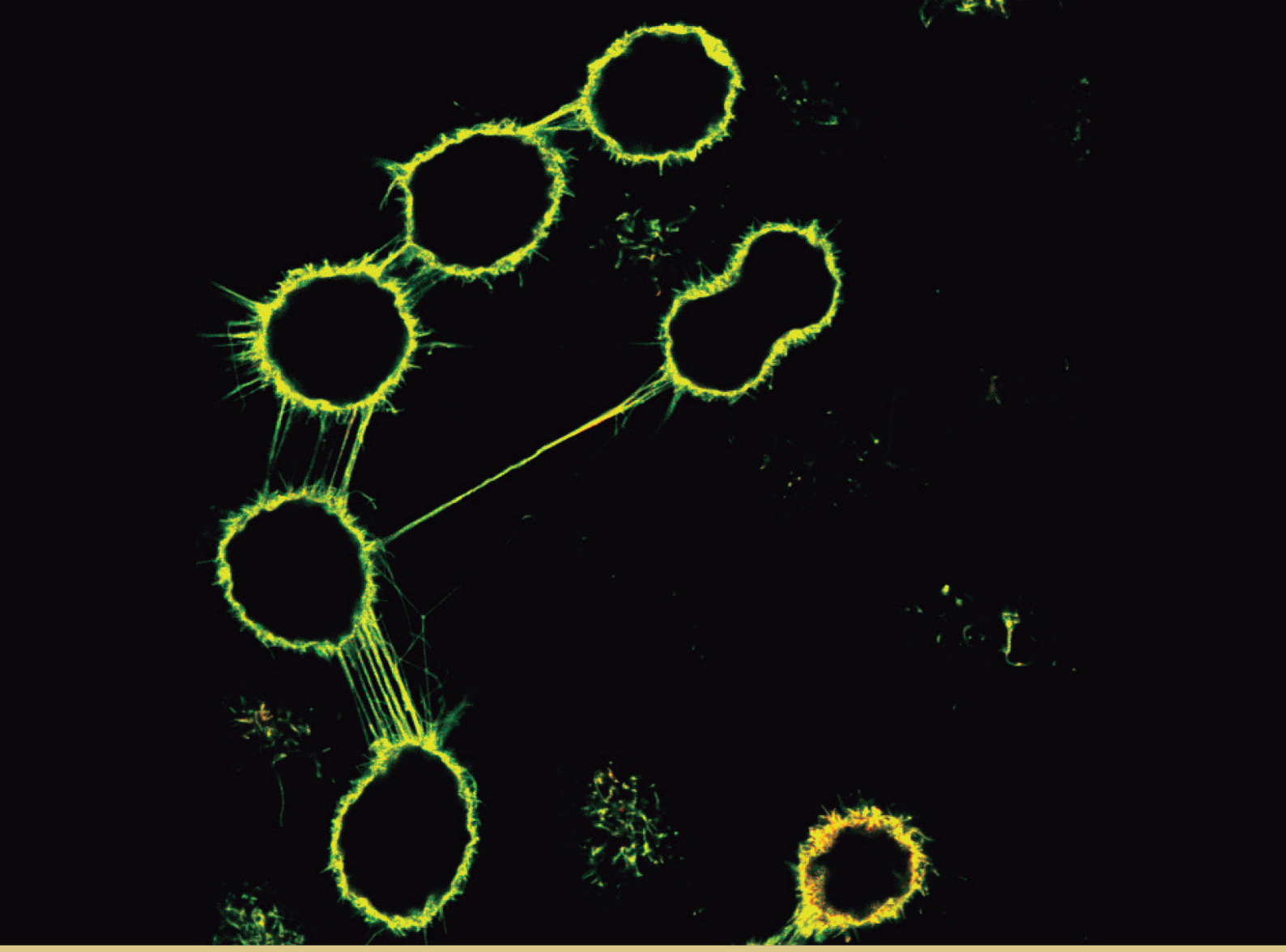

Miguel Jorge Macedo Lemos

ROLE OF ACTIN REGULATORY PROTEINS IN TUNNELING NANOTUBES FORMATION AND INTERCELLULAR TRANSFER IN HELA CELLS

Dissertação para obtenção do Grau de Mestre em Investigação Biomédica sob a orientação científica da Doutora Chiara Zurzolo e coorientação do Doutor Henrique Manuel Paixão dos Santos Girão e apresentada à Faculdade de Medicina da Universidade de Coimbra

Maio de 2017

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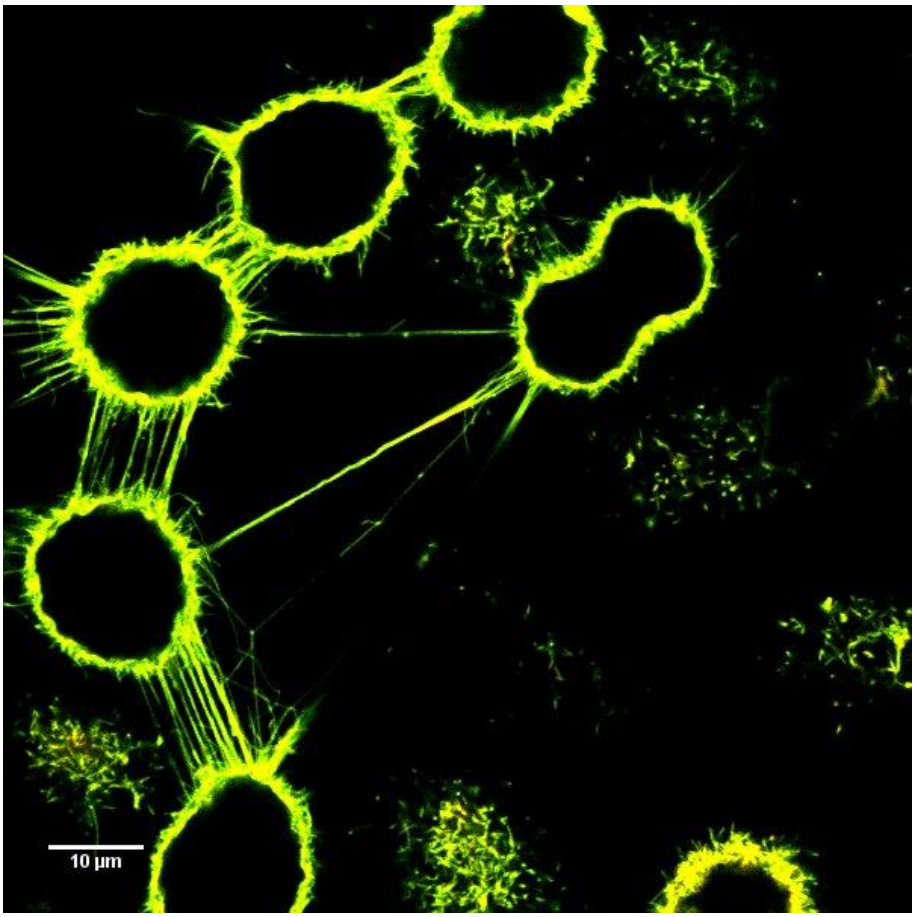

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On the front page:

Tunneling nanotube formation between HeLa cells (Nap1 Knockdown) stained with wga488 (green) and phalloidin (red).

Role of Actin Regulatory Proteins in Tunneling Nanotubes Formation and Intercellular Transfer in HeLa cells

Miguel Jorge Macedo Lemos

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica. Este trabalho foi realizado no grupo *Membrane Traffic and Pathogenesis* do Institut Pasteur, em Paris, sob a orientação científica da Doutora Chiara Zurzolo e coorientação do Doutor Henrique Manuel Paixão dos Santos Girão.

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2017

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“If opportunity doesn’t knock,

build a door.”

MILTON BERLE

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ABBREVIATIONS

AD – Alzheimer's disease

Akt – protein kinase B

Arp2 – actin-related protein 2

Arp2/3 – Actin-related protein complex 2 and 3

Arp3 – actin-related protein 3

ARPC1-5 – actin-related protein complex subunit 1-5

ARPC2 – Actin-related protein complex subunit 2

ATP – adenosine triphosphate

Aβ – amyloid-β

BSA – bovine serum albumin

CAD cells – Catecholaminergic-a-differentiated mouse neuronal cell line

CDC42 – cell division control protein 42 homolog

DAAM – disheveled-associated activator of morphogenesis

DMEM – Dulbecco's Modified Eagle Medium

EGF – epidermal growth factor

EGFr – epidermal growth factor receptor

ENA/VASP – enabled/vasodilator-stimulated phosphoprotein

EPS8 – epidermal growth factor receptor pathway 8

FBS – fetal bovine serum

FH1 – formin homology 1 domain

FH2 domain – formin homology domain

FHOD – formin homology domain-containing protein

Fix1 – fixative solution 1

Fix2 – fixative solution 2

FMN – formin

FMNL – formin-like protein

HD – Huntington's disease

HIV – Human immunodeficiency virus

Htt – huntingtin

INF – inverted formin

IRSp53 – insulin receptor substrate p53

KD - Knockdown

mDia1 – Diaphanous-related formin-1

mDia2 - Diaphanous-related formin-2

mTor - mechanistic target of rapamycin

Nap1 – Nck-associated protein 1

NPFs – nucleation promoting factors

NRK cells – Normal rat kidney epithelial cells

N-WASP – neural WASP

PBS – phosphate-buffered saline solution

PC12 cells – rat neuronal pheochromocytoma cells

PD – Parkinson's disease

PI3K - phosphatidylinositol-3-kinases

PrP^{C} – normal conformer prion protein

PrP^{Sc} – abnormal disease-specific conformation of prion protein

PVDF – Polyvinyl difluoride

Rac1 – Ras-related C3 botulinum toxin substrate 1

Ras – ras protein family

RhoA – Ras homolog gene family, member A

RIPA buffer – radioimmunoprecipitation assay buffer

TBS – tris buffered saline solution

TNTs – Tunneling nanotubes

VCA domain – verprolin central acidic domain

WASP – Wiskott-Aldrich Syndrome Protein

WAVE - WASP-family verprolin homologous protein

WB – Western blot

WGA – wheat germ agglutinin

WH2 motif – WASP homology 2 motif

α-syn – α-synuclein

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RESUMO

Os túneis de nanotubos (TNTs) são canais compostos por elementos do citoesqueleto de actina que ligam o citoplasma de células distantes e que surgiram como um novo mecanismo de comunicação intercelular de longa distância. Estas estruturas dinâmicas promovem a transferência de componentes celulares como moléculas citoplasmáticas, proteínas, vesículas e organelos. Os TNTs podem ser "sequestrados" por diferentes agentes patogénicos para se propagarem entre células, estando este mecanismo implicado na progressão do cancro e doenças neurodegenerativas, representando assim um potencial alvo terapêutico.

Os TNTs são formados a partir de células que estiveram previamente em contacto, ou de extensões semelhantes a filopodia, formando-se em direção a células vizinhas. A polimerização de actina tem um papel importante neste último tipo de formação dos TNTs, demonstrado ser predominante em células CAD, uma linha celular neuronal. Perceber os mecanismos de formação dos TNTs e a sua relação com a filopodia é importante para descobrir as funções fisiológicas de ambas estas estruturas, principalmente sabendo que a filopodia, contrariamente aos TNTs, não permite a transferência de diferentes componentes celulares entre células distantes.

O principal objetivo desta tese é investigar o papel das proteínas ARPC2, mDia1 e Nap1 na formação de TNTs. Estas proteínas fazem parte de três conjuntos de proteínas que regulam a polimerização dos filamentos de actina - o complexo Arp2/3, as forminas e o complexo WAVE, respetivamente. Foi usada uma abordagem farmacológica e genética combinada com técnicas de biologia molecular e celular para perceber as funções das proteínas ARPC2, mDia1 e Nap1. A formação de TNTs, a dinâmica do citoesqueleto de actina e o papel dos TNTs na transferência de vesículas marcadas entre células em co-cultura foram avaliados recorrendo à microscopia confocal.

Neste trabalho, mostramos que a transferência de vesículas marcadas entre células HeLa foi maioritariamente devido ao contacto entre células e esta transferência encontra-se aumentada nas células knockdown para a proteína ARPC2.

Em suma, os nossos resultados fornecem novos dados sobre a remodelação do citoesqueleto de actina, bem como a sua arquitetura, envolvidos na formação de TNTs nas células HeLa.

Palavras-chave: Túneis de nanotubos; citoesqueleto de actina; proteínas reguladoras do citoesqueleto de actina; filopodia; transferência intercelular; células HeLa.

ABSTRACT

Tunneling Nanotubes (TNTs) are F-actin containing channels that connect the cytoplasm of remote cells and recently emerged as a new mechanism for long-range intercellular communication in many cell types. These dynamic structures mediate the transfer of a wide variety of cellular materials such as cytoplasmic molecules, plasma membrane components, proteins, vesicle and organelles. In addition, TNTs can be "hijacked" by various pathogens to propagate across cells and are also implicated in cancer and neurodegenerative diseases, thus representing promising therapeutic targets.

TNTs are formed either after cells previously in contact detach from each other, or from the extension of filopodia-like protrusions toward neighboring cells. Actin polymerization plays an important role in this later type of TNT formation which was demonstrated to be the predominant formation type in CAD cells, a neuronal cell line. Understanding the mechanisms of TNT formation and the relation with filopodia is of utmost importance to uncover their physiological functions, particularly since filopodia, unlike TNTs are not able to mediate the transfer of different cargoes between remote cells.

The aim of this project is to investigate the role in TNT formation and function of three actin binding proteins - ARPC2, mDia1 and Nap1 – that are part of three major actin regulatory protein complexes such as Arp2/3 complex, formins and WAVE complex, respectively. A combination of pharmacological and genetics approaches combined with molecular and cellular biology techniques were used to target the roles of ARPC2, mDia1 and Nap1 proteins, and TNT formation and actin dynamics were monitored by confocal microscopy. In parallel, the role of TNTs in the transfer of labeled vesicles between cells in co-culture was also assessed by confocal microscopy.

In this work, we demonstrated that the labeled vesicle transfer between HeLa cells in co-culture was mainly due to cell-to-cell contact and was increased in ARPC2 knockdown (KD) cells.

Taken together, our data provides new insight regarding actin cytoskeleton remodeling and architecture underlying TNT formation in HeLa cells.

Key-words: tunneling Nanotubes; actin cytoskeleton; actin regulatory proteins; filopodia; intercellular transfer; HeLa cells.

CHAPTER 1

Introduction

1.1 – The discovery of Tunneling nanotubes

In 2004, Rustom and colleagues described a new mechanism of cell-to-cell communication in animal cells, showing evidences in rat neuronal pheochromocytoma (PC12) and rat kidney NRK cells connected by an ultrafine intercellular structure with a diameter of 50 to 200 nm and a variable length (Rustom, 2004). These structures were referred as Tunneling Nanotubes (TNTs) and since then, TNTs have been targeted by several research studies in order to better understand their role in long-range communication.

The work developed by Rustom and his colleagues was fundamental in order to distinguish TNTs from similar cell connections. TNTs are dynamic structures formed *de novo* within a few minutes, which are thin F-actin-based membranous channels allowing the communication between distant cells.

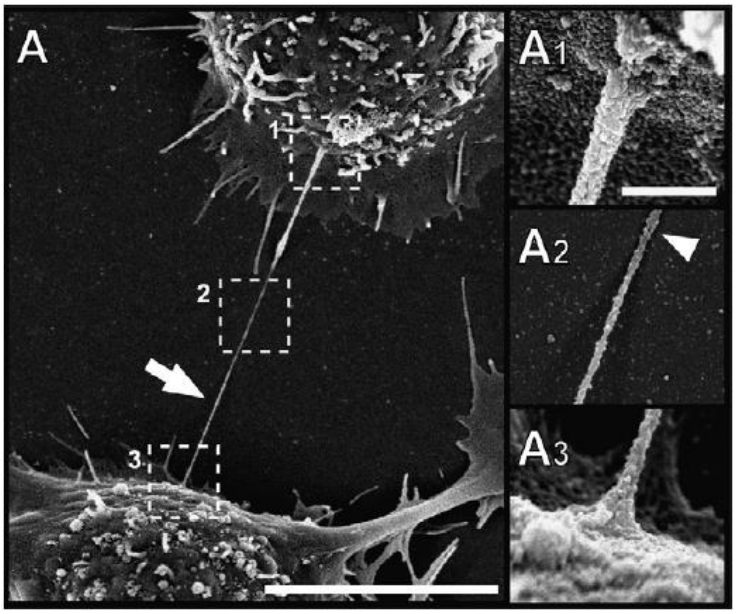


Figure 1 – Structure of Tunneling Nanotubes (TNTs). Image of TNT-connected PC12 cells through observation in electron microscopy in different scale bars. Modified from (Rustom, 2004).

These structures began to be compared to similar actin-rich filopodia-like extensions called cytonemes, observed in wing imaginal disc cells in *Drosophila* (Ramírez-Weber & Kornberg, 1999). Both cytonemes and TNTs were described as tunnel channels providing open conduits for the transfer of cellular components and soon distinguished as bridges that do not connect or connect the cell cytoplasm, respectively

(Sherer & Mothes, 2009). As in relation to other filopodia cell extensions, TNTs are also composed by filamentous actin and can extend over several lengths mediating the long-range intercellular transfer of cellular components and transmission of depolarization signals (Björn Onfelt, Purbhoo, Nedvetzki, Sowinski, & Davis, 2005). Their structural integrity is sensitive to chemical fixation, mechanical stress, and even to prolonged light exposure. However, the most strikingly characterization of TNTs is that they hover in the medium without making contact to the substratum (Rustom, 2004).

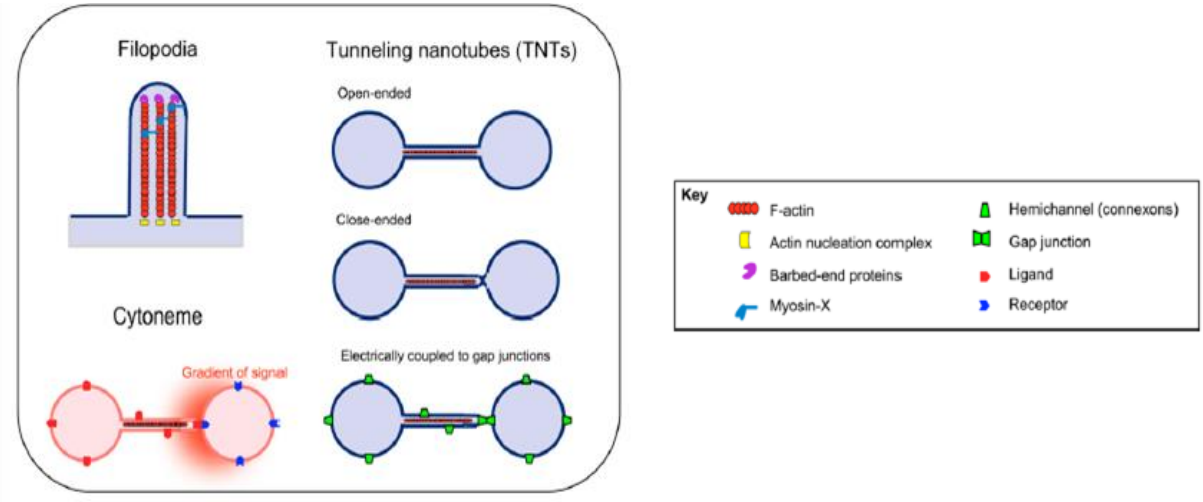


Figure 2 – Schematic representation of filopodia, cytonemes and tunneling nanotubes. Filopodia are exploratory projections containing bundles of F-actin. Cytonemes are F-actin projections and are able to mediate the transfer of cargoes from cell to cell and rely on specific ligand-receptor interaction for their formation. TNTs differ from filopodia and cytonemes because they mediate the continuity between the cytoplasm of remote cells without touching the substrate. TNTs have been reported in the literature as: open-ended, close-ended and electrically coupled to gap junctions. Modified from (S. Abounit & Zurzolo, 2012).

After these findings, TNTs and similar structures have been reported in different types of cells (H. H. Gerdes, Bukoreshtliev, & Barroso, 2007). Their role in intercellular communication has been studied through the last decade revealing a high level of heterogeneity in morphology and structure in different cell lines (Figure3). Subsequent studies described the presence of TNTs between cells *in vitro,* including fibroblasts, epithelial cells, immune cells and neurons (Davis & Sowinski, 2008; H. H. Gerdes & Carvalho, 2008). Also, TNTs have been observed *in vivo* between myeloid cells in mouse cornea, neural crest in chicken embryo and human mesothelioma cells (Lou et al., 2012; Seyed-Razavi, Hickey, Kuffová, McMenamin, & Chinnery, 2013). Interestingly, most of

the findings in TNTs *in vivo* were from studies in embryos, which might suggest an important role of these structures during the development of multicellular organisms (H.-H. Gerdes & Rustom, 2014).

Cell type	Length	Thickness	Cytoskeleton
PC12	Avg. 6 μm*	50 - 200 nm	actin, no microtubules
HEK293	N/A	< 500 nm	actin, no microtubules
Jurkat T cells	Avg. 22 μm, max 100 μm	< 380 nm	actin, no microtubules
ARPE-19	Avg. 44 μm, max 120 μm	50 – 300 nm	actin, no microtubules
NRK	Max 70 μm	N/A	actin, no microtubules
HeLa	Avg. 17.7 μm, max 40 μm	N/A	actin, no microtubules
Cardiac myoblast H9c2 cell	Max 100 μm	< 1000 nm (AFM)	actin and microtubules
Human lung carcinoma A549	Max 105 μm	400 – 1500 nm	actin and microtubules
Human monocyte-derived macrophages	N/A	700 nm	actin, microtubules **
Primary neurons and astrocytes	Avg. 7.1 μm	N/A	microtubules, actin ***

Figure 3 – The diversity of TNTs. Adapted from (H.-H. Gerdes & Rustom, 2014)

TNTs are not empty membranous channels. They are mainly composed by cytoskeleton filaments (Figure 3) and for that reason F-actin is found in TNTs along its entire length (Rustom, 2004). These facts suggest that the actin cytoskeleton plays an important role in TNT formation. To prove this theory, F-actin depolymerization drugs, such as cytochalasin B, were shown to disrupt TNT formation (Bukoreshtliev et al., 2009). Besides actin, TNTs that connect immune cells, cardiomyocytes and immature neurons to astrocytes are also composed by microtubules (H. H. Gerdes, Rustom, & Wang, 2013; B. Onfelt et al., 2006; Y. Wang, Cui, Sun, & Zhang, 2011). The function of microtubules in TNTs remains to be investigated. However, microtubules could serve as tracks for cargo transportation through kinesin/dynein mechanisms and these filaments shows a higher stiffness when compared to actin-filaments, providing a different set of transportations and a longer lifetime to the TNT, respectively (H.-H. Gerdes & Rustom, 2014).

1.2 – Functions of tunneling nanotubes

1.2.1 – Physiologic and pathologic implications of tunneling nanotubes

As a novel biological tool for intercellular communication, TNTs allow the direct transfer of plasma membrane components, proteins, various organelles such as endosomes, lysosomes, Golgi complex and mitochondria, cytoplasmic molecules, calcium as well as pathogens such as bacteria, HIV particles and prion proteins (Davis & Sowinski, 2008; Karine Gousset et al., 2009; Koyanagi, Brandes, Haendeler, Zeiher, & Dimmeler, 2005; Björn Onfelt et al., 2005; Rustom, 2004; Watkins & Salter, 2005). In a more summarized way, TNTs establish routes to intercellular signaling underlying development, immune responses and electrical conduction between remote cells, and it may be crucial for tissue homeostasis and regeneration.

Figure 4 – The functional properties of tunneling nanotubes between distant cells. Adapted from (Sisakhtnezhad & Khosravi, 2015).

Since the last decade, several studies were performed to investigate the direction of cargoes transportation. It became crucial to understand whether the unidirectional transfer occurs when a donor cell transmits information to the acceptor

cell or if the bidirectional transfer occurs when both cells exchange materials (Marzo, Gousset, & Zurzolo, 2012).

The reason for uni- and bidirectional transfer was reported to be dependent on the structural components (actin *versus* microtubules) or on specific signals that stimulate TNT formation and are responsible for directing the cargo transfer. For instance, bidirectional transfer of mitochondria and viruses was found when both actin and microtubules cytoskeleton were present in TNTs (Arkwright et al., 2010; He et al., 2010). On the other hand, the transfer of calcium and labeled endocytic vesicles seemed to be unidirectional when TNTs only contains actin (Karine Gousset et al., 2009; Gurke, Barroso, & Gerdes, 2008; Rustom, 2004).

1.2.3 – Tunneling nanotubes and diseases

Being a novel tool in intercellular communication between distant cells, TNTs begun to be investigated in several studies in order to understand their role in pathologic and physiologic situations (Figure 4). The research developed by Lou and colleagues showed that TNTs provided a mechanism of cell-to-cell communication in cancer environments. TNT formation was observed between malignant and their surrounding cells, which might allow tumor development, invasion and metastasis by transferring cellular contents such as proteins, Golgi vesicles and mitochondria among cells (Lou et al., 2012). The presence of TNTs in tumor cells could be examined to prevent tumor initiation and progression. (Sisakhtnezhad & Khosravi, 2015).

Moreover, another important finding in the study of TNTs was their ability to mediate the intercellular transfer of pathogens. TNTs can be “hijacked” by bacteria, viruses or prions in order to facilitate their spreading in an infected organism (Davis & Sowinski, 2008; Karine Gousset et al., 2009; Karine Gousset & Zurzolo, 2009; B. Onfelt et al., 2006). In fact, the spreading of HIV particles in primary human macrophages was shown to increase TNT formation (Eugenin, Gaskill, & Berman, 2009). The transfer of bacterial and viruses’ particles occurs along the thin nanotubes constituting important routes for pathogen spreading and the rearrangement of host-cell cytoskeleton.

In addition, several studies demonstrated the ability of TNTs to mediate the transfer of exogenous and endogenous membrane-associated prion proteins between cells in culture (Karine Gousset et al., 2009). Prions are infectious agents, capable to self-propagate from one cell to another, and that accumulate in a misfolded form (PrP^{Sc}) in the Central Nervous System, generating transmissible spongiform encephalopathies including scrapie in sheep and Creutzfeldt-Jakob disease in humans (Kimura, Hase, & Ohno, 2012). Moreover, Gousset and coworkers described that PrP^{Sc} was able to transfer through TNTs in bone-marrow dendritic cells, follicular dendritic cells and primary neurons (cerebellar granular neurons and primary hippocampal neuros).

However, the spreading and transmission of disease by propagation of protein misfolding was thought to be peculiar to prion proteins. The “prion-like” mechanisms underlying the pathological spreading of misfolded proteins is also observed neurodegenerative diseases including amyloid-β (Aβ) and tau in Alzheimer’s disease (AD), α-synuclein (α-syn) in Parkinson´s disease (PD), huntingtin (Htt) in Huntington’s disease (HD) (Saida Abounit, Delage, & Zurzolo, 2015; Costanzo & Zurzolo, 2013). Interestingly, several studies also showed that amyloidogenic proteins such as Aβ, tau, α-syn, and Htt can be transferred between distant cells through TNTs (Saïda Abounit et al., 2016; Costanzo & Zurzolo, 2013).

Overall, these *in vitro* studies disclose the importance of TNTs in diseases suggesting that these structures may serve as potential therapeutic targets.

1.3 – Proposed models for tunneling nanotubes formation

To date, time-lapse recording studies showed that TNTs are dynamic and transient structures, formed *de novo* and two different mechanisms have been proposed to explain their formation. Both require plasma membrane and cytoskeleton remodeling: Actin driven mechanism (Figure 5) and cell dislodgment (Figure 7).

1.3.1 – Actin-driven mechanism

This first proposed model for TNT formation was initially described by Rustom and colleagues (Rustom, 2004). The authors suggested that either one or both of the

connected cells have to induce the outgrowth of filopodia-like protrusions containing F-actin (Figure 5).

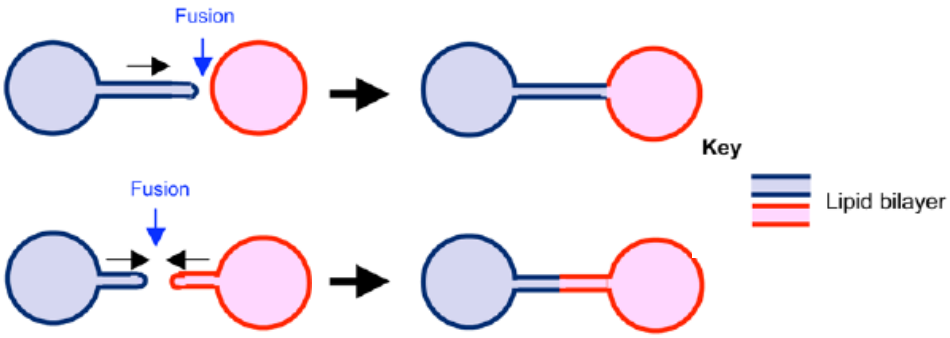


Figure 5 – Schematic representation of the actin-driven protrusion model. One or both cells extend filopodia-like protrusions towards a target cell. Modified from (S. Abounit & Zurzolo, 2012).

As in filopodia, the actin cytoskeleton plays a very important role in TNT formation. Although it is still not fully understood, the molecular basis of TNT formation share some similarities regarding classical filopodia, and their extension might involve the actin cytoskeleton regulatory proteins to initiate the actin nucleation (Hase et al., 2009). For this reason, actin polymerization could promote TNT elongation toward a target cell. Adhesion and membrane fusion molecules (Figure 6) have crucial roles to physically connect both cells through an open TNT connection (S. Abounit & Zurzolo, 2012).

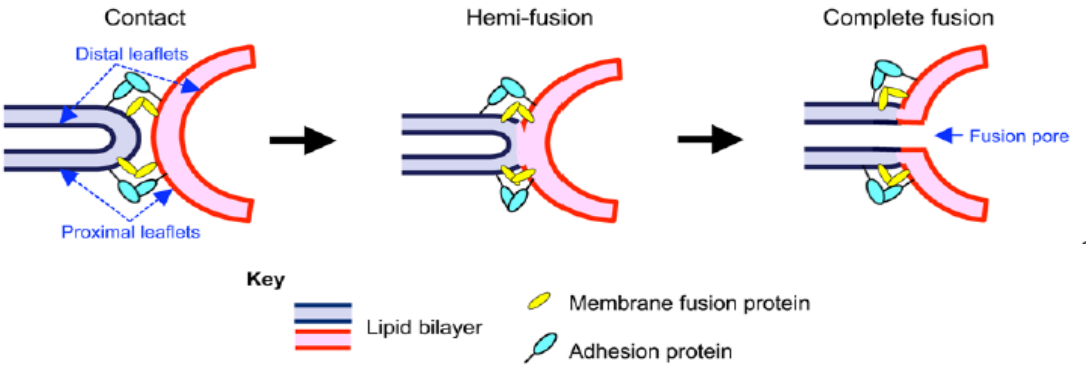


Figure 6 – Schematic representation of the model for cell-to-cell fusion. The contact between two lipid bilayers might rely on the function of adhesion molecules such as cadherins. Membrane fusion proteins might be also needed to induce the membrane curvature. Modified from (S. Abounit & Zurzolo, 2012).

The actin-driven mechanism of TNT formation has been demonstrated in several cell lines such as neuronal cells (PC12), mouse catecholaminergic neuronal CAD cells and also in NRK cells (H. H. Gerdes et al., 2007; K. Gousset, Marzo, Commere, & Zurzolo, 2013; Rustom, 2004). However, due to the lack TNT-specific protein markers and also to the highly dynamism of these structures, it became more difficult to observe the TNT formation by microscopic observation.

1.3.2 – Cell-dislodgment mechanism

This second proposed mechanism of TNT formation relies on the migration of two cells previously in contact. The subsequent migration of the cells in opposite ways can form a TNT (Figure) either from one or both cells that was previously attached (Sowinski et al., 2008). However, once the cells were initially attached, it is still unknown if the adhesion molecules were essential in this type of TNT formation.

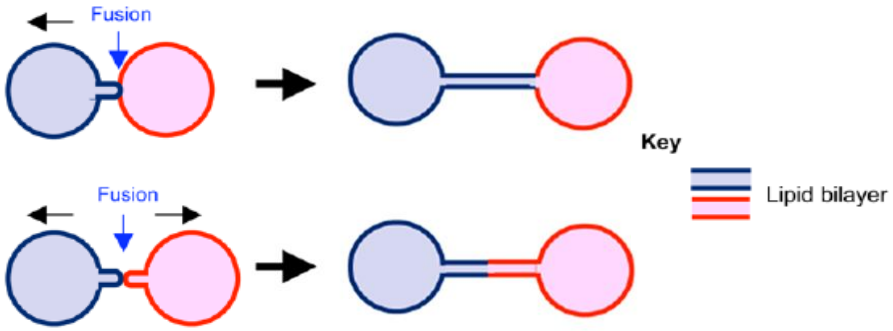


Figure 7 – Schematic representation of the cell-dislodgment model. Modified from (S. Abounit & Zurzolo, 2012).

1.3.3 – Molecular machinery involved in Tunneling nanotube formation

In order to understand the specific conditions and factors that enhance TNT formation, several studies on the molecular level were performed in different cell types.

Recent studies revealed that stress conditions like inflammation, serum starvation, glucose-rich low pH growth medium, hypoxia, H_2O_2, temperature, bacterial toxins and ultra-violet radiation can induce TNT formation between distant cells (Arkwright et al., 2010; Chinnery, Pearlman, & McMenamin, 2008; H.-H. Gerdes & Rustom, 2014; Kabaso, Lokar, Kralj-Iglič, Veranič, & Iglič, 2011; Sisakhtnezhad &

Khosravi, 2015; X. Wang & Gerdes, 2012). It was also demonstrated that, in stressful conditions, cells could release certain metabolites into the culture which would trigger the formation of TNTs.

The work developed by Wang and coworkers (2011) showed evidence of astrocytes and neurons connected through TNTs after being treated with H_2O_2, suggesting that TNT formation constitutes a defense mechanism by the stressed cells (Y. Wang et al., 2011). In the same work, they found that activation of the transcription factor p53, the epidermal growth factor (EGF) and the Akt/PI3K/mTor pathway was essential for TNT formation. Interestingly, this later signaling pathway was shown to have a critical role in the actin polymerization and in the regulation of the production of cellular protrusions, cell polarization and adhesion (Hemmings & Restuccia, 2012; Rosich et al., 2014).

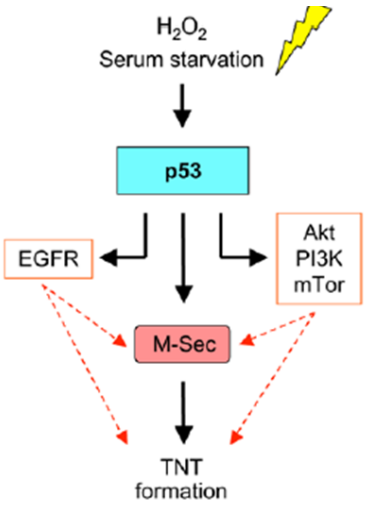


Figure 8 – Example of the molecular machinery involved in TNT formation. Modified from (S. Abounit & Zurzolo, 2012).

Another study developed by Hase and colleagues showed that M-Sec, a protein highly expressed in myeloid lineages of mouse and human origin, could be a promotor of TNT formation in Raw264.7 macrophage cell line. There was evidence showing that an interaction between M-Sec and the active form of the small Ras-like GTPase proteins was required for TNT formation in HeLa cells (Hase et al., 2009). In contrast, a dominant-

negative form of CDC42 could interact with M-Sec and negatively regulate the TNT formation (Hase et al., 2009).

TNTs have been described between several cell lines which do not express M-Sec. Gousset and colleagues demonstrated a role for myosin-X-induced dorsal filopodia in TNT development and consequent increase in vesicle transfer in a mouse neuronal cell line (CAD cells) (K. Gousset et al., 2013).

These facts suggest that different mechanisms of TNT formation can exist in different cell types (Sisakhtnezhad & Khosravi, 2015). Even though all these studies were able to find a relationship between protein complexes that are known to have an important role on the actin cytoskeleton remodeling, the signaling pathways regarding TNT formation remains poorly understood. Thus, is still necessary to identify specific TNT markers which are not involved in filopodia formation in order to better understand the pathophysiologic implications of TNTs formed between cells.

1.4 – The actin cytoskeleton

As mentioned above, TNTs are actin-enriched filopodia like protrusions and for that reason, the remodeling of the actin cytoskeleton plays a vital role in their formation.

Actin is a ubiquitous protein present in all the eukaryotic cells and its amino acids are conserved from yeast to human. The actin cytoskeleton can be found in two distinct states: the monomeric (G-actin) and filamentous state (F-actin) (Melak, Plessner, & Grosse, 2017). This dynamic transition between G-actin and F-actin allows the cell to coordinate the homeostatic balance in response to extracellular stimuli (Lee et al 2014).

F-actin filaments are asymmetric and polar structures, as all subunits are oriented in the same direction. They are composed by two biochemically different ends known as "barbed" (positive) end and "pointed" (negative) end. Under normal conditions, the monomer addition to the actin filaments occurs at the barbed end. However, the irreversible hydrolysis of the bound ATP destabilizes the actin filaments leading monomer dissociation at the pointed end (Amann & Pollard, 2000). The continuously polymerization at the barbed end and depolymerization at the pointed end constitutes a process known as actin filament treadmilling. This particular specificity of the actin cytoskeleton plays an important role in cellular processes including membrane protrusion (Borisy & Svitkina, 2000).

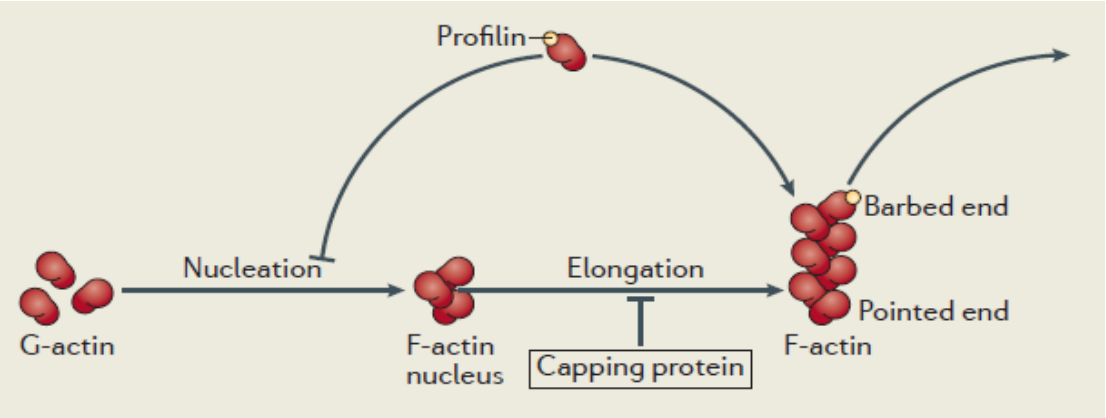


Figure 9 – Schematic representation of the actin polymerization machinery. Modified from (Krause & Gautreau, 2014).

The stability and mechanical properties of the actin filaments can be regulated by accessory proteins such as profilins, that can bind to the polymer, determining the state of polymerization. In addition, capping proteins such as the epidermal growth factor receptor pathway 8 (EPS8) can also bind to the positive end, thus regulating the elongation of the F-actin filaments (Thomas D. Pollard & Borisy, 2003).

1.4.1 – Actin-binding proteins

The properties and functions of actin structures are dependent on a large variety of actin-binding proteins. Some of these proteins lead to a higher organization of the actin structures while others modulate the dynamic and treadmilling of the F-actin filament and subsequently the remodeling of actin cytoskeleton.

For instance, profilin a small actin-monomer-binding protein, has a higher affinity for ATP-actin monomers and most of the non-polymerized actin are bound to this protein (Thomas D. Pollard, 2016). Profilin binds to the G-protein and promotes elongation at the barbed ends. In the opposite way, proteins from the ADF/cofilin family bind both G- and F-actin and are predominantly bounded to pointed end, causing depolymerization (Blanchoin, Pollard, & Mullins, 2000).

The nucleation of actin filaments from actin monomers is unfavorable due to the extreme instability of the small actin oligomers. To overcome this obstacle, cells use nucleating factors for *de novo* actin polymerization such as the actin-related protein 2/3 complex (Arp2/3 complex) and formins to produce actin filament branches and to initiate unbranched filaments, respectively (Thomas D. Pollard, 2016).

In addition to these large actin-binding protein families, there are also other proteins that play a crucial role on the maintenance of the formed actin filaments. For example, the enabled/vasodilator-stimulated phosphoprotein (ENA/VASP) protein family and also the Fasicin protein family. ENA/VASP proteins are required to promote the association of actin filaments as directly antagonize capping activity in barbed ends from capping proteins such as EPS8 (Pasic, Kotova, & Schafer, 2008; Vaggi et al., 2011). Furthermore, ENA/VASP proteins has F-actin bundling and anti-branching activity, indicating a crucial role for filopodia formation (Schirenbeck et al., 2006).

1.4.2 – Arp2/3 complex and branching nucleation

The first nucleator factor discovered was the Arp2/3 protein complex (Machesky, Atkinson, Ampe, Vandekerckhove, & Pollard, 1994) which is composed of seven subunits: Arp2, Arp3 and ARPC1-5 (Rotty, Wu, & Bear, 2012). This protein complex is able to bind alongside the "mother" actin filament and promotes the elongation and nucleation of a new "daughter" filament, resulting in a ± 70° branch (Goley & Welch, 2006).

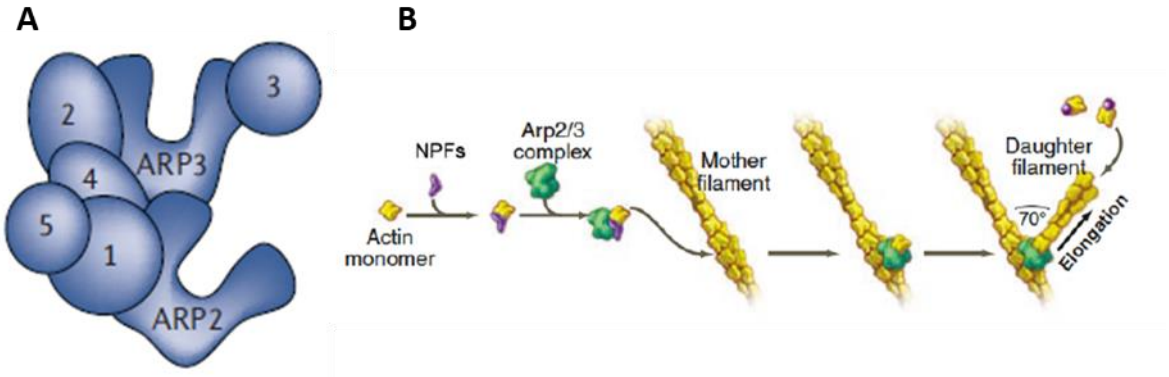


Figure 10 – The Arp2/3 protein complex. **A)** Schematic representation of the subunit organization of the Arp2/3 complex. ARP2, ARP3 and ARP complex 1 (ARPC1) through ARPC5. Adapted from (Goley & Welch, 2006). **B)** Branching nucleation by Arp2/3. Modified from (T. D. Pollard & Cooper, 2009).

Although Arp2/3 complex consists of seven subunits, only Arp2 and Arp3 are structurally similar to the actin monomer. Both of these subunits serve as an active site for polymerization. The association of Arp2/3 complex with an existing actin filament is crucial for the formation of branched actin structures. As the new branching polymerization occurs, the plasma membrane is pushed forward to generate lamellipodia and filopodia (Rouiller et al., 2008).

This protein complex is not an efficient nucleator (Higgs, 2001). For this reason, proteins called nucleation promoting factors (NPFs), together with free actin monomers are required to promote a rapid actin polymerization (Pollard et al., 2007) (Thomas D. Pollard, 2007).

1.4.3 – Regulation and function of NPFs

NPFs are proteins that regulate the activity of the protein complex Arp2/3. Indeed, a several number of NPFs were identified to activate the Arp2/3 complex, thus inducing the actin polymerization in branches. One example of NPFs is the Wiskott-Aldrich Syndrome Protein (WASP)-family proteins (Chen et al., 2010). This protein family is composed by WASP, neural WASP (N-WASP), Scar/WAVE 1,2,3, WASH, WHAMM and JMY (Welch et al 2010). All these proteins have a verprolin central acidic domain (VCA) at the C-terminus which is able to activate the Arp2/3 complex. When binding of VCA domain to Arp2/3 brings Arp2 and Arp3 near to the "mother" actin filament, the complex becomes activated, promoting actin polymerization (Rodal et al., 2005). In addition, the VCA domain contains a WASP-homology 2 motif (WH2) which is able to bind free actin monomers and bring them near the Arp2/3 complex in order to initiate the nucleation.

N-WASP and WAVE proteins are the most studied concerning their activity in actin cytoskeleton remodeling. In order to become activated, N-WASP and Scar/WAVE proteins can bind to others from the Rho GTPase protein family such as CDC42 or Rac1. Through this interaction, WASP proteins become active, leading to the activation of the Arp2/3 complex (Ibarra, Pollitt, & Insall, 2005).

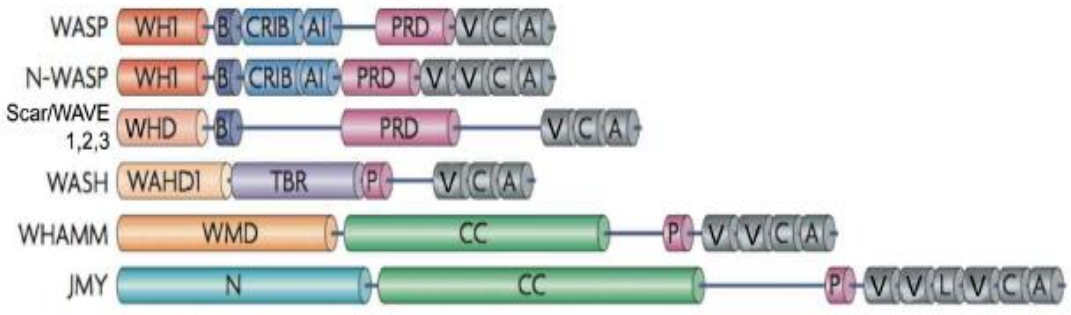


Figure 11 – Wiskott-Aldrich Syndrome Protein (WASP) family proteins. Adapted from (Campellone & Welch, 2010).

Scar/WAVE proteins are the major NPFs responsible for the activation of Arp2/3 complex in lamellipodium formation. These proteins are composed by four subunits, namely, Nap1, Sra1/PIR121, Abi1/2 and HSPC300. In resting conditions, the Scar/WAVE regulatory complex is inactive. The small GTPase Rac1 protein and negatively charged phospholipids were identified to promote conformational changes in the WAVE subunits, leading to the Arp2/3 complex activation (Pollitt & Insall, 2009). WAVE complex can be at the leading edge of the membrane protrusion in response to Rac activation. Once the cells are depleted of WAVE complex, they do not produce membrane ruffles in response to growth factors or active Rac, showing that the actin polymerization is compromised (Beli, Mascheroni, Xu, & Innocenti, 2008; Steffen et al., 2004).

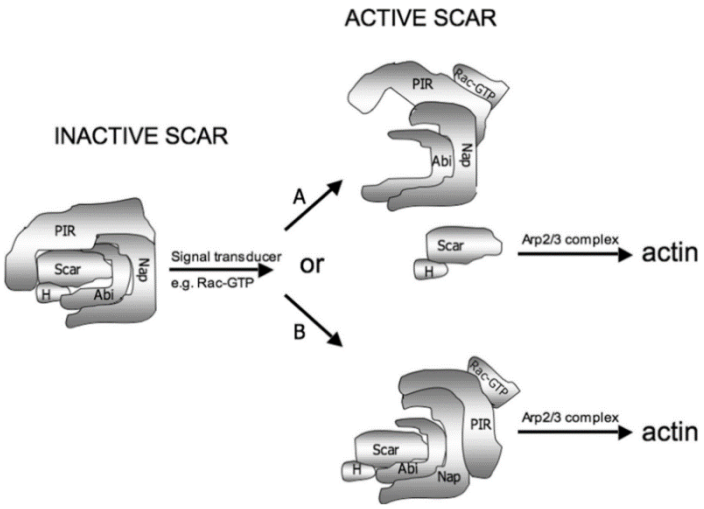


Figure 12 – Models for regulation of Scar/WAVE proteins. Adapted from (Ibarra et al., 2005).

1.4.4 – The formin protein family as actin nucleators

Since 2002, the formin protein family have been studied as important regulators of actin polymerization and some of the mechanisms involved have already been discribed (Goode & Eck, 2007; Higgs, 2005; Kovar, 2006).

Cells rely on this protein family to promote the actin assembly for the contractile ring that separates cells during cytokinesis and for the unbranched actin filaments that are crucial for the formation of filopodia (Wallar & Alberts, 2003).

In contrast to Arp2/3 complex, formins are able to nucleate the actin filaments in the absence of additional factors, solely through their formin homology 2 (FH2) domains (Aspenström, 2010). Several studies established the existence of different formins but the presence of FH2 domain is the common characteristic of these protein family (Cvrckova, Novotny, Pickova, & Zarsky, 2004).

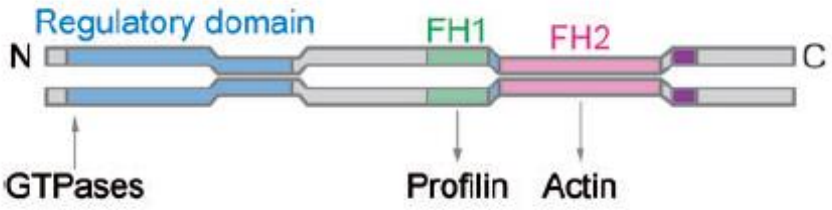


Figure 13 – Formin domain map. Adapted from Aspenstrom et al., 2009

Through phylogenetic analysis of FH2 doamin, the formin protein family was classified into different subfamilies: diaphanous (Dia), disheveled-associated activator of morphogenesis (DAAM), formin-like protein (FMNL), formin homology domain-containing protein (FHOD), inverted formin (INF), formin (FMN) and Delphilin (Li & Higgs, 2005). FH2 domains form circle-shaped dimer that stabilize spontaneously formed G-actin filaments, thus promoting the nucleation and actin polymerization (Schönichen & Geyer, 2010). The formin dimers are associated with F-actin in the barbed end and after the nucleation they move alongside the growing barbed end allowing new actin monomer nucleation and prevent capping proteins for terminating the elongation. In order to have a higher rate of elongation the interaction between FH2 domain and the formin homology 1 (FH1) domain needs to be enhanced together with profilin-actin. FH1 domain has a higher affinity to actin monomers which can be transferred to the barbed end to promote the actin polymerization (Kovar, 2006).

How the mechanisms of these formins are controlled is still not clear. It is possible that there are binding partners which can modulate the formin activity. However, several studies provide evidence that formins and the Arp2/3 protein complex activities and localization in the cells can be modulated by the Rho GTPase protein family (Jaffe & Hall, 2005).

1.5 – Actin-based protrusive structures

After the nucleation and elongation, the actin filaments allow cells for a dynamic remodeling of cell shape, polarity and formation of membranous protrusions in response to microenvironment stimuli. This remodeling includes formation of lamellipodia and filopodia and the main role of these structures in the cell is to act as sensory and guidance organelles (Mattila & Lappalainen, 2008). Lamellipodia is the branched actin network, largely assembled by the Arp2/3 complex which is activated by the WAVE complex (Koestler et al., 2013) while filopodia are tight bundles of polymerized actin that form finger-like protrusions (Mattila & Lappalainen, 2008).

As mentioned above, TNTs are actin enriched structures and the actin cytoskeleton may play an important role on their formation. Although filopodia and TNTs share similar morphologies, such as the presence of thin polymerized actin filaments, a recent study showed that these two membranous protrusions may be regulated in an opposite way (Delage et al., 2016).

A current working model to explain the filopodia formation relies on the elongation of new filopodia actin filaments derived from lamellipodial networks and that extend from the root to the tip of the membrane protrusion (Mattila & Lappalainen, 2008). Actin lamellipodia filaments are formed by the Arp2/3 complex and subsequently the formed filaments start to converge and are bundled together by actin bundling proteins such as fascin (Svitkina et al., 2003). Also, ENA/VASP proteins prevent the capping of barbed ends of the actin filaments, thus promoting a rapid elongation by Arp2/3 complex and also formins (Gupton & Gertler, 2007). It is also important to note that most of the studies on filopodia have focused on substrate-attached filopodia which may have distinct properties compared to dorsal filopodia (non-attached) (Bohil, Robertson, & Cheney, 2006).

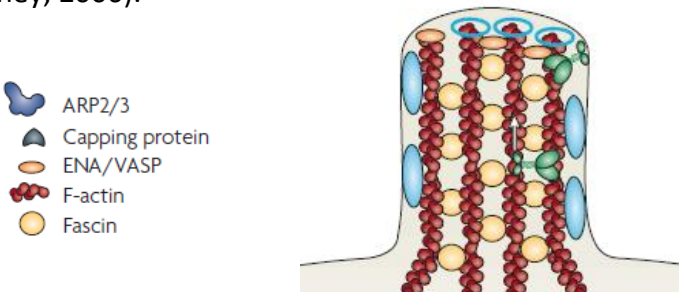


Figure 14 – Schematic representation of filopodia formation. Modified from (Mattila & Lappalainen, 2008).

1.5.1 – Tunneling nanotubes versus filopodia

TNTs and filopodia share similar structures and both require actin for their formation (Lokar, Iglič, & Veranič, 2010). Several studies demonstrated that proteins involved in filopodia formation such as VASP and fascin decrease the number of TNT-connected CAD cells. On the other hand, Myosin-X, a dorsal filopodial inducer, increases the number of TNTs and their functions CAD cells, suggesting dorsal filopodia may be a TNT precursor (K. Gousset et al., 2013).

In addition, Delage and colleagues also demonstrated that CDC42, IRSp53 and VASP proteins negatively regulate the TNT formation in CAD cells, while EPS8, an actin capping protein, has an opposite effect (Figure 15) (Delage et al., 2016).

Taken together, evidence collected so far suggests that even though TNTs and filopodia are similar in their structures, the mechanisms underlying TNT and filopodia formation are most likely different.

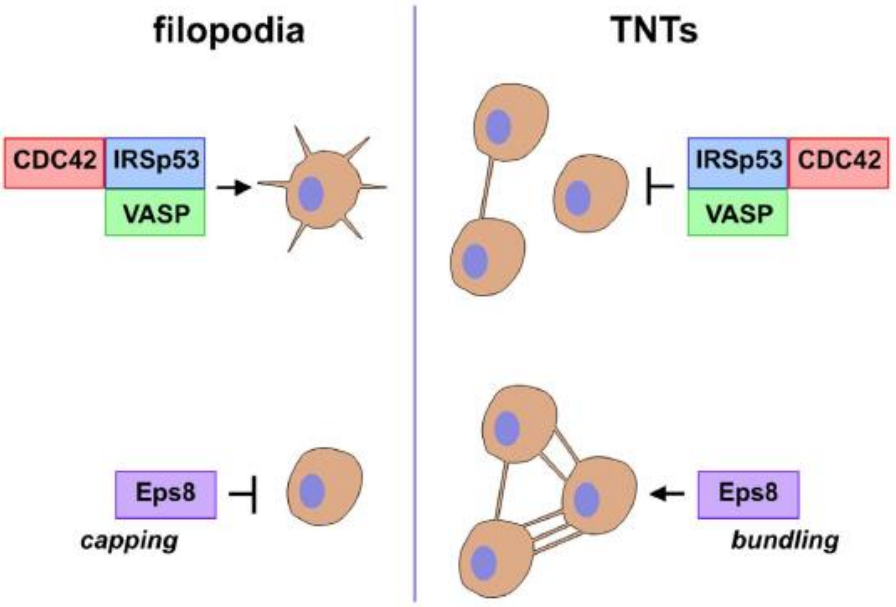


Figure 15 - Schematic representation of filopodia and TNT formation oppositely regulated by the same actin regulatory protein complexes. Adapted from (Delage et al., 2016).

CHAPTER 2

Aims

2.0 – Aims

Considering the structural similarities between TNTs and filopodia and also that both are regulated by proteins that have fundamental roles in the cytoskeleton remodeling, the group has developed studies in order to shed light on the role of some proteins in the formation of TNTs in CAD cells, a mouse neuronal cell line.

The main goal of my Master's thesis was to investigate whether the differential mechanisms that lead to TNT and filopodia formation in CAD cells are also present in other cell lines. We consider this is an important step to understand if the mechanisms of TNT formation are cell type-dependent.

For this purpose, we have taken advantage of the works reported by Inocentti's group. In recent studies, they showed that the depletion of several proteins presented in the three-major actin regulatory family proteins (Formins, Arp2/3 complex and WAVE protein family) caused important alterations in the lamellipodia/filopodia formation machinery. They used three different protein knockdowns (KD) (ARPC2, mDia1 and Nap1) in HeLa cells and subsequently treated both control and KD cells with EGF. Remarkably, the cells depleted of these proteins did not form lamellipodia upon EGF treatment but started to form filopodium-like protrusions (Beli et al., 2008; Isogai et al., 2015).

Therefore, in the first step, we examined the presence of TNTs in control and KD HeLa cells. In the second step, we evaluated the formation of TNTs between control and KD cells in the presence of various EGF treatments to promote cytoskeleton remodeling.

Finally, we also evaluated the function of formed TNTs by performing transfer experiments with labeled vesicles in HeLa cells in co-culture.

These results allow us to understand whether these proteins, which are known to play an important role in filopodia formation, also have an impact on TNT formation in HeLa cells.

CHAPTER 3

Materials and Methods

3.0 – Materials and methods

3.1 – Cell cultures

Control and stable mDia1, Arpc2 and Nap1 knockdown Human Hela cell lines were kindly provided by Mettelo Innocenti (Netherlands Cancer Institute, Amsterdam, Netherlands) and were maintained in culture in Gibco® Dulbecco's Modified Eagle Medium High Glucose + Na pyruvate + Glutamax (Life Tecnhologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2.5 µg/ml puromycin (Invitrogen). Cells were kept at 37°C under a humidified atmosphere with 5% CO_2 in sterile T25 flasks. All HeLa cells were passed three times a week in a concentration of 1:4 or 1:5 during the week and 1:6 or 1:7 during the weekend.

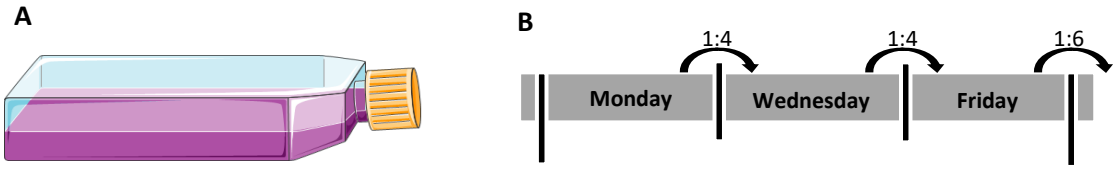


Figure 16 – Hela cells culture. **A)** Example of T25 flasks where HeLa cells were plated. **B)** Schematic representation of the experimental cell culture protocol for HeLa control and ARPC2, mDia1 and Nap1 knockdown cells.

3.2 – Protein extraction and Western blot

HeLa control and knockdown cells were plated in 6-multiwell plates with 300.000 per well and kept in culture for 48 hours at 37°C in the incubator. When confluent, all cell lines were washed twice with ice-cold phosphate-buffered saline (PBS in mM: 137 NaCl; 2,7 KCl; 10 Na_2HPO_4; 1,8 KH_2PO_4, pH 7,4) and whole cell extracts were lysed in RIPA buffer (50 mM Tris HCl, pH 7,9; 150 mM NaCl; 0,1% Triton X-100; 0,5% DOC; 0,1% SDS) supplemented with protease inhibitors (3 µl in 1ml, Roche) on ice. Then, lysates were sonicated (Cycle: 0.4; Amplitude: 40%) and centrifuged at the max speed for 3 min at 4°C. The supernatant was collected and stored at -20°C until protein quantification. The protein concentration was determined by Bradford protein assay, following the instructions of the manufacturer (Bio-Rad). For Western blot, 50 µg of the protein samples were resuspended in 5x sample buffer and boiled for 5 minutes at 99°C. Samples were size fractionated with 10% SDS-polyacrylamide gel electrophoresis (SDS-

PAGE) (table 1). Then, proteins were transferred to polyvinyl difluoride (PVDF) membranes (GE Healthcare Life Sciences). The membranes were blocked with 5% low-fat milk in PBS for one hour at room temperature, with agitation. After the blocking, the membranes were incubated with primary antibodies against respective proteins of interest (Table 2) overnight at 4°C. On the next day, membranes were washed with PBS with 0,1% tween for three times, 10 minutes each at room temperature. Afterwards, the membranes were incubated with the secondary antibodies (Table 3) prepared in the blocking solution for one hour at room temperature. The membranes were washed three times in PBS-T and protein bands were visualized using Amersham ECL prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) under the chemiluminescence imager (Amersham Imager 600, GE Healthcare Life Sciences). If the membranes were needed to probe for other proteins, a stripping protocol was performed. For that purpose, membranes were washed with the stripping solution (Thermofisher) for ten minutes and then washed three times with water, 5 minutes each. The membranes were blocked in 5% milk in PBS, followed by an overnight incubation with the primary antibody. The remaining steps until revealing the membranes were the same as the ones mentioned above.

Table 1 – Gel formulation

Formulation (1 gel)	10 %Resolving gel (20ml)	5% Stacking Gel (10 ml)
Water	9,6 ml	7,29 ml
40 %Acrylamide	5 ml	1,25 ml
1,5 M TRIS Ph8.8	5 ml	1,25 ml
10% SDS	200 µl	100 µl
10% APS	200 µl	100 µl
TEMED	20 µl	10 µl

Table 2 – Primary antibodies for Western blot

Antibody	Host	Supplier	Reference	Dilution	Protein Band Size (kDa)
ARPC2 (p34)	Rabbit	Millipore	07-227	1:2000	34 kDa
mDia1	Mouse	Santa Cruz	sc-373895	1:1000	150 kDa
WAVE2	Mouse	Santa Cruz	sc-10394	1:5000	80 kDa
α-tubulin	Mouse	sigma	T9026	1:2000	55 kDa

Table 2 – Primary antibodies for Western blot

Antibody	Host	Supplier	Reference	Dilution
Anti-mouse IgG Horseradish Peroxidase conjugate	sheep	Ge healthcare	Nxa931v	1:5000
Anti-rabbit IgG Horseradish Peroxidase conjugate	donkey	Ge healthcare	Na934v	1:5000

3.3 – Tunneling nanotubes at the basal condition

60.000 cells per well were plated in 4-well multidish with a final volume of 500 µl. The cells were incubated for twenty-four hours at 37°C and then fixed with 300 µl of Fixative 1 (Fix1) (2% paraformaldehyde (PFA); 0,05% glutaraldehyde and 0,2 M HEPES in PBS) for twenty minutes at room temperature. Then, the Fix1 solution was removed and the same amount of Fixative 2 (Fix2) (4% PFA and 0,2 M HEPES in PBS) was added for another twenty minutes before proceeding to membrane staining for TNTs imaging. Cells were stained with a fluorescent membrane dye wheat germ agglutinin (WGA, 1 mg/ml stock solution) 1:300 (v/v) in 1x PBS for twenty minutes at room temperature in the dark. WGA is a lectin-binding protein that binds to glycolipids and glycoproteins. After this procedure, the cells were washed, with PBS, three times and then we labeled F-actin with fluorophore-conjugated phalloidin 1:200 (v/v) in 1x PBS for twenty minutes at room temperature in the dark. Finally, the coverslips were washed, mounted with Aqua-Poly/Mount mounting medium and stored at 4°C in the dark before imaging.

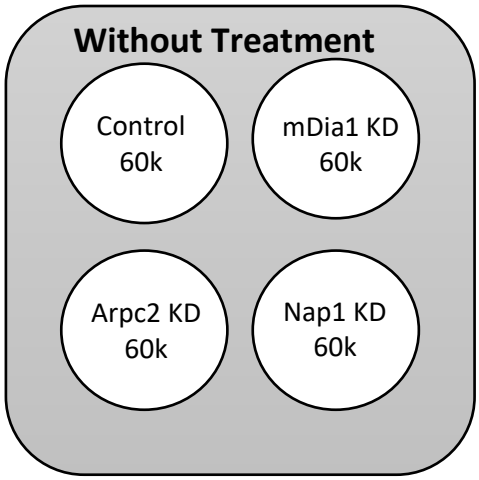
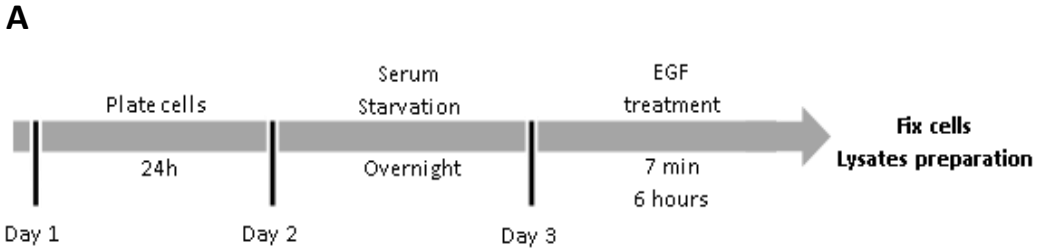


Figure 17- Schematic representation of disposition and amount of Control and Arpc2, mDia1 and Nap1 knockdown HeLa cells plated in a 4-multiwell plate for TNTs observation without any treatment.

3.4 – Tunneling nanotubes analysis after EGF treatment

HeLa control and knockdown cells were plated in a total amount of 30.000 cells in 1.9 cm^2/well 4-multiwell plate with medium for twenty-four hours and kept in the incubator. Subsequently, all cells were serum starved with DMEM medium supplemented with 0,1% of heat-inactivated FBS and kept overnight at 37°C. On the next day, a working solution of EGF 100 ng/ml was prepared from the stock (1 mg/ml) and two different periods of the EGF treatment were performed: 7 minutes and 6 hours. After the treatment, cells were either immediately fixed with Fix1 and Fix2 or re-incubated with the culture medium for an additional hour before fixation.

After the fixation procedure, cells were stained with WGA 488 1:300 (v/v) in 1x PBS for twenty minutes at room temperature in the dark. Then, all cells were washed three times with PBS and subsequently, F-actin was labeled with rhodamine phalloidin 1:200 (v/v) in 1x PBS for twenty minutes. Finally, the coverslips were washed, mounted with Aqua-Poly/Mount mounting medium and stored at 4°C in the dark before imaging.



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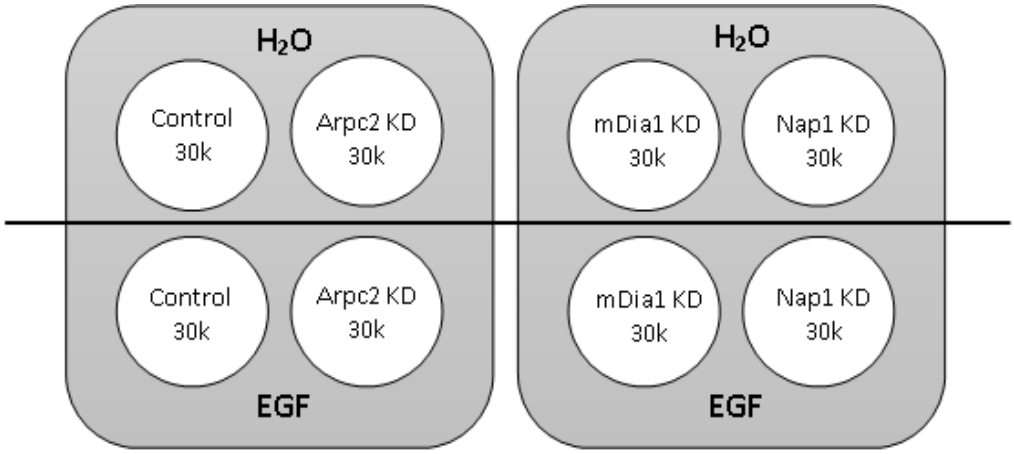


Figure 18 – EGF treatment in HeLa control and knockdown cells. **A)** Schematic representation of the EGF treatment experimental protocol in HeLa cells. **B)** Disposition and amount of Control and Arpc2, mDia1 and Nap1 knockdown HeLa cells plated in a 4-multiwell plate for EGF treatment and TNTs observation.

3.4.1 – Western blot after EGF treatment

After protein concentration was determined, samples were prepared for Western blotting by adding sample buffer supplemented with proteinase inhibitor and phosphatases inhibitors (10 mM NaF and 1 mM Na_3VO_4), at 4°C followed by the same protocol as the one above mentioned. Gel electrophoresis was carried out in a 10% polyacrylamide resolving gel with a 5% polyacrylamide stacking gel. The proteins were transferred to a PVDF membranes with a constant current of 0,25A for two hours at 4°C. Prior to the incubation with the primary antibody, membranes were blocked in a solution containing 5% low-fat milk in a Tris Buffered Saline (1x) with 1% tween 20 (TBS-T) solution, for one hour in room temperature with agitation. Primary antibodies (Table) were diluted in a solution of 5% milk in TBS-T and membranes immersed in it overnight at 4°C. After washed with TBS-T, membranes were incubated in a solution containing the corresponding horseradish peroxidase conjugated secondary antibody (Table) diluted in 5% milk in TBS-T for one hour at room temperature. Membranes were revealed using the same materials and methods above mentioned in the HeLa cells characterization.

Table 4 – Primary antibodies for Western blot after EGF treatment

Antibody	Host	Supplier	Reference	Dilution	Protein Band Size (kDa)
EGFr	Rabbit	cell signalling	4267t	1:1000	175 kDa
P-EGFr	Rabbit	cell signalling	3777t	1:1000	175 kDa

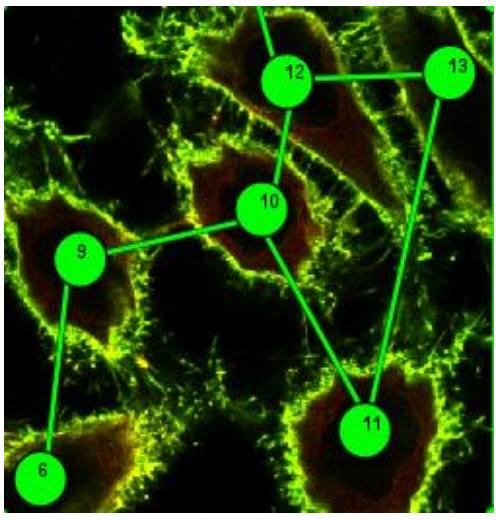
Table 5 – Secondary antibodies for Western blot after EGF

Antibody	Host	Supplier	Reference	Dilution
Anti-mouse IgG Horseradish Peroxidase conjugate	sheep	Ge healthcare	Nxa931v	1:5000
Anti-rabbit IgG Horseradish Peroxidase conjugate	donkey	Ge healthcare	Na934v	1:5000

3.5 – Tunneling nanotube detection and analysis

To evaluate the number of TNT-connected cells, image stacks (0,25 µm) covering the whole cellular volume were acquired using a confocal microscope (Zeiss LSM700). Cells connected with TNTs were manually marked with ICY image analysis software (http://icy.bioImageanalysis.org/) and percentage of TNT-connected cells was calculated.

A



B

A1 | Number of cel

	A	B
1	Number of cell:	17
2		
3	Number of cell class 0 :	17
4	Number of cell class 1 :	0
5	Number of cell class 2 :	0
6	Number of cell class 3 :	0
7		
8	Number of TNT forming cell of class 0 :	12
9	Number of TNT forming cell of class 1 :	0
10	Number of TNT forming cell of class 2 :	0
11	Number of TNT forming cell of class 3 :	0
12		
13	Average TNT connexion for cell class 0 :	1,176471
14	Average TNT connexion for cell class 1 :	0
15	Average TNT connexion for cell class 2 :	0
16	Average TNT connexion for cell class 3 :	0

Figure 19 – TNTs analysis and quantification. **A)** Example of one analyzed image in ICY image analysis software, showing numbered TNT-connected cells. **B)** Excel file resulted from the analysis of the image presented in A).

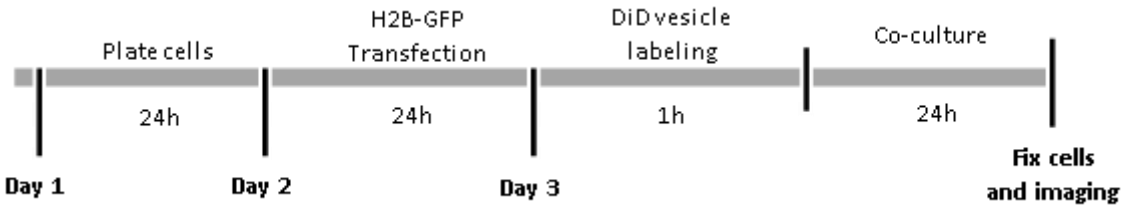
3.6 – Transfer experiments

3.6.1 – HeLa acceptor cells transfection

Both HeLa control and Arpc2 knockdown cells were plated in two 25-cm^2 culture flasks each with 1.000.000 cells plated on the day before the experiment. One flask is donor (with labeled vesicles) and the other one is acceptor (transfected with H2B-GFP). On the second day, two 15-ml falcon tubes were prepared with 1,5 ml of serum-free Opti-MEM (Gibco). 10 µl of Lipofectamine 2000 were added in the first tube and 2µg of the plasmid H2B-GFP in the other. After that, tube 2 was added to tube 1 and waited for five minutes at room temperature before adding to the flask serving as the acceptor. Cells were kept in the incubator for 24 hours. On the next day, a 1:3000 (v/v) dilution of DiD (1 mg/ml stock solution) in HeLa growth medium was added to the donor cell flask. The DiD solution was added when the cells reached approximately 80% of confluency with 3 ml of DMEM medium for half an hour in a 37°C incubator. Then, the DiD containing medium was removed and 3 ml of HeLa medium was added for another half an hour. Finally, all the acceptor and donor cells were detached with trypsin and mixed at a ratio of 2:1 (donor : acceptor, total amount 70.000 cells per well). Both control and Arpc2 knockdown cells were plated in a 1.9 cm^2/well non-treated 4-multiwell plate with HeLa medium for twenty-four hours and kept in the incubator.

A medium transfer control was used to eliminate the possibility that DiD labeled vesicles transfer to acceptor cells resulted from secretion. Medium from the co-cultured wells, as mentioned above, was collected 24h after the incubation and centrifuged to remove debris. The supernatant was added to H2B-GPF acceptor cells for another twenty-four hours in the incubator.

A



B

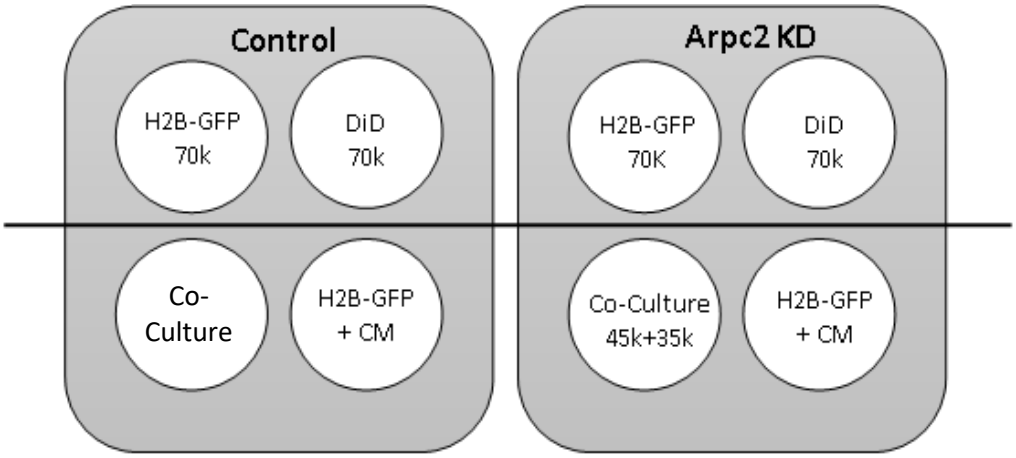


Figure 20 – Transfer experiments in HeLa control and knockdown cells. **A)** Schematic representation of the transfer experiment protocol in HeLa cells. **B)** Disposition and amount of Control and Arpc2, mDia1 and Nap1 knockdown HeLa cells plated in a 4 multiwell plate for DiD vesicle transfer observation.

3.6.2 – DiD transfer experiment quantification and analysis

All the cells were fixed with Fixative solution 1 and Fixative solution 2 and stained with Alexa Fluor 594 conjugated WGA (1:300) for 20 minutes in the dark at room temperature. To stain the entire cell volume a 1:3000 diluted solution of HCS CellMask™ Blue was used for one hour in the dark. All the samples were mounted on microscope slides with Aqua-Poly/Mount mounting medium. The whole cellular volume was imaged by acquiring Z-stacks images with an inverted microscope (Zeiss LSM700) controlled by Zen software. To assess the percentage of transfer, Spot Detector plugin on ICY image analysis software (http://icy.bioImageanalysis.org/) and CellProfiler (http://cellprofiler.org/) were used.

3.6 – Vinculin-positive focal adhesion quantification

For immunofluorescence labeling of vinculin, 15.000 cells were plated in a 1.9 cm^2/well 4 multiwell plate with culture medium and kept in the incubator for 24 hours. In the next day, HeLa control and Arpc2, mDia1 and Nap1 knockdown cells were serum starved overnight with DMEM + 0,1 % FBS and treated with 7 minutes of EGF according

to the previous experiments. Cells were immediately fixed with 4% PFA in PBS for 15 min at room temperature. All the samples were quenched with 50 mM NH_4Cl for 15 min, then subjected to a blocking and permeabilization solution with 2% BSA (w/v) in PBS containing 0,075% of saponin for one hour at room temperature. Afterwards, cells were incubated for one hour with mouse anti-vinculin antibody (V9264, Sigma) diluted to 1:500 in PBS supplemented with 2% BSA and 0,075% of saponin, followed by 3 washes with PBS and incubated with AlexaFluor®-488 conjugated goat anti-mouse antibody (Invitrogen) diluted to 1:500 in PBS containing 2% of BSA and 0,075% of saponin. Cells were washed with PBS and stained for 20 min with a solution of AlexaFluor-647 conjugated WGA. Samples were mounted with Aqua-Poly/Mount mounting medium. The bottom of the cell, in contact with the coverslip was imaged with an inverted confocal microscope (Zeiss LSM700). Displayed images correspond to stack projections (0,25 µm). Vinculin-positive peripheral adhesions were detected and counted automatically using ICY image analysis software. Spot detector plugins were used to enumerate all the vinculin spots at the cell periphery and the mean of vinculin positive puncta per cell was quantified.

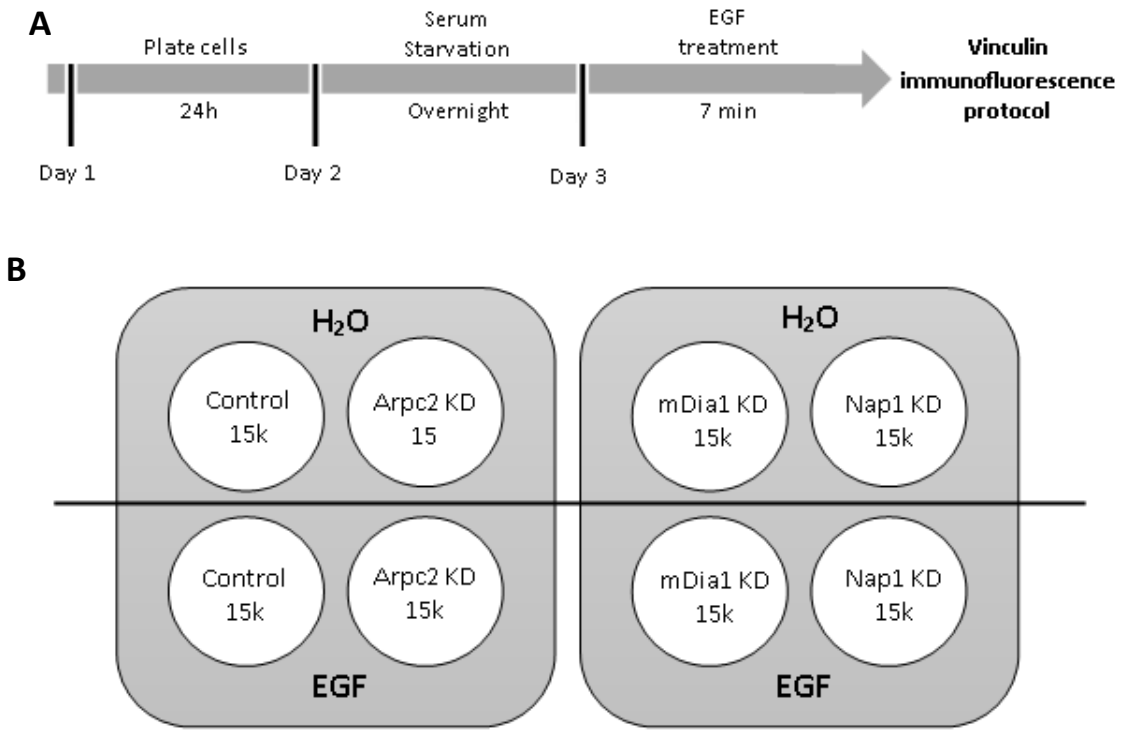


Figure 21 – Filopodia formation in HeLa control and knockdown cells. **A)** Schematic representation of the vinculin-positive focal adhesion quantification experiment in HeLa cells. **B)** Disposition and amount of Control and Arpc2, mDia1 and Nap1 knockdown HeLa cells plated in a 4- multiwell plate for vinculin filopodia observation.

3.7 – Statistical analysis

Results are presented as mean ± SD. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software). Data was analyzed using the non-parametric unpaired Student's *t* test, followed by non-parametric Mann-Whitney test. Differences were considered significant for $p<0,05$.

CHAPTER 4

Results

4.0 – Results

4.1 – ARPC2, mDia1 and Nap1 protein levels in control and knockdown HeLa cells.

To start the experimental part of this project, Western blots (Wb) were performed to evaluate the expression levels of Arpc2, mDia1 and Nap1 proteins in control and knock-down (KD) HeLa cells.

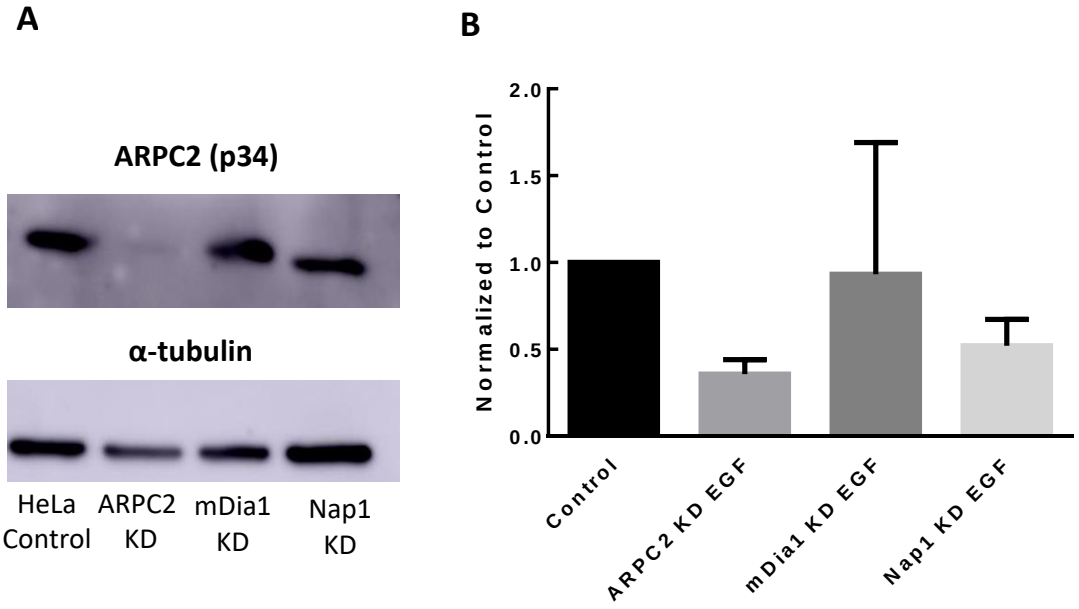


Figure 22 – Arpc2 protein levels were decreased in Arpc2 knockdown HeLa cells. A) Arpc2 levels in HeLa cells after protein extraction without any treatment. Representative Western blot showing that the level of Arpc2 was reduced in the Arpc2 KD cells. α-tubulin served as an internal control. **B)** Quantification of the protein expression levels from the Wb (n=2). The levels are compared and normalized to HeLa control cells. Bar graphs show SD.

First of all, 300.000 cells were seeded at day 1 and total lysates were collected at day 2. 50 µg of the total lysates were loaded and were detected with antibodies recognizing Arpc2, WAVE2 and mDia1, respectively.

We observed decreased Arpc2 density in Arpc2 KD HeLa cells (Figure 22A). The deletion of this protein reduced the protein levels without affecting significantly the expression of mDia1 and the WAVE-complex subunit Nap1 (Figure 22B).

Similarly, silencing Nap1 led to a decreased expression of WAVE but not the mDia1 and Arpc2 levels (Figure 23).

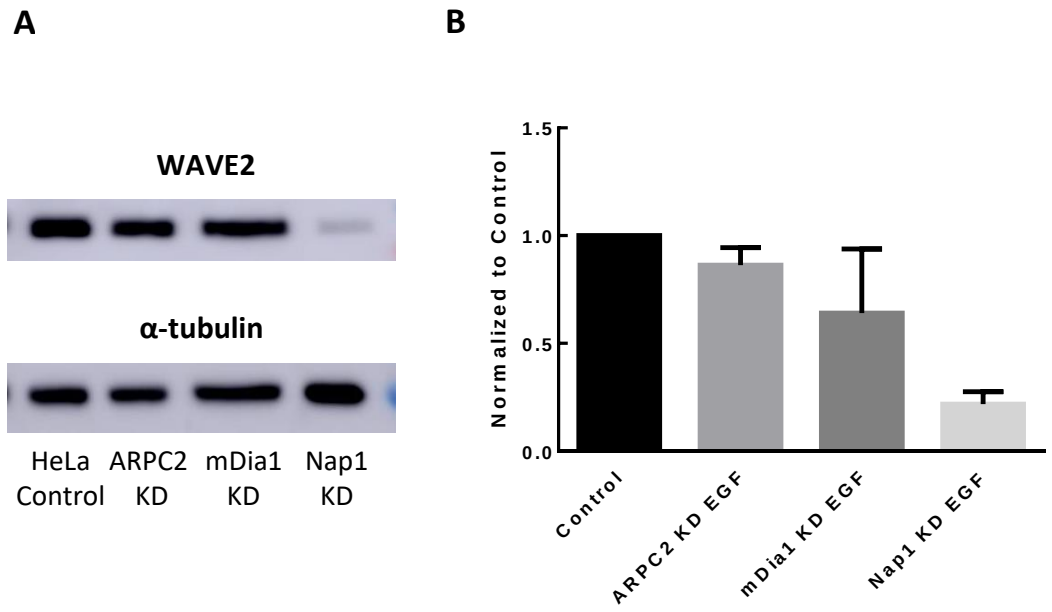


Figure 23 – WAVE2 protein levels were decreased in Nap1 knock-down HeLa cells. A) WAVE2 levels in HeLa cells after protein extraction without any treatment. Representative Western blot showing that the level of WAVE2 was reduced in the Nap1 KD cells. α-tubulin served as an internal control. **B)** Quantification of the protein expression levels from the WB (n=2). The levels are compared and normalized to HeLa control cells. Bar graphs show SD

Finally, we evaluated the effect of the deletion of mDia1 protein. In figure 24, we can observe that mDia1 level was significantly reduced in mDia1 knock-down HeLa cells. Moreover, the absence of Arpc2, but not, Nap1 resulted a slightly reduction of mDia1

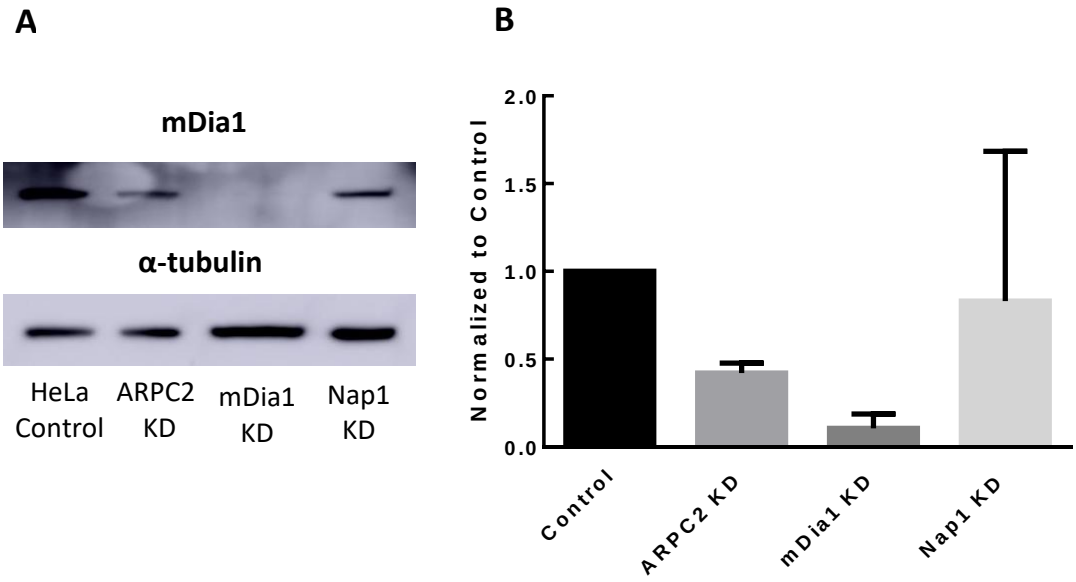


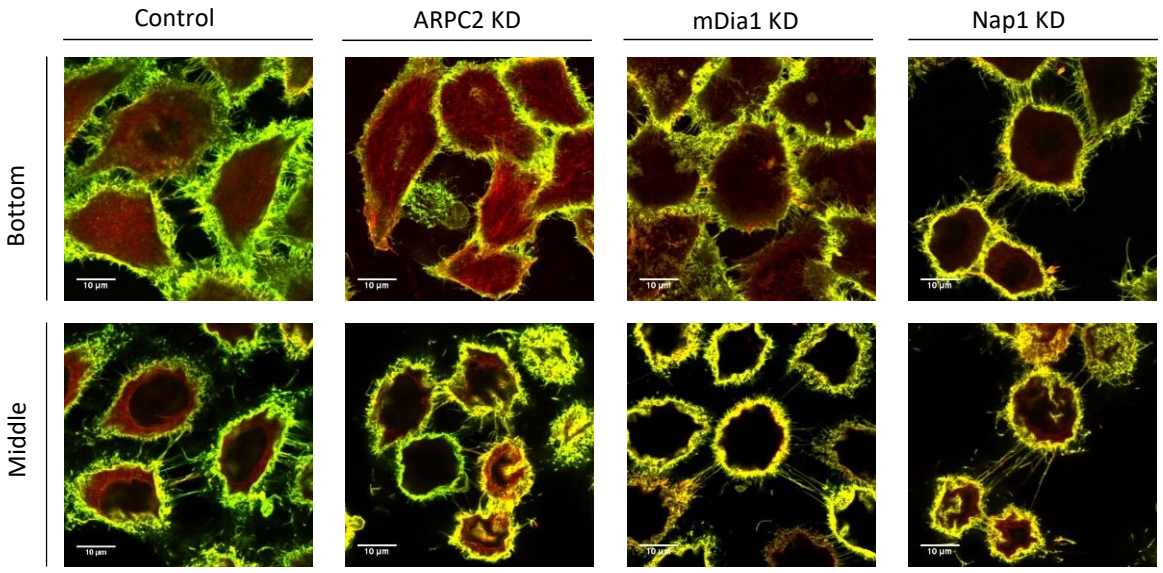
Figure 24 – mDia1 protein levels were decreased in mDia1 knock-down HeLa cells. A) mDia1 levels in HeLa cells after protein extraction without any treatment. Representative Western blot showing that the level of mDia1 was reduced in the mDia1 KD cells. α-tubulin served as an internal control. **B)** Quantification of the protein expression levels from the WB (n=2). The levels are compared and normalized to HeLa control cells. Bar graphs show SD.

4.2 – HeLa cells form tunneling nanotubes

After assessing expression levels of ARPC2, mDia1 and Nap1 proteins in knockdown cells, we then examined whether these cells were able to form TNTs. In order to determine the mechanisms involved in TNT formation at the basal condition, cells were plated at several different densities (60.000 and 70.000 cells per coverslip) and fixed after 24h without any treatment. We found that 60.000 cells per coverslip (10 mm glass diameter) was the best density to observe TNTs in HeLa cells. Interestingly, even though in the absence of the major actin regulators, Arpc2, mDia1 and Nap1 knock-down HeLa cells were able to form TNTs between remote cells.

Quantification analysis revealed that, approximately 70% of both control and knock-down cells were connected with TNTs (Figure 25B), suggesting that in the absence of ARPC2, mDia1 and Nap1 did not affect the formation of TNTs in the basal conditions in HeLa cells. It is also important to note that the morphology of wild-type and knock-down cells at the basal state was similar.

A



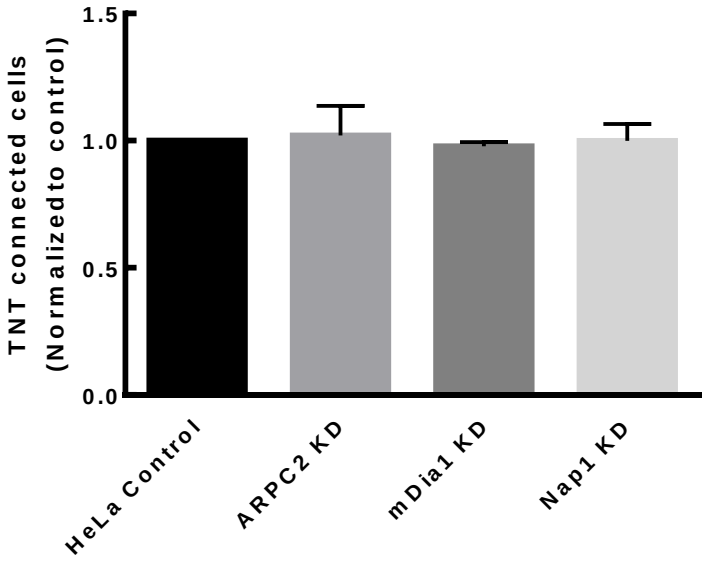


Figure 25 – HeLa control and knockdown cells are able to form TNTs. A) Representative confocal Z-stack images showing TNT-connected cells without any treatment. Sixty thousand cells were plated on 4-multiwell plates and fixed twenty-four hours later. The images are shown in two different focus areas. At the top, representative images of the cells in the lowermost position, attached to the substrate. Below, are representative images of the same cells in a central position of the Z-stack. Cells were labeled with WGA-Alexa Fluor©-488nm (green) and Phalloidin (red) was used to stain F-actin, allowing the observation of TNTs and cell limits and observed by confocal microscopy. Scale bar: 10 µm. **B)** Quantification of TNT-connected cells without any treatment. Data represent the mean (±SD), normalized to control cells (HeLa control) arbitrarily set at 1, at least 2 independent experiments were performed.

4.3 – EGF promotes filopodia formation in ARPC2, mDia1 and Nap1 knockdown HeLa cells

Based on the work of Innocenti and his colleagues, after 7 min of EGF treatment, ARPC2, mDia1 and Nap1 HeLa cells form filopodia instead of lamellipodia/ruffles (Beli et al., 2008; Innocenti et al., 2004; Isogai et al., 2015). Therefore, we evaluated the formation of TNTs in HeLa cells after the treatment of EGF for 7 min (Figure 26).

4.3.1 – EGF incubation in HeLa cells promotes EGF receptor activation after 7-minutes treatment

We started by plating 30.000 cells of control and ARPC2, mDia1 and Nap1 knockdown HeLa cells at day 1. After serum starvation overnight at day 2, cells were treated with EGF for 7 minutes. The serum starvation in the cells promotes an efficient response with EGF, inducing the EGF receptor activation at the HeLa cells plasma membrane.

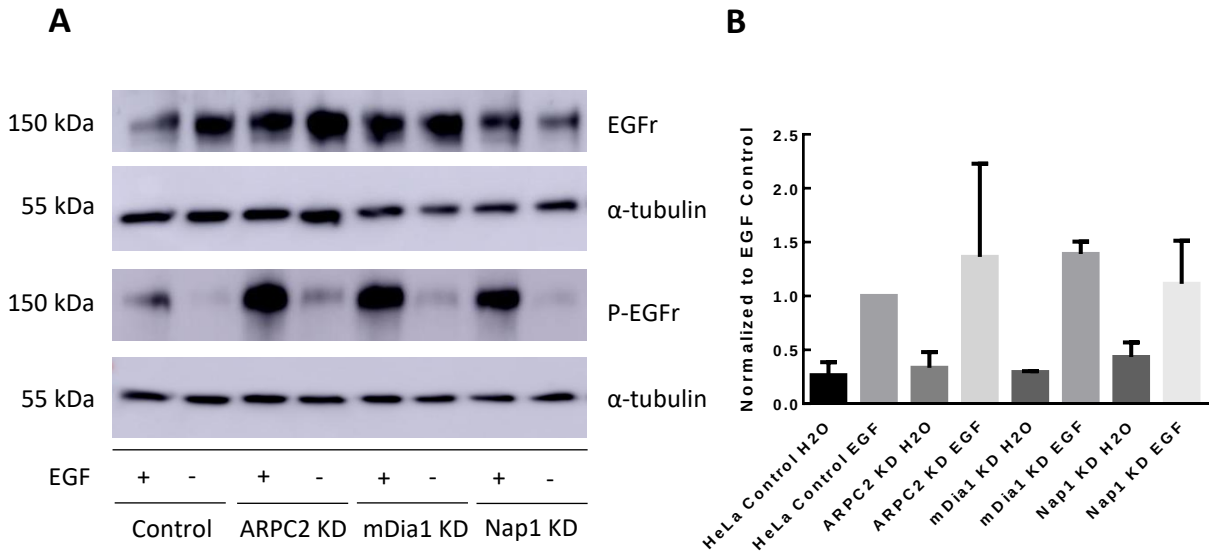


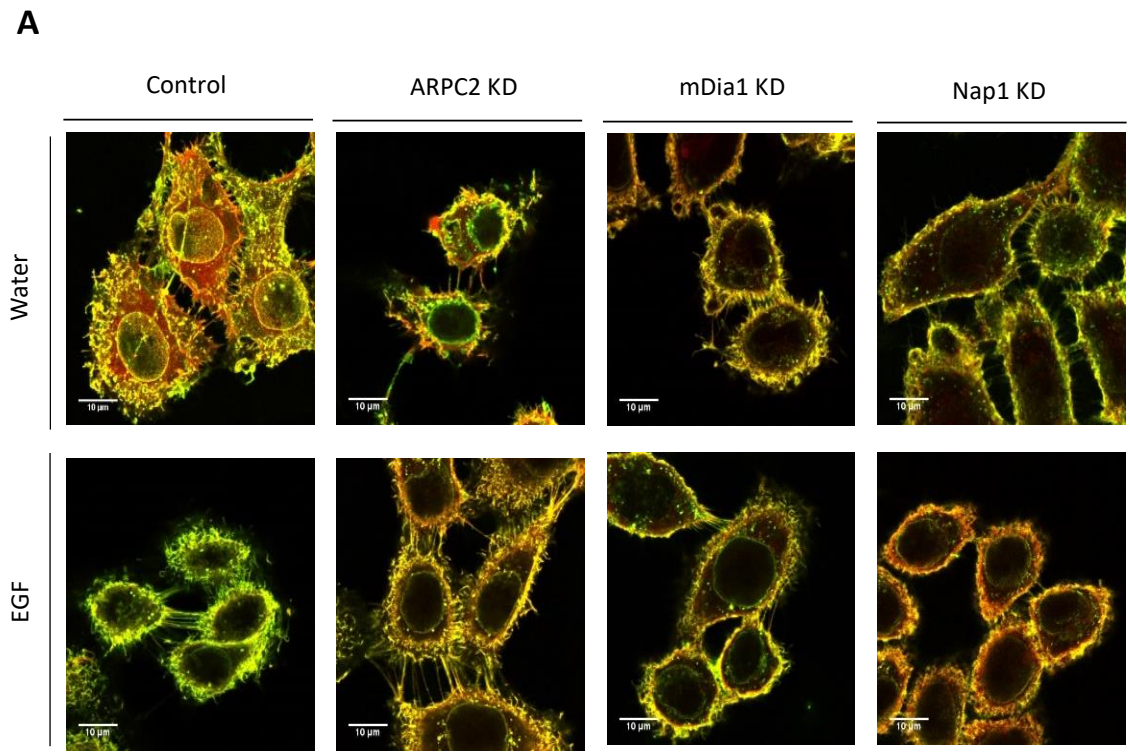
Figure 26 – EGF receptor activation after 7-minute EGF treatment in HeLa cells. A) Total EGF and phosphorylated EGF receptor levels in HeLa cells after seven-minute treatment of EGF in HeLa control and Arpc2, mDia1 and Nap1 knock-down cells. Representative Western blot showing that the total EGF receptor levels were identical in all the cell lines when treated with EGF (+) and water (-) used as control. In opposite, P-EGF receptors are significantly increased in cells treated with EGF when compared to water, showing the activation of the EGF receptors upon EGF incubation for seven minutes. α-tubulin served as an internal control. **B)** Quantification of the protein expression levels from the Wb (n=2). The levels are compared and normalized to HeLa control cells treated with EGF. Bar graphs show mean +-SD.

After the EGF incubation, the activation of EGF receptors was measured in control and Arpc2, mDia1 and Nap1 knock-down HeLa cells by Western blot (Figure 26A). The phosphorylation of EGFR shows that they become active and are able to initiate a cascade of reactions promoting different responses by the cells. Therefore, using an antibody recognizing the phosphorylation site would reveal the activation of EGF receptor. Cells were treated either with EGF (+) or water (-) as control and from the

Wb results, we observed that the expression levels of the total EGF receptors did not reveal significant changes in all the cells. After the treatment, a significant increase in the phosphorylated receptor levels was observed only in the cells treated with EGF proving their activation.

4.3.2 – EGF incubation in HeLa cells increases the number of TNT-connected cells after 7-minute EGF treatment.

After confirming EGF receptors activation in HeLa cells, we then evaluated the number of TNT-connected cells by imaging. All cell lines were serum starved overnight followed by a seven-minute treatment of EGF or water. Cells were then fixed with a fixative solution 1 and fixative solution 2.



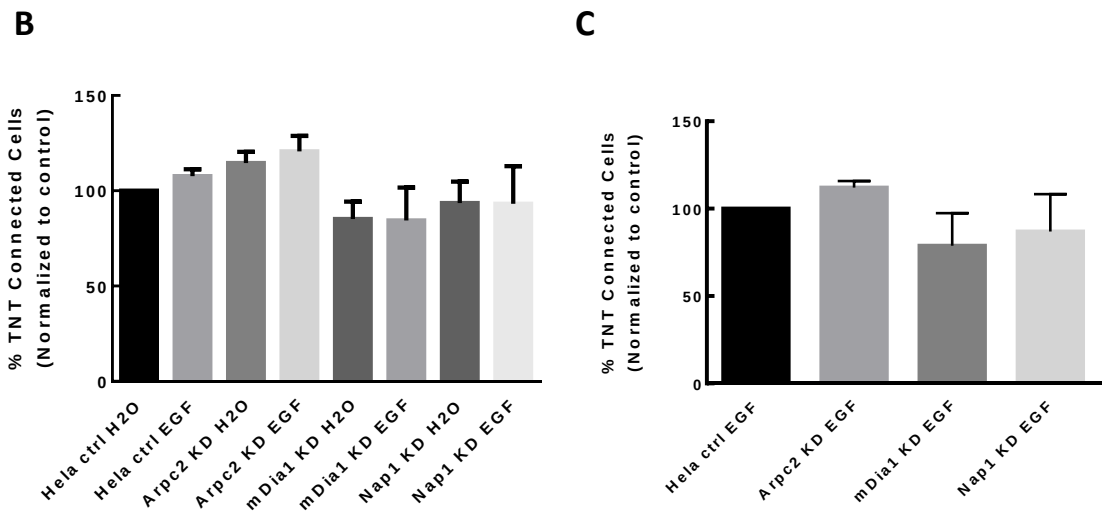


Figure 27 – TNT-connected cells are slightly increased in Arpc2 knockdown cells after seven-minute EGF treatment. A) Representative confocal Z-stack projected images showing TNT-connected cells after seven-minute EGF treatment. Thirty thousand cells were plated on 4-multiwell plates and submitted to serum starvation followed to EGF treatment. Cells were labeled with WGA-Alexa Fluor©-488nm (green) and Phalloidin (red) was used to stain F-actin, allowing the observation of TNTs and cell limits and observed by confocal microscopy. Scale bar: 10 µm. **B)** Quantification of TNT-connected cells in HeLa control and ARPC2, mDia1 and Nap1 knockdown cells after 7-min EGF treatment. Data represent the mean (±SD), normalized to control cells (HeLa control EGF) arbitrarily set at 100%, of at least 2 independent experiments. **C)** Quantification of TNT-connected cells showing a slightly increase in Arpc2 KD cells compared to control. Data represent the mean (±SD), normalized to control (HeLa control H20) arbitrarily set at 1, at least 2 independent experiments were performed.

Quantification analysis revealed that the number of TNT-connected cells was slightly higher in ARPC2 knockdown HeLa cells. However, mDia1 and Nap1 knockdown cells did not reveal any significant change in the number of TNT-connected cells upon EGF treatment.

4.3.3 – EGF treatment in HeLa cells increases the number of TNTs after a seven-minutes treatment, followed by a one-hour later fixation.

Because the increase of the TNT-connected cells after 7 min treatment of EGF was not significant, we speculated that seven-minutes might not be sufficient for the TNT formation. Therefore, a different protocol was used, in which the cells were also subjected to an EGF treatment for seven minutes but fixed one hour later.

Quantification analysis showed that Arpc2 knockdown cells had a higher trend, though not significant, to increase the TNT-connected cells when compared to the control.

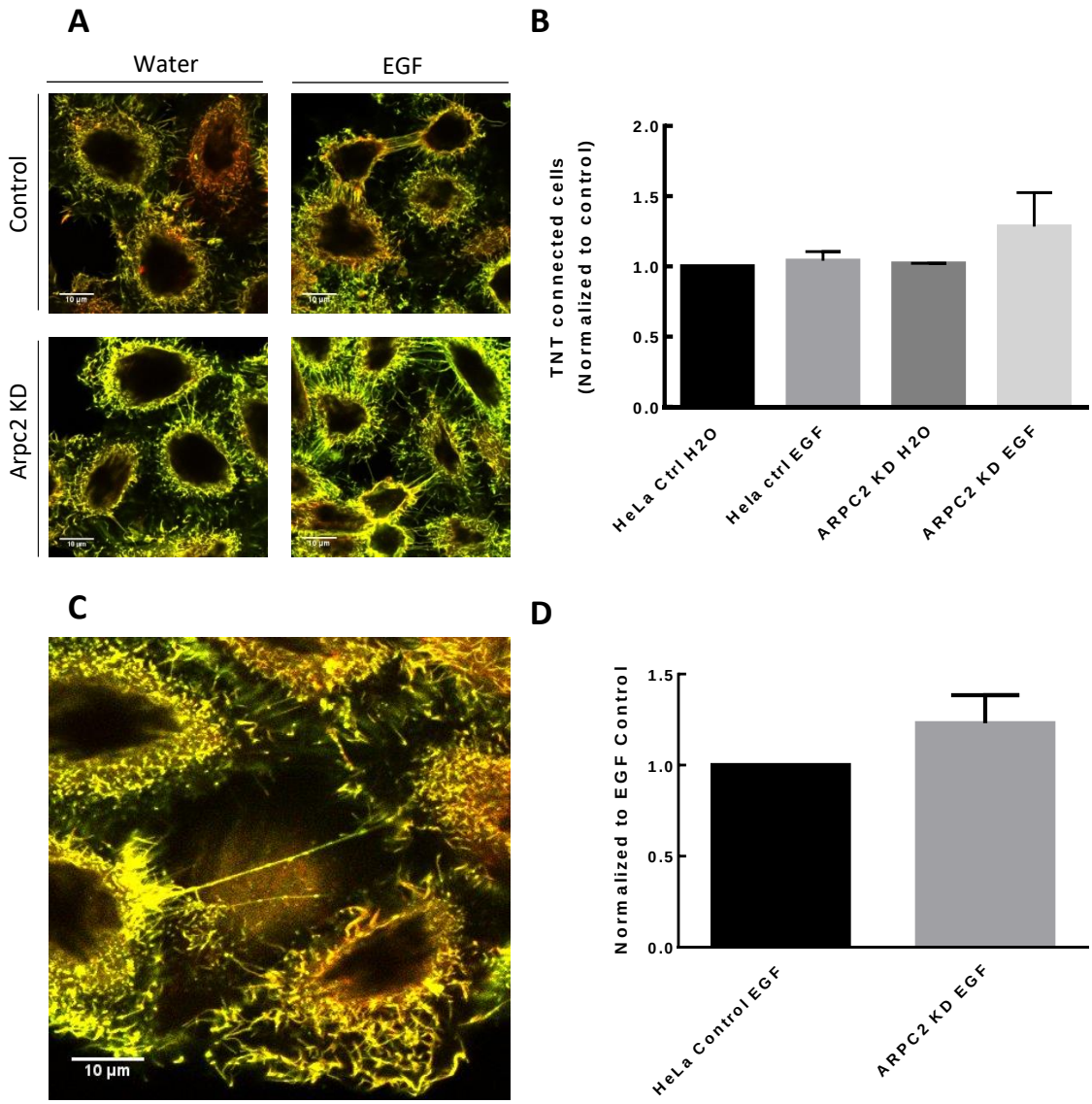


Figure 28 - TNT-connected cells are slightly increased in Arpc2 knockdown cells after seven-minute EGF treatment followed by a one hour later fixation protocol A) Representative confocal Z-stack images showing TNT-connected cells after EGF treatment. Thirty thousand cells were plated on 4-multiwell plates and submitted to serum starvation followed to EGF treatment. After the treatment, new medium was added to the cells left in the incubator and fixed one hour later. Cells were labeled with WGA-Alexa Fluor©-488nm (green) and Phalloidin (red) was used to stain F-actin, allowing the observation of TNTs and cell limits and observed by confocal microscopy. Scale bar: 10 µm. **B)** Quantification of TNT-connected cells after EGF treatment. Data represent the mean (±SD), normalized to control cells (HeLa control H2O) arbitrarily set at 1, of at least 2 independent experiments. **C)** Representative confocal Z-stack image showing a highly visible TNT formed between two Arpc2 KD cells after the EGF treatment. Scale bar: 10 µm. **D)** Quantification of TNT-connected cells after EGF treatment. Data represent the mean (±SD), normalized to control cells treated with EGF (HeLa control EGF) arbitrarily set at 1, at least 2 experiments were performed.

4.3.4 – EGF receptor in HeLa cells has a lower expression after a six-hour treatment.

The results above prompted us to speculate that perhaps we need an even longer time to see the changes in the number of TNT-connected cells. Therefore, we then decided to treat the cells with EGF for 6 hours.

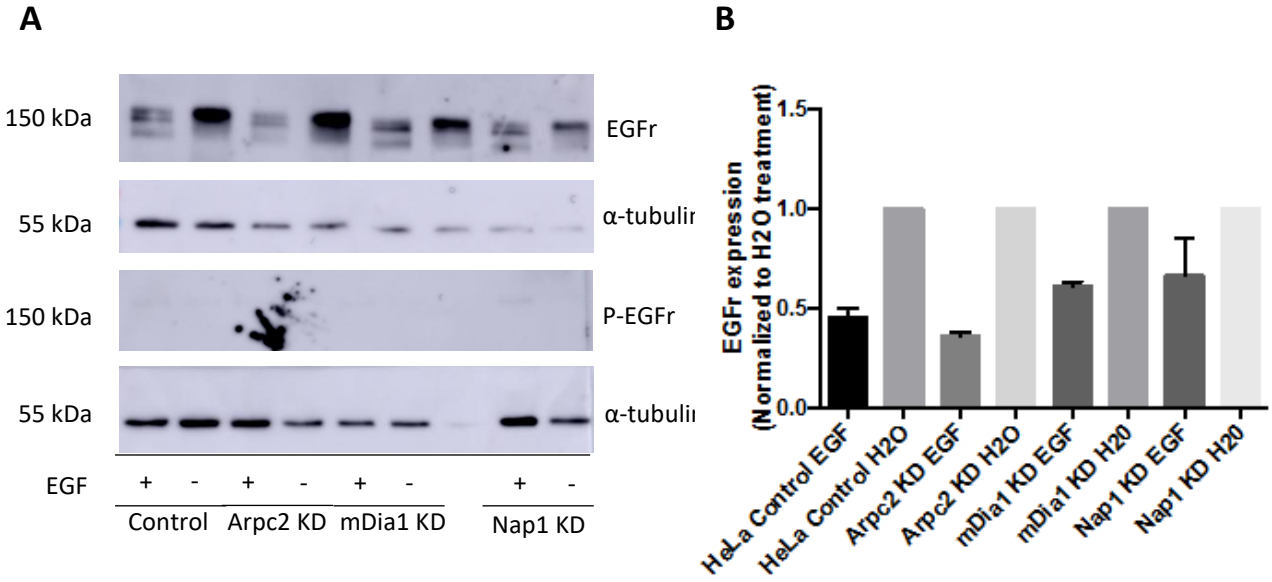


Figure 29 - EGF receptor activation after 6-hrs EGF treatment in HeLa cells. A) Total EGF and phosphorylated EGF receptor levels in HeLa cells after six-hour treatment of EGF in HeLa control and Arpc2, mDia1 and Nap1 knock-down cells. Representative Western Blot showing that the total EGF receptor levels were decreased in cells treated with EGF (+) compared to the cells treated with water (-) used as control. P-EGF receptors expression were significantly decreased after the treatment in all the cell lines. α-tubulin served as an internal control. **B)** Quantification of the protein expression levels from the WB (n=2). The levels are compared and normalized to all the cells treated with water. Bar graphs

HeLa control and knock-down cells were plated with 300.000 cells per well (10mm glass diameter) and after a serum starvation overnight period, cells were applied a six-hour incubation of EGF. Lysates were collected and the activation of EGF receptors were measured by Western blot.

The data presented in figure 29 shows a decrease in the expression levels of the total EGF receptor in the cells treated with EGF. Also, the levels of the phosphorylated receptors were significantly decreased after 6-hour incubation of EGF, meaning that these receptors were subjected to the degradation mechanisms after the activation.

4.3.5 – EGF treatment for six hours increases the number of TNT-connected cells in ARPC2 knockdown cells

We then wanted to assess the TNT formation after this time period of EGF incubation. All cells lines were plated with 30.000 cells per coverslip (10mm glass diameter) and subjected to further EGF treatment as mentioned in previous experiments. Cells were fixed and the TNT-connected cells were analyzed by imaging (Figure 29A).

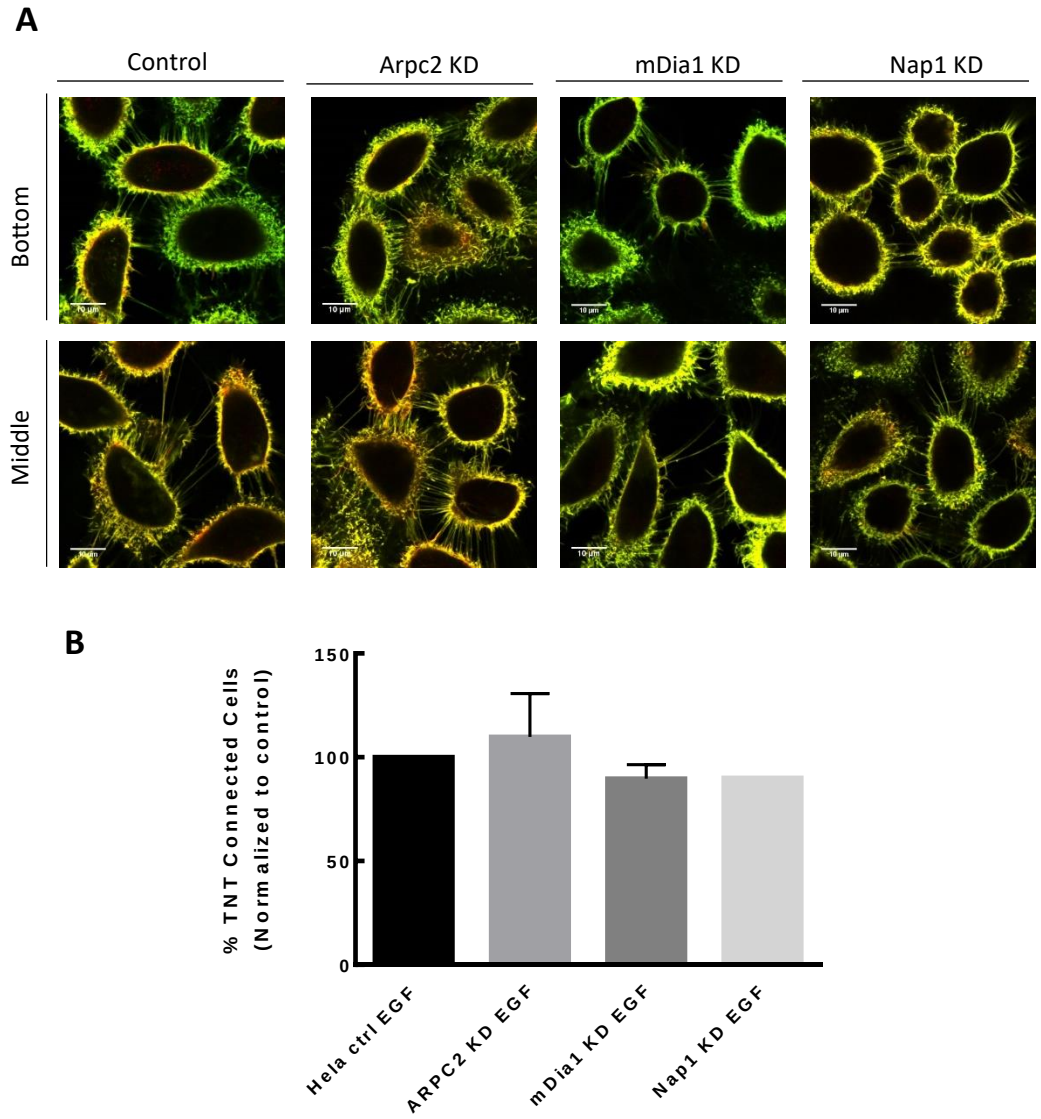


Figure 29 – TNT-connected cells are slightly increased in Arpc2 knockdown cells after six-hour EGF treatment. A) Representative confocal Z-stack images showing TNT-connected cells after six-hour EGF treatment. Thirty thousand cells were plated on 4-multiwell plates and submitted to serum starvation followed to EGF treatment. Cells were labeled with WGA-Alexa Fluor©-488nm (green) and Phalloidin (red) was used to stain F-actin, allowing the observation of TNTs and cell limits and observed by confocal microscopy. Scale bar: 10 µm. **B)** Quantification of TNT-connected cells without any treatment. Data represent the mean (±SD), normalized to control cells (HeLa control) arbitrarily set at 1, at least 2 independent experiments were performed.

Quantification analysis showed the same trend to increase the number of TNT-connected cells in the ARPC2 knockdown cells. Based on these results, we observed that the EGF treatment only promoted TNT formation in HeLa ARPC2 knockdown cells, suggesting an important role of the Arp2/3 complex in the formation of these structures.

4.4 – HeLa cells can form functional TNTs that mediate the transfer of labeled vesicles between cells in co-culture

TNTs are cellular connections that can transfer a wide range of cargos between distant cells (Saïda Abounit et al., 2016). Therefore, if there is an increase of TNT-connected cells, we expect to also observe an increase of transferred vesicles. For this purpose, the lab has established a protocol to test the transfer of labeled vesicles in several different cell lines, including CAD cells (Abounit et al., 2015).

Due to the trend noticed in the ARPC2 knockdown cells in increasing the number of TNT-connected cells, we decided to develop a transfer experiment only in the HeLa control and ARPC2 knockdown cells. Thus, we aimed to understand if the increase in the number of TNTs was also related to a higher number of vesicles transferred between cells in co-culture.

Control and ARPC2 knockdown cells were plated with 1.000.000 cells in two different T25 flasks at day 1. In the next day, one T25 flask of both cell line was selected as acceptor cells in order to start the transfection of H2B-GFP for 24 hours. At day 3, HeLa control and ARPC2 knockdown donor cells were incubated with DiD far-red in order to label all the vesicles. After that, cells were mixed in a concentration of 2:1 (donor cells : acceptor cells) and kept in co-culture for subsequent fixation 24 hours later. To determine whether the transfer was mediated by cell-cell contact or by secretion, we also collected the medium only from the donor cells and replaced it in the H2B-GFP acceptor cells.

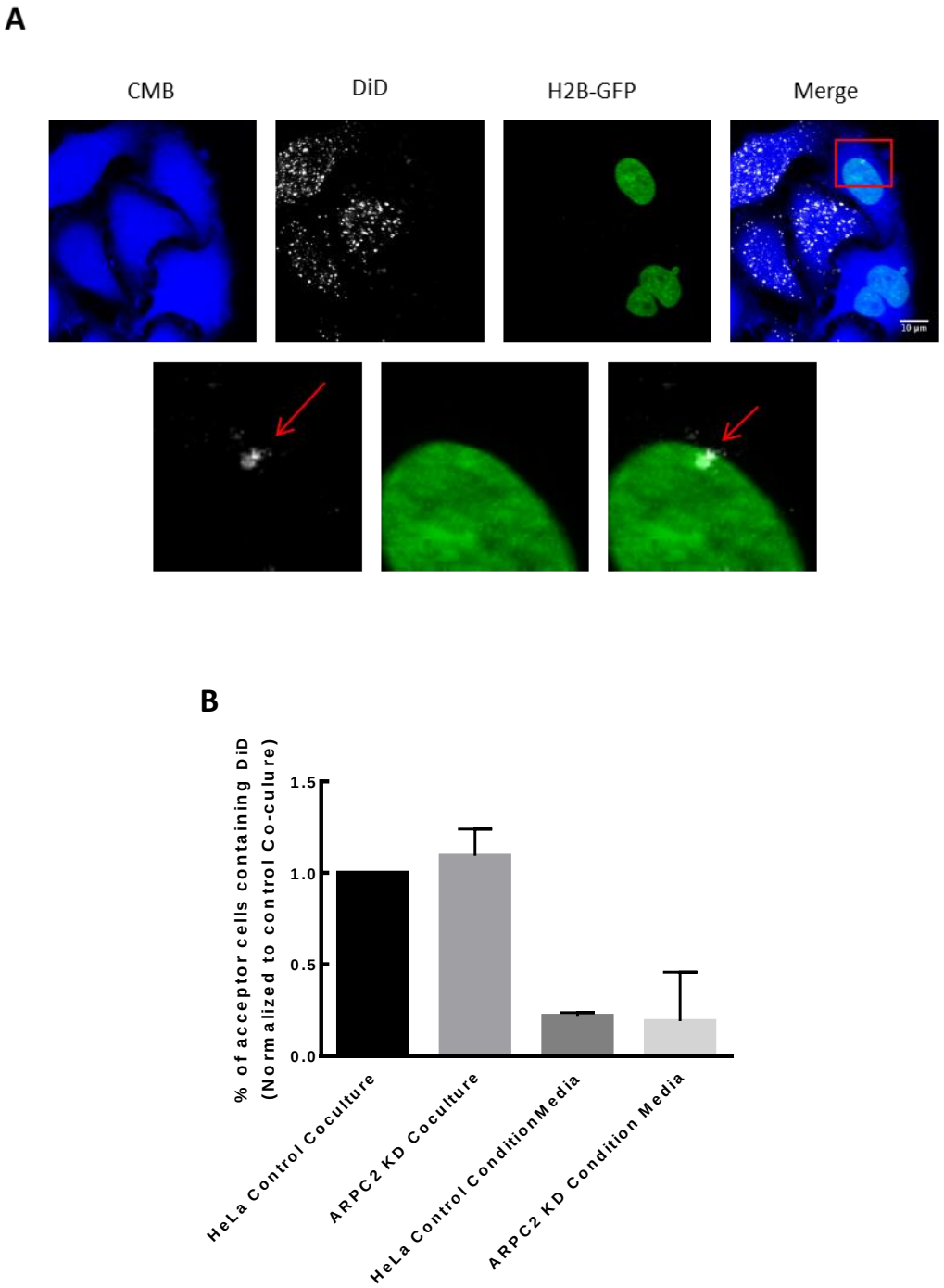
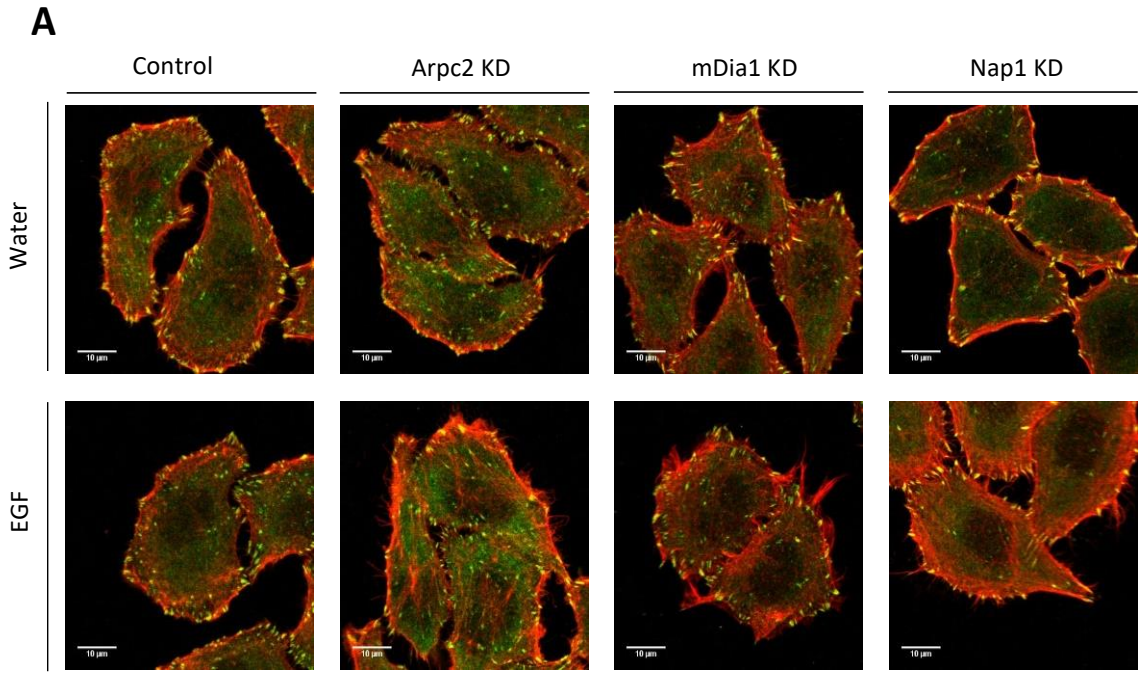


Figure 30 – DiD vesicles transfer occurs in HeLa cells and is mediated by cell-cell contact. A) Representative confocal Z-stack images showing acceptor (H2B-GFP) and donor (DiD) cells after transfer experiment in HeLa cells. Cells were plated in co-culture (2:1 = donor cells : acceptor cells) on 4-multiwell plates and kept at 37°C in the incubator. Cells were labeled with WGA-Alexa Fluor©-594nm (red) and HCS CellMask (Blue) to stain the entire cellular volume. Images were observed by confocal microscopy. Scale bar: 10 µm. **B)** Quantification of the number of cells having DiD labeled vesicles normalized to HeLa control Co-culture. Data represent the mean (±SD), normalized to control cells (HeLa control) arbitrarily set at 1, at least 2 independent experiments were performed.

Even though the data here shown was not significant, the same tendency was maintained in ARPC2 knockdown cells, showing a higher rate of transferred vesicles. We noticed that the transfer mediated by the condition media was lower compared to the transfer mediated by cell-cell contact. These results suggested that the DiD labeled vesicles could be transferred through cell-to-cell contact. Also, due to the trend noticed in the Arpc2 Knock-down cells in increasing the number of TNT-connected cells, we speculated to the transfer of did was through TNTs.

4.5 – EGF treatment in HeLa cells does not promote any differences in vinculin-positive filopodia

As mentioned above, the EGF treatment promotes filopodia formation in Arpc2, mDia1 and Nap1 knock-down cells. It is important to note that these proteins are part of several protein complexes playing an important role in actin polymerization and filopodia formation. We focused our interest on the substrate-attached filopodia. Contrary to TNTs and dorsal filopodia, substrate-attached filopodia display vinculin-positive focal adhesions at their tips (Bohil et al., 2006). Therefore, we wanted to address whether attached filopodia was altered after EGF treatment by counting the vinculin positive filopodia. For this purpose, 15.000 cells of each cell line were plated per coverslip (10mm) and subjected to a seven-minute EGF treatment.



B

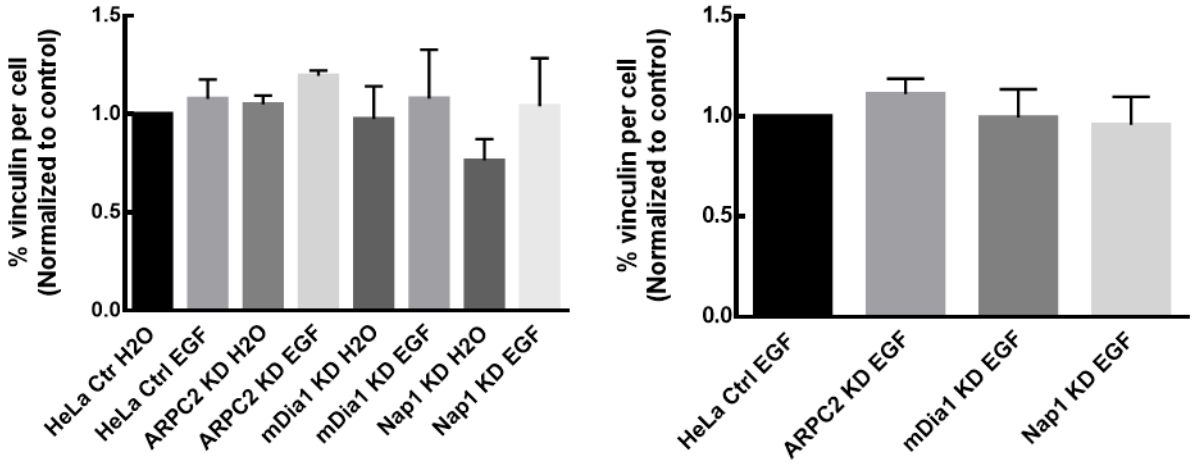


Figure 31 – Vinculin-positive focal adhesions did not alter after EGF treatment in HeLa cells. A) Representative confocal Z-stack images of HeLa control and ARPC2, mDia1 and Nap1 knockdown cells. For immunofluorescence labeling of vinculin, 15.000 cells were plated in 4-plate multiwell and subsequently treated with seven minutes of EGF. Mouse anti-vinculin primary antibody was used followed by goat anti-mouse AlexaFluor 488 (green) secondary antibody. Cells were labeled with WGA-Alexa Fluor©-647nm (red) to stain the plasma cell membrane. Images were observed by confocal microscopy. Scale bar: 10 µm. **B)** Quantification of the number of vinculin positive focal adhesions in HeLa cells. Data represent the mean (±SD), normalized to control cells (HeLa control) arbitrarily set at 1, at least 2 independent experiments were performed.

All the cells were serum starved overnight and subsequently treated with EGF for seven minutes. Afterwards, cells were fixed immediately after the treatment and the vinculin-positive focal adhesions were analyzed by imaging (Figure 31A). From the figure 31B, we observed that there were no significant changes in the number of vinculin-positive filopodia among treated and non-treated cells, suggesting that the EGF treatment did not cause any changes in the substrate-attached filopodia.

CHAPTER 5

Discussion

5.0 – Discussion

The dynamics of actin cytoskeleton are considered vital for several processes such as cell migration, invasion, and the nucleation of actin filaments. The latter is crucial for the formation of membranous protrusions such as lamellipodia, filopodia and TNTs. Unlike filopodial protrusions, TNTs are able to mediate the transfer of different cargoes and are not attached to the substrate but hovering between two distant cells (Rustom, 2004).

A recent study from Dr. Zurzolo's lab proposed that the formation of TNTs and filopodia in CAD cells could be regulated by the same actin binding proteins, but in an opposite way (Delage et al., 2016). Yet, whether the similar pattern of TNT and filopodia formation could be observed in other cell types remains to be investigated.

Therefore, in this work, we focused on the role of ARPC2, mDia1 and Nap1 proteins in TNT formation in HeLa cells. These three proteins are known to be involved in the regulation of lamellipodia and filopodia machinery in HeLa cells (Isogai et al., 2015). Previous experiments conducted in HeLa cells revealed that upon EGF stimulation, ARPC2, mDia1 and Nap1 knockdown cells did not form lamellipodia but filopodia (Beli et al., 2008; Innocenti et al., 2004; Isogai et al., 2015).

In basal conditions, we have shown that HeLa control and ARPC2, mDia1 and Nap1 knockdown cells are able to form TNTs (approximately 70% of the cells connected with TNTs, with a slightly increase of TNTs in ARPC2 KD cells).

After EGF treatment in control and ARPC2, mDia1 and Nap1 knockdown cells, we observed a tendency to increase approximately 15% in the number of TNT-connected ARPC2 knockdown cells, when compared with basal conditions. In fact, this tendency was observed along increasing time-periods of EGF treatment, suggesting a role for ARPC2 protein in TNT formation. ARPC2 is a subunit of Arp2/3 complex and the association of this complex with an existing actin filament is crucial for the formation of branched actin structures. The actin polymerization in branches is known to generate lamellipodia structures at the plasma membrane. Loss of function studies with subunits of this protein complex showed that lamellipodia formation was abrogated, leading to filopodia formation promoted by other actin-binding proteins such as the formin protein

family (Di Nardo et al., 2005). Consistent to our hypothesis, we found that Arp2/3 complex may have an inhibitory role in TNT formation under normal and EGF treatment conditions.

Moreover, the increase of TNTs formation is often correlated with the function of TNTs (Saïda Abounit et al., 2016; K. Gousset et al., 2013). We observed an increase in the transfer of DiD labeled vesicles between ARPC2 knockdown cells, when compared to control cells, supporting the data that ARPC2 knockdown cells had slightly more TNTs. We were also able to show that this transfer was mediated by cell-to-cell contact, highlighting further the role of arp2/3 complex in TNT formation and the role of these structures in cargo transfer between distant cells.

In addition, Nap1, which is a subunit of the WAVE complex, can regulate the activity of the Arp2/3 complex (Steffen et al., 2004). For this reason, we would expect to see an increase of TNTs in HeLa cells upon Nap1 knockdown. However, we did not observe significant changes in TNTs in Nap1 knockdown cells in both basal and EGF treated conditions. One explanation for this observation could be that the expression level of WAVE complex was still detected through Western blot analysis (Figure 22). These remaining residues could be sufficient to maintain the number of TNTs in the Nap1 knockdown cells. Comparing to the situation for the filopodia formation upon EGF treatment, Nap1 knockdown cells form significantly higher amount of filopodia (Beli et al., 2008). This data may imply that the synthesis of TNTs might be more tightly regulated than filopodia.

In contrast to the Arp2/3 complex, the formin protein family promotes the polymerization of linear actin filaments (Thomas D. Pollard, 2007). mDia1 is a member of the formin family and was recently shown together with Arp2/3 complex to form lamellipodia/ruffles in HeLa cells (Isogai et al., 2015). Furthermore, HeLa cells depleted of mDia1 did not ruffle upon EGF stimulation but formed filopodium-like protrusions. In our results, we did not observe an increase in TNT-connected mDia1 knockdown cells after an EGF treatment. These results suggest that mDia1 may not be important for the formation of TNTs or that there are other formins partially compensating the function.

Although filopodia are normally thought to arise from the leading edge of attached cells, recent studies demonstrated that filopodia protrusions can also arise from the dorsal surface of many cells (Bohil et al., 2006). Contrarily to TNTs and dorsal filopodia, the substrate-attached filopodia display vinculin-positive focal adhesions at their tips (Schaffer et al., 2010). We use this specificity to assess the filopodia formation among HeLa control and ARPC2, mDia1 and Nap1 knockdown cells. Our results showed that EGF treatment had no significant effect on substrate-attached filopodia in all the knockdown cells when compared to control cells. The work developed by Innocenti's group showed that the manually counting phalloidin-positive protrusions as the number of filopodia in knockdown cells increased significantly compared to control cells (Beli et al., 2008; Isogai et al., 2015). In this situation, no distinction was made between attached and dorsal filopodia. Taken together, our data and the published data may suggest that the increase in filopodia observed in Isogai's and Beli's work could be due to an increase in dorsal filopodia formation.

Interestingly, a study on myosin-X, a dorsal filopodia inducer, showed that overexpression of this protein is required for promoting TNT formation and intercellular vesicle transfer in CAD cells, suggesting that dorsal filopodia might be a TNT precursor (K. Gousset et al., 2013). Since the overexpression of myosin-X was done for 24 hours, it is possible that the time points we used for EGF treatment in the experiments were not long enough to form TNTs properly.

Overall, our data suggests that ARPC2 may play an important role in the TNT formation in HeLa cells. More experiments need to be done in order to confirm the differential mechanisms for TNTs and filopodia formations.

CHAPTER 6

Conclusion

6.0 – Conclusion

Throughout the last years, TNTs emerged as an important mechanism of intercellular communication, facilitating the uni- and bi-directional transfer of cellular components between distant cells. TNTs can be “hijacked” by several pathogens such as bacteria and viruses and the observation of TNT-like structures *in vivo* as further increased the importance that these structures as key players in pathophysiologic conditions such as cancer, immune response and neurodegenerative diseases

In this work, we demonstrate that HeLa cells can form TNTs and proteins from the Arp2/3 complex can have an inhibitory role in TNT formation and also in intercellular transfer between HeLa cells in co-culture.

Understanding the function of the actin regulatory proteins involved in TNT formation represents a great challenge in research nowadays. Our results provide information that may help to unveil some of the mechanisms and functions of TNTs, particularly in pathological conditions, thus constituting a new way for therapy to limit or eradicate the spreading of diseases and infections in our organism.

CHAPTER 7

Future Perspectives

7.0 – Future Perspectives

To further complete this work:

Knowing that the EGF treatment promotes the increase in the TNT-connected cells, it would be interesting to perform experiments and assess the transfer of DiD labeled vesicles in both control and ARPC2, mDia1 and Nap1 knockdown cells after EGF stimulation. However, the timepoints we tested in the experiments were not long enough to properly form TNTs. For this reason, other EGF treatments should be tested in order to understand what could be the appropriate timepoint allowing the actin cytoskeleton remodeling and subsequently TNT formation in HeLa cells.

Once an appropriate time point is decided, it would be interesting to assess the differences in the number of TNT-connected cells between control and knockdown cells and investigate the signaling pathways involved in the reorganization of actin cytoskeleton mainly in the knockdown cells. For instance, WASP and WAVE proteins as well as the formin protein family can be regulated by Rho GTPase proteins, thus promoting the formation of filopodia or lamellipodia (Mattila & Lappalainen, 2008) and the EGF treatment in the knockdown proteins could affect the activation levels of such proteins.

Future working hypothesis:

It will also be important to study the role of the Diaphanous-related formin mDia2 in TNT formation. Increasing evidence suggested that mDia2 has an important role in filopodia formation (Beli et al., 2008; Yang et al., 2007). Indeed, the overexpression of mDia2 was shown to increase filopodia formation and its depletion led to defects in filopodia formation in mammalian cells (Schirenbeck, Bretschneider, Arasada, Schleicher, & Faix, 2005). It was also shown that WAVE and Arp2/3 complexes work in-concert in promoting membrane ruffling and inhibiting mDia2-inducing filopodia. It would be interesting to assess the role of mDia2 in a knockdown situation and also after an EGF treatment to better understand the relationship between filopodia and TNTs in HeLa cells.

Once we find a protein that significantly regulates TNT formation, we can assess the speed of generation and transfer of vesicles between cells by using live imaging

techniques. It would also be interesting to develop studies to evaluate whether there are differences in actin cytoskeleton between TNTs and filopodia in a higher resolution by using electron microscopy.

CHAPTER 8

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