts in a cascade mechanism to regulate TNT formation. This indicates that TNT formation and neurite outgrowth may act in a similar, but not exact pathway. The molecules identified here that have a role in TNT formation, constitute potential molecular targets for therapies aiming to block the spreading of pathogens that transfer through TNTs.

This study proves that proteins that have a role in vesicle trafficking and neurite outgrowth, such as Rab proteins, are also involved in TNT formation.

