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Investigating junctional filopodia exchange

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Metastasis is the leading cause of cancer-related deaths, driven by the ability of cancer cells to migrate from the primary tumour to other parts of the body. Filopodia, finger-like actin-rich protrusions, play a crucial role in migratory processes by facilitating interactions with the extracellular matrix (ECM) and neighbouring cells. While ECM-sensing filopodia have been extensively studied, the role of filopodia at cell-cell junctions, known as junctional filopodia, remains largely unknown. Previous unpublished results from our research group revealed that DCIS.com cells can internalise junctional filopodia. Three key proteins, Pacsin2, caveolin1 and dynamin2, were identified at filopodia invaginations on the receiving cell's membrane, suggesting their involvement in the internalisation process. These proteins are important in endocytic processes and are also involved in trans-endocytosis, a direct intercellular exchange of materials, suggesting that a similar mechanism could drive filopodia internalisation.

This study aimed to investigate whether junctional filopodia can be internalised in other cell types and whether Pacsin2, caveolin1 and dynamin2 are involved in this process. MYO10, a motor protein localised at the ends of filopodia, was used as a marker to visualise filopodia tips. U2OS cells expressing MYO10 were co-cultured with U2OS cells that had been transfected with fluorescent markers for Pacsin2, caveolin1, and dynamin2. High-resolution confocal microscopy was used to image both fixed and live cells. Additionally, to investigate Pacsin2's role in more detail, an inducible silencing of Pacsin2 was made with shRNA.

Results revealed that junctional filopodia can be internalised in U2OS cells. Live imaging showed the recruitment of Pacsin2 and caveolin1 to filopodia contact sites, suggesting their involvement in coating the emerging filopodia. The attempt to silence Pacsin2 was unsuccessful as no difference in protein levels was detected. Despite several challenges, our findings align with previous results, highlighting the need for further research into filopodia-mediated intercellular communication. Understanding filopodia dynamics could provide insights into novel pathways involved in cancer cell migration and metastasis, possibly leading to the identification of new therapeutic targets.

Keywords: junctional filopodia, Pacsin2, trans-endocytosis

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Etäpesäkkeet ovat suurin syy syöpäkuolleisuuteen, mikä johtuu syöpäsolujen kyvystä irtautua pääkasvaimesta ja levitä muihin kehon osiin. Filopodit ovat sormimaisia, pääosin aktiinista koostuvia ulokkeita, jotka ovat hyvin tärkeitä solujen liikkumiseen liittyvissä prosesseissa, mahdollistaen vuorovaikutuksen soluväliaineen ja viereisten solujen kanssa. Soluväliaineen kanssa vuorovaikuttavia filopodeja on tutkittu laajasti, mutta solujen välisissä liitoksissa olevien filopodien, niin sanottujen liitosfilopodien (eng. junctional filopodia), toiminta on edelleen suurelta osin tuntematon. Tutkimusryhmämme aiemmat julkaisemattomat tulokset osoittavat, että DCIS.com- solut voivat ottaa sisäänsä liitosfilopodeja. Kolme proteiinia, Pacsin2, kaveoliini1 ja dynamiini2, havaittiin filopodien invaginaatioissa vastaanottavan solun solukalvolla, mikä viittaa näiden proteiinien osallistumiseen filopodien internalisaatioon. Nämä proteiinit ovat tärkeitä endosyyttisissä prosesseissa, ja ne osallistuvat myös trans-endosytoosiin, jossa solut vaihtavat materiaalia suoraan keskenään. Tämä viittaa siihen, että samanlainen mekanismi voisi ohjata filopodien sisäänottoa.

Tämän tutkielman tavoitteena oli selvittää, voidaanko liitosfilopodeja sisään ottaa muissakin solutyypeissä ja tutkia Pacsin2:n, kaveoliini1:n ja dynamiini2:n osuutta tässä prosessissa. Filopodien kärkien visualisoimiseksi käytettiin merkkiaineena MYO10:tä, joka on filopodien päihin pakkautuva moottoriproteiini. MYO10:tä ilmentäviä U2OS-soluja kasvatettiin yhdessä U2OS-solujen kanssa, joihin oli transfektoitu Pacsin2:n, kaveoliini1:n tai dynamiini2:n fluoresoivat merkkiaineet. Korkean resoluution konfokaalimikroskopiaa käytettiin sekä fiksoitujen että elävien näytteiden kuvantamiseen. Pacsin2:n roolia tutkittiin lisäksi tarkemmin indusoitavalla shRNA-hiljennyksellä.

Tulokset osoittivat, että U2OS-solut voivat ottaa liitosfilopodeja sisäänsä. Elävien solujen kuvantaminen paljasti, että Pacsin2 ja kaveoliini1 kertyvät niihin solukalvon kohtiin, joissa viereisen solun filopodit tunkeutuvat soluun, ja osallistuvat muodostuvan invaginaation päällystämiseen. Pacsin2 hiljennys ei onnistunut, eikä proteiinipitoisuuksissa havaittu eroja. Useista haasteista huolimatta tulokset ovat yhdenmukaisia aiempien tutkimusten kanssa, mikä korostaa tarvetta lisätutkimuksille filopodien solujenvälisestä viestinnästä. Filopodien toiminnan syvällisempi ymmärtäminen voi tarjota uusia oivalluksia syöpäsolujen migraatioon ja etäpesäkkeisiin liittyvistä mekanismeista, ja siten auttaa uusien hoitokohteiden löytämisessä.

Avainsanat: filopodit, Pacsin2, trans-endosytoosi

Abbreviations

AMT	Amoeboid-to-mesenchymal transition
APO	Apochromat
Arp2/3	Formin and Actin related protein complex 2/3
Caveolin1	Caveolin-1
CAT	Collective-amoeboid transition
Cdc42	Cell division control protein 42 homolog
CS2	Corrected System 2
DCIS.com	Ductal carcinoma in situ cell line
DMEM	Dulbecco's Modified Eagle's Medium
Dynamin2	Dynamin-2
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
EMT	Epithelial-to-mesenchymal transition
Ena/VASP	Enabled/vasodilator-stimulated phosphoprotein
Eph	Ephrin
F-actin	Filamentous actin
F-BAR	Fes/CIP4 homology-Bin/Amphiphysin/Rvs
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HC	High contrast
I-BAR	Inverse-Bin1-Amphiphysin-Rvs domain
IRSp53	Insulin receptor substrate 53 kDa isoform B
LB	Lysogeny broth medium
MAT	mesenchymal-to-amoeboid transition
MET	mesenchymal-to-epithelial transition
MYO10	Unconventional myosin-X
NPF	Nucleation-promoting factor
N-WASP	Neural Wiskott-Aldrich syndrome protein
Pacsin2	Protein kinase C and casein kinase substrate in neurons 2
PBS	Phosphate-buffered saline

PL	Plan
Rac1	Ras-related C3 botulinum toxin substrate 1
Rpm	Revolutions per minute
RT	Room temperature
SDS-PAGE	Sodium dodecyl-sulphate-polyacrylamide gel electrophoresis
SH3	SRC Homology 3
shRNA	Short hairpin RNA
TagRFP	Tagged red fluorescent protein
TetRep	Tetracycline repressor protein
U2OS	Human osteosarcoma cell line

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1. Literature review

1.1 Cell migration

Cell migration is a fundamental cellular process, essential not only in various physiological activities, including tissue repair, embryonic development, and immune responses but also in pathological processes. Migration can be categorised into single-cell and collective migration, both having distinct cellular mechanisms and interactions with the environment. Cells alter their migratory strategies based on their phenotype and the properties of the surrounding microenvironment (Merino-Casallo et al., 2022). External environmental cues significantly influence cell shape, polarity, migration and interactions with other cells and the extracellular matrix (ECM).

Cancer, a group of diseases characterised by uncontrollable cell growth, has the potential to invade and spread to other parts of the body, forming metastases. The ability of cancer cells to metastasise remains the primary cause of mortality among patients with solid tumours, accounting for up to 90% of cancer-related deaths (Chaffer & Weinberg, 2011). Metastasis relies on cell migration, which allows cancer cells to breach physical barriers, invade surrounding tissues, and spread to distant locations. This dynamic process is regulated by complex interactions between cancer cells and their microenvironment.

The metastatic cascade begins with local invasion, where the cancer cells within the primary tumour lose their adhesion to the surrounding cells and undergo epithelial-to-mesenchymal transition (EMT) (Fares et al., 2020) (**Figure 1**). This process equips the cancer cells with increased motility and invasive properties, allowing them to penetrate the basement membrane that separates the tumour from the surrounding tissue. The next step is intravasation, during which the cancer cells enter the bloodstream or lymphatic system. The circulation can be a harsh environment, where many cancer cells fail to survive. However, groups of cells often survive and adhere more effectively to the endothelium (Friedl & Gilmour, 2009). This leads to extravasation, where cancer cells exit the circulation to invade distant tissues. The final step of the metastatic cascade is colonisation, where the cells must adapt, survive, and proliferate in the new microenvironment. This process often involves reversing EMT through a mesenchymal-to-epithelial transition (MET) to regain cell-cell interactions and create a secondary tumour.

Understanding the mechanisms of cell migration is crucial, as it provides essential insights into how cancer cells metastasise. By elucidating the complex interactions between cancer cells and their microenvironment, researchers can develop effective therapeutic strategies to improve patient outcomes and advance cancer therapy.

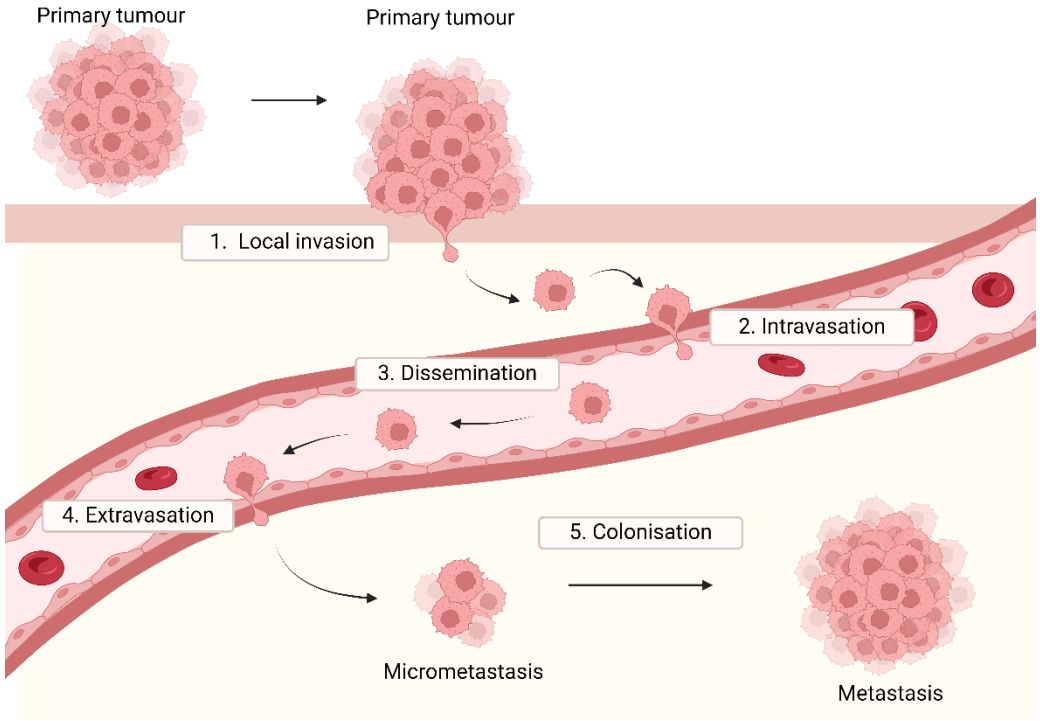

Figure 1. Illustration of the steps in the metastatic cascade. *There are five steps in the metastatic cascade: 1) Local invasion, where tumour cells break through the basement membrane and invade surrounding tissue; 2) Intravasation into the bloodstream; 3) Dissemination and survival in the circulation; 4) Extravasation to a secondary tissue; 5) Colonisation of the new environment. Modified from Bui et al. (2021).*

1.1.1 Single-cell migration

In single-cell migration, individual cells move independently through their microenvironment. This mode of migration allows cells to integrate into surrounding tissues, or travel longer distances to other parts of the body, which is especially relevant for cancer cells during metastasis (Madsen & Sahai, 2010). Single-cell migration occurs in two primary modes: amoeboid and mesenchymal (**Figure 2**).

Amoeboid migration is characterised by weak adhesions to the surrounding environment, allowing faster movement compared to mesenchymal migration. This mode can be further

subdivided into blebby and pseudopodial modes of migration (Lämmermann & Sixt, 2009). Blebby migration relies on the generation of myosin II-powered blebs, which enable fast changes in cell morphology and direction while propelling the cell forward (Yoshida & Soldati, 2006). In pseudopodial migration, cells extend protrusions at the leading edge, forming adhesions to the ECM (Friedl & Wolf, 2010). Although these adhesions are stronger than those in blebby migration, they are still relatively weak. Despite the weakness, these adhesions make pseudopodial migration faster, making it the preferred mode for leukocytes and other motile cells as they quickly navigate through various environments (Kameritsch & Renkawitz, 2020).

Mesenchymal migration relies heavily on cell-ECM adhesions, resulting in slower movement than in amoeboid migration. These adhesions are crucial for transmitting forces between extracellular and intercellular spaces. Actin polymerisation at the leading edge drives the formation of protrusions like filopodia, which probe the surrounding environment and facilitate cell-ECM adhesions. Integrins are essential proteins at the tips of filopodia that connect cells to the ECM and transmit signals from the ECM to the cell, affecting cell behaviour, adhesion, and migration (Kechagia et al., 2019). Adaptor proteins, like talin (Calderwood et al., 1999), link integrins at adhesion complexes to actin microfilaments, enabling cytoskeletal binding to the substrate. Integrins initiate the formation of nascent adhesions which can mature into focal adhesions (Wong et al., 2014). Through these focal adhesions, actomyosin stress fibre contractions generate the tensile forces necessary to propel the cell body forward (Cohen et al., 2004; Paňková et al., 2010)

A critical aspect of single-cell migration is initiating and regulating cell polarisation. Polarisation involves the asymmetric stiffening of actomyosin networks, ECM remodelling and the asymmetric distribution of cellular components. Key polarity regulators include the Rho family GTPases such as Rac1 and Cdc42, which coordinate cytoskeletal dynamics and membrane trafficking, stabilise protrusions at the leading edge and organise retraction at the rear (Nobes & Hall, 1995; Dow & Humbert, 2007; Warner et al., 2019).

Notably, cells do not only use one mode of migration. Instead, they can transition between different modes depending on the context and requirements of their environment. For instance, cells can shift between blebby and pseudopodial modes within amoeboid migration. They can also transition from pseudopodial to mesenchymal migration through the amoeboid-to-mesenchymal transition (AMT) or reverse the process via the mesenchymal-to-amoeboid transition (MAT) (Wolf et al., 2003; De Wever et al., 2004). During MAT, cells' ability to adhere

to the ECM through integrins weakens, leading to increased cellular motility as they obtain amoeboid characteristics (Carragher et al., 2006).

Additionally, the downregulation of integrins can lead to a collective-amoeboid transition (CAT), where the loss of cell adhesions causes cells to switch from collective migration to amoeboid migration, allowing continuous movement even in the absence of strong intercellular and cell-ECM interactions. This transition highlights the dynamic nature of migration strategies in response to changing conditions (e.g., collective migration).

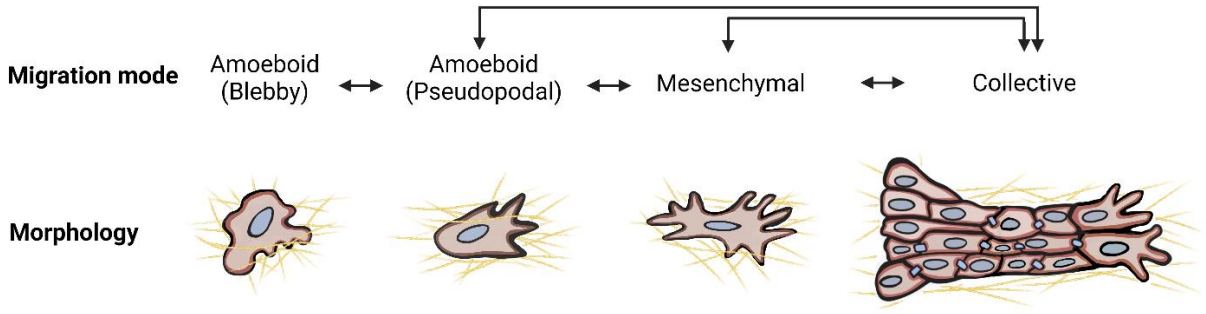

Figure 2. Schematic representation of cell migration modes, transitions, and morphologies. *Cell migration is categorised into single-cell and collective migration. Single-cell migration can be further divided into amoeboid and mesenchymal migration. The amoeboid migration includes two subtypes: blebby and pseudopodal migration. Each mode of migration is characterised by distinct features and cell morphologies. Cells can transition between different migration modes, with the most recognised transitions being mesenchymal-to-amoeboid and collective-to-single-cell migration, as indicated by brackets. Modified from Friedl & Wolf (2010).*

1.1.2 Collective migration

Collective migration plays a vital role in processes such as wound repair and embryonic development, and it is also involved in cancer invasion. In this mode of cell migration cells move as a cohesive group rather than as individuals (**Figure 2**). Like single cells, collectively migrating cells use actin- and myosin-based protrusions for movement but in collective migration, the cells form various structures, such as clusters, ducts, strands, or sheets. Friedl and Gilmour (2009) describe three main characteristics of collective migration. Firstly, the cells

must remain functionally and physically connected to maintain the integrity of cell-cell junctions. Secondly, the cells continuously remodel their cytoskeleton to produce traction and protrusion forces, crucial for migration and maintaining cell-cell adhesions. Thirdly, moving cell clusters modify the surrounding ECM by regulating its composition or degrading it to create pathways for migration.

In collective migration, cells move in a coordinated manner, communicating through chemical and mechanical signals to maintain group cohesion and direction. Strong cell-cell interactions are crucial for coordinated movement, and they help to establish front-rear asymmetry, distinguishing leader cells from followers. These adhesions are mediated by various proteins, including adherens junction proteins such as cadherins and integrins, tight junction proteins, and gap junction proteins (Goodenough & Paul, 2009; Hartsock & Nelson, 2008). Cadherins, a superfamily of calcium-dependent transmembrane proteins, are particularly important for maintaining the structural integrity of the cell group and controlling cell signalling.

The extracellular domains of cadherins mediate calcium-dependent binding between cells and the intracellular domains link cadherins to the actin cytoskeleton (Maître & Heisenberg, 2013). Epithelial cadherin (E-cadherin) is the most recognised cadherin and it is an essential component of the direction-sensing mechanism of collective migration (Cai et al., 2014). Loss or dysfunction of E-cadherin is often associated with increased cancer cell invasion and metastasis, ultimately affecting patient survival (Friedl & Gilmour, 2009; Nguyen-Ngoc et al., 2012).

At the leading edge, the leader cells generate sufficient force to pull the group forward through protrusions and focal adhesions, similar to single-cell migration. Meanwhile, the follower cells are packed together tightly to maintain the structural integrity of the cell group. They can also contribute to the movement through the “rear-wheel drive” mechanism, where they form an actomyosin ring that contracts, squeezing the cells forward and pushing the entire group in a coordinated manner (Shellard et al., 2018).

1.1.3 Collective migration in cancer invasion

Despite the traditional view of invasion as a single-cell process, most solid tumours exhibit characteristics of collective invasion (Friedl et al., 2012). While single-cell migration provides

adaptability and speed, collective migration proves to be a powerful force in cancer invasion often resulting in a worse clinical prognosis (Hart, 2009; Hou et al., 2011; Lintz et al., 2017). Invading as a cohesive group offers several advantages. Leader cells can remodel and degrade ECM components, creating paths for follower cells to migrate through (Mayor & Etienne-Manneville, 2016). Additionally, leader cells can undergo partial or transient EMT, acquiring characteristics that help to guide the direction and movement of the entire cell cluster (Friedl & Gilmour, 2009).

Collective invasion is not only a hallmark of primary tumour expansion but also a critical mechanism in metastasis, where clusters of cancer cells break away from the primary tumour, intravasate into the bloodstream or lymphatic system and establish secondary tumours at distant sites. This form of migration can offer a survival advantage to cancer cells, as in a group they are often more resistant to apoptosis and can withstand the mechanical stresses of the metastatic route better than single cells (Wang et al., 2016).

Just like in collective migration, effective collective invasion is achieved by maintaining strong cell-cell adhesions. An example is the collective invasion observed in epithelial tumours, where E-cadherin and β-catenin positive cell-cell junctions are crucial for maintaining cancer cell group integrity and coordinated movement (Friedl & Mayor, 2017). However, tumours often exhibit reduced expression of cell-adhesion genes (Nguyen-Ngoc et al., 2012). Loss of E-cadherin can trigger EMT, weakening the cell junctions and causing cells to detach from the primary tumour and go through a collective-amoeboid transition (CAT) (Thiery, 2002; Friedl et al., 2012).

Cancer invasion is highly plastic, allowing multiple types of migration to coexist within a single tumour. By harnessing the combined power of individual and collective movement, along with the use of filopodia and other protrusions to navigate and probe the surrounding tissue, cancer cells can effectively move through complex environments and establish distant metastases. Understanding the mechanisms behind collective migration in cancer invasion is essential for developing therapeutic strategies that target these pathways. By disrupting the coordinated interactions between cancer cells or cells and the ECM, it might be possible to slow down the invasive and metastatic potential of tumours, thereby improving patient outcomes.

1.2 Filopodia

Filopodia are dynamic, finger-like actin-rich membrane protrusions extending from the cell surface. They probe the surrounding environment, form adhesions, and transduce different cues and signals to the cell. Filopodia play a crucial role in cell migration processes (Jacquemet et al., 2015) like wound healing (Wood et al., 2002), angiogenesis (Gerhardt et al., 2003) and tumour progression (Jacquemet et al. 2017). Filopodia can be found at two locations: at the cell edge, where they interact with the extracellular matrix (ECM), and at cell junctions, where they are known as junctional filopodia (**Figure 3**). While cell-ECM filopodia have been extensively studied, the specific functions of junctional filopodia remain less understood, highlighting a critical area for future research.

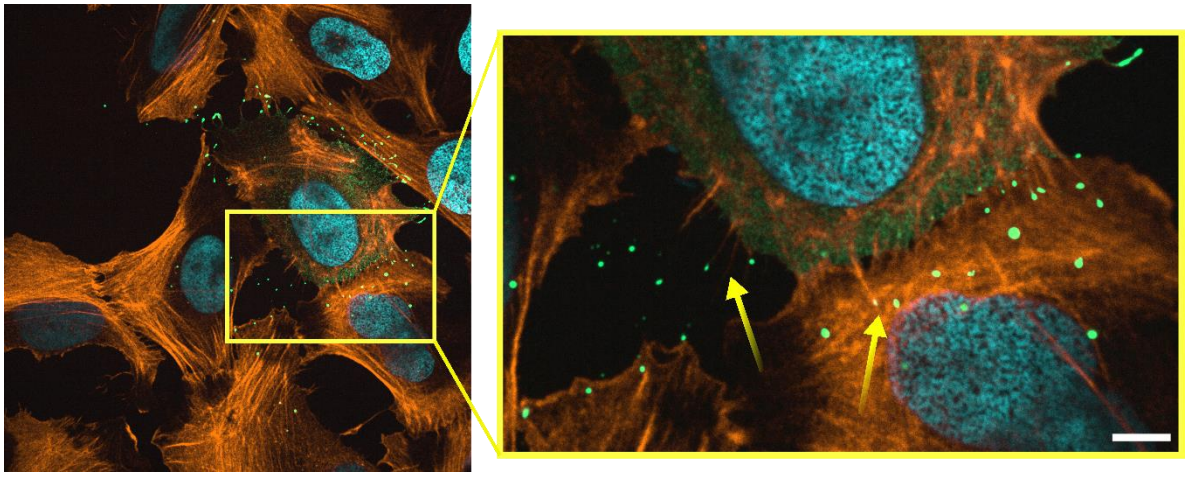

Figure 3. ECM-sensing filopodia and junctional filopodia. *The figure shows parental osteosarcoma (U2OS) cells with U2OS cells expressing MYO10, a motor protein that accumulates to filopodia tips, aiding their visualisation. MYO10 is shown in green, the actin cytoskeleton in amber and nuclei in blue. The magnified area highlights filopodia at two locations: ECM-sensing filopodia at the cell edge and junctional filopodia at cell-cell junctions, as indicated by arrows. The scalebar is 5µm.*

1.2.1 Structure and Formation

The inside of the filopodium consists of bundled F-actin filaments with their barbed ends towards the cell membrane (Blake & Gallop, 2023). Filopodia formation requires the coordinated action of various proteins (**Figure 4**), and it begins with the activation of the Actin-related protein complex 2/3 (Arp2/3) by the Wiskott-Aldrich syndrome protein (N-WASP). This initiates the formation of a small cluster of actin filaments which acts as the initial platform for

further growth (Lee et al., 2010). Actin monomers are added to the barbed ends of growing filaments by elongators such as Enabled/vasodilator-stimulated phosphoproteins (Ena/VASP) and formins located at the filopodia tip (Mallavarapu & Mitchison, 1999). The continuous polymerisation at the front and depolymerisation at the base allow filopodia to probe and sense their surrounding environment. Various signalling pathways and regulatory proteins, including Rho GTPases (e.g., Cdc42) and actin-binding proteins (e.g., profilin, cofilin), regulate the dynamics of actin polymerisation and bundling to ensure proper filopodial formation and maintenance (Cisterna et al., 2024; Hylton et al., 2022; Nobes & Hall, 1995). Fascin is a key bundling protein that tightly packs the acting filaments together creating the bundles that form the core of the filopodium (Vignjevic et al., 2006).

This actin structure acts as a route for motor proteins to move along the filaments, transporting cargo to the tips of the filopodia (Jacquemet, et al. 2015). One of these motor proteins is myosin-X (MYO10), a unique member of the myosin superfamily, which accumulates at the filopodia tips and is essential for their formation (Mattila & Lappalainen, 2008). MYO10 transports key regulators to the filopodia tips and participates in actin reorganisation. MYO10 ensures stable filopodial growth by facilitating the convergence and bundling of actin filaments at the leading edge (Tokuo et al., 2007). Pokrant et al. (2023) discovered that MYO10 is also essential for Ena/VASP clustering at the tip of actin-filament bundles. Additionally, MYO10 interacts with integrins at the filopodial tips, promoting integrin activation and enhancing cellular adhesion (Miihkinen et al., 2021).

Cell membrane remodelling is essential for filopodia formation. The membrane is reshaped and expanded around the growing bundle of actin filaments, creating the thin, finger-like structure characteristic of the filopodium. Proteins containing the Inverse-Bin1-Amphiphysin-Rvs (I-BAR) domain can generate an outward force on the membrane to form tubular compartments. For example, the membrane-bound Insulin receptor substrate 53 kDa isoform B (IRSp53) is an I-BAR protein that participates in filopodia formation by stabilising the membrane. IRSp53 interactions with the membrane recruit actin-elongating proteins like VASP, facilitating filopodial elongation (Scita et al., 2008).

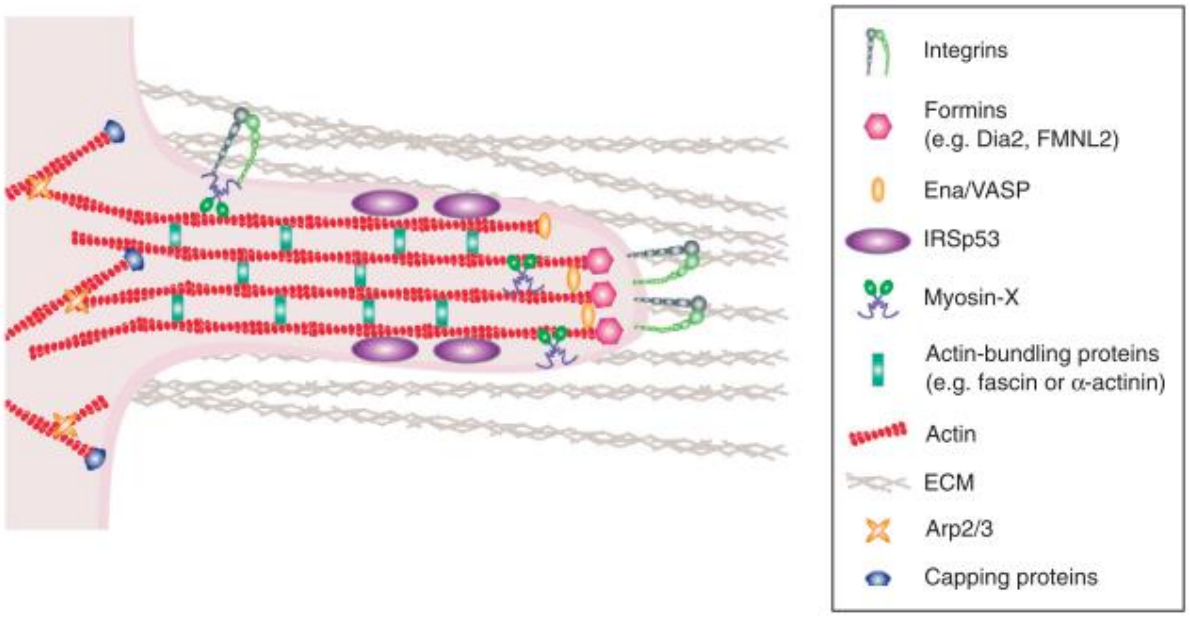

Figure 4. Schematic representation of filopodia structure. *The formation of filopodia requires multiple proteins. Arp 2/3, formins, and Ena/VASP drive the actin polymerisation at the filopodia tip, while the bundling protein fascin binds the actin filaments together. The I-BAR protein IRSp53 stabilises the membrane while capping proteins and Arp 2/3 regulate actin dynamics. The motor protein MYO10 transports cargo along the actin filaments to the tip, interacting with integrins to enhance cellular adhesion (Jacquemet et al., 2015).*

1.2.2 Filopodia in cellular interactions

Filopodia function as dynamic environmental sensors, enabling cells to detect and respond to cues from the surrounding microenvironment. A key function of filopodia is to facilitate cell adhesion and migration. They initiate contact with the ECM through adhesion molecules such as integrins, transmitting signals that influence cell behaviour, adhesion, and migration. Integrins located at the tips of filopodia form nascent adhesions, which can mature into focal adhesions depending on substrate rigidity (Wong et al., 2014). Additionally, integrin-mediated calcium signalling within filopodia not only stabilises these nascent adhesions but also promotes their maturation into strong focal adhesions. These adhesions anchor the cell to the ECM, generating the traction forces necessary for migration. (Jacquemet et al., 2016).

Filopodia are also crucial in establishing and regulating cell-cell adhesion through adherens junctions. They facilitate the initial contact and fusion of cadherin receptors, linking them with

the actin cytoskeleton to strengthen these junctions (Hayer et al., 2016). Junctional filopodia, which form at cell-cell contacts, are particularly important in the early stages of adherens junction formation, where they help to connect neighbouring cells and maintain tissue integrity.

Effective cell migration is the result of a dynamic two-way interaction between the filopodia and the ECM. Filopodia exert mechanical forces on ECM components, like hyaluronan and fibronectin, and actively remodel the surrounding environment to create pathways for movement (Kyykallio et al., 2020; Summerbell et al., 2020). Simultaneously, these ECM components provide structural cues and regulatory signals that guide filopodial extension, orientation, and stability, facilitating coordinated cell movement.

1.2.3 Filopodia in cancer invasion

In cancer, filopodia's functions extend beyond normal cellular processes and become critical in tumour progression and metastasis (Jacquemet et al., 2015). Filopodia act as sensory antennas allowing cancer cells to probe their environment, identify invasion pathways and navigate through complex tissue structures. They mediate the adhesion of cancer cells to the ECM and the endothelium of distant tissues, which is essential for processes such as extravasation and metastasis. Cancer cells often exhibit an increased number and length of filopodia which is associated with poor clinical prognosis (Jacquemet et al., 2017). This increased filopodia density facilitates invasion and metastasis by promoting ECM degradation, enhancing migration through tight spaces, and creating new adhesion sites.

Several proteins involved in the formation and structure of the filopodia are central to cancer development. For instance, fascin, an actin-bundling protein, is strongly associated with cancer progression and metastasis (Machesky & Lia, 2010). High fascin expression, often observed at the invasive edge of tumours, correlates with low levels of E-cadherin, which weakens cell-cell adhesion and potentially promotes cancer cell detachment and metastasis (Zou et al., 2010). Interestingly, in premalignant breast cancer, filopodia interactions with the ECM can sometimes support intact basement membranes, potentially inhibiting cancer cell invasion (Peuhu et al., 2022). This highlights the complex role of filopodia in different stages of cancer progression.

Although filopodia are known for their roles in cancer metastasis, their full impact on cancer progression remains unknown. Gaining a better understanding of how junctional filopodia

contribute to cancer dynamics could reveal fundamental mechanisms of cancer progression and help to identify new therapeutic targets. Disrupting the formation or function of filopodia could offer a promising way to reduce the invasive and metastatic potential of cancer cells, creating opportunities for new therapies and potentially improving patient outcomes (Jacquemet et al., 2016).

1.3 Trans-endocytosis and filopodia internalisation

1.3.1 Trans-endocytosis

Trans-endocytosis is a relatively understudied form of endocytosis that facilitates direct intercellular exchange of materials (Sakurai et al., 2014; Generous et al., 2019). This process involves protrusions, such as filopodia, which act as pathways for material transfer (Sanderlin et al., 2019; Gaitanos et al., 2016; Valenzuela & Perez, 2020).

Trans-endocytosis can occur when Ephrin (Eph) receptors on the cell's surface bind to ephrin ligands on a neighbouring cell, forming an intercellular bond that can be removed through trans-endocytosis. The activation of Eph receptors triggers Rac-dependent membrane ruffling, which forms protrusions that engulf the ephrin-expressing cell surface (Marston et al., 2003). This process leads to the internalisation of full-length ephrin proteins. Bidirectional trans-endocytosis of the ligand-receptor complex is specific to the B subfamily of Eph receptors, while in the A subfamily, trans-endocytosis occurs only with the ephrin ligand (Gaitanos et al., 2016; Valenzuela & Perez, 2020).

In addition to mediating intercellular communication, several studies have identified trans-endocytosis as a mechanism for pathogen transmission (Bishai et al., 2013; Sanderlin et al., 2019; Miernikiewicz & Dąbrowska, 2022). Sanderlin et al. (2019) revealed that the bacteria *L. monocytogenes* utilises actin-rich double-membrane protrusions that extend from the host cell to the recipient cell, facilitating the spread of bacteria without the need for them to escape the host cell. As the protrusion enters the recipient cell, it becomes surrounded by a double membrane vacuole. The rupture of this vacuole releases the bacteria into the recipient cell. A similar trans-endocytosis process occurs with the bacteria *Shigella flexneri*, although in this case, it is highly dependent on the motor protein Myosin-X found in filopodia (Bishai et al., 2013).

Furthermore, proteins such as Pacsin2 and caveolins are crucial for the trans-endocytosis of *L. monocytogenes*, as they are involved in several steps, including the regulation of protrusion engulfment (Sanderlin et al., 2019). Caveolins coordinate the formation of caveolae, specialised membrane invaginations that facilitate material transfer between cells. Pacsin2, an F-BAR protein, interacts with caveolin1 to regulate membrane sculpting, supporting the structural integrity of both caveolae and filopodia involved in the trans-endocytosis process (Hansen et al., 2011; Sanderlin et al., 2019).

1.3.2 Internalisation of junctional filopodia

Despite the growing body of research on filopodia, their role in cell-cell communication is still not fully understood and continues to be an area of active investigation (Ruhoff et al., 2024). Through connections with neighbouring cells, filopodia participate in processes such as synaptic formation in neurons and developmental signalling between cells (Ventura et al., 2022; Wit & Hiesinger, 2023). Junctional filopodia, which have received relatively little attention, could reveal previously unknown roles and further improve our understanding of cell-cell interactions.

In collective cell migration, epithelial leader cells initiate contact with follower cells by extending double-membrane protrusions from their rear. These protrusions can be engulfed by neighbouring cells, resulting in the fusion of cadherin receptors and the establishment of stable cell-cell adhesions (Hayer et al., 2016). This aligns with unpublished results from our group, the Cell Migration Lab, which suggest that junctional filopodia can be internalised by neighbouring ductal carcinoma *in situ* cells (MCF10DCIS.com or DCIS.com). This filopodia internalisation process could facilitate the exchange of signalling molecules or intracellular components between cells (Sakurai et al., 2014).

The Cell Migration Lab's unpublished results indicate that Pacsin2, caveolin1, and dynamin2 are recruited at the junctional filopodia internalisation sites, suggesting that trans-endocytosis may be the mechanism involved. In DCIS.com cells, Pacsin2 localises at the invaginated membrane where filopodia are received from neighbouring cells, enhancing membrane invagination by binding to the neck regions of caveolae. Caveolins further contribute by coating these invaginations, while dynamin2 is found at cleavage sites, facilitating membrane scission through interactions with Pacsin2. These findings build on existing literature (Marston et al.,

2003; Sanderlin et al., 2019; Valenzuela & Perez, 2020) and suggest that junctional filopodia may play a critical role in cell-cell communication through trans-endocytosis.

Understanding the mechanisms behind the internalisation of junctional filopodia can provide valuable insights into the dynamics of cell communication and offer potential therapeutic targets to prevent cancer progression. Therefore, trans-endocytosis is a critical area of research in understanding both normal cellular processes and the pathological mechanisms that drive cancer progression.

1.4 Pacsin2

Protein kinase C and casein kinase substrate in neurons (Pacsins), also known as synaptic dynamin-associated proteins (syndapins), form a protein family involved in numerous processes including intercellular trafficking, cell signalling, and the regulation of actin cytoskeleton (Dumont & Lehtonen, 2022). They are also important in clathrin-dependent and independent endocytic pathways, endosomal recycling, and endosome-to-Golgi trafficking (Dumont & Lehtonen, 2022)

There are three distinct Pacsin proteins: Pacsin1, Pacsin2 and Pacsin3. While they share structural similarities, they are distributed differently across tissues (Modregger et al., 2000). Pacsin1 is primarily associated with neuronal functions and is a key factor in neuromorphogenesis, synaptic plasticity and receptor trafficking in the brain (Plomann et al., 1998). Pacsin3 is abundant in muscle tissue (Sumoy et al., 2001), whereas Pacsin2, crucial for caveolae-dependent endocytosis, is present in most tissues (Ritter et al., 1999). This thesis will focus exclusively on Pacsin2.

1.4.1 Structure of Pacsin2

Pacsin2 consists of arc-shaped dimers that organise into a spiral structure with a central opening, facilitated by both, tip-to-tip and lateral wedge loop interactions between monomers (Bai & Zheng, 2013) (**Figure 6**). Pacsin2, along with other Pacsin proteins, has a highly conserved domain structure characterised by a Fes-CIP4 homology Bin-Amphimysin-Rvs161/167 (F-BAR) domain located in the N-terminal region and Src-homology 3 (SH3) domain in the C-

terminal region (Plomann et al., 1998; Qualmann et al.., 1999; Ritter et al.., 1999; Sumoy et al., 2001) Additionally, both Pacsin1 and Pacsin2 have a variable central region containing asparagine-proline-phenylalanine (NPF) motifs, with Pacsin1 and Pacsin2 having two and three motifs, respectively (Qualmann & Kelly, 2000).

BAR domains, including the F-BAR domains, allow proteins to recognise and modify membrane curvature to create high-curvature membrane structures. Through oligomerisation via the F-BAR domain, Pacsin2 can wrap around membrane invaginations, forming neck-like structures that support, for example, clathrin-coated pits. The F-BAR domain also interacts with actin filaments and proteins such as caveolin1 (Senju et al., 2011).

The NPF motifs and SH3 domains facilitate the main interactions with other proteins. For instance, the SH3 domain interacts with dynamin2, a GTPase involved in membrane scission, and the Wiskott-Aldrich syndrome protein (N-WASP), which stimulates the Arp2/3-complex involved in actin polymerisation (Modregger et al., 2000; Yao et al., 2014). The NPF motifs bind to proteins containing Eps15 homology-domain (EHD), such as EHD1, which plays a role in vesicle recycling (Braun et al., 2005).

Pacsin2 can be regulated through autoinhibition, where the SH3 domain interacts with the F-BAR domain, maintaining the protein in a closed conformation. Phosphorylation of residues in the central region can trigger a conformational change, activating Pacsin2 and enabling it to participate in its various cellular functions (Senju et al., 2011).

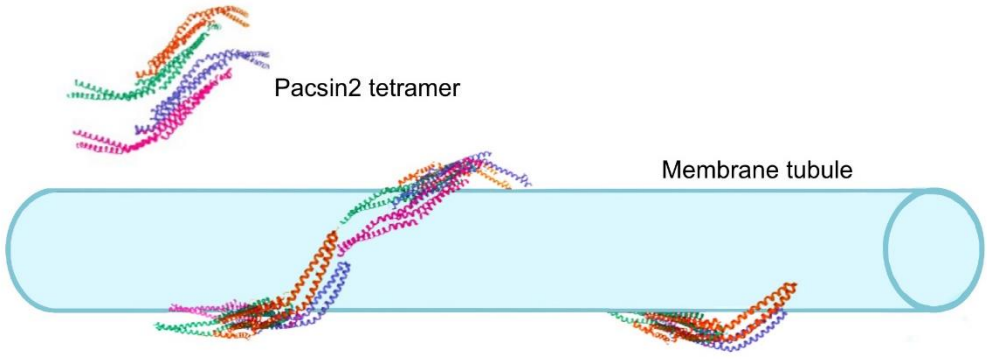

Figure 6. Molecular structure and tubulation of Pacsin2. *Pacsin2 forms dimers that organise into tetramers that wrap around membrane tubules. Modified from Bai & Zheng (2013) and Anna Nylund (2020). The Pacsin2 crystal structure (PDB ID: 3Q0K reported by Bai et al. (2012) was obtained from the RCSB Protein Data Bank (www.rcsb.org).*

1.4.2 Pacsin2 in Filopodia Dynamics

Pacsin2 regulates cytoskeletal dynamics, particularly actin polymerisation, via its interactions with the N-WASP and Arp2/3-complex (Yao et al., 2014). Interestingly, Kostan et al. (2014) demonstrated that Pacsin2's F-BAR domain can directly bind to actin filaments. The reorganisation of the actin cytoskeleton is essential for the formation of cellular protrusions like filopodia and lamellipodia, which facilitate cell movement by interacting with the extracellular environment. Besides regulating the actin cytoskeleton, Pacsin2 also stabilises the neck regions of outgoing protrusions, such as filopodia, and promotes the formation of tubular membrane invaginations (Shimada et al., 2010). While Pacsin2 contributes to protrusion formation in the donor cell, its primary function lies in membrane remodelling processes within the receiving cell (Sanderlin et al., 2019).

1.4.3 Pacsin2 in membrane dynamics

Pacsin2, together with proteins like caveolin1, regulates endocytic processes from caveolar invaginations (Senju et al., 2011). When Pacsin2 undergoes phosphorylation, it detaches from the membrane, allowing dynamin2 to take place at the invagination site where it completes the endocytosis process by cleaving the vesicles from the neck regions through its interactions with Pacsin2's SH3 domain (Senju & Suetsugu, 2015). In addition to its involvement in caveolar endocytosis, Pacsin2 also participates in clathrin-dependent endocytosis (CDE), particularly in the later stages when clathrin-coated vesicles are cleaved from the plasma membrane (Taylor et al., 2011).

Recent unpublished results from the Cell Migration Lab highlight Pacsin2's involvement in the internalisation of junctional filopodia, where it stabilises filopodia and promotes membrane curvature at the cell-cell contact sites. Together with Pacsin2, caveolin1 enhances these membrane invaginations, while dynamin2 mediates the membrane scission. The coordinated localisation of Pacsin2, caveolin1, and dynamin2 at the filopodia invagination sites suggests a mechanism similar to the one observed in trans-endocytosis, where these three proteins collaborate to facilitate protrusion engulfment (Sanderlin et al., 2019). However, these

interactions are not only present in trans-endocytosis and therefore further research is needed. Uncovering the role of Pacsin2 in filopodia dynamics could significantly increase our understanding of intercellular communication and cell migration.

1.5 Aims

Building on unpublished results from the Cell Migration Lab regarding the recruitment of Pacsin2, caveolin1 and dynamin2 to the filopodia contact sites, and their established roles in trans-endocytosis, this thesis proposes the hypothesis that these three proteins are key players in the internalisation of junctional filopodia through trans-endocytosis (**Figure 5**). While the function of these proteins is relatively well-known, the specific mechanism of junctional filopodia internalisation remains unknown, especially in other cell lines than DCIS.com. To address this gap, this study will use the human osteosarcoma U2OS cells to investigate the mechanisms involved in junctional filopodia exchange.

The specific aims of this thesis are to:

1. Determine whether U2OS cells can internalise junctional filopodia
2. Examine the roles of Pacsin2, caveolin1, and dynamin2 in the endocytosis of junctional filopodia
3. Investigate the role of Pacsin2 in junctional filopodia

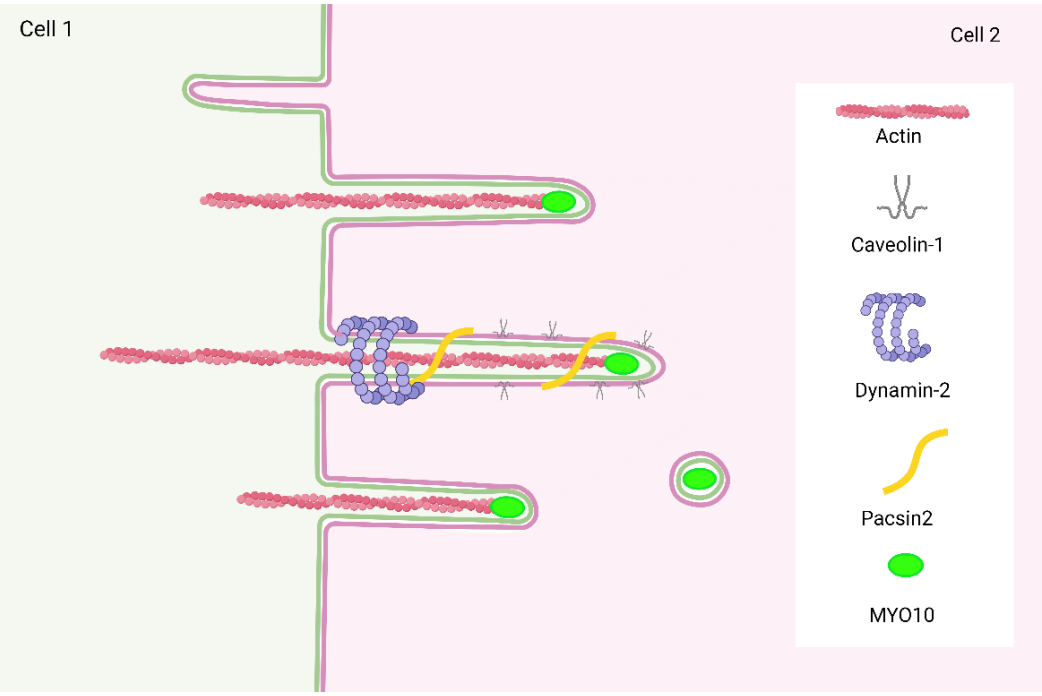

Figure 5. Schematic representation of potential events during junctional filopodia internalisation. *At cell-cell junctions, actin-rich filopodia from one cell extend towards a neighbouring cell. Primary results from The Cell Migration Lab indicate that the extending filopodia induce Pacsin2- and caveolin1-mediated invaginations at the receiving cell's membrane. Dynamin2 mediates membrane scission, leading to the internalisation of the filopodia tip and the associated proteins such as MYO10. The involvement of Pacsin2, caveolin1 and dynamin2, suggests that trans-endocytosis could be the mechanism involved in junctional filopodia internalisation.*

2. Materials and Methods

2.1 Cell culture

To study the filopodia internalisation, human osteosarcoma cells (U2-OS) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Saint Louis, USA, #D6171) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France, #S1810), 2 mM L-glutamine (Biowest Nuaillé, France, #X0550) and 100 U/µl Penicillin-Streptomycin (Sigma-Aldrich, #P0981). Cells were cultured at 37 °C with 5% CO_2 and 95% humidity.

2.2 Cell transfection

2.2.1 Plasmid amplification

The needed plasmids (Table 1) were amplified using E. coli strain DH5α for transformation. 50 µl of bacteria were combined with 10 ng of plasmid DNA and incubated on ice for 30 min, followed by a heat shock at 42°C for 30 seconds, and a 2 min incubation on ice. To recover the transformed cells, 500 µl of lysogeny broth (LB) medium was added and the mixture was incubated at 37 °C for 1h. After incubation, 100 µl of the mixture was spread onto LB agar plates containing 2% agar and 100 µg/mL of the appropriate antibiotics and incubated overnight at 37°C. A control plate was made using non-transformed bacteria. The following day, if the control was negative, individual colonies were picked and grown overnight at 37 °C on a shaker (220 rpm) in 5 ml of LB media supplemented with the same antibiotic at a concentration of 100 µg/ml. 100 µl of the culture was then added into 1 l Erlenmeyer flask with 100 ml of LB media supplemented with the same corresponding antibiotic (1:1000) and then incubated overnight at 37 °C on a shaker (220 rpm). The following day, the cultured bacteria were centrifuged at 6000 x g for 4 min at 4°C. The supernatant was discarded, and the plasmids were purified using the NucleoBond Xtra Midi EF kit (Macherey-Nagel, Düren, Germany, #740420.50). The concentrations of the purified plasmids were determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

Table 1. DNA constructs

Plasmid	Encoded Product	Origin
MYO10ΔFERM mScarlet	MYO10ΔFERM mScarlet	Gift from Ivaska Lab
Caveolin-RFP	Caveolin1-RFP	Gift from Ivaska Lab
Dynamin2 EGFP	Dynamin2-GFP	Gift from Ivaska Lab
MYO10 GFP	MYO10-GFP	Gift from Ivaska Lab
PACISN2 N-EGFP	Pacsin2-GFP	Gift from Ivaska Lab
pMDL.g/pRRE	Gag-Pol polyprotein	Addgene #12251
pRSV-Rev	Rev protein	Addgene #12253
pMD2.g	VSV-G	Addgene #12259
pRSIT16-U6Tet-(sh)-CMV-TetRep-2A-TagRFP-2A-Puro	Inducible shRNA, TetRep, TagRFP, Puromycin resistance	Cellecta #CVSHC-PX

2.2.2 Transient transfections

U2OS cells were transfected with Lipofectamine 3000™ reagent (Thermo Fisher Scientific, L3000015). Two Eppendorf tubes were prepared for each transfected plasmid by adding 250 µl of Opti-MEM (Gibco, Texas, USA, #31985070) to each tube. Lipofectamine and P3000 were added to separate tubes in a 1:50 ratio. 2 µg of plasmid was added to the P3000 tube. After 5 min incubation at room temperature (RT), the lipofectamine tube with Opti-MEM-lipofectamine mixture was transferred to the P3000 tube. After 20 min incubation RT the mix was added to cells plated on a 6-well-plate. The cells were collected from a confluent 10 cm culture dish by trypsinisation and plated on a 6-well plate with 250 000 cells/well. The cells were incubated overnight under normal culture conditions.

2.3 Co-culturing cells

To study the filopodia exchange, U2OS cells were transfected with fluorescent-tagged proteins to visualise the internalised filopodia and the structures around it. MYO10 helps to visualise the filopodia tips which is why transfected U2OS was co-cultured with U2OS expressing MYO10 (MYO10GFP, MYO10dFERM mScarlet). All transfections were done using lipofectamine and the samples were stained for actin. To see if the junctional filopodia of U2OS can be internalised, U2OS MYO10GFP was co-cultured with parental U2OS and stained for F-actin. The involvement of Pacsin2, dynamin2 and caveolin1 in the endocytosis of junctional filopodia was examined by co-culturing U2OS MYO10GFP cells with U2OS transfected with these fluorescent-tagged proteins.

2.3.1 Sample preparation

For fixed samples, sterile coverslips were added to a 24-well plate (Greiner Bio-One™, Kremsmünster, Austria, 662160) and coated with 0.01% Poly-L-Lysine (Merck Life Science, Darmstadt, Germany, #A-005-C), incubated for 45 minutes at room temperature (RT), and then washed three times with PBS. For live samples, a µ-Slide 8 Well Glass Bottom Ibidi dish (Ibidi,

Grafelfing, Germany, #80827-90) was used. Both fixed and live samples were subsequently coated with Fibronectin (Sigma-Aldrich, #341631) diluted 1:100 in PBS and incubated for 1.5-2 hours at 37°C or overnight at 4°C. After incubation, the wells were washed three times with PBS.

After coating, trypsinised (Biowest, Nuaillé, France, #L0931) transfected cells were mixed and plated onto the prepared coverslips at a density of 250,000 cells per coverslip for fixed samples, or directly into the wells at 175,000 cells per well for live samples. To achieve the desired final density, appropriate media volumes were added (1 ml/well for fixed and 250 µl/well for live). The cells were incubated for 24-48 hours at 37°C. U2OS cells were grown in Dulbecco's Modified Eagle′s Medium (DMEM) (Sigma-Aldrich, #D6171) supplemented with 10% fetal bovine serum (FBS) (Biowest, Nuaillé, France, #S1810), 2 mM L-glutamine (Biowest Nuaillé, France, #X0550) and 100 U/µl Penicillin-Streptomycin (Sigma-Aldrich, #P0981).

For fixed samples, the cells were then fixed with 4 % PFA (Thermo Fisher Scientific, #28908) for 10 min RT. The coverslips were washed three times with PBS and transferred into a wet chamber. The coverslips were treated with 1 M Glycine for 1 h RT, washed three times with PBS, and incubated with 0,025% saponin (Thermo Fisher Scientific, #11453098) for 1h RT. After incubation, the actin cytoskeleton was stained with Alexa Fluor™ Plus 647 Phalloidin (Invitrogen, Massachusetts, USA, #A12379) diluted 1:1000 in PBS and incubated for 2 hours at RT in the dark. The coverslips were washed three times with PBS + 0,025 % saponin and mounted on slides with Mowiol 4-88 (Sigma-Aldrich, #475904). After drying overnight at RT in the dark the samples were ready to image.

For live samples, two hours before live imaging, the media was changed to F-actin dye Sir-Actin (Spirochrome, Stein am Rhein, Switzerland, #SC001) diluted 1:1250 in fresh media. Just before imaging, the media was changed to ensure optimal conditions.

2.3.2 Image acquisition and processing

The images were obtained with high-resolution confocal microscopes. The fixed images were obtained with a Marianas spinning-disk system equipped with a Yokogawa CSU-W1 scanner on a Zeiss Axio Observer Z1 inverted microscope, controlled by SlideBook 6 software. For

imaging, the Orca Flash 4 sCMOS camera (2,048 × 2,048 pixels) was used with a 63x oil immersion objective (NA 1.4, Plan-Apochromat).

Live imaging was performed with a Leica Stellaris 8 confocal microscope. A tuneable white light laser (440-790 nm) was used with a 63x oil immersion objective (NA 1.4, HC Plan-Apochromat, CS2). Confocal detection was achieved using a Leica's HyD (Hybrid Detector) system, consisting of two HyD S and HyD X detectors for standard high-sensitivity imaging with improved dynamic range and weak signal detection, as well as one HyD R detector for near-infrared detection. The system was controlled with Leica's LAS X software.

The images were processed and analysed with Fiji (Schindelin et al., 2012). Brightness and contrast adjustments were applied to each channel, followed by the selection of appropriate Look-Up Tables (LUTs) to optimise colour representation. Filter such as Median 3D was applied if necessary to enhance image clarity. The multi-channel images were then merged, and figures were made using Inkscape (Harrington, 2004–2005).

2.4 Silencing Pacsin2

A PACSIN2 inducible silencing was created using shRNA by incorporating a short hairpin RNA sequence targeting the PACSIN2 gene. When induced, the shRNA is transcribed and processed, which leads to the degradation of PACSIN2 mRNA, effectively silencing the expression of the PACSIN2 gene. This inducible system allows for controlled timing of gene knockout, which is crucial because previous results suggest that knocking out PACSIN2 stops the cell cycle, making it unsuitable for creating a stable cell line. The shRNA was delivered into cells using a lentivirus-based system.

The viral production work was conducted in a biosafety level 2 (BSL II) laboratory. To produce the viral material, HEK293T cells were seeded at 2,000,000 cells/dish with DMEM + 10 % FBS. The cells were grown until they reached >80% confluence. The media was then aspirated, and the cells were washed briefly with PBS before adding 8000 µl Opti-MEM™ (Gibco, #31985070) serum-free media. DNA packaging plasmids (pMDL.g/pRRE, pRSV-Rev, pMD2.g) and the transfer plasmid (pRSIT16-U6Tet-(sh)-CMV-TetRep-2A-TagRFP-2A-Puro) (Table 1) were mixed thoroughly with 500 µl OptiMEM, with plasmid amounts specified in Table 2. The transfer plasmid (pRSIT16-U6Tet-(sh)-CMV-TetRep-2A-TagRFP-2A-Puro) was

ordered pre-constructed, with the shRNA sequence targeting Pacsin2 inserted into the cloning site. The plasmid map is provided in the attachments (Attachment 1). Afterwards, 50 µl of Lipofectamine 3000™ reagent (Thermo Fisher Scientific, L3000015) was added and the transfection mixture was incubated for 15 minutes at 37 °C. The mixture was added to the cells and incubated overnight at 37 °C and 5 % CO_2. The following day, the media was changed to an appropriate media for the cell line to be transduced. 24 hours after the media change, the viruses were collected from the media using a syringe and a 0,45 µm syringe filter. The viruses that were not used immediately were stored at – 80 °C in 1 ml aliquots.

DCIS.com cells were seeded in a 6-well plate at a low confluence (20-40%) on the day of infection. The virus suspension was diluted to achieve a final 8 µg/ml concentration. Polybrene (Sigma-Aldrich, 638133) was added to the diluted virus to a final concentration of 8 µg/ml with a total volume of 2 ml to enhance infection efficiency. The media was aspirated from the cells, and the diluted virus was added. After 24 hours, the media was changed, and after 72 hours, the antibiotic selection was initiated using puromycin (InvivoGen, Toulouse, France, 58-58-2).

The cells had to be negative for lentivirus before proceeding to cell sorting, which took 5-10 passages. The cells were then sorted using fluorescence-activated cell sorting (FACS) with the SH800S Cell Sorter (Sony Biotechnology Inc., California, USA). In this process, individual cells pass through a narrow tube where a laser detects their fluorescent signal, allowing sorting based on fluorescence intensity resulting in two distinct groups: the silenced cells and the remaining non-silenced cells. The transfected cells were identified and sorted by red fluorescence, as the transfer plasmid contained a red fluorescent marker.

After sorting, the cells were allowed to rest for several days. PACSIN2 silencing was then induced with doxycycline in a 1:1000 ratio (Sigma-Aldrich, 24390-14-5). Following induction, the cells were lysed at 24 and 72 hours, and the success of the knockout was validated using Western blot.

Table 2. Plasmid amounts for viral packaging and transfection

Plasmid	**Per 10 cm dish (ng)**
pRSIT16-U6Tet-(sh)-CMV-TetRep-2A-TagRFP-2A-Puro	15000
pMDL.g/pRRE	4455
pRSV-Rev	6435
pMD2.g	2772

2.5 Cell lysis

During cell lysis, all procedures were done on ice with ice-cold reagents. The medium was discarded, and the wells were washed three times with PBS. Lysis buffer (25 mM HEPES, 150 mM NaCl, 1 % Triton-X-100, 0,1 % SDS) with protease inhibitor (Thermo Fisher Scientific, #A32953) was added in wells and the cells were scraped. All liquid was collected in Eppendorf tubes, which were then centrifuged at +4 °C at 16 000 g for 1-2 minutes. The supernatant was collected to new Eppendorfs, and the sample buffer (4 % SDS, 20 % glycerol, 0,004 % bromophenol blue, 0,125 M Tris-Cl, 10 % DTT) was added in a 1:1 ratio. The samples were boiled at + 95 °C for 5 min before use or kept at -20°C until later use.

2.6 SDS-PAGE and Western blot

The proteins were separated using sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Ready-made NovexTM WedgeWellTM 4-12% Tris-Glycine gels (Invitrogen, Massachusetts, USA, #XP04120BOX) were used, and the samples were run in a Mini Gel Tank (Invitrogen). The proteins were transferred using the Mini Blot module (Invitrogen) onto an AmershamTM ProtranTM supported 0.45 µm nitrocellulose blotting membrane (Cytiva, Massachusetts, USA, #10600016).

The success of the transfer was tested with Ponceau S (0,1% Ponceau S in 5 % acetic acid), followed by blocking non-specific binding sites on the membrane with StartingBlock™ blocking buffer (Thermo Fisher Scientific, #37538) for 30 minutes at RT. The primary antibody, Pacsin2 rabbit (Sigma-Aldrich, HPA049854), was diluted 1:1000 in a mixture of blocking buffer and PBS (1:1), added to the membrane and incubated overnight at +4°C. The membrane was washed three times with PBS for 5 minutes on a roller. The secondary antibody, Alexa Fluor™ 488 anti-rabbit (Thermo Fisher Scientific, AB-143165), was diluted 1:5000 in the blocking buffer-PBS-mixture (1:1) and incubated for 2h at RT. After washing the membrane three times with PBS for 5 minutes the membrane was also stained for GFP and α-tubulin (Hybridoma Bank (DSHB), Iowa, USA, AB-1157911) which was used as a loading control.. After immunostaining, the membrane was imaged using iBright FL1000 (Thermo Fisher Scientific).

To estimate Pacsin2 protein levels, the intensity profiles of each Western blot lane were analysed using histograms created with ImageJ software. The peak area of each histogram provided a semi-quantitative assessment of Pacsin2 protein levels. Pacsin2 protein measurements were normalised to the intensity of the loading control signal.

3. Results

3.1 Filopodia exchange in U2OS cells

Previous unpublished results from The Cell Migration Lab showed that junctional filopodia can be internalised in the DCIS.com cells (**Figure 7A**). However, this process has not been studied on other cell types. To address this, we aimed to investigate whether U2OS cells can also engage in junctional filopodia internalisation. U2OS cells expressing MYO10-GFP were co-cultured with parental U2OS cells for 24 hours, followed by fixation and staining for F-actin. Imaging was performed using a spinning disk confocal microscope.

We observed that junctional filopodia can penetrate neighbouring cells even without full internalisation and that U2OS cells are capable of internalising junctional filopodia. ECM-sensing filopodia were observed at the cell-extracellular matrix (ECM) interface (**Figure 7B(a)**), interacting with the surrounding environment. In **Figure 7B(b)**, a junctional filopodium enters a neighbouring cell, with its tip containing MYO10 visible inside the cell. However, full internalisation has not yet happened, as MYO10 remains attached to the filopodium. In contrast, **Figure 7B(c)** demonstrates a fully internalised filopodium, where MYO10 has completely detached from the filopodium and is located within the neighbouring cell, confirming that U2OS cells are capable of internalising junctional filopodia. These observations suggest a stepwise process, where filopodia initially penetrate neighbouring cells before fully detaching and becoming internalised.

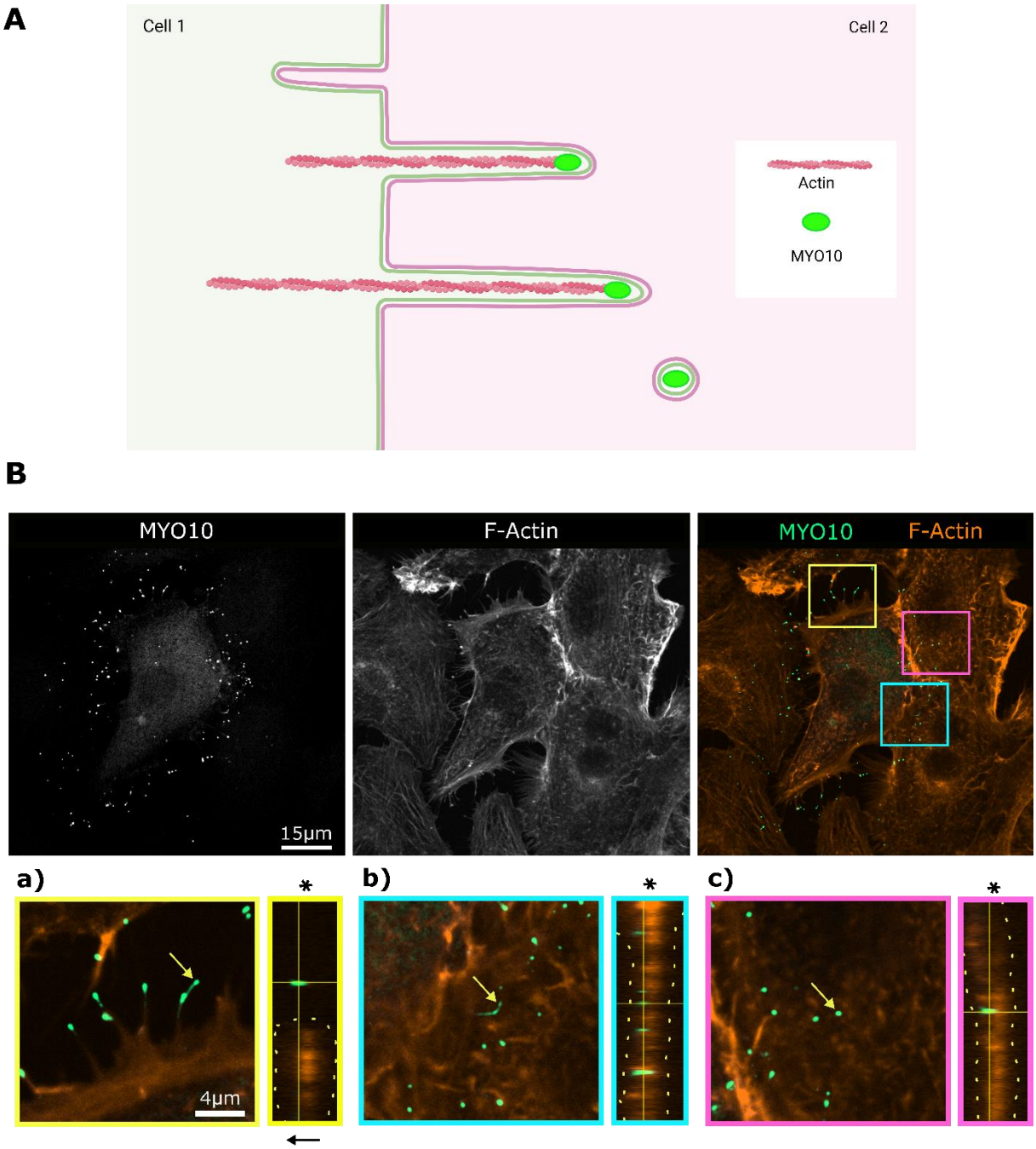

Figure 7. Junctional filopodia exchange in U2OS cells. *A) Schematic representation of junctional filopodia internalisation. U2OS cells expressing MYO10 (cell 1) and adjacent parental U2OS cells (cell 2) extend filopodia towards each other, creating an invagination at the receiving cell's membrane. The tip of the filopodium is internalised with MYO10 expression visible inside the receiving cell, detached from the filopodium. B) U2OS cells expressing MYO10-GFP were co-cultured with parental U2OS cells for 24 h on fibronectin. The cells were then fixed and stained for F-actin and imaged using a spinning disk confocal microscope (63x magnification). MYO10 and F-actin are shown separately and as a merged image in the top row. Coloured boxes represent regions of interest. a) Cell-ECM filopodia at the cell-ECM interface. b) Intracellular filopodia, with the MYO10 signal visible along the length of the filopodium. c) Internalised junctional filopodia, where MYO10 is detached from the filopodium and*

located inside the neighbouring cell. Yellow arrows highlight points of interest, and orthogonal views from the y/z plane are shown on the right side of each image, with yellow lines also indicating the points of interest. A black arrow points toward the top of the cell, and the dotted yellow line outlines the cell. The asterisk (*) indicates the sample thickness of 5.4 µm.

3.2 The involvement of Pacsin2 and caveolin1 in the internalisation of junctional filopodia

3.2.1 Recruitment of Pacsin2

Considering Pacsin2's established role in membrane invaginations and trans-endocytosis (Shimada et al., 2010; Hansen et al., 2011; Sanderlin et al., 2019), along with previous results suggesting its recruitment at filopodia contact sites (**Figure 8A**), we wanted to further investigate its role in the internalisation of junctional filopodia. To do so, we co-cultured U2OS cells transiently expressing MYO10ΔFERM mScarlet with U2OS cells expressing Pacsin2-GFP for 24 hours. MYO10ΔFERM, a modified version of MYO10 lacking the FERM domain, was chosen due to the unavailability of functional red Pacsin2 or MYO10 plasmids. Previous data from our group suggests that the lack of the FERM domain does not impact the functionality of filopodia.

Live imaging using a confocal microscope revealed filopodia expressing MYO10ΔFERM mScarlet entering the neighbouring cell and the Pacsin2-GFP signal gradually intensifying along the length of the filopodia (**Figure 8B**). This accumulation of Pacsin2 suggests that it may be recruited to the filopodia during their extension, potentially facilitating their internalisation into adjacent cells.

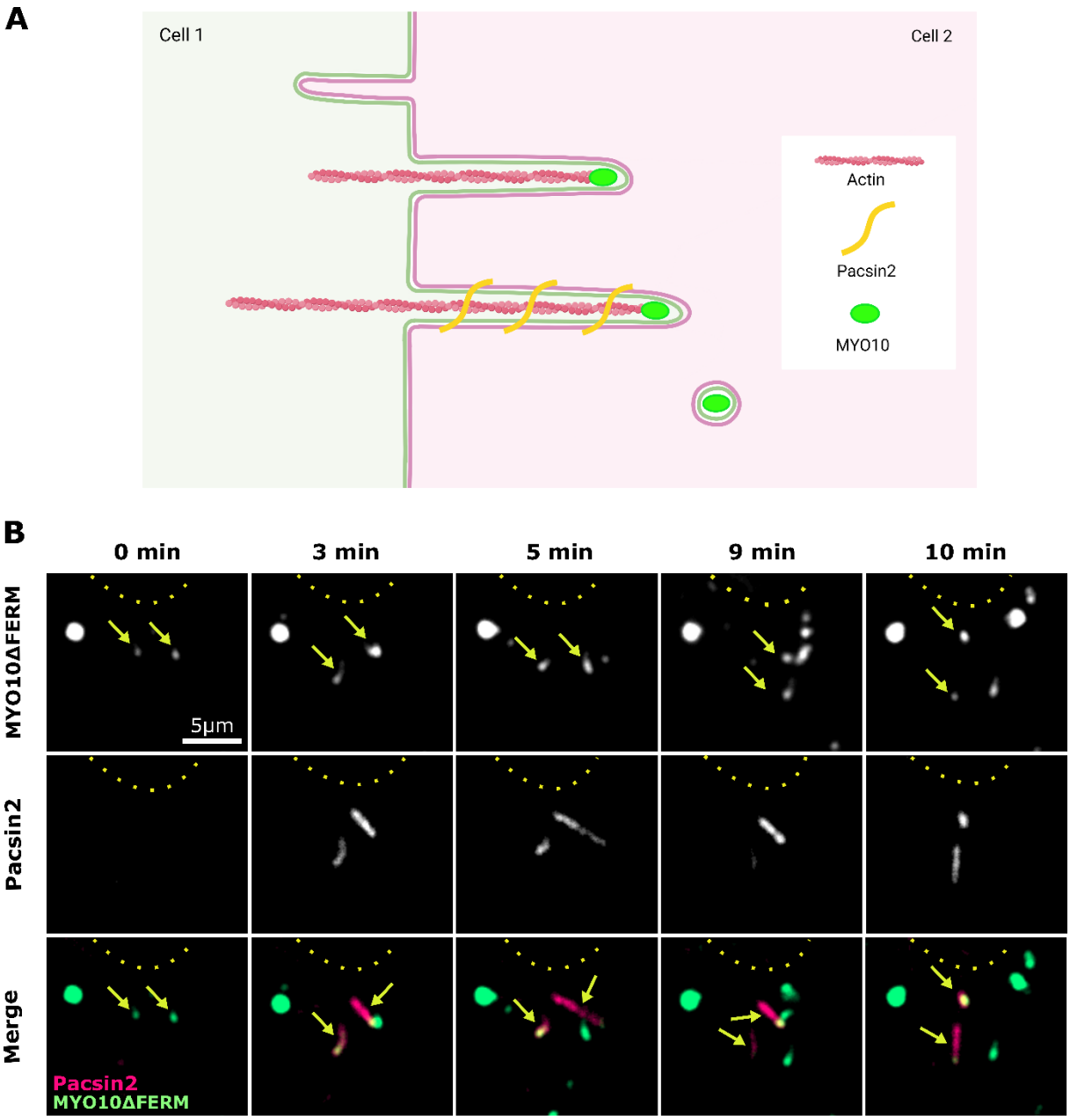

Figure 8. Pacsin2 coating of junctional filopodia. *A) Schematic representation of the experimental setup and Pacsin2 coating of filopodia. U2OS cells expressing MYO10ΔFERM (cell 1) and adjacent U2OS cells expressing Pacsin2 (cell 2) extend filopodia towards each other, creating an invagination at the receiving cell's membrane. Pacsin2 is recruited to the contact site where it coats the invagination. B) U2OS cells expressing MYO10ΔFERM mScarlet were co-cultured with U2OS cells expressing Pacsin2-GFP for 24 hours on fibronectin. Live imaging captured filopodia as they emerged, penetrated the neighbouring cell, and became progressively coated with Pacsin2. Yellow arrows highlight key areas of interest, and the yellow dotted line outlines the borders of neighbouring cells. The top cell expresses MYO10ΔFERM, and the bottom cell expresses Pacsin2. Images were acquired using a Leica confocal microscope at 63x magnification.*

3.2.2 Recruitment of Caveolin1

Given the known role of caveolin in endocytosis and trans-endocytosis (Busija et al., 2017; Hansen et al., 2011; Sanderlin et al., 2019), along with its previously reported localisation at filopodia contact sites (**Figure 9A**), we attempted to further explore its potential involvement in the internalisation of junctional filopodia. We co-cultured U2OS cells transiently expressing MYO10-GFP with U2OS cells expressing Caveolin1-RFP and after 24 hours the cells were imaged live using a confocal microscope. We observed caveolin1 coating the intercellular filopodia (**Figure 9B**). The fluorescence signal of caveolin1 consistently surrounds the filopodia during both their extension and retraction phases. MYO10 retracted with the filopodia, suggesting that the tip was not internalised. Additionally, caveolin1 is not evenly expressed in all filopodia, as other MYO10 fluorescence can be seen in the images, indicating that internalisation is not a universal event for all filopodia.

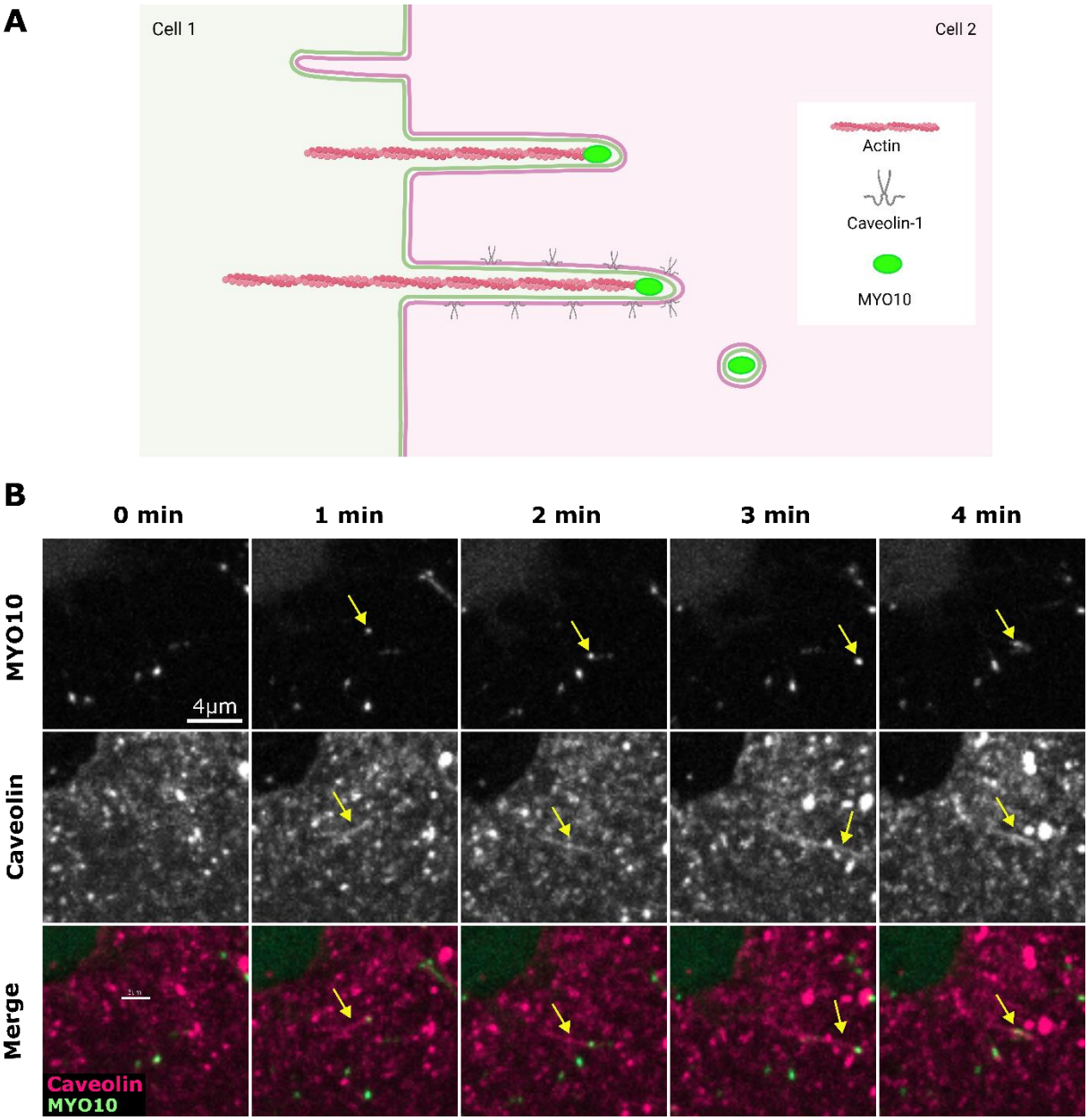

Figure 9. Caveolin1 coats intracellular filopodia. *A) Schematic representation of the experimental setup and the caveolin1 coating of filopodia. U2OS cells expressing MYO10 (cell 1) and adjacent U2OS cells expressing caveolin1 (cell 2) extend filopodia toward each other, inducing an invagination at the receiving cell's membrane. Caveolin1 is recruited to the contact site where it coats the forming invagination. B) U2OS cells expressing MYO10-GFP were co-cultured with U2OS cells expressing Caveolin1 RFP for 24 hours on fibronectin. Live imaging shows caveolin1 coating the total length of the intracellular filopodium as it extends and retracts. Yellow arrows indicate the points of interest. Images were acquired using a Leica confocal microscope at 63x magnification.*

3.2.3 Caveolin1 colocalises with MYO10

While investigating caveolin1's recruitment on internalised filopodia, U2OS cells expressing MYO10-GFP and Caveolin1-RFP were co-cultured for 24 hours and imaged live using a confocal microscope. Interestingly, several MYO10 puncta were observed to colocalise with caveolin1-coated vesicles (**Figure 10**). However, colocalisation was not observed in all caveolin1-positive vesicles, suggesting that MYO10 could be internalised in some but not all vesicles.

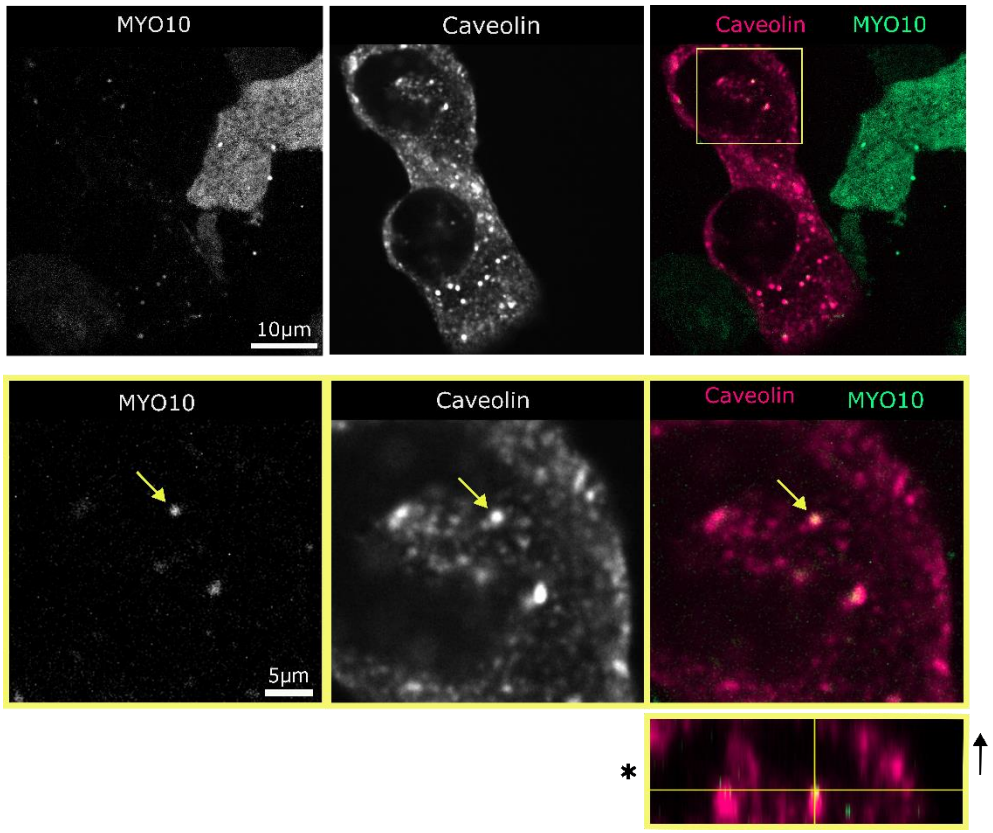

Figure 10. Caveolin1 colocalisation with MYO10. *U2OS cells expressing Caveolin1-RFP were co-cultured with U2OS cells expressing MYO10-GFP for 24 hours on fibronectin. Live imaging was performed using a Leica confocal microscope at 63x magnification. The region of interest is magnified in the yellow square and the yellow arrows indicate points of interest. An orthogonal view of the x/z plane is shown at the bottom of the merged image, with yellow lines marking the same point of interest. A black arrow points towards the top of the cell, and an asterisk (*) indicates a sample thickness of 6 µm.*

3.3 Unsuccessful silencing of Pacsin2

To further investigate the role of Pacsin2 in junctional filopodia, we attempted to develop an MCF10DCIS.com cell line with inducible Pacsin2 silencing using a shRNA construct delivered via lentivirus. After transduction, cells were sorted using FACS to isolate those that were successfully transduced. The silencing was then induced with doxycycline and the samples were collected 24- and 72-hours post-induction. The silencing's success was confirmed by Western blot analysis.

The silencing of Pacsin2 turned out to be unsuccessful. Despite sorting the transfected cells with FACS, no fluorescence was observed at either time point following doxycycline induction (**Figure 11A**). Western blot analysis confirmed that there is no difference in the Pacsin2 levels compared to the non-induced sample or the parental cell control (**Figures 11B & 11C**). Although changes in relative protein levels can be observed in parental cells (**Figure 11C**), these are probably only due to normal variation, which is likely to disappear with more repeats. Due to time constraints, no repeats were conducted, and therefore no statistical analysis was performed.

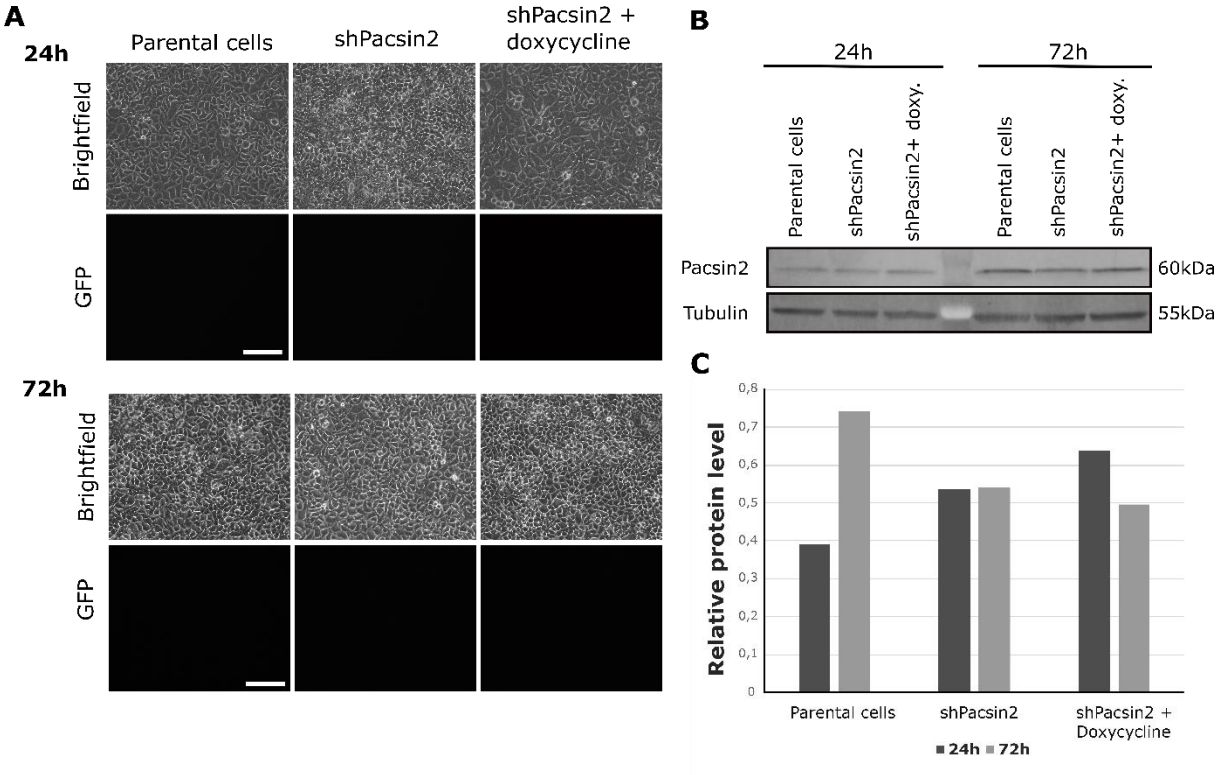

Figure 11. Unsuccessful silencing of Pacsin2. *Inducible silencing of Pacsin2 was attempted in DCIS.com cells using shRNA. Cells were sorted by FACS, and the success of the silencing was validated via western blot. Cells were lysed at 24- and 72-hours post-induction. Parental cells (control), uninduced shPacsin2, and shPacsin2 after doxycycline induction were analysed. A) Brightfield and red fluorescence images of samples at both time points showing no fluorescent cells. Images were captured with a Leica DM IRBE microscope (10x magnification). The scalebar is 600 µm. B) Western blot membrane of Pacsin2 silencing shows the three samples at 24- and 72-hour time points, with α-Tubulin as a loading control. C) Bar chart showing the relative Pacsin2 protein levels in each sample at both time points. Due to the lack of repeats, no statistical analysis is presented.*

4. Discussion

4.1 Internalisation of junctional filopodia

While most studies on filopodia focus on cell-ECM filopodia, the function of junctional filopodia remains largely unknown. Unpublished data from The Cell Migration Lab reveals that junctional filopodia can be internalised in DCIS.com cells. This phenomenon has not been observed in other cell types, and therefore the first aim of this thesis was to investigate whether U2OS cells also engage in junctional filopodia exchange. Our findings confirm that junctional filopodia are internalised as MYO10 is detached from the filopodium and is visible within the receiving cell. Interestingly, the internalisation appears to be selective as some filopodia tips are fully internalised, while others remain intracellular and are not internalised.

Unfortunately, these results were not quantifiable due to the lack of data. Technical difficulties, time constraints and the unavailability of functional plasmids prevented us from providing a proper quantitative analysis. A critical issue was the lack of a functional plasmid, such as F-actin expressing Lifeact (Riedl, et al., 2008), that could have helped to visualise the filopodia and to distinguish MYO10-negative receiving cells from MYO10-positive ones. As a result, we cannot be entirely sure that the MYO10 observed within the receiving cells originated from the MYO10-positive donor cells.

Despite the challenges and the lack of quantification, we provide evidence that junctional filopodia are internalised in U2OS cells, confirming a previously observed process in DCIS.com cells. The selective nature of this internalisation, where some filopodia tips are fully

internalised while others are not, adds valuable insights into filopodia dynamics but also raises questions about how frequently the internalisation process occurs. This selective internalisation could also be related to mechanisms similar to those described by Sorkin and Von Zastrow (2009), where selective endocytosis enables cells to regulate signalling and the exchange of materials effectively. Nevertheless, filopodia internalisation opens interesting possibilities for how cells might regulate communication and material transfer. Further research should focus on determining whether this internalisation represents a mechanism for transferring filopodial tip material or if it is simply a by-product of cell-cell interactions.

4.2 Recruitment of Pacsin2 and Caveolin1

The second aim of this thesis was to investigate the roles of Pacsin2, caveolin1 and dynamin2 in junctional filopodia internalisation. These three proteins are known players in various cellular processes related to membrane dynamics and endocytosis (Hansen et al., 2011; Senju & Suetsugu; Sanderlin et al., 2019). Pacsin2 and caveolin1 mediate membrane curvature and stabilise forming invaginations, while dynamin2 completes the endocytosis by cleaving out the vesicles (Gundu et al., 2022). Live imaging data revealed that Pacsin2 and caveolin1 coat the filopodia as they penetrate neighbouring cells. The recruitment of Pacsin2 and caveolin1 in U2OS cells aligns with previous results from the Cell Migration Lab on DCIS.com cells, indicating that this event is not exclusive to DCIS.com cells. These findings also align with several other studies that demonstrate Pacsin2 and caveolin1 localising at membrane invaginations (Hansen et at., 2011; Sanderlin et al., 2019; Senju et al., 2011).

The observed recruitment of Pacsin2 and caveolin1 closely resembles their roles in the trans-endocytosis of *L. monocytogenes*-containing protrusions (Sanderlin et al., 2019). However, the presence of Pacsin2 and caveolin1 alone does not confirm that junctional filopodia undergo trans-endocytosis. Instead, our results suggest that these proteins may be involved in maintaining the structural integrity of filopodia invaginations during intercellular interactions, rather than directly facilitating their internalisation. This is supported by the absence of internalised MYO10 together with Pacsin2 and caveolin1. Finally, the lack of data regarding the interaction between these proteins and filopodia during internalisation suggests that alternative mechanisms could cause filopodia and MYO10 internalisation.

4.3 Colocalisation of caveolin1 and MYO10

While it is known that filopodia can be internalised, the fate of the internalised material post-scission remains unclear. Specifically, whether the internalised tip remains within a caveolin1-coated vesicle or undergoes further processing is unknown. The colocalisation of MYO10 and caveolin1 suggests that the filopodium's internalised tip could remain in a caveolin1-coated vesicle, indicating a potential role for caveolin1 in stabilising or trafficking this material. This observation is interesting but not entirely unexpected, as caveolin1 is known to participate in vesicle formation and trafficking (Matveev et al., 2001; Parton & Del Pozo, 2013; Busija et al., 2017). However, due to challenges with actin and membrane staining, it remains uncertain whether these MYO10 puncta are truly internalised or still attached to the filopodium.

If the MYO10-associated tip material indeed remains within a caveolin1-coated vesicle, it could suggest a role for caveolin1 in the vesicle-mediated processing of internalised filopodia tips. Whether the internalised tip material undergoes degradation, recycling, or participates in further intracellular signalling events, processes typically associated with caveolae-mediated endocytosis (Parton & Del Pozo, 2013), remains to be explored in future studies. Notably, previous research on trans-endocytosis demonstrated that caveolin1-coated vesicles ruptured after bacterial internalisation (Sanderlin et al., 2019), raising questions about whether a similar vesicle rupture is unique to bacterial infection or could also occur during filopodia internalisation.

Since no quantification was performed, we cannot draw definitive conclusions about the interaction between caveolin1 and MYO10. Nevertheless, these findings align with previous unpublished results indicating caveolin1’s recruitment around intracellular filopodia, suggesting that junctional filopodia may play a role in material exchange between cells. Overall, these observations underscore the need for further research to clarify filopodia internalisation mechanisms and determine the fate of the internalised material.

4.4 Unsuccessful silencing of Pacsin2

The third aim of this thesis was to investigate the unknown role of Pacsin2 in junctional filopodia by using inducible shRNA-mediated silencing. This strategy was chosen based on

previous observations from the Cell Migration Lab, where constitutive Pacsin2 silencing arrested the cell cycle. Unfortunately, the attempt to silence Pacsin2 in DCIS.com cells was not successful, as confirmed by both, fluorescence microscopy and Western blot analysis. Despite sorting the transfected cells by FACS, the lack of fluorescence following doxycycline induction, coupled with the unaltered Pacsin2 protein levels, suggests that the shRNA construct or its delivery method may have been ineffective. Other possibilities for the failed silencing include low transduction efficiency, issues with the doxycycline induction system, or technical difficulties in the experimental setup.

Further optimisation of the shRNA construct, alternative sequences or delivery methods, and refining the induction conditions could significantly increase the effectiveness of gene silencing. Given the critical role Pacsin2 has in membrane dynamics (Hansen et al., 2011; Senju et al., 2011), overcoming these technical challenges in future experiments is essential for uncovering its complete function in junctional filopodia. Successful silencing of Pacsin2 could elucidate its role in pathological conditions, such as cancer metastasis, where filopodia promote invasive behaviour (Jacquemet et al., 2017).

4.5 Technical challenges and future directions

Dynamin2's key role in endocytosis, facilitating membrane scission (Itoh et al., 2005; Prichard et al., 2022), along with the unpublished results suggesting its involvement in junctional filopodia internalisation, led us to explore its potential role further. However, despite multiple attempts to investigate the role of dynamin2 in the internalisation of junctional filopodia, no concrete results were obtained. This was primarily due to technical challenges, including issues with the plasmids, cell line, and transfection procedures. These obstacles were not unique to the dynamin2 experiments but also affected the studies involving Pacsin2 and caveolin1. Additionally, the inability to successfully visualise the internalisation process highlights several key areas for improvement in future experiments.

One critical problem was the lack of plasmids and the quality of those available. Some plasmids were defective and did not express the intended structures or proteins. In future studies, sequencing plasmids to confirm their integrity, or ordering new plasmids, would be essential. Another significant improvement would be the creation of stable cell lines that express these proteins. Although generating stable cell lines by viral transduction can be time-consuming and

not always successful, it would dramatically increase imaging efficiency, reduce the time spent on transfections, and minimise the associated risks.

Another challenge was related to the U2OS cell line. Previous studies in the Cell Migration Lab used the DCIS.com cell line because of its ability to form distinct junctions and generate numerous filopodia. However, the DCIS.com cells have challenging transfection requirements. Therefore, to investigate filopodia exchange in another cell type, the U2OS cell line was selected for easier and more efficient transfections. Despite this advantage, U2OS cells caused problems due to their tendency to grow on top of each other, making it difficult to visualise the cell junctions. Additionally, problems with the live staining of actin and membrane affected the filopodia visualisation. While different staining methods could give more reliable results, selecting another cell line that produces numerous filopodia and forms a clear monolayer might be a better model for future research. Human lung carcinoma cell lines such as H1299 or A549 could serve as effective alternatives, as they are known to grow as monolayers and are widely used in research. These cell lines could provide clearer insights into filopodia dynamics and improve the reproducibility of the experiments.

While this thesis provides insights into junctional filopodia internalisation and the roles of Pacsin2 and caveolin1 in intracellular interactions, multiple challenges prevented us from obtaining enough data for quantitative analysis. Addressing these challenges and optimising the research conditions would be beneficial, as our findings emphasise the potential and the need for further research into filopodia-mediated intercellular communication.

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7. Appendices

7.1 Map of the transfer plasmid pRSIT16-U6Tet-sh-CMV-TetRep-2A-TagRFP-2A-Pur