

Investigating the role of End-Binding Protein-2 in epithelial tissue architecture

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i. Abbreviations

+TIP	Plus-end tracking protein
2D	Two dimensional
ACF7	Actin cross-linking factor-7
ADP	Adenosine-5'-diphosphate
APC	Adenomatous polyposis coli
Arp2/3	Actin-related protein 2/3 complex
ATP	Adenosine-5'-triphosphate
AU	Arbitrary units
Ca ⁺⁺	Calcium ions
CAP-Gly	Cytoskeleton-associated Protein Glycine-rich
CDK1	Cyclin-dependent kinase 1
CH	Calponin homology domain
CMV	Cytomegalovirus promotor
CRC	Colorectal cancer
DAPI	4',6-diamadino-2-phenylindole
DAPLE	Dishevelled-associated protein with a high frequency of leucine residue
DMEM	Dulbecco's modified eagle medium XVIII
DMSO	Dimethyl sulfoxide
EB1/2/3	End binding protein 1/2/3
EBH	End binding homology domain
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
F-actin	Actin filaments
FBS	Foetal Bovine Serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FH2	formin homology 2 domain
FN1	Fibronectin-1
G418	Geneticin [®] selective antibiotic
G-actin	Globular actin
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4

MAP	Microtubule associated protein
MDCKII	Madin darby canine kidney II
mDia-1/2	Mammalian diaphanous-related formin-1/2
MET	Mesenchymal epithelial transition
MG ²⁺	Magnesium ions
MMP1/7	Matrix metalloproteinase-1/7
MTs	Microtubules
MTOC	Microtubule organising centre
neoR	neomycin resistance gene
NMII	Non-muscle myosin 2
PBS	Phosphate buffered saline
Par3	Partioning defective-3
pEMT	Partial epithelial mesenchymal transition
PMLC	Phosphorylated myosin light chain
RhoA	Ras homolog family, member A
ROI	Region of interest
RT-PCR	Reverse transcription polymerase chain reaction
SxIP	Ser-x-Ile-Pro motif
TCF4	Transcription factor 4
TKI	Tyrosine kinase inhibitor
UJT	Unjamming transition
WT	Wildtype
ZO-1	Zona occludins-1

ii. Abstract

Microtubules (MTs) and their dynamic instability govern several cellular processes such as a cell's intrinsic polarity, differentiation, division, migration, organisation, and protein/molecule trafficking. The End-Binding protein family (EB1-3) are one group of MT plus-end tracking proteins, also known as +TIPs, that play a role in MT dynamics. EB1 and EB3 demonstrate functional redundancies and associate strongly with the plus-ends of MTs. EB2, on the other hand, appears to be more divergent in function and associates more strongly along the MT lattice. EB2 demonstrates a role in epithelial differentiation, cell migration and MT dynamics and has been linked to cancer progression and more recently with Fibroblast Growth Factors (FGFs) and their receptors (FGFRs). This investigation has demonstrated that EB2 localises along the MT lattice while FGFR3 accumulates at the cell peripheries in TC7 cells. TC7 cells were used as a model for epithelial colorectal cancer cells and are derived from the Caco-2 cell line. Inhibition of all FGFRs resulted in a significant increase in the mean EB2 fluorescence intensity compared to the DMSO control; therefore, suggesting a role for FGF signalling in the regulation of EB2 expression.

Preliminary data (Mogensen lab) and published data suggest that an upregulation in EB2 is associated with loss of normal tissue architecture, increased cell migration and MT dynamics. In this study the possible mechanisms behind the effect of high EB2 expression on cell shape and unjamming is explored in partially polarised MDCKII cells. Unjamming describes a mechanism in which cells of an epithelial tissue transition from a jammed 'solid-like' state, to an unjammed 'fluid-like' state. When jammed, cells are closely packed together and have reduced motility. During the transition to an unjammed state, cells become more motile, and their apical surface area increases. Here shape index analysis indicated that EB2 overexpression promotes unjamming. Some other cellular changes included, the polarity marker ZO-1, displaying more undulating labelling. Therefore, suggesting that high EB2 expression compromises junction integrity and function. A reduction in junctional mDia-1 was also seen, suggesting that actin nucleation at adherens junctions is affected by overexpression of EB2. In addition, junctional GEF-H1 was decreased when EB2 was overexpressed, and activation of Rho resulted in the partial rescue of cell shape index. However, no change in junctional pMLC was seen when EB2 was overexpressed, suggesting that actomyosin contraction is possibly affected by abnormal actin and non-muscle myosin organisation.

Interestingly, MDCKII cells overexpressing EB2 revealed more dynamin-2 at the junctions compared to control. Previous data by Ben Rix (Mogensen lab) showed a greater spread in

E-cadherin across junctions, suggesting increased E-cadherin internalisation. Dynamin inhibition led to a partial rescue of cell shape, and a decrease in junctional E-cadherin. Therefore, suggesting that EB2 overexpression results in the disruption of normal dynamin-mediated E-cadherin endocytosis. Perhaps, indicating that this disruption allows the permanent changes in junction length which allows the increase in shape index and thus contribute to further unjamming. However, previous research also implicates dynamin-2 with actomyosin contraction at junctions (Chua et al., 2009). Thus, proposing several mechanisms at work.

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Table 9. Key findings

Chapter 1.

Introduction

1.1. The cytoskeleton

In order to survive and proliferate a cell must achieve certain cellular functions. One way in which eukaryotic cells accomplish such processes is via a dynamic cytoplasmic filament network called the cytoskeleton. This network consists of three major filamentous components: actin filaments, intermediate filaments and microtubules (MTs). The cytoskeleton plays pivotal roles in several cellular functions including organising cell components, aiding compartmentalisation of a cell, mediating physical and biochemical reactions with other cells and the external environment and coordinating the forces necessary for morphogenesis and movement (Alberts et al., 2007). MTs, actin and intermediate filaments organise themselves into their own networks within the cytoskeleton, allowing cells to prevent deformation from internal and external forces. In addition, the dynamic behaviour of actin filaments and MTs, via polymerization and depolymerization, mediates cell morphogenesis as seen, for example, during differentiation and polarisation (Alberts et al., 2007).

Actin filaments have the smallest diameter (7-8 nm) of the three filaments that constitute the cytoskeleton and can exist as single cables, bundles or complex networks. Actin filaments are employed during cell division, specifically cytokinesis, whereby actin rearranges into a ring that separates the dividing cell into two daughter cells upon contraction (Pelham and Chang, 2002). In addition, actin filaments play a role in cell motility and muscle contraction; thus, demanding a sustained high quantity of polymerized actin. Intermediate filaments, like the name suggests, have an intermediate diameter of the three filament types of approximately 10 nm and form rope-like structures. One important role that intermediate filaments play in epithelial tissue is providing mechanical strength and resilience. They do this by extending through the cell's cytoplasm, joining to cell-cell junctions such as desmosomes (Geisler and Leube, 2016). Finally, MTs, these filaments have the largest diameter of approximately 25 nm. MTs play a role in several processes, for instance, they are one of the three main components of the mitotic spindle. MTs assemble spindle fibres, during mitosis, that tether chromosomes, via kinetochores, to opposite centrosomes, allowing for an equal segregation of genetic material between the two daughter cells (O'Connell and Khodjakov, 2007).

Epithelial tissue is a major tissue type in all animals, lining the external surfaces such as skin and internal surfaces such as organs, cavities and glands. While providing protection, the tissue is also semi-permeable, allowing the transport of solutes (Fletcher and Mullins, 2010). Epithelial cells form polarised sheets and tissues, however the mechanism in which cells change shape in response to mechanical and biochemical stimuli is not completely understood. The cytoskeleton is the key mediator in changes in cell-cell adhesion, cell shape and junctional length. Under normal physiological conditions, the tissue architecture is in a

homeostatic state, although disruption of this is common in pathological scenarios such as cancer. The overall aims of this study are to investigate whether FGF signalling influences expression of the MT associated protein EB2 and to explore possible mechanism responsible for elevated EB2 expression leading to unjamming and loss of normal tissue architecture.

1.2. Actin and myosin II structure and function

Actin is an abundant multi-functional protein found in all eukaryotic cells. Actin's monomeric form is denoted as globular-actin (G-actin) with a molecular weight of 46 kDa. The structure of G-actin includes a cleft functioning as an active site situated between two domains. The active site allows ATP and Mg²⁺ to bind (Wang et al., 2010). This binding site highlights the minus, slow-growing end of the protein, also described as the pointed end; while, the opposite end is the plus, fast-growing end, also known as the barbed end (Figure 1A). G-actin is characterised as active and therefore assembly competent when ATP is bound. Upon hydrolysis, ATP is exchanged for ADP, making the G-actin inactive. G-actin monomers assemble in a head-to-tail fashion, and form double helical actin filaments, also known as F-actin, which can then assemble into more complex bundles and networks. This arrangement gives actin filaments their intrinsic polarity (Wang et al., 2010, Merino et al., 2018) (Figure 1B). The actin network is highly dynamic, similar to that of MT, whereby actin filaments undergo rapid assembly, disassembly, and reorganization. It is this rearrangement of actin that plays an important role in cell shape and motility.

Polymerisation of G-actin into F-actin is a multi-step process including, activation, nucleation and elongation. G-actin assembles into trimers or tetramers that go on to bind nucleation factors which encourage actin polymerisation. Formins are a large (120-200 kDa) family of multi-domain proteins that stimulate the nucleation of linear actin filaments. Formins are also involved in elongation and bundling of parallel actin filaments (Kovar et al., 2006, Rao and Zaidel-Bar, 2016) (Figure 1D). Currently, there are 15 formin members in mammals (Chesarone et al., 2010). Formins dimerise to form a catalytic homodimeric formin homology 2 (FH2) domain that is responsible for binding to the plus-end of G-actin and stimulating the actin polymerisation process. While the FH1 domain recruits G-actin, bound to profilin, to feed into the growing actin filament (Pruyne et al., 2002). The results by Rao and Zaidel-Bar (2016) confirmed that the formin mDia-1 (mammalian Diaphanous-related formin-1), localises at epithelial adherens junctions and play a key role in actin polymerisation and E-cadherin stabilisation at the cell junctions.

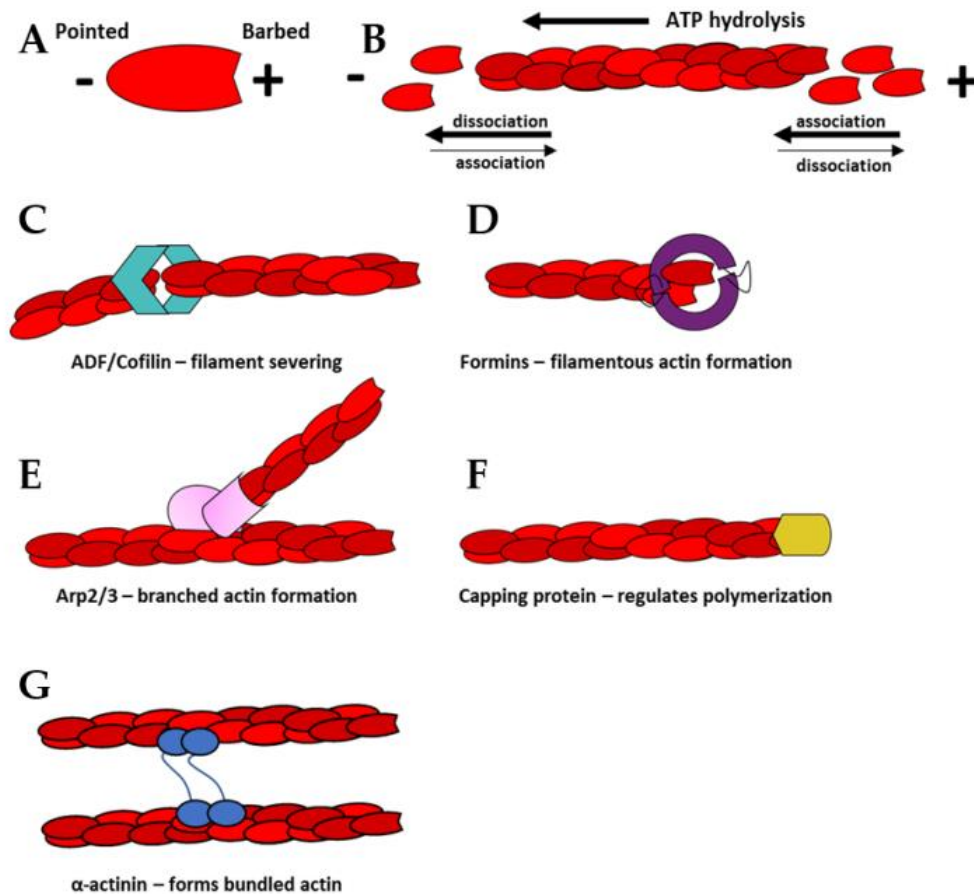


Figure 1. An overview of actin polymerization and common factors. (A) An actin monomer (G-actin) has a barbed or plus-end where ATP and magnesium ions bind and a pointed or minus-end. (B) Actin association and dissociation occur at both ends of a growing actin filament. Actin polymerisation occurs at a greater rate from the plus-end of an actin filament. (C) Cofilin (aqua), an actin depolymerizing factor, severs actin filaments. This generally occurs towards the pointed end of the actin filament. (D) Formins (purple), encourage polymerization at the plus-end by directly associating with G-actin and assisting in its addition. (E) The Arp2/3 complex (pink) mediates branching of an actin network by binding to F-actin and stimulating the nucleation of daughter filaments. (F) Capping proteins (yellow) bind at the plus-end and prevent further actin filament growth or loss (G) Bundling proteins, such as α -actinin (blue), link parallel actin filaments into bundles. Adapted from Cronin et al. (2022).

In contrast to formins, the actin-related protein 2/3 complex (Arp2/3) is one nucleating factor that encourages the polymerisation of non-parallel actin filaments leading to a branched dendritic network as seen at the leading edge during mesenchymal cell migration. The Arp2/3 complex is highly conserved amongst most eukaryotes and consists of seven polypeptides. The Arp2/3 associates with the minus-end of G-actin, providing a cap and increasing its stability. F-actin assembly is encouraged from the plus-end of the G-actin (Pollard, 2007, Vinzenz et al., 2012). This branching process is activated by nucleation promoting factors such as the WAVE complex and results in the nucleation of daughter filaments at a characteristic

~70° angle to the pre-existing filament (Pollard, 2007, Rotty et al., 2013). From here, the Arp2/3 complex may remain at the minus-end of the growing actin filament, acting as a stabiliser, or may be removed by an actin depolymerizing factor such as cofilin; thus, highlighting the dynamic nature of actin assembly and disassembly (Hsiao et al., 2015).

1.3. Myosin, cell-cell junctions and Rho GTPases

Myosins are a superfamily of motor proteins involved in several cellular processes (Figure 2). For example, myosins can mediate intracellular transport by 'walking' along actin filaments, aid muscle contraction by inducing actin filament sliding within the sarcomere and mediate changes in cell shape and motility by engaging with actin filaments (Vicente-Manzanares et al., 2009). In order to carry out such functions myosins require energy from ATP hydrolysis that occurs at a catalytic site commonly found in the amino-terminal (head) region of the molecule (Figure 3A). Binding of the myosin carboxy-terminal (tail) region varies depending on myosin type. Myosins such as myosin V, bind their C-terminal domains to cargo for intracellular transport, while the C-terminal domain of non-muscle myosin II (NMII) bind to actin filaments (Hammer and Sellers, 2011, Brito and Sousa, 2020).

NMII is part of the myosin-2 class, the largest class of myosins, and has three isoforms (NMIIA, NMIIB and NMIIC) (Figure 2). NMII consists of 6 peptides arranged in pairs, two heavy chains (230 kDa), two regulatory light chains (20 kDa) and two essential light chains (17 kDa) (Figure 3A). Despite its name NMII are present in muscle cells where they play a role in muscle tension and myofibrillogenesis (Sanger et al., 2010). In non-muscle cells, such as epithelial cells, NMII plays an essential role in cell shape and motility by cross-linking with actin filaments in actomyosin structures, generating tension to maintain cell shape.

Cell-cell adhesion is key for epithelial tissue integrity. E-cadherin is one of the main adhesion molecules at cell junctions. The stability of E-cadherin is mediated through F-actin, however, the mechanism behind actin polymerisation at junctions is not completely known. Previous studies have identified formin activity, via RhoA signalling, as possible drivers of actin polymerisation at junctions (Rao and Zaidel-Bar, 2016, Acharya et al., 2017). For example, Rao and Zaidel-Bar (2016) showed that mDia-1 and Fmn13 enhance actin polymerisation and contribute to stabilisation of E-cadherin at junctions. Depletion of mDia-1 resulted in weakened cell-cell adhesion during migration and would repair while Fmn13 depletion led to reduced F-actin and E-cadherin at junctions (Rao and Zaidel-Bar, 2016).

Cadherins are important proteins at adheren junctions - an adhesion complex within the apical junction complex. Tight and adheren junctions make up the apical junction complex. At adheren junctions, two E-cadherin molecules in neighbouring cells join via their N-terminals,

forming an adhesion complex between the two plasma membranes. This binding is a homophilic interaction and results in an E-cadherin dimer. The interaction between the two cadherins is dependent on Ca^{++} binding within the hinge region of each E-cadherin protein (Alberts et al., 2007, Nelson, 2008). Rac signalling is then thought to be inhibited by partitioning defective 3 (PAR3) and α -catenin which also activates Rho signalling and therefore stabilises the forming junction (Matsuzawa et al., 2016). Within the cytoplasm of each neighbouring cell, the E-cadherin cytoplasmic tail is bound to P120, and α -catenin that associates with β -catenin. α -catenin and β -catenin function as adaptor proteins for actin bundles to attach to this multi-protein complex (Alberts et al., 2007, Ishiyama et al., 2013).

The tight junctions are the most apical and include transmembrane proteins such as occludins, in order to create a semi-permeable barrier to mediate transport of ions and molecules (Citi, 2019). In addition, members of the zonula occludens (ZO) family, ZO-1 in particular, can be found in tight junctions and provide a link between the transmembrane proteins and the actin cytoskeleton via their C-terminal tail (Fanning and Anderson, 2009, Tornavaca et al., 2015).

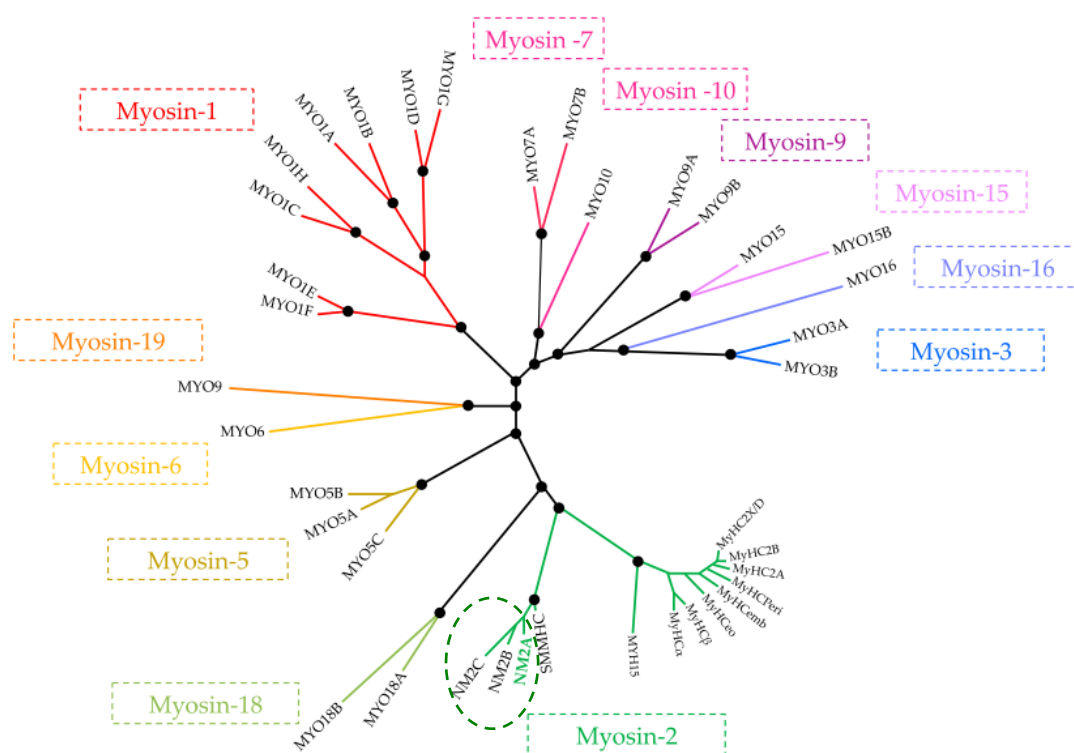


Figure 2. Schematic representation of the human myosin superfamily phylogenetic tree. The 38 members were phylogenetically grouped by motor domain sequences into 12 classes. The green dashed circle highlights the three non-muscle myosin isoforms in the myosin-2 class. Adapted from Brito and Sousa. (2020).

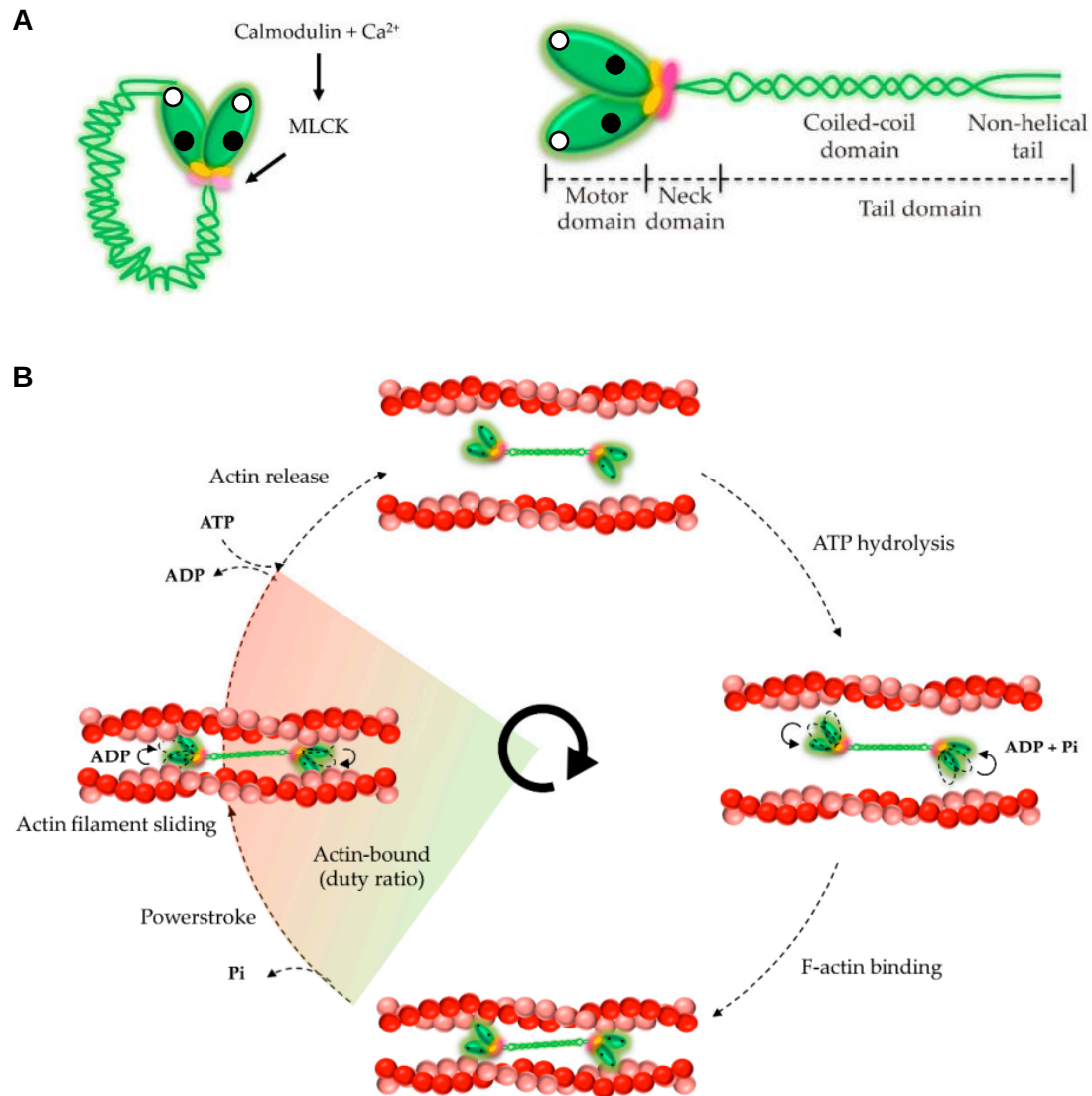


Figure 3. Non-muscle myosin 2 (NMII) structure and actin-sliding cycle. A) Inactive (left) and active (right) NMII molecule with two unphosphorylated regulatory light chains (RLC, pink), two essential light chain (ELC, yellow) and two heavy chains (green). NMII consists of the motor, neck and tail domain. ATP-binding sites (black dot) and actin-binding (white dot) are in the head domain. B) A model for NMII-actin sliding. ATP binds to the binding sites on NMII causing NMII to dissociates form actin. ATP hydrolysis allows reattachment of NMII to actin. The release of a phosphate as a result of ATP hydrolysis stimulates a conformational change, also known as the power stroke, that leads to contraction of actin filaments. The highlighted section indicates where NMII is strongly bound to actin, also known as duty ratio. The exchange of ADP for ATP allows the cycle to restart. Adapted from Brito and Sousa. (2020).

Actin filaments associate into bundles that then organise to form a dense circumferential belt spanning around the apical region of epithelial cells, which binds to adheren junctions via cadherin adhesion receptors. This belt, also known as the zonal adherence belt, joins neighbouring cells, enhancing tissue stability and acts as a pathway for mechanical stimuli between cells. The NMII is a key component of the cortical actin belt. NMII generates a

mechanical force that leads to contraction in which actin filaments slide in opposite directions (Figure 3B). This contraction 'zips up' the adherens junctions, tightening them and maintaining tension (Vicente-Manzanares et al., 2009, Efimova and Svitkina, 2018, Leite et al., 2020, Cronin and DeMali, 2021). Of the three NMII isoforms, NMIIA and NMIIIB have been found to localise at adherens junctions (Smutny et al., 2010).

The Rho GTPase family, including Rho, Rac and Cdc42 for example, describes a group of signalling G proteins that are a part of the Ras superfamily. Rho GTPases switch from inactive when GDP-bound to active when GTP-bound and play a central role in contraction, cell-cell adhesion and migration via the cytoskeleton, specifically via actomyosin (Arnold et al., 2017).

In a mature epithelial tissue, tight and adherens junctions comprise the apical junction complex in epithelial cells and are connected to the underlying actomyosin cytoskeleton. Actomyosin's dynamic instability is vital for junction formation, junction function and integrity. Rho GTPases are a primary player in the regulation of the actomyosin cytoskeleton dynamics through polymerisation and organisation of actin and myosin II, and thus the associated processes (Arnold et al., 2017).

Activation of Rho GTPases occurs through guanine exchange factors (GEFs) that catalyse the exchange of GDP for GTP. Meanwhile GTPase activating proteins (GAPs) deactivate Rho GTPases (Joo and Olson, 2021). Once activated, Rho GTPases can signal to downstream effectors. For example, activated RhoA signals to formins, such as the mDia family, which encourages actin nucleation at the apical junctions.

Another example includes the Rho-associated coiled-coil kinase (ROCK) which stimulates the phosphorylation of myosin light chain, a precursor to myosin engagement to actin and then contraction (Arnold et al., 2017). Whereas, during migration, Rho signalling mediates stress fibre formation, this, with Rac1 and Cdc42 activation, allows the establishment of lamellipodia and filopodia at the leading edge of the cell. While actomyosin contraction at the trailing edge of the cell is occurring for retraction (Panvov et al., 2005).

1.4. Microtubules

MTs are hollow, cylindrical structures composed of α - β tubulin heterodimers arranged in a head-to-tail arrangement, that govern the MT's intrinsic polarity (Figure 4). During MT assembly, α - β tubulin heterodimers come together to form protofilaments, 13 protofilaments join laterally to form a β -lattice that then rolls into its final non-symmetrical cylindrical shape with a distinct seam (Akhmanova and Steinmetz, 2008) (Figure 4). MTs are polar with α -tubulin exposed at the minus- or slow growing-end and β -tubulin at the plus- or fast-growing-end. In

relatively flat cells MTs are arranged in a radial array with their minus-ends anchored to the microtubule organising centre (MTOC), and the plus-ends extending into the cytoplasm (Alberts et al., 2007). In cells with a radial array, the MTOC is likely to be the centrosome however there are non-centrosomal MTOCs.

1.4.1. Microtubule dynamics

The head to tail arrangement of α - β heterodimers gives MTs two distinct ends, plus and minus, with distinct behaviours. While the minus-ends of MTs are often associated with an MTOC, the plus ends are continuously in a state of growing and shrinking, therefore demonstrating a dynamic instability (Figure 5). Dynamic instability is governed by four main parameters: MT growth rate, shrinking rate, catastrophe frequency and rescue frequency (Bowne-Anderson et al., 2013). Catastrophe describes the frequency in which MT ends convert from a growing state to a paused or shrinking state, whereas rescue is the frequency from a shrinking to a paused or growing state (Bowne-Anderson et al., 2013) (Figure 5).

During MT polymerisation, when a MT is 'growing', GTP is bound to the β -subunits making the MT assembly competent, allowing further association of GTP-tubulin heterodimers (Akhmanova and Steinmetz, 2008). This can form a GTP cap that leads to MTs adopting a straighter, more stable conformation. Once the GTP-tubulin heterodimer is incorporated GTP is hydrolysed to GDP (Geyer et al., 2015). GDP-bound tubulin results in a more curved and unstable MT. MTs in this state are more likely to undergo depolymerisation (Akhmanova and Steinmetz, 2008, Nawrotek et al., 2011) (Figure 5). Due to their polarity and dynamic capability, MTs are involved in several cellular processes: polarity, division, migration, organisation, and trafficking of cellular components.

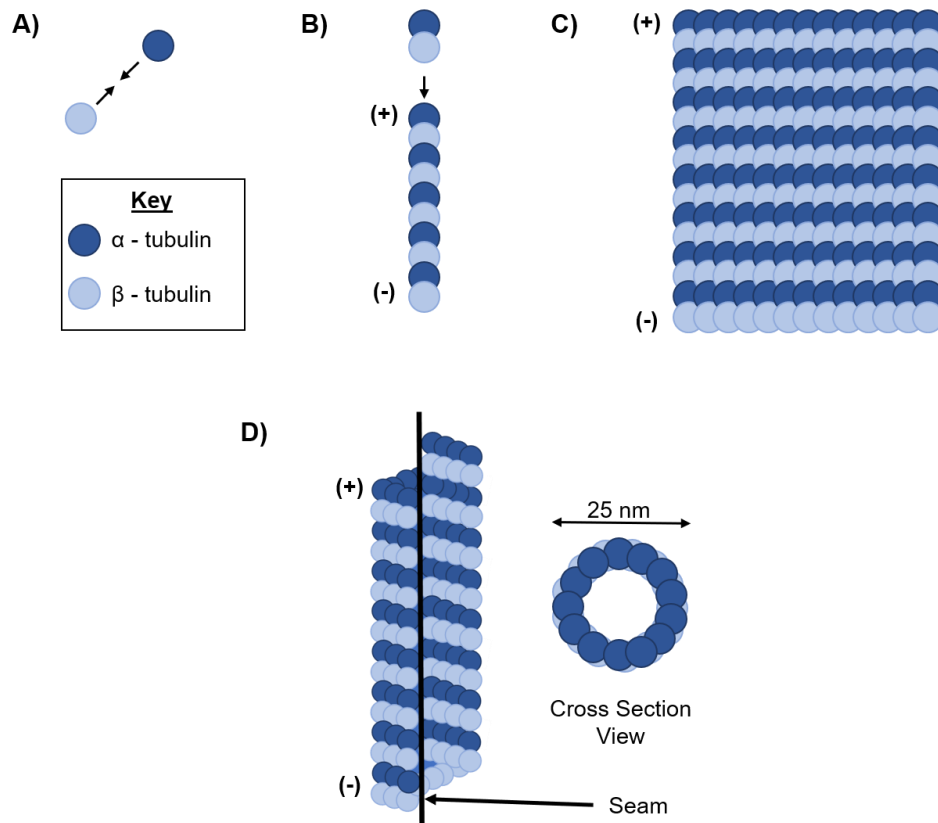


Figure 4. Microtubule structure. A) Tubulin of α - and β - types form an α - β -tubulin heterodimer. This gives MTs a plus- fast growing end and a minus- slow growing end which makes the MTs intrinsically polar. GTP binds α - and β - tubulin however, GTP hydrolysis occurs on β -tubulin bound GTP. B) Protofilament assembly: Several α - β heterodimers combine in a head-to-tail fashion to form a single protofilament. C) 13 protofilaments bind laterally to form a β -lattice. D) The β -lattice undergoes a conformational change, folding into its final cylindrical shape forming a MT. (Based on Alberts et al., 2007).

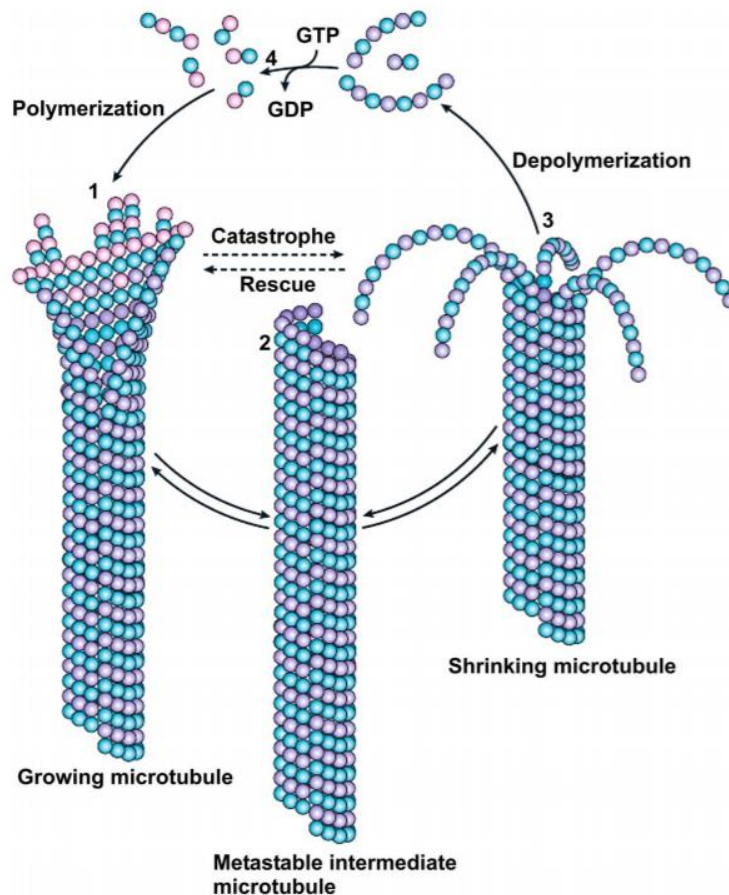


Figure 5. Microtubule dynamic instability. MT dynamic instability is mediated by the intrinsic ability of β -tubulin to hydrolyse GTP to GDP. A GTP-tubulin pool mediates MT polymerisation (1). When GTP is bound to beta-tubulin the heterodimer and protofilament is stable, straight and assembly competent. Formation of a GTP-cap stabilises the MT further. (2) From here the MT can be in a paused, growing or shrinking state. Next, depolymerisation involves the loss of the GTP-cap (3) and GTP hydrolysis to GDP which leads to a conformational change resulting in curved protofilaments that are unstable and a shrinking state. (4). Catastrophe describes when MT shrinking frequency dominates over growing whereas rescue describes when the growing frequency dominates over shrinking. (Adapted from Akhmanova and Steinmetz, 2008).

1.4.2. Microtubules and end-binding proteins

In addition to tubulin's intrinsic ability to hydrolyse GTP, there are other factors contributing to MT dynamic instability such as microtubule associated proteins (MAPs). MAPs can be sub-classified into those that are capable of binding to MT plus-ends via specific sequence motifs: CH, EBH, SxIP and CAP-Gly, for example (Akhmanova and Steinmetz, 2008). These MAPs govern MT dynamics and are termed MT plus-end tracking proteins, also known as +TIPs. The mammalian end-binding (EB) protein family consisting of EB1, EB2 and EB3 are highly conserved +TIPs, with a molecular weight of approximately 32-39 kDa (Figure 6). They are encoded by the MAPRE1-3 genes, respectively (Su and Qi, 2001, Slep et al., 2005). The EB

family, through MT dynamics, play a role in processes such as cell division, differentiation, and migration (Kumar and Wittmann, 2012).

All EB proteins have a similar structure consisting of multiple domains with distinct functions; however, it is their calponin homology (CH) domain at the N terminal region that allows their binding to MTs (Figure 6). Before EBs can bind to MTs, they must first form stable homo or heterodimers in the cell cytoplasm via their coiled-coil and EBH domains at their C-terminus (Sen et al., 2013). EB1 and EB3 are able to form both homo and heterodimers whereas EB2 can only form homodimers. The possible dimers include: EB1/EB1, EB2/EB2, EB3/EB3 and EB1/EB3 (Komarova et al., 2009). The dimerization process creates a hydrophobic cavity that facilitates the EBs associations with MTs and other +TIPs that possess a Cap-Gly motif via the acidic EEYF/Y domain at the C-terminus (Bu and Su, 2003, Honnappa et al., 2005, Komarova et al., 2009). It is through these specific sequence motifs, SxIP for example, that +TIPs bind and interact with MTs and other +TIPs, such as the cytoplasmic linker associated proteins (CLASPs) (Akhmanova and Steinmetz, 2008) (Figure 6).

EB1 and EB3 have been observed to influence MT dynamics and govern the association of MTs with cellular components including organelles, actin filaments, the cell cortex and kinetochores during cell division (Vaughan, 2005, Lansbergen and Akhmanova, 2006). In addition, EB1/3 are capable of inhibiting MT catastrophe (Figure 5) and therefore encourage MT growth via MT dynamics whereas EB2 cannot (Komarova et al., 2009). EB1 and EB3 are functionally redundant and are most strongly associated with the plus-end of MT due to their high affinity for GTP-tubulin (Maurer et al., 2011). EB2, on the other hand, is more divergent and has a weaker affinity to the plus-end of MT, it is therefore outcompeted for the plus-end position by EB1/3. As a consequence, EB2 is more often found bound along the lattice associated with GDP-tubulin (Roth et al., 2018).

Interestingly, EB2's affinity for MTs has been observed to be significantly reduced when phosphorylated by the kinases Aurora B and CDK1, resulting in a cytoplasmic localisation (not associated with MTs) (Iimori et al., 2016). The phosphorylation occurs several times within the amino terminus and at the serine/threonine residues in the linker region between the CH and EBH domain (Figure 6). Additionally, Iimori et al. (2016) revealed that when a non-phosphorylatable EB2 was expressed, the MTs of the kinetochore were more stable and less dynamic, therefore hindering MT reorganisation into the spindle network. Therefore, concluding that phosphorylation of EB2 by Aurora B and CDK1 reduced EB2's binding affinity to MTs and increased MTs dynamics. Thus, suggesting temporal phosphorylation of EB2 to be a prerequisite for initiation of mitosis, and a player in genomic stability as a result (Iimori et al., 2016). Consequently, an inappropriate expression of EB2 may disrupt the proper

progression through the cell cycle and result in genomic instability: this is an enabling characteristic of cancer as described by Hanahan and Weinberg (2011).

Goldspink et al. (2013) found that EB2 depletion leads to less dynamic MTs, therefore, suggesting that EB2 expression is a driver for MT dynamics. Additionally, Zhong et al. (2021) found that when EB2 was overexpressed in Hepatocellular carcinoma (HCC) cells, levels of acetylated α -tubulin and detyrosinated α -tubulin, markers for stable MTs, were significantly decreased. Moreover, EB2 knockdown resulted in an increase in the markers for stable MTs (Zhong et al., 2021). Thus, further suggesting that EB2 expression promoted MT dynamics.

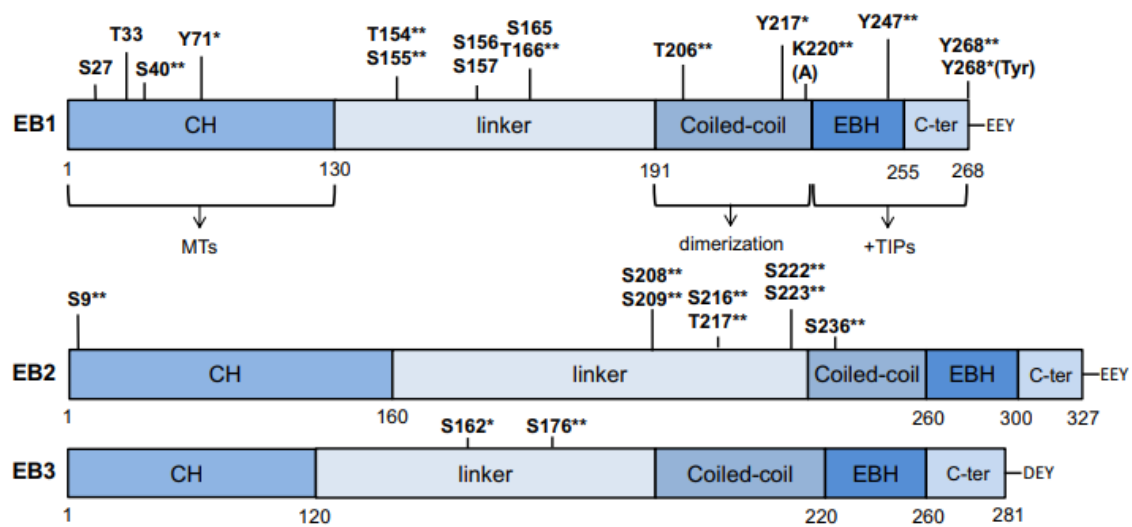


Figure 6. The EB family structure. The figure represents the structure and domains of human EB1, EB2 and EB3 with the sites for post-translational modifications. CH indicates the globular calponin homology domain required for microtubule binding at the N-terminus. The coiled-coil domain governs dimerisation of EB proteins in parallel. EBH specifies the end-binding homology domain that partly overlaps the C-C domain. The EBH domain also possesses conserved residues that allow binding of +TIPs containing the SxIP motif and dimerisation activity via their collective hydrophobicity. Finally, the EEY/F domain demonstrates a conserved sequence motif that specifically targets CAP-Gly domains in other +TIPs, allowing binding. ** indicates phosphorylation with the kinase identified, * indicates phosphorylation with no kinase identified. K220(A) indicates a site of acetylation and Y268(Tyr) Detyrosination. Adapted from Nehlig et al. (2017).

1.5. EB2 and cancer

As with many proteins, EB2's pathological expression may contribute to cancer onset and development. Findings by Abiatari et al. (2009) indicated an upregulation in MAPRE2 gene expression in three highly nerve-invasive pancreatic cell clones from the cell lines, Panc-1,

Colo357 and T3M4. The results also indicated that MAPRE2 protein (EB2) levels were upregulated in highly nerve invasive pancreatic lines, with increased granular cytoplasmic MAPRE2 expression which co-localised with actin filaments. In addition, actin cortical and transverse stress fibres appeared considerably denser in the highly nerve invasive pancreatic lines.

A similar EB2 upregulation has been demonstrated in cancer of the liver. Zhong et al. (2021), for instance, revealed that EB2 expression was consistently upregulated in 8 HCC cell lines (HepG2, Hep3B, HCCLM3, PLC/PRF/5, SMMC7721, Huh7, MHCC97 -H and MHCC97 -L) and 60 HCC tissues compared to normal, healthy liver cells and tissues, respectively. Moreover, EB2 overexpression, *in vitro*, demonstrated an increase in cell proliferation rate, colony numbers, cell migration and invasion ability; while EB2 knockdown resulted in the opposite effects (Zhong et al., 2021). Similarly, *in vivo*, EB2 upregulation demonstrated parallel oncogenic effects of increased tumour growth rate, tumour size and intrahepatic metastasis nodules, while EB2 knockdowns demonstrated the reverse (Zhong et al., 2021). Furthermore, patients with EB2 upregulation HCC cancers also presented with more tumour nodules, larger tumours overall, macrovascular and microvascular invasion and more advanced Edmondson-Steiner grade, compared to low EB2 expressing HCC cancers. Thus, the evidence strongly suggests that high EB2 expression correlates with more malignant tumour characteristics and poorer HCC patient prognosis.

In addition, Yue et al. (2014) concluded that through EB2 association with Mitogen-activated protein kinase kinase kinase kinase 4 (MAP4K4) and other proteins, focal adhesion disassembly is promoted in cultured mouse and Human Keratinocyte cells (HaCaT cells). Focal adhesion disassembly is a vital step required for cell retraction and integrin detachment from the extracellular matrix and therefore an essential process for cell migration (Yue et al., 2014). A pivotal step in cancer progression is metastasis – the invasion of a tissue different to that of the cancer origin, and recolonisation into a secondary tumour: a hallmark of cancer (Hanahan and Weinberg, 2000). Thus, pathological overexpression of EB2 may promote cell migration and therefore, metastasis, aiding colorectal cancer (CRC) progression.

1.6. Apico-basal epithelial polarisation, differentiation and EBs

The gut mucosa, for example, is lined with a simple columnar epithelium that mediates the exchange of molecules and ions in and out of the body. During development gut epithelial cells must polarise, elongate and differentiate to achieve their required structure and function. Apico-basal polarisation is characteristic of columnar epithelial cells and is vital for normal tissue architecture and function. Apico-basal polarised cells possess an apical membrane that

faces the lumen or exterior of the body and an opposite basolateral membrane associated with the ECM or underlying cells (Manninen, 2015). The apical and baso-lateral domains are connected through the apical junction complex that is comprised of tight and adheren junctions. The apico-basal polarisation process is not completely understood, however key protein complexes and interactions have been elucidated over the years.

Initiation of the apico-basal polarisation process is mediated by extrinsic and intrinsic stimuli from the ECM, neighbouring cells, junction formation and the positioning of polarity proteins or complexes. Cues from the ECM, for example, that are mediated by integrins, a ubiquitous family of ECM receptors, allow the positioning of intracellular polarity protein complexes. The three main polarity complexes include, the Par-complex, Crumbs-complex and Scribble-complex (Pieczynski and Margolis, 2011). The Par-complex is comprised of Par3, Par6, Cdc42 and aPKC, whereas the Crumbs-complex includes Crumbs, Stardust, PATJ and Lin-7 (Goldstein and Macara, 2007, Bulgakova and Knust, 2009). The Par- and Crumbs complex are involved in establishing an apical domain and position the cell-cell junctions (tight and adheren junctions) between the apical and basolateral domain. The Par- and Crumbs-complex are semi-redundant in function. While the primary role of the Scribble-complex is to establish the basolateral domain and consists of the proteins, Scrib, Dlg and Lgl (Riga et al., 2020).

Differentiation describes a process whereby a dividing cell changes in form and/or function to become more specialised. During epithelial differentiation, MTs reorganise in a stepwise process, however, it is not completely understood. Typically, undifferentiated epithelial cells are relatively flat and have a radial MT array. Here MTs are anchored by their minus-ends at a centrally located centrosome with the plus-ends elongating outward towards the cell periphery (Goldspink et al., 2013) (Figure 7). In this case, the centrosome is functioning as the MTOC; perhaps the most well-known example of an MTOC. The centrosome is defined as a non-membrane bound organelle consisting of a pair of centrioles encompassed by pericentriolar material.

During the apico-basal differentiation process, cells lose their radial array and adopt an apico-basal array through MT reorganisation. MTs are released from the centrosomal MTOC and are captured at the apical cell periphery, also known as cortical capture (Sanchez and Feldman, 2017) (Figure 7). The centrosome may still function as a MT nucleator but is inactivated as an MTOC, while the non-centrosomal MTOC replaces the role of MT anchorage near the apex of the cell (Noordstra et al., 2016).

One theory for the mechanism in which MTs reorganise during apio-basal differentiation is that they release from the centrosome and are captured at adheren junctions (Bellett et al., 2009, Mogensen, 1999). During differentiation, the centrosome's affinity for MTs is reduced,

this is thought to be due to the loss of centrosomal associated proteins such as pericentrin and centrin (Muroyama et al., 2016). Another possible factor in MT release from the centrosome is MT cleavage via the enzyme katanin (Dong et al., 2017). The inactivation of the centrosome and its release of MTs then leads to the MTs reorganisation and re-anchoring at non-centrosomal MTOCs near the apex of epithelial cells (Muroyama et al., 2016). The cortical capture process is hypothesised to be mediated by several proteins such as EB1 and CLIP-170. These proteins are suggested to localise at the MT plus-end and then to cortical receptors on the cell periphery (Bellett et al., 2009, Goldspink et al., 2013).

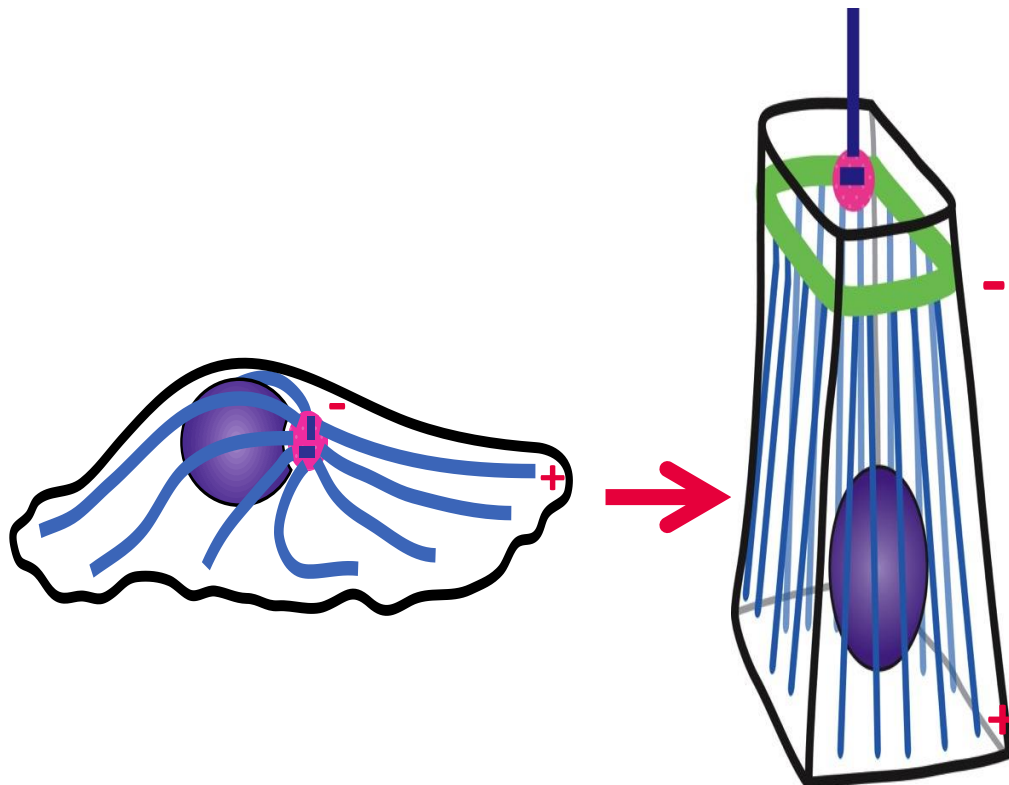


Figure 7. Apico-basal Epithelial Differentiation. The schematic presents an undifferentiated relatively flat epithelial cell (left) with a radial MT array (blue). The MTs are anchored by their minus-ends to the centrosome (pink), close to the nucleus (purple), serving as a MT organising centre,. During epithelial differentiation the cell elongates, polarises and the MTs detach from the centrosome and re-anchor at apical non-centrosomal MT organising centres, producing an apico-basal MT array (right). The green band indicates the the non-centrosomal MTOC associated with apical adherens junctions. The vertical purple line indicates the primary cilium. (Diagram courtesy of MM Mogensen).

A study investigating the EB family, by Bu and Su (2001), concluded that overexpression of EB1 significantly stimulated bundle formation and MT acetylation, which also showed an increased resistance to Nocodazole compared to the control. Perhaps, suggesting a link between the EB family and MT stability. Komarova et al. (2009) similarly investigated the relationship of the EB protein family and MT dynamics. Dynamic instability is governed by four main parameters: MT growth rate, shrinking rate, catastrophe frequency and rescue

frequency (Bowne-Anderson et al., 2013). The results revealed that depletion of all three EBs (EB1/2/3) led to cell death, however the cells survived when both EB1 and EB3 were depleted simultaneously. Surprisingly, depletion of EB1 and EB2 together, did not lead to a change in MT growth rate or the MT rescue frequency in cultured cells (Komarova et al., 2009). MT rescue indicates the frequency from a shrinking to a paused or growing state (Bowne-Anderson et al., 2013). Thus, indicating that both EB1 and EB3 were not essential for these processes, and perhaps suggesting EB2 to be a key component. However, overexpression of EB2 did not rescue the depletion effects (Komarova et al., 2009). Depletion of EB1 and EB3 did lead to a decrease in MT length and an increase in pausing events during MT growth that was often followed by MT shortening, in addition, MT catastrophe rate increased 10-fold (Komarova et al., 2009). MT catastrophe describes the frequency in which MT ends convert from a growing state to a paused or shrinking state (Bowne-Anderson et al., 2013). Therefore, suggesting that the EB family do play a role in MT dynamics: a key player in epithelial differentiation.

In addition, Goldspink et al. (2013) investigated the role of EB2 in MT organisation during apico-basal epithelial differentiation. Mouse inner medullary kidney (mIMCD-3) cells were grown until fully confluent and partially polarised. The results revealed that during apico-basal differentiation, EB2 expression decreased while EB1 expression remained the same. Furthermore, as cells became more confluent and differentiated, the expression of de-tyrosinated tubulin increased (Goldspink et al., 2013) (Figure 8A). Therefore, the results suggest a negative correlation between apico-basal differentiation and EB2 expression with the added effect of an increase in MT stability. The results also revealed that as the *in vitro* epithelial cells increased in confluency and became more differentiated, EB1 relocated from the MT plus-ends to the MT lattice. This was followed by a co-localisation with ACF7, which is a cytoskeletal crosslinking protein that links MT and actin filaments (Kodama et al., 2003, Goldspink et al., 2013).

In order to further explore a potential role for EB2 in MT organisation, Goldspink et al. (2013) depleted EB2 in human retinal pigment epithelial cells (ARPE-19), Osteosarcoma cells (U2OS), colorectal adenocarcinoma cells (TC7) and colorectal carcinoma cells (HCT116). The cells demonstrated a significantly larger cell size; presenting a 3.7-fold increase, on average, compared to the controls (Goldspink et al., 2013). Also, MTs transitioned from individual curved filaments to straight and bundled with significantly fewer MT crossover events. An increase in de-tyrosinated tubulin, a marker for increased MT stability, was also observed. Detyrosination describes a reversible post-translational modification whereby the terminal tyrosine is removed from α -tubulin (Kreiss, 1987). Thereby, detyrosinated tubulin is an indicator for long-lived MTs which would therefore be less dynamic and more stable (Bulinski and

Gundersen, 1991). This was subsequently verified with cold treatment, indicating that the EB2 depleted cells had the most resistant MTs; thus, indicating increased stability compared to the controls (Goldspink et al., 2013). An increase in MT stability and decrease in dynamicity was likewise concluded by live time-lapse imaging with a significant increase in MT pausing and decrease in shrinking, while no difference in growth was observed (Goldspink et al., 2013). Thus, suggesting that the absence of EB2 correlates with increased MT stability and decreased MT dynamics.

Furthermore, analysis of intestinal crypt epithelial tissue, *in situ*, revealed a distinct EB2 expression pattern within intestinal crypt-villi units. EB2 expression was highest within the basal stem cell region at the base of the crypt where cells are most proliferative and then decreased with progression out of the crypt and into the villus where cells are terminally differentiated (Figure 8B). Again, presenting a negative correlation between EB2 expression and differentiation (Goldspink et al., 2013). Interestingly, cells at the bottom of the crypt, that had high EB2 expression, also demonstrated non-bundled curved MTs; this is a likely indicator that the MTs are more dynamic. Highly dynamic MTs would serve the stem cell's purpose of an increased proliferative power and therefore undergo MT reorganisation, such as for spindle formation in order to divide more frequently. Then, moving up the crypt, as cells differentiated, MTs were organised in apico-basally oriented bundles (Goldspink et al., 2013). The *in situ* data here mirrored the results found *in vitro*.

In conclusion, the results gained by Goldspink et al. (2013) from *in vitro* epithelial monolayers and *in situ* intestinal crypt epithelial tissue analysis suggest that in early stages of epithelial apico-basal differentiation, high EB2 plays a role in MT dynamics which is critical for MT reorganisation while its downregulation or depletion encourages MT stability, bundle formation, EB1 re-localisation, ACF7 recruitment and MTs-actin co-alignment.

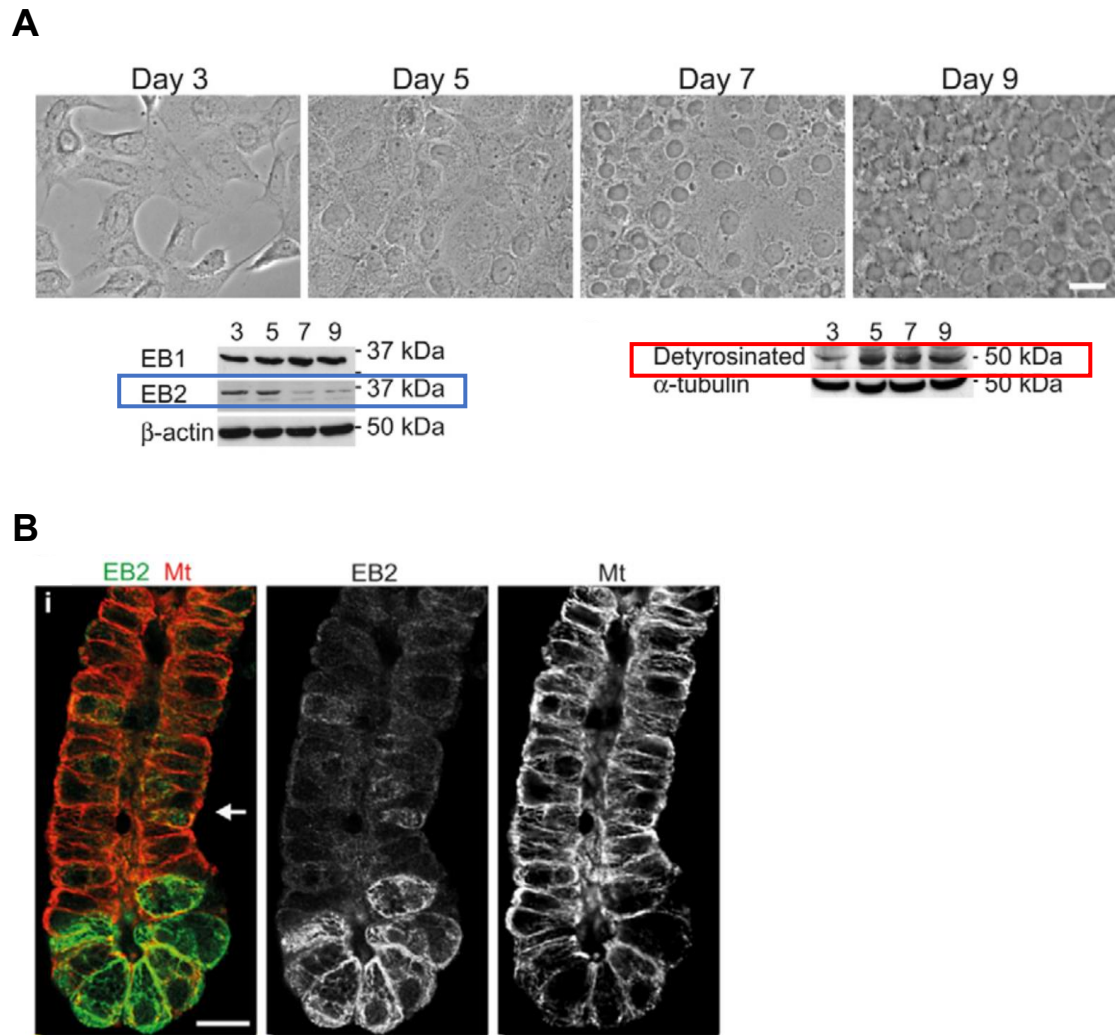


Figure 8. EB2 expression and epithelial differentiation. A) Phase-contrast images of mouse inner medullary kidney (mIMCD-3) cells at different time points and stages of confluency/differentiation. The images highlight the cells increasing confluency and the corresponding western blot analyses show a decrease in EB2 expression at increasing time points (blue box) while an increase in de-tyrosinated tubulin (red box). Scale bar = 20 μ m. B) Lateral view of an adult mouse small intestinal crypt immuno-labelled for α -tubulin (red) and EB2 (green), indicating highest EB2 expression in cells at the base of the crypt. The white arrow indicates the transit-amplifying region. Scale bar = 5 μ m. Adapted from Goldspink et al. (2013).

1.7. Fibroblast growth factors and their receptors

Previous research as indicated a potential link between EB2 expression, FGFs and FGFRs. Mammalian fibroblast growth factors (FGFs) are a 22-member family of potent mitogenic growth factors that function as key signalling molecules in several biological processes. Such processes may be categorized during embryonic developmental or homeostasis and regeneration in an adult organism, including cell differentiation, proliferation and migration

(Ornitz and Itoh, 2015). FGFs can be subdivided depending on whether they have an endocrine, paracrine or intracrine function. Endocrine and paracrine FGFs constitute 18 out of the 22 FGFs and initiate such processes by binding to their receptors (FGFR1-4) via the third and final immunoglobulin (Ig)-like loop of the extracellular ligand-binding domain (Powers et al., 2000). All FGFRs possess a characteristic intracellular kinase domain, joined to the extracellular Ig-loops via a relatively small transmembrane domain (Powers et al., 2000, Ornitz and Itoh, 2015). Furthermore, to accommodate such a large ligand to receptor ratio, FGFRs, through heterodimerisation and alternative RNA splicing, can allow diverse FGF-FGFR binding complex possibilities depending on location, the nucleus or cytoplasm (Antoine et al., 2005, Eswarakumar et al., 2005, Ornitz and Marie, 2015). Additionally, individual FGFs may bind more than one FGFR member. In particular, the IIIb and IIIc isoforms of FGFRs are one method of increasing the FGFR variants that involves alternative splicing of exons in the ligand-binding domain (Eswarakumar et al., 2005). Generally, IIIb and IIIc FGFR1-3 isoforms are expressed in epithelial and mesenchymal tissue, respectively (Scotet and Houssaint, 1995, Wuechner et al., 1996). However, when it comes to FGFR3, the location of its isoform is not exclusively tissue specific. Like with FGFR1-2, the expression of the FGFR3 IIIb isoform is restricted to epithelial tissue, though, IIIc expression can be found in both epithelial and mesenchymal tissue (Scotet and Houssaint, 1995).

Once an FGF is bound to its specific FGFR, downstream intracellular signalling pathways are stimulated; predominantly the RAS/ Mitogen activated protein (MAP) kinase pathway that is associated with processes such as proliferation, differentiation and apoptosis (Chen et al., 2017). Other downstream pathways including PI3 kinase/AKT pathway or PLC γ pathway are possible (Beenken and Mohammadi, 2009, Yun et al., 2010). Several early studies identified the association of pathological expression of FGFs, FGFRs and common carcinomas such as those of the breast and intestine (Halaban, 1991, Wilkinson et al., 1993, Anandappa et al., 1994).

1.7.1. FGFR3's potential link to EB2 expression

FGFR3 expression has previously been confirmed in the gut, specifically the intestinal crypts. Interestingly, FGFR3 expression was revealed to be highest in the bottom half the crypt, where the stem cells lie, and lowest at the top of the crypt (Vidrich et al., 2004, Arnaud-Dabernat et al., 2007) (Figure 9A-B). This expression pattern corresponds to that of EB2 found by Goldspink et al. (2013), concluding EB2 decreased with confluency. However, *in vitro* studies such as that by Kanai et al. (1997) found that FGFR3 expression increased with Caco-2 confluency (Figure 9C). Arnaud-Dabernat et al. (2007) also showed FGFR3 expression

increased with confluency in transimmortalized mouse intestinal cells (M-ICc12). The negative correlation between EB2 expression and confluency was likewise seen by Goldspink et al. (2013) in mouse inner medullary kidney (mIMCD-3) cells (Figure 8). In addition, Szarama et al. (2012) revealed that when mouse cochlear explants were treated with SU5402 there was a significant upregulation in MAPRE2. SU5402 is a tyrosine kinase inhibitor of all FGFRs (Mohammadi et al., 1997). In addition to decreased MT bundles in mouse cochlear pillar cells when subject to SU5402 (Szarama et al, 2012). Therefore, the results may suggest that in undifferentiated cells, such as those in the base of intestinal crypts and inner ear hair cells, EB2 expression is high and MTs are more dynamic. Then, as epithelial cells progress through differentiation, EB2 expression decreases, FGFR3 expression increases and MTs become more stable. Perhaps indicating a negative correlation between EB2 and FGFR3 expression with an effect MT dynamics.

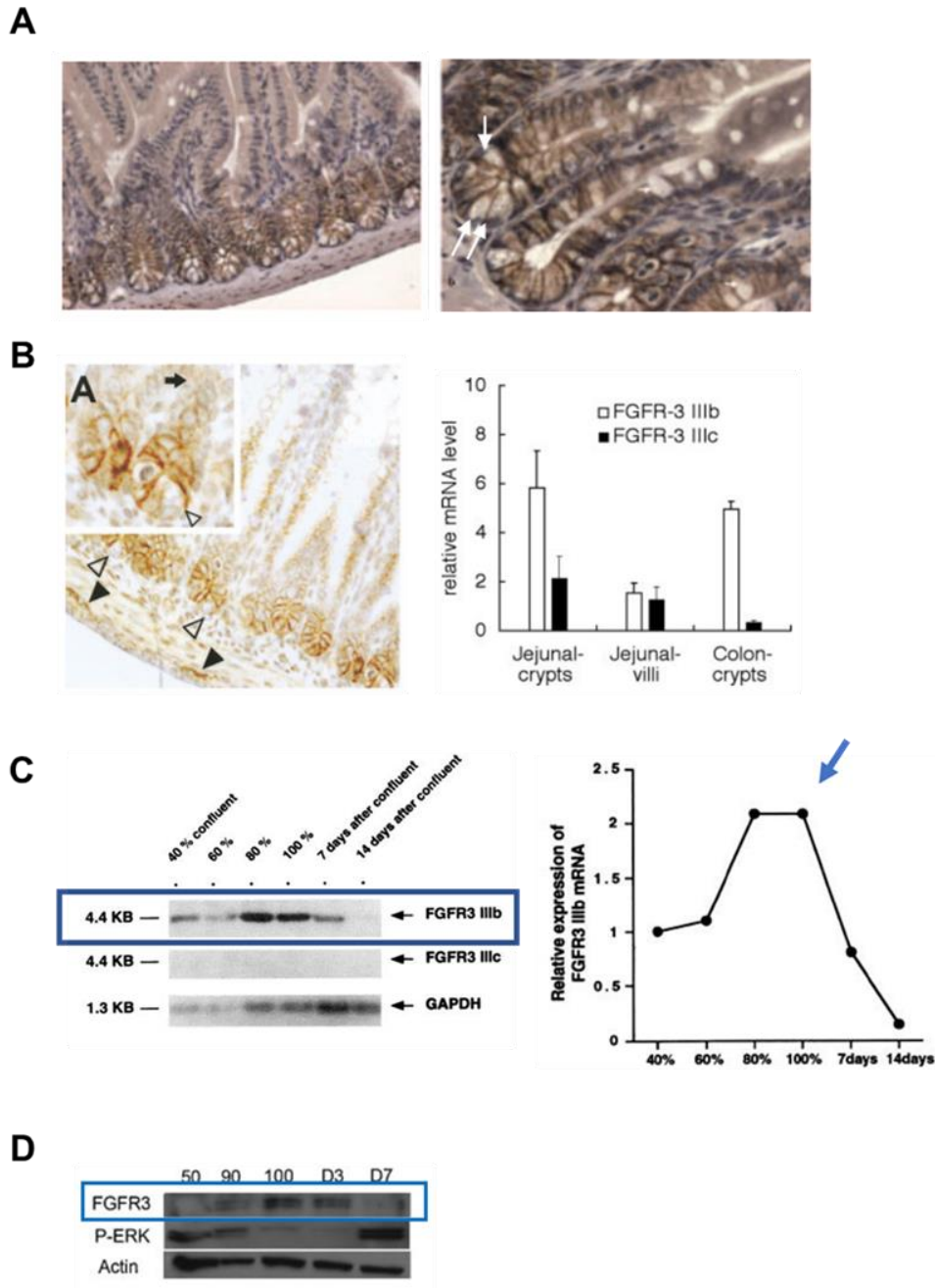


Figure 9. EB2 and FGFR3 expression. A) Mouse adult small intestine labelled for FGFR3 (dark brown) revealing localisation in the lower half of the intestinal crypts while differentiated cells, Paneth and goblet cells, for example (white arrows) lacked FGFR3 labelling (Adapted from Arnand-Dabernat et al., 2008). B) Positive FGFR3 labelled epithelial (gold) localised in the lower half of proximal jejunal crypts from a 14-day-old mouse (left). Bar chart indicating the expression of FGFR3 mRNA isoforms in intestinal crypts and villi determined by RTPCR (right). (Adapted from Vidrich et al., 2004). C) Northern blot analysis (left) and relative mRNA levels (right) demonstrating FGFR3 IIIb expression in Caco-2 cells highlighting an initial increase as confluency increases but a decrease from 7 days post 100% confluency (blue box and arrow). (Adapted from Kanai et al., 1997). D) Western blot analysis of FGFR3 (blue box) in M-ICc12 cells revealed an increase with increasing confluency, then a decrease at Day 7 (D7) post 100% confluency (Adapted from Arnand-Dabernat et al., 2008).

1.8. Jamming and unjamming

The cells of an epithelial tissue are able to undergo collective migration; however, the underlying mechanisms of their coordination remain unclear. One hypothesis is that the process involves jamming, unjamming and transitioning between the two states (Figure 10A). Epithelial cells are often in a homeostatic state where they are compacted next to one-another in single or multiple layers. Though these cells may move, it is not significant and so they are 'caged in their position' – this has been described as a solid-like state or jammed. On the other hand, some cells can be more dynamic in their shape and movements, during migration, wound healing and disease, for example, thus, presenting a liquid-like or unjammed state (Farhadifar et al., 2007, Tambe et al., 2011, Park et al., 2015, Atia et al., 2018, Kim et al., 2020). The more unjammed epithelial cells become, the longer and more variable the cell shape is, and cells in an unjammed state often migrate in multicellular groups (Kim et al., 2020).

In epithelial biology it is hypothesised that cells are in a balance between oppositional tensile and adhesion forces along the cell-cell junctions (Figure 10A). Tensile forces are contractile and therefore decrease the perimeter of the cell-cell junction, whereas adhesion proteins, such as E-cadherin, increase the perimeter. It is suggested that tensile forces dominating over adhesive forces is characteristic of jamming, whereas adhesive forces dominating over tensile forces allows a system to become more fluidlike and unjammed (Farhadifar et al., 2007, Bi et al., 2015, Park et al., 2016). The shift of cells from a jammed to unjammed state is defined as the unjamming transition (UJT) and assumes the energy barrier between cells falls to zero for its occurrence (Farhadifar et al., 2007, Bi et al., 2015, Park et al., 2016). The UJT from jammed to unjammed is mediated by several factors such as cellular crowding, cortical tension and cell-cell adhesion. Furthermore, the UJT is marked by a critical change in cell shape that can be calculated by the shape index equation (Figure 10C) with a threshold factor of $P_0 = 3.813$ (arbitrary units). Below this factor ($P_0 < 3.81$), the system is considered jammed (solid-like); whereas, above this factor ($P_0 > 3.81$), the system is classified as unjammed (fluid-like) (Figure 10B).

One of the earliest papers to suggest that jamming may occur was by Trepap et al. (2009). The team investigated the physical forces during collective migration in monolayers of Madin–Darby Canine Kidney cells, strain II, (MDCKII) in substrate. The distribution of forces was concluded to be parallel to that of jammed inanimate material such as sand and grain (O'Hern et al., 2002, Ostojic et al., 2006). Force distribution in such granular systems is hypothesised to present three characteristic features, including close packing, structural disorder and long-

range force transmission (Coppersmith et al., 1996). All three characteristics were apparent in MDCKII cells, *in vitro*, therefore indicating the possibility of epithelial cells being jammed (Treat et al., 2009).

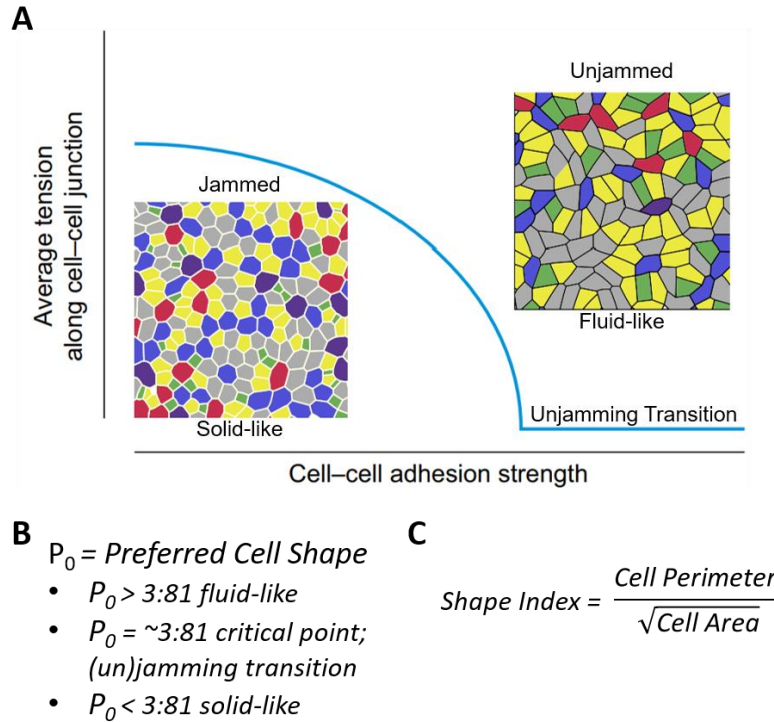


Figure 10. Jamming ↔ Unjamming Transition. (A) Jammed epithelial cells are also referred to as being in a solid-like state while unjammed cells are fluid-like. The balance between tensile and adhesion forces determines the state of cells. The transition between jammed and unjammed is described as the unjamming transition (UJT). (B) The UTJ is marked by a critical change in preferred cell shape (P_0), in arbitrary units, with a threshold factor of $P_0 = 3.813$. (C) The preferred cell shape is calculated using the shape index equation. (Adapted from Park et al., 2016).

Results by Tambe et al. (2011) likewise suggest that when transmitted across cell-cell junctions, intracellular forces, govern the collective migration of monolayers of epithelial cells *in vitro*. Similar results were demonstrated in endothelial and breast cancer cells and concluded that unjamming occurs before EMT (Tambe et al., 2011). Meanwhile, Angelini et al. (2011) demonstrated that confluent MDCK cell layers undergo collective migration in a manner parallel to the glass transition – a characteristic of a jammed system. Therefore, the evidence supports the notion that epithelial cells *in vitro* are a jammed system (Treat et al., 2009, Tambe et al., 2011, Serra-Picamal et al., 2012, Park et al., 2015).

However, the evidence so far still poses the question – does jamming/unjamming occur *in vivo*? Atia et al. (2018) investigated cell shape and shape variability during the formation of

the ventral furrow in *Drosophila melanogaster* embryos. The results suggested that through cell-cell interactions, as a cell's shape decreases and becomes less variable, the system becomes more jammed, whereas cell elongations and increased variability relates to unjamming (Atia et al., 2018). Similar conclusions were made by Mongera et al. (2018) whereby the jamming transition (liquid- to solid-like state) was apparent during the vertebrae axis elongation of posterior tissues in *Danio rerio* (zebrafish). For instance, cells of the mesodermal progenitor zone were characteristically unjammed (liquid-like state) while those of the presomitic mesoderm were jammed (solid-like) (Mongera et al., 2018). Therefore, strongly suggesting the presence of jamming and unjamming in biological systems, *in vivo*.

Moreover, jamming and unjamming has not just been suggested in physiological conditions such as cell migration and embryonic development. Recent studies have considered jamming, unjamming and the transition between the two in pathological scenarios such as cancer. These studies propose that the transition from solid-like, jammed and non-migratory phenotype, to fluid-like, unjammed and migratory phenotype in a cancer setting led to an increase in cancer progression and cancer cell migration (Park et al., 2016, Oswald et al., 2017, Atia et al., 2018). For example, Kim et al. (2020) investigated cell shape and shape variability of several breast cancer cell lines of varying invasiveness: MCF10A, MCF10A.14-3-3z, MCF10.ErbB2, MCF10AT, and MCF10CA1a. All cell lines demonstrated the predicted characteristics of unjamming regarding their shape and variability (Atia et al., 2018, Kim et al., 2020). Therefore, supporting previous findings indicating that unjammed cells are more migratory (Bi et al., 2015, Park et al., 2015, Kim et al., 2020).

More recently, it is hypothesised that the UJT does not work in isolation. The Epithelial Mesenchymal Transition (EMT), specifically partial EMT (pEMT), and the UJT both facilitate collective migration, dynamics and remodelling of cells. However, the distinctions between these two processes are unclear. Therefore, the question remains do the UJT and EMT facilitate one-another?

1.9. Partial Epithelial Mesenchymal Transition – pEMT

Polarised epithelial cells are strongly adhered to one another by intercellular adhesion complexes; meanwhile mesenchymal cells have weaker adhesion forces (Figure 11). The EMT is an evolutionary conserved mechanism that was first observed during embryonic development. However, EMT is now apparent in regeneration/ wound healing and cancer progression (Steinestel et al., 2014). In cancer, EMT describes the transition of carcinoma cells whereby epithelial markers are suppressed while mesenchymal markers are adopted

(Dongre and Weinberg, 2019, Yang et al., 2020) (Figure 11). In general, EMT allows the development of carcinoma cells into a more aggressive phenotype. For example, the transition to a mesenchymal state facilitates a decrease in cell-cell adhesions and an increase in the cell's invasiveness and therefore metastases (Kalluri and Weinberg, 2009, Ye and Weinberg, 2015). Moreover, there is a disruption to the cell's basement membrane, rearrangement of the cell's cytoskeleton and loss of their apico-basal polarity (Hanahan and Weinberg, 2011) (Figure 11).

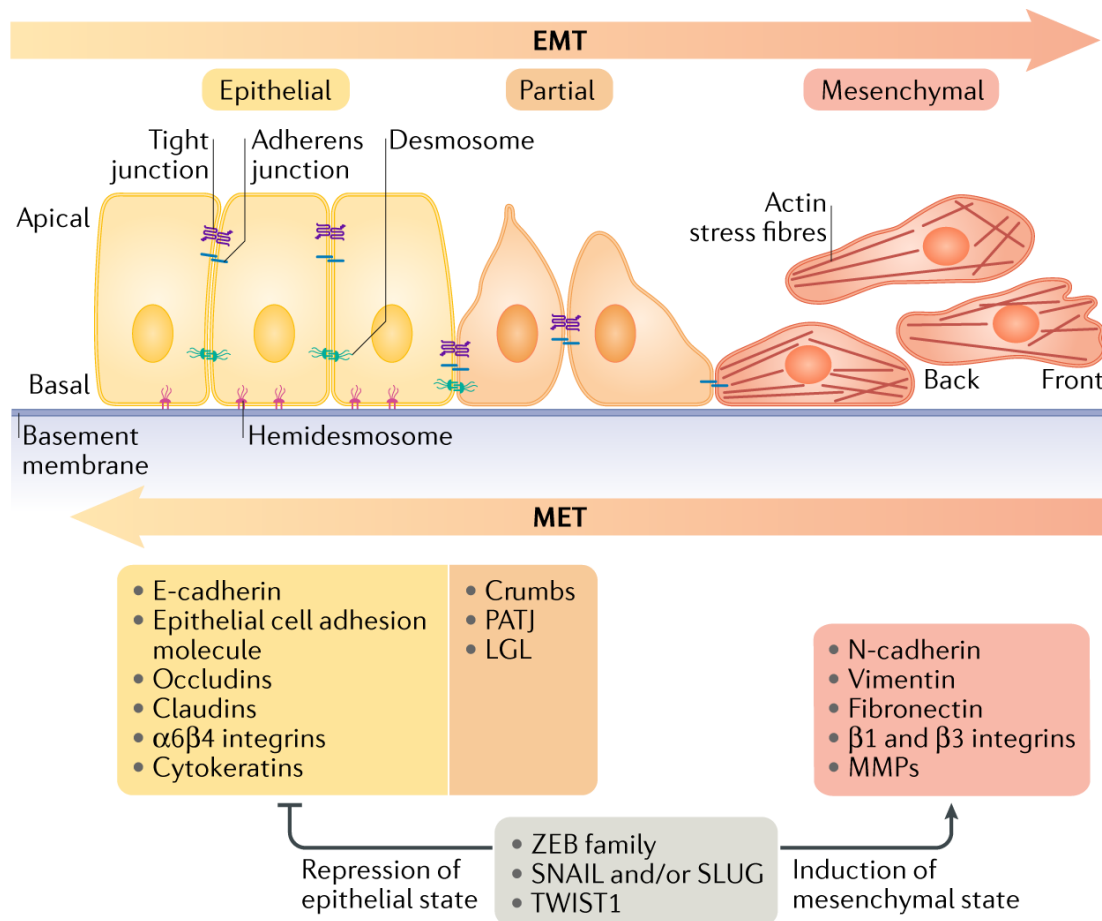


Figure 11. Model for Epithelial to Mesenchymal Transition (EMT). The schematic shows cells in their typical epithelial states, highlighting important cell-cell and cell-membrane contacts and the transition into a typical mesenchymal state. The yellow box indicates key markers for the epithelial state. Initiation of EMT leads to the upregulation of transcription factors, highlighted in the grey box. These inhibit expression of epithelial molecules and activate expression of mesenchymal molecules (pink box). During EMT, cell-cell junctions disassemble, the cytoskeleton reorganises, cells become more migratory, apico-basal polarity is lost while a front-to-back polarity is adopted. However, the EMT process is fluid and thus intermediate phenotypes are possible, this describes partial EMT (pEMT). EMT is reversible via mesenchymal to epithelial transition (MET). (Adapted from Dongre and Weinberg., 2019).

In addition to the epithelial and mesenchymal state, there is an intermediate state now recognised as pEMT. This is characterised as incomplete EMT, which involves the partial loss

of epithelial and gain of mesenchymal markers (Figure 11). pEMT has been observed in several cancer types including breast, lung, pancreatic and colorectal (Bronsert et al., 2014, Koliijn et al., 2015, Zacharias et al., 2018). It remains unclear whether pEMT indicates an intermediate/transitional state of EMT or specifies its own end-state. Moreover, investigations by Pastushenko et al. (2018) concluded that there are several distinguishable states, early, intermediate and late, for example, within pEMT itself. These states were characterised by distinct expression of surface proteins such as vascular Cell Adhesion Molecule-1 (VCAM-1), Integrin beta-3 (ITGB3) and Integrin alpha-V (ITGAV), *in vivo*. Also, during the UJT, E-cadherin expression remains unchanged therefore, maintaining barrier function. In contrast, during EMT or pEMT, E-cadherin expression is decreased, disturbing cell-cell junctions and compromises barrier function (Mitchel et al., 2020).

Aiello et al. (2018) investigated EMT, *in vivo*, using a mouse model of pancreatic ductal adenocarcinoma. Interestingly, the results demonstrated that the majority of cells analysed presented a pEMT phenotype. Furthermore, epithelial protein markers appeared to be lost via internalisation in contrast to transcriptional repression generally associated with complete EMT (Aiello et al., 2018). Another difference found was that cancer cells with the pEMT phenotype tended to undergo collective migration as opposed to single-cell migration - a characteristic of complete EMT (Aiello et al., 2018). Therefore, suggesting another mechanism whereby carcinoma cells lose their epithelial characteristics, gain mesenchymal features and disseminate.

The changes that occur during pEMT and EMT such as altered protein expression, distribution and function can be used as biomarkers to determine the state cells are in. The transforming growth factor- β (TGF- β) is suggested to be one of the initial promoters of EMT, therefore perhaps a reliable indicator of pEMT, promoting loss of epithelial markers such as E-cadherin and gain of mesenchymal markers such as N-cadherin and vimentin (Pickup et al., 2013) (Figure 11). In addition, TGF- β , a player in cell proliferation, is often mutated in cancer and therefore stimulates hyperproliferation of cancer cells: a hallmark of cancer (Hanahan and Weinberg, 2000, Bellam and Pasche, 2010). TGF- β induces the transcription factor Snail that likewise leads to epithelial marker repression and mesenchymal marker promotion. Twist, another transcription factor, facilitates the transition from epithelial to mesenchymal by governing migration and a switch from E-cadherin to N-cadherin expression known as the cadherin exchange (Vahtinen et al., 2001). However, markers for EMT and pEMT are not synonymous.

Chaturvedi et al. (2019) investigated EMT in colorectal cancer using Caco-2 cells as a model. The role of Neogenin1 (NEO1) was explored due to its role in tumour suppression and adheren

junction maintenance. NEO1 is a cell surface protein involved in cell growth, differentiation and cell-cell adhesion. NEO1 depleted Caco-2 cells demonstrated a more mesenchymal phenotype; being more migratory with a decrease in F-actin stress fibres, MT density and an increase in MT growth rate and basal lamellipodia (Chaturvedi et al., 2019). An upregulation in mesenchymal markers Fibronectin-1 (FN1) and matrix metalloproteinase-1 (MMP1) was also confirmed. FN1 is a glycoprotein found at the cell surface of the ECM that is involved in cell adhesion and migration, while MMP1 plays a role in ECM degradation. However, the tight junctions between cells that were subject to NEO1 depletion persisted (Chaturvedi et al., 2019), perhaps suggesting pEMT.

1.10. Hypothesis and aims

Hypothesis:

This study hypothesises that FGF signalling influences EB2 expression in gut epithelial cells and that EB2 upregulation leads to unjamming and loss of normal tissue architecture.

This investigation aims to show that:

1. FGF18/FGFR3 influences the expression level of EB2 by:
 - Determining which FGFRs and FGFs are expressed in TC7 cells using RT-PCR analysis
 - Inhibiting all FGFRs with SU5402 and investigating any effects on EB2 expression using fluorescence intensity analysis.
2. EB2 overexpression leads to unjamming by:
 - Confirming that MDCKII cells overexpressing EB2 (MDCKII^{mChEB2}) have a greater cell shape index than control MDCKII (MDCKII^{mChEMPTY}) cells by analysing the apical cell perimeter and area in cells labelled with a junctional marker.
 - Determining whether the MT and actin organisation is altered in MDCKII^{mChEB2} compared to MDCKII^{mChEMPTY} cells.
 - Determining whether the level of activated non-muscle myosinII (non-muscle myosinII contraction) is altered in MDCKII^{mChEB2} compared to MDCKII^{mChEMPTY} cells by analysing the fluorescence intensity levels in cells immunolabelled for phosphorylated myosin light chain (pMLC).

- Determining whether E-cadherin localisation at cell-cell junctions is altered in MDCKII^{mChEB2} compared to MDCKII^{mChEMPTY} cells by analysing the fluorescence intensity levels at cell junctions in cells immunolabelled for E-cadherin
- Determining whether Rho activation can rescue the cell shape index in MDCKII^{mChEB2} cells

Chapter 2.

Materials and Methods

2.1. Cell lines

2.1.1. Colorectal cancer – TC7 cells

The TC7 cell line is an epithelial colorectal cancer cell line derived from the Caco-2 cell line which was first isolated from an adenocarcinoma of a 72-year-old Caucasian male. TC7 cells pose as a model for the Adenomatous polyposis coli (APC) truncated mutation. TC7s contain a genetic change at codon 1367 (CAG-TAG), resulting in a stop codon, on a single APC allele while the other allele is lost completely (Ilyas et al., 1997). Such mutations in the APC gene can lead to cellular β -catenin accumulation (Ilyas et al., 1997). TC7 cells also contain β -catenin mutation (GGC-GCC) on codon 245, encoding a missense mutation (Ilyas et al., 1997). TC7 cells are characterised as having a more homogenous population structure and further developed intracellular junctions compared to other enterocytes of the small intestine (Turco et al., 2011). As the TC7 cells increase in confluency, so too do the enterocytic differentiation characteristics. MDCKII cells were sub-cultured according to protocol (see 2.2.1.) twice a week with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Foetal Bovine Serum (FBS), 1% (2mM) L-glutamine and 1% (100 units/ml) penicillin/streptomycin, hereafter referred to as supplemented media.

2.1.2. Madin-Darby Canine Kidney - MDCKII cells

MDCKII cells were used to generate a model of epithelial tissue as they readily formed polarised layers in 2D culture. MDCKII cells are a canine non-cancerous epithelial cell line derived from the MCDK heterogenous parent cell line derived from an adult female Cocker Spaniel in 1958. The Mogensen lab previously transfected wildtype MDCKII cells (MDCKII^{WT}) with a vector containing mCherry unattached plasmid denoted as EMPTY in this investigation (MDCKII^{mChEMPTY}) or a plasmid containing the mouse MAPRE2 gene encoding the EB2 protein (MDCKII^{mChEB2Hi}) in the multiple cloning site (MCS). The vector also consisted of a highly active cytomegalovirus promotor (CMV) to drive high expression. Both plasmids, mCherry-EMPTY and mCherry-EB2, contained a neomycin resistance (neoR) gene to be used as a positive selection marker with Geneticin® (G418, Sigma-Aldrich, Cat. No. A1720, 10 μ l per 1 ml). The MDCKII^{mChEMPTY} cells functioned as a negative control to ensure any effects revealed were due to the EB2 overexpression rather than the transfection and mCherry expression. MDCKII^{WT} were also used in initial shape index analysis as a negative control for the MDCKII^{mChEMPTY} cells. However, due to distinct differences in growth patterns and speed, the analysis proceeded with the MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} only. All MDCKII cell lines were sub-cultured according to protocol (see 2.2.1.) twice a week with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Foetal Bovine Serum (FBS), 1% (2mM)

L-glutamine and 1% (100 units/ml) penicillin/streptomycin, except media for MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cell lines contained G418 in addition.

The original plan for this investigation was to create stable cell lines overexpressing human MAPRE2 in TC7 cells. However, due to delays caused by the Covid-19 pandemic, the laboratory we were collaborating with did not have the human MAPRE2 vector ready in time.

2.2. Cell culture and passaging

Under aseptic conditions, cells were grown in T-75 cm² flasks containing supplemented media. The cells were incubated in a humidified incubator at 37 °C, 5% CO₂ and the medium replaced every 4 days. When the cells reached approximately 80% confluency, in log phase growth, they were subjected to a 2 ml wash with prewarmed (37 °C) Trypsin. Then, the cells were detached after a 15-20-minute incubation with 3 ml Trypsin. Once the cells were detached, the solution was neutralised in fresh media (3 ml) to mitigate the trypsin toxicity. A 10 µl aliquot was used to perform a cell count using a haemocytometer to determine the required volume for seeding (see 2.2.2.). The suspension was then centrifuged for 5 minutes at 1,000 rpm and the resulting pellet was resuspended in 2 ml of fresh media. The appropriate volume of resuspension was then transferred to a new T-75 flask containing 10 ml fresh media to accommodate a 1:15-20 split ratio for culture maintenance and incubated in a humidified incubator at 37 °C, 5% CO₂.

2.2.1. Seeding, freezing and thawing cells

Under aseptic conditions, 3 glass coverslips (15 mm) were placed in a 35 mm petri dish. The appropriate volume of cell suspension (calculated in 2.2.1.) was added to new supplemented media, to give 2 ml per dish at 500,000 cells per ml. The seeded cells were then placed in a humidified incubator at 37 °C, 5% CO₂. Additionally, 24-well plates containing glass coverslips were used to seed cells at the same volume, 500,000 cells per ml. 500 µl of cell suspension was inserted into each well. This became the preferred method as coverslips did not move as much as they did in the 35 mm dishes, meaning that it minimised disruption to the monolayer as cells increased in confluency.

To avoid phenotype selection associated with high passage numbers, cells of low passage numbers were propagated in T-75 cm² flasks to approximately 60-70% confluency and frozen down under aseptic conditions. To freeze, cells were trypsinised and counted according to protocol (see 2.2.1.). The cells were then resuspended in the appropriate quantity of DMEM supplemented with 10% (v/v) FBS, 1% (2mM) L-glutamine and 1% (100 units/ml)

penicillin/streptomycin containing 10% dimethylsulphoxide (DMSO), to act as a cryo-preservant, to give 1 million cells per 1 ml. The cell suspension was transferred into cryovials (Corning®, Cat. No. 430488) as 1 ml aliquots. The cryovials were then transferred to a Mr Frosty™ containing isopropanol which was then stored at -80 °C for 24 hrs. For short term storage, of up to 3 months, cells were stored at -80 °C; meanwhile, long term storage was carried out in a cryogenic storage dewar for liquid nitrogen.

To thaw cells, the cryovials containing cells were rolled between the hands until liquid. Supplemented media was prewarmed in a 37°C water bath for approximately 20 minutes. Under aseptic conditions, 1 ml of cells was added to 9 ml of supplemented media in a sterile 15 ml falcon tube. The cell solution was then spun in a centrifuge at 400 rpm for 5 minutes. The supernatant was discarded to remove the DMSO, and the cell pellet was resuspended in 10 ml of new supplemented media. The resuspension was transferred to a T-75 cm² and placed in a humidified incubator at 37 °C, 5% CO₂. Once cells reached approximately 70% confluency they were passaged according to protocol (see 2.2.).

2.3. Methanol fixation, immuno-labelling and widefield fluorescence microscopy

Seeded cells were washed in and fixed with -20 °C 100% methanol and placed in a -20 °C freezer for 4 minutes. All washes were carried out on a rocker at room temperature. The methanol was promptly removed and replaced with PBS for a 10-minute wash; followed by 2x 10-minute washes in PBS+1% G.S. Next, the cells were permeabilised by a 1% NP-40 incubation for 3 minutes. The cells were blocked for 30 minutes in PBS+10% G.S. Next, the cells were incubated with the appropriate primary antibodies (Table 1) prepared in PBS+1% G.S for 1.5 hours at room temperature or overnight at 4 °C. This was followed by another 3 washes in PBS+1% G.S. for 10 minutes each. Next, the secondary antibody solution was prepared using PBS+1% G.S and cells were subject to a secondary antibody incubation for 30 minutes (Table 2). The cells then underwent another set of 3 washes in PBS, for 10 minutes each, followed by staining with DAPI (Sigma-Aldrich) at a 1:10,000 dilution giving a 300 nM concentration. Finally, the cells were washed 3 more times in PBS for 5 minutes each and mounted on a microscope slide with hydro-mount as mounting medium. The slides were stored at 4 °C and visualised under a widefield fluorescence upright Zeiss Axiovert 200M microscope or the Observer 7 microscope using Axiovision software (Zeiss) to produce multichannel immuno-fluorescence images using 20x (air) and 63x (oil) objectives. Alternative fixation methods were trialled (not shown) such as the use of formalin and fixing cells at room temperature. However, fixing cells at -20 °C produced the best immuno-labelled images.

Table 1. Primary Antibodies. The primary antibodies, their dilutions, origin species and manufacturing information used to immuno-label the TC7 and MDCKII cells.

Primary antibody	Dilution	Species origin	Manufacturer	Product no.
α -tubulin	1:500	Rabbit	Abcam	AB15246
β -catenin	1:2000	Rabbit	Sigma-Aldrich	C2206
β -actin	1:2000	Mouse	Sigma-Aldrich	A1978
β -tubulin	1:300	Mouse	Sigma-Aldrich	T4026
Dynamin-2	1:300	Rabbit	Abcam	AB3457
EB2	1:300	Rat	Absea	010614A11
E-cadherin	1:500	Mouse	BD BioSciences	610181
FGFR3	1:300	Rabbit	Abcam	AB180908
GEF-H1	1:500	Rabbit	Abcam	AB201687
mDia-1	1:200	Mouse	BD BioSciences	610849
mDia-2	1:200	Rabbit	Abcam	AB12319
PAR3	1:500	Rabbit	Millipore	07-33D
pMLC (S20)	1:300	Rabbit	Abcam	AB2480
YL1/2 (Tyrosinated Tubulin)	1:150	Rat	Abcam	AB11316
ZO-1	1:200	Rabbit	Thermo Fisher	61-7300

Table 2. Secondary Antibodies. The secondary antibodies used, their conjugated fluorophore and wavelength, dilutions, origin species and manufacturing information. All secondary antibodies were raised in goat.

Secondary antibody	Fluorophore colour	Dilution	Species origin	Manufacturer	Product no.
Alexa 488	Green		Rat		A11006
Alexa 488	Green		Rabbit		A11008
Alexa 568	Visible red	1:1000	Rat	Thermo Scientific	A11077
Alexa 568	Visible red		Rabbit		A11011
Alexa 568	Visible red		Mouse		A11031

2.4. FGFR inhibition assay

SU5402 (sigma) was dissolved in DMSO according to manufacturer's protocol to a concentration of 100 mM and stored in 10 μ l aliquots at -20 °C. This stock was then further

diluted in supplemented media to concentrations 10 μ M, 5 μ M and 2 μ M. The two negative controls included an equivalent DMSO dilution and media only. Seeded cells (250,000 per dish) at a 70-80% confluency were subject to an overnight (18.5 hr) incubation. After the incubation, the cells were fixed, immuno-labelled and imaged (see 2.3.), a fluorescence intensity analysis was performed (see 2.5.1.), followed by statistical analysis (see 2.6.).

2.5. Fluorescence intensity analysis using CellProfiler™ software

CellProfiler™ is a cell image analysis workflow program that allows the automatic identification of primary (nuclei) and secondary (cell borders, for example) objects within a microscope image and performs chosen bespoke analyses in the form of 'pipelines' on every 'accepted' cell, giving built in reproducibility. For the fluorescence intensity analysis on TC7 cells the following pipelines and modifications to default settings were made:

- (1) Identify Primary Objects (nuclei): Typical diameter of objects, in pixel units (Min, Max): 40-80, Threshold strategy: Adaptive, Thresholding method: Otsu, Two or Three class thresholding: Three-class, Threshold correction factor: 1.75, Method to distinguish clumped objects: Shape, Method to draw dividing lines between clumped objects: Shape.
- (2) Identify Secondary Objects (cells): Select the method to identify the secondary objects: Propagation, Threshold strategy: Global, Thresholding method: Otsu, Two or Three class thresholding: Three-class, Threshold correction factor: 0.7.
- (3) Measure Object Size Shape:
- (4) Measure Object Intensity:
- (5) Export to Spreadsheet:

2.6. Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis on TC7 cells

2.6.1. RNA extraction and purification from TC7 cells

RNA was extracted and purified using the Quick-RNA™ Miniprep Kit by Zymo Research (catalogue numbers R1054 and R1055). All steps were performed at room temperature and centrifugation occurred at 10,000 g for 30 seconds, the flow-through was discarded unless specified. TC7 cells were grown until 90% confluency in T-75 flasks. 600 μ l of RNA lysis buffer was directly applied to the cell monolayer and the cells were removed by scraping. For purification, the lysed sample was transferred into a Spin-Away™ Filter (yellow) in a collection tube and centrifuged to remove the majority of genomic DNA. Then, 99.9% ethanol was added

to the flow-through (1:1) and mixed. Next, the sample was transferred into a Zymo-Spin™ IIICG column (green) in a collection tube and centrifuged. 400 µl RNA wash buffer was added to the column and centrifuged. A reaction mix of 75 µl digestion buffer and 5 µl DNase I was prepared in a nuclease-free tube, gently inverted to mix and applied directly to the column matrix. This was incubated for 15 minutes to remove trace DNA. 400 µl RNA prep buffer was then added to the column and centrifuged; then, 700 µl RNA wash buffer was added to the column and centrifuged. Following this, 400 µl RNA wash buffer was added to the column and centrifuge. The column was then transferred into a nuclease-free tube and 100 µl DNase/RNase-free water was applied directly to the column matrix and centrifuged for RNA elution. The eluted RNA was then stored at -80 °C until required.

2.6.2. RT-PCR reaction

RNA from TC7 cells was extracted and purified (according to 2.6.1.). The RNA was subjected to one PCR cycle at 42°C for 15 minutes, then, one cycle at 95°C for 5 minutes using Ready-to-go beads (Pharmacia™) and primers to amplify DNA from mRNA. Next, 32 cycles at 95°C for 30 secs, 55°C for 30 secs, and 72°C for 1 minute were undertaken with ligand-specific primer pairs. A preliminary reaction was performed to test the RNA quality using the β -actin primer sequences, Forward: GAAATCGTGCGTGACATTAAGGAG and Reverse: ATACTCCTGCTTGCTGATCC ACAT (Appendix). Then, 50 µl of reaction products and 30 µl loading dye were resolved alongside a Thermo Scientific™ GeneRuler™ 1 kb Plus DNA Ladder on 1% agarose gel. This was carried out by my co-supervisor Dr Hajihosseini during the COVID-19 lockdown.

2.7. Jamming-unjamming analysis

MDCKII cells were sub-cultured according to the protocol (see 2.2.1). The MDCKII cell lines MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} were trypsinized and counted according to the protocol (see 2.2.1 and 2.2.2). A cell suspension of 500,000 cells per ml was produced from each cell line in supplemented media and 500 µl of cell suspension was transferred into individual wells in a 24-well plate containing a glass coverslips. MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} were kept in media containing Geneticin® (G418, Sigma-Aldrich, Cat. No. A1720, 10 µl per 1 ml) at every stage. The media was replaced every 2 days until confluency when the media was switched to 5% (v/v) FBS on Day 2. On the appropriate Day (8, 10 or 14) the cells were methanol-fixed, immuno-labelled for E-cadherin or β -catenin to highlight the cell peripheries (Table 1), stained for DAPI and mounted according to protocol (see 2.3.). The slides were stored at 4 °C and visualised using a Zeiss Observer 7 inverted microscope and

Zeiss ZEN blue 3.0 imaging software to produce multichannel immuno-fluorescence images using a 63x (oil) objective. The junctional labelling was used to determine cell perimeter and area, which was then used to calculate the shape index and determine the state of cells, jammed or unjammed, either via ImageJ or CellProfiler™ (See 2.8. Image analysis method development).

2.8. Image analysis method development

The image processing programme ImageJ, also known as FIJI, is one commonly used for cellular analysis such as fluorescence intensity and cell shape. Since ImageJ is a widely used and accepted method of analysis, there are many resources and learning opportunities among peers for its use. One of the uses of ImageJ in this investigation was the determination of cell perimeter and area of different MDCKII lines. E-cadherin or β -catenin immuno-fluorescence labelling was used to visualise the cell periphery. One major advantage of using ImageJ in this way is that the freehand selection tool allowed the tracing of the cell outline with high accuracy in the E-cadherin or β -catenin channel. Once the cell perimeter and area were obtained, the shape index could be calculated, using the formula $\text{shape index} = \text{perimeter} / \sqrt{\text{area}}$, to determine the jammed or unjammed state (Figure 6). This method was adapted from Park et al. (2015). However, this method does possess some disadvantages. For example, the process of tracing cell peripheries one cell at a time can be very time consuming and labour intensive. As a consequence, the sample size is dictated by the time available. Additionally, as with any human-mediated method, there is the risk of subconscious bias, choosing larger or smaller looking cells, for instance. One way this investigation attempted to tackle any subconscious bias for certain cell phenotypes was to methodically trace every complete cell periphery in an image before proceeding to the next image, marking on a duplicate image which cells had or had not been traced.

CellProfiler™ is another program that allows the analysis of cell images in a workflow manner, using modules that analyse varying parameters, to form a pipeline that is then applied to every image uploaded. All analysis starts with the automatic identification of a primary object, usually the nuclei. From here, a secondary object can be identified. For the shape index analysis in this investigation, the cell periphery was the secondary object, via the E-cadherin or β -catenin fluorescence labelling. The next module in the pipeline would be 'Measure Object Size Shape' producing an output outlining the perimeter and area of every whole cell identified within the images. This method of analysis has the advantage of being automated and unbiased. For instance, once the settings in each module have been set to give an effective capture of the cell, i.e., outlining the nucleus and cell border true to the image, several images can be

analysed within minutes. Therefore, the sample size is not dictated by time constraints or labour of an individual. Additionally, if the settings within each module are correct, then the program will measure every whole cell in an image giving an unbiased approach. However, there are also disadvantages of this method. There are settings within each module that need to be modified with every image set to gain a true capture of your cells in order to determine accurate measurements. While there are example data sets and tutorials available, every image varies considerably; therefore, this investigation found that the accuracy of the capture was not satisfactory for every image. This led to the troubleshooting of settings in a trial-and-error method that, at times, resulted in an unsatisfactory capture. In addition, any part of the images that are not in focus or have low fluorescence can lead to CellProfiler™ not capturing the cell border accurately and thus affecting the measurements given. It was therefore decided that ImageJ was the preferred method to use for the cell shape analysis. Due to this, no circularity and eccentricity data was collected for Day 8 and Day 14 cells.

2.9. Rho activation in MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} Cells

To assess the effects of RhoA on MDCKII^{mChEB2Hi} cells, the cells were subject to a 30-minute Shp-2 phosphatase mediated Rho activation by a Rho activator I (G-switch™, Cat no. CN01 from Cytoskeleton). The Rho activator I was reconstituted in DMSO to give a stock solution of 0.2 units/μl. The stock was then diluted in supplemented media to give working concentrations of 1 unit/ml (0.5%) and 2 unit/ml (1%). The same method was used with DMSO to prepare a negative control. MDCKII cell lines, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} were sub-cultured, trypsinized and counted according to protocol (see 2.2. and 2.2.1). A cell suspension of 500,000 cells per ml was produced from each cell line in supplemented media and 500 μl of cell suspension was transferred into individual wells of a 24-well plate containing glass coverslips. MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} were kept in media containing G418 (10 μl/ml) at every stage. The media was switched from 10% FBS (v/v) to 5% (v/v) FBS on Day 2. Cells had a media wash and change every other day. Cells were then subject to a 24-hr 'serum starve' with 0.1% DMSO, 1% (v/v) FBS supplemented media. After the serum starve, cells were treated with 500 μl of 1 unit/ml (0.5%) or DMSO (0.5%) supplemented media, as a negative control, and placed in a humidified incubator for 30 minutes at 37 °C, 5% CO₂. The cells were then methanol-fixed, immuno-labelled, stained for DAPI and mounted according to the protocol (see 2.3.). Cells were immuno-labelled for either pMLC and E-cadherin or mDia-1 and β-catenin (Table 1). E-cadherin and β-catenin labelling were used to identify cell borders. The slides were stored at 4 °C and visualised using a Zeiss Observer 7 inverted widefield microscope and Zeiss ZEN blue 3.0 imaging software to produce multichannel immuno-fluorescence images using a 63x (oil) objective. A fluorescence intensity analysis of

junctional pMLC was carried out using ImageJ. The Region Of Interest tool (ROI) allowed set rectangles (2µmX10µm) to be placed in the E-cadherin channel for unbiased measurements of the fluorescence intensity in the pMLC channel (Appendix 8).

2.10. Dynamin-2 inhibition with Dynasore in MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} Cells

To investigate the effects of Dynamin-2 inhibition on E-cadherin distribution, Dynasore was used to inhibit the downstream endocytic pathways of dynamin, resulting in the suppression of coated vesicle formation. Dynasore was resuspended in DMSO to give a stock concentration of 80 nM, this was then diluted further (1:1000) in serum free DMEM media to produce a working concentration of 80 µM (0.1%). The same method was used with DMSO to prepare a negative control. MDCKII cell lines, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} were sub-cultured, trypsinized and counted according to protocol (see 2.2.1 and 2.2.2). A cell suspension of 500,000 cells per ml was produced from each cell line in supplemented media and 500 µl of cell suspension was transferred into individual wells of a 24-well plate containing glass coverslips. MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} were kept in media containing G418 (10 µl/ml) at every stage. The media was switched from 10% FBS (v/v) to 5% (v/v) FBS on Day 2. Cells had a media wash and change every other day. Cells were then subject to a 24-hr 'serum starve', with 0.1% DMSO, 1% (v/v) FBS supplemented media on Day 9. After the serum starvation, on Day 10, cells were treated with 500 µl of the Dynasore suspension (80 µM) or 0.1% DMSO supplemented media, as a negative control, and placed in a humidified incubator for 1 hr at 37 °C, 5% CO₂. The cells were then methanol-fixed, immuno-labelled, stained for DAPI and mounted according to protocol (see 2.3.). Cells were immuno-labelled for either E-cadherin and Dynamin-2 or E-cadherin and β-catenin (Table 1). The slides were stored at 4 °C and visualised using a Zeiss Observer 7 inverted microscope and Zeiss ZEN blue 3.0 imaging software to produce multichannel immuno-fluorescence images using 63x (oil) objectives. A fluorescence intensity analysis of junctional E-cadherin and analysis of E-cadherin intensity across junctions was carried out using ImageJ (Appendix 8 and 9).

2.11. Statistical analysis

All statistical analyses were performed using the IBM SPSS® Software. First, to test for normality, the Shapiro-Wilk test was conducted on the data sets (P=0.05) with the null hypothesis: there is no statistically significant difference between the data set being tested and a normally distributed data set. If P>0.05, the null hypothesis would be accepted, concluding a normal distribution for the data, allowing a parametric two-tailed unpaired t-test to be conducted, utilising the group's means. However, if P<0.05 then the null hypothesis

would be rejected, concluding that data set would not be normally distributed and therefore the non-parametric equivalent test was adopted – a Mann Whitney-U test, using the group's mean ranks. Whether a parametric or non-parametric test was performed the null hypothesis remained: there is no such statistically significant difference between the two groups being tested.

Chapter 3.

Potential role of FGF signalling in EB2 expression

3.1. Overview

Previous studies have elucidated a link between FGFs, their receptors and EB2 in various epithelial cell lines (Vidrich et al., 2004, Arnaud-Dabernat et al., 2007, Szarama et al., 2012). Here, TC7 cells were used as a model for differentiated, partially polarised epithelial monolayers. The aim of this investigation was to determine which FGFs and FGFRs were expressed in TC7 cells via an RT-PCR analysis. Then, the effect of inhibiting all FGFRs in TC7 cells with SU5402 was explored to investigate the effects on EB2 expression using fluorescence intensity analysis of EB2 immunolabelled cells.

3.2. Introduction

3.2.1. EB2, FGFR3 and epithelial differentiation

FGFs and their receptors play a key role in the signalling for many processes in epithelial cells. When investigating the crypt-villus units in the small intestine, Goldspink et al. (2013) found that EB2 expression was highest in the stem cell population at the base of the crypt (Figure 4A); a similar expression pattern was found for FGFR3 expression in the gut (Vidrich et al., 2004, Arnaud-Dabernat et al., 2007) (Figure 8A-B). Conversely, results by Kanai et al. (1997) indicated that increasing FGFR3 expression correlated with increasing confluency in Caco-2 cells. However, once partial/full epithelial polarisation was reached (Day 7) a dramatic decrease in FGFR3 expression was observed (Figure 9C). A similar pattern of increasing FGFR3 expression with increasing confluency, followed by a decrease upon reaching full confluency was also evident in transimmortalized mouse intestinal cells (M-ICc12) (Figure 9D) (Arnaud-Dabernat et al., 2007). A reverse relationship was demonstrated in mouse inner medullary collecting duct cells (mIMCD-3) with EB2 decreasing in expression with increasing confluency (Goldspink et al., 2013) (Figure 8A). Perhaps this indicates that during early stages of epithelial differentiation, FGFR3 is upregulated, and this leads to subsequent EB2 downregulation and, once achieved, FGFR3 expression is downregulated.

Another study conducted by Szarama et al. (2012) likewise indicated a potential connection between FGFR3 and EB2 expression. Here, analysis of the cochlea of FGFR3 knockout mice suggested that FGFR3 influences MT stability. FGFR3 knockout resulted in a significant reduction in MT number and subsequently an altered morphology of the supporting cells in the organ of Corti within the cochlea compared to control cells (Szarama et al., 2012). These supporting cells are noted for containing large apico-basal MT arrays and expressing low levels of EB2 (Goldspink et al., 2013). Also, inhibition of all FGFRs with SU5402 in mouse cochlear explants resulted in upregulation of genes involved in MT stability including MAPRE2

and the serine/threonine-protein kinase MARK2 which is involved in the regulation of cell polarity and MT dynamics (Szarama et al., 2012). These findings indicate an association between FGF signalling and MAPRE2 gene expression with MT stability. Therefore, suggesting that FGFR3 mediates changes in MT stability by downregulating EB2.

3.2.2. FGFR3, a potential receptor for FGF18

FGFR3-IIIb expression is restricted to epithelial tissue, though IIIc expression can be found in both epithelial and mesenchymal tissue (Murgue et al., 1994, Scotet and Houssaint, 1995). In colorectal cancer, FGF3-IIIb is downregulated, but its rescue has been suggested to inhibit cancer progression to more malignant phenotypes, possibly by promoting differentiation (Jang, 2005). FGF3-IIIb is downregulated by mutations mediating defective splicing, therefore producing a nonsense mutation within the FGFR3-IIIb mRNA (Holzmann et al., 2012). Meanwhile, FGR3-IIIc may be upregulated or maintained depending on the tumour. Disruption of FGFR-IIIc expression in CRC by a dominant-negative FGFR3-IIIc construct, resulted in inhibition of tumour growth and survival, *in vitro* and *in vivo*; whereas no such effect was observed with the respective FGFR3-IIIb mutant (Sonvilla et al., 2010). This, therefore, strongly suggests that FGFR-IIIc expression is associated with CRC progression. This switch to the FGFR-IIIc isoform in CRC has been associated with progression of carcinomas to a more malignant phenotype, sensitising the tissue to several more potential ligands such as FGF18 which is commonly upregulated in CRC (Table 3) (Yan et al., 1993, Ellsworth et al., 2002, Liu et al., 2002, Sonvilla et al., 2008, Sonvilla et al., 2010).

Table 3. The ligand-binding pattern of FGFR3 isoforms IIIb and IIIc. Adapted from Zhang et al. (2006), Holzmann et al. (2012).

Ligand binding	
IIIb	IIIc
FGF1, FGF9, FGF16	FGF1, FGF2, FGF4, FGF5, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF20, FGF21

The results by Vidrich et al. (2004) found that in mouse cells, both IIIb and IIIc FGFR3 expression was exclusive to the epithelial stem cells at the base of the crypt and their adjacent undifferentiated progenitor cells, *in vivo*. However, levels of FGFR-3 IIIb mRNA were 3-fold higher than FGFR-3 IIIc mRNA. Vidrich et al. (2004) demonstrated that FGFR3 expression

was highest at 7-21 days post birth in the undifferentiated epithelial cells at the base of the intestinal crypt, followed by a decline with low expression persisting in adult mice. Furthermore, adult mouse small intestine sections that were subjected to radiation demonstrated a significant increase in FGFR3 expression when regenerating. Therefore, suggesting FGFR3 activated signalling modulates small intestinal crypt formation and their cell composition by proliferation and differentiation via stem cells and their undifferentiated progenitors. Previous studies have provided evidence for a role for FGFR3 in both proliferation and differentiation (Colvin et al., 1996, Mueller et al., 2002).

Arnaud-Dabernat et al. (2007) investigated the consequences of FGFR3 deficiency in mice using cells from the small intestinal crypt-villus unit. FGFR3^{-/-} and WT mice treated with a FGFR3-neutralising antibody demonstrated a similar consequence: a significant increase in crypt stem cell proliferation. Thus, suggesting that FGFR3 acts as a negative regulator of cell proliferation. Moreover, administration of FGF18 in wildtype mice decreased cell proliferation; thus, strongly suggesting FGF18 as a ligand for FGFR3 in the mouse gut (Arnaud-Dabernat et al., 2007). In addition, ERK activation was increased in FGFR3^{-/-} mice therefore, demonstrating an inverse correlation. However, this contrasts with the findings by Chen et al. (2017) in lung cancer whereby knockdown of FGF18 led to inhibition of the ERK signalling pathway, suppression of cell proliferation and migration. Perhaps indicating that FGF18 binding to FGFR3 leads to inhibition of ERK signalling and suppression of cell proliferation.

Furthermore, overexpression of FGFR3 in the human epithelial colorectal adenocarcinoma cell line Caco-2, resulted in a significant decrease in expression of enterocytic differentiation markers and an increase in mature Paneth marker genes (Brodrick et al., 2011). In addition, β -catenin mRNA expression was significantly increased, followed by increased TCF4/ β -catenin-regulated transcriptional activity; known to stimulate Paneth cell markers (Brodrick et al., 2011). Therefore, the findings suggest a contribution of FGFR3 overexpression to the stimulation of TCF4/ β -catenin pathways in Paneth cell differentiation (Brodrick et al., 2011). A converse result was shown by Vidrich et al. (2009) who found that when FGFR3 expression was knocked out in mice, the quantity of β -catenin in the cell, especially in the nucleus, was significantly decreased. Similarly, cyclin D1 and matrix metalloproteinase-7 (MMP7) expression was significantly reduced in FGFR3^{-/-} mice, that pose as downstream targets of the TCF4/ β -catenin pathway (Vidrich et al., 2009). Therefore, a significant reduction in the differentiation from stem cell progenitors to Paneth cells was also observed (Vidrich et al., 2009). Furthermore, FGFR3^{-/-} mice demonstrated a significant decrease in the quantity of stem cells and an approximate 50% reduction in the number of intestinal crypts. In addition, when FGFR3 was activated in Caco-2 cells, β -catenin and Tcf-4 signalling was reduced (Vidrich et al., 2009). This suggests that FGFR3 expression is strongly associated with stem

cell differentiation via the TCF4/ β -catenin signalling pathways. Therefore, FGFR3 expression may influence the cell composition of intestinal crypts in the developing mouse intestine; perhaps suggesting a similar link may be apparent in humans.

FGF2 is well evidenced in its role of stimulating angiogenesis in both physiological and pathological conditions within endothelial cells (Giuliani et al., 1999, Compagni et al., 2000, Javerzat et al., 2002). However, results by (Antoine et al., 2005) indicate that other FGFs and FGFRs have a role to play in angiogenesis. Complete expression profiling via RT-PCR on Human umbilical vein endothelial cells (HUVECs) revealed the presence of secreted FGF5, 7, 8, 16 and 18. Additionally, FGFR3IIIc was expressed to equivalent levels to that of FGFR2IIIc, a well characterised FGFR in endothelial cells. In HUVECs, FGF8 was consistently expressed in the A, B, E and F isoforms (Antoine et al., 2005). FGF8 is considered the most potent and presents 100% identity between human and mouse (Blunt et al., 1997, Antoine et al., 2005). Previous data confirms binding of FGF8 to FGFR2IIIc and FGFR3IIIc (Blunt et al., 1997). Antoine et al. (2005) similarly verified expression of FGFR1-3, exclusively in the IIIc isoform, in endothelial cells. However, expression of FGFR3IIIc was observed to be highest, thus, posing as a favoured receptor. Earlier studies by Mattila et al. (2001), for example, demonstrated that FGF8B overexpression in breast cancer cells (S115) stimulates tumour formation and neo-angiogenesis. Furthermore, FGF8 belongs to the FGF8/17/18 subfamily (Itoh and Ornitz, 2011, Ornitz and Marie, 2015) and FGF18 exhibits the highest homology to FGF8B; therefore, demonstrating a comparable binding specificity (Hu et al., 1998). Consequently, suggesting FGFR3IIIc as a favoured receptor on endothelial cells for the FGF8, 16 and 18 (Antoine et al., 2005).

3.2.3. FGF18 upregulation and cancer

FGF18 is classified as a paracrine FGF, part of the FGF8/17/18 subfamily (Itoh and Ornitz, 2011, Ornitz and Marie, 2015). The FGF8/17/18 subfamily have been shown to specifically bind FGFR4 and other select mesenchymal isoforms of FGFR1-3 (Zhang et al., 2006). Under normal physiological conditions, FGF18 activity facilitates limb development via skeletal growth (Moore et al., 2005). For example, FGF18 mediated activation has been demonstrated to negatively regulate chondrocyte proliferation and differentiation (Ellsworth et al., 2002, Liu et al., 2002). While FGFs frequently play a critical role in normal physiology, their absence can be a contributor to pathological conditions. For instance, FGF18 null mice demonstrated defects in their bones and cartilage; including slower osteogenic differentiation, increased proliferative zones and delayed suture fusion (Liu et al., 2002). Nevertheless, there is considerable evidence to suggest a link between the inappropriate expression of FGFs, their

receptors and cancer progression. In particular, the increase in cancer characteristics such as an increased proliferation rate, equalling tumour growth, enhanced invasiveness, neo-angiogenesis and increased motility (Antoine et al., 2005, Beenken and Mohammadi, 2009, Turner and Grose, 2010). Specifically, upregulation of FGF18 expression has been revealed in colorectal, lung and ovarian cancers with emphasis on an association between increased FGF18 mRNA and protein with tumorigenesis (Sonvilla et al., 2008, Koneczny et al., 2015).

Overexpression of FGF18 was observed to stimulate proliferation in mouse embryonic fibroblasts (NIH 3T3) *in vitro*, whereas *in vivo* overexpression of FGF18 resulted in stimulation of various tissues of epithelial and mesenchymal origin (Hu et al., 1998). However, the liver and small intestine demonstrated the most significant increase in cell proliferation (Hu et al., 1998). Sonvilla et al. (2008) revealed that FGF18 expression positively correlates with colon oncogenesis. HUVECs treated with equivalent levels of vascular endothelial growth factor (VEGF) or recombinant FGF18, demonstrated parallel tube formation; indicating a neoangiogenic function of FGF18 (Sonvilla et al., 2008). Therefore, the results by Sonvilla et al. (2008) suggest that FGF18 has a stimulating effect on tumour progression in the cancer cells themselves, but also in the tumour microenvironment. Furthermore, artificial overexpression of FGF18 significantly increased the proliferation rate in two colorectal tumour cell lines, LT97 and Caco-2, which are normally characterised by their slow growth and low levels of endogenous FGF18 (Sonvilla et al., 2008). Meanwhile, in another colorectal tumour cell line, SW480, which demonstrated high amount of endogenous FGF18, cell survival during starvation was enhanced via prevention of apoptosis induction (Sonvilla et al., 2008). SiRNA depletion of FGF18 in SW480 cells significantly reduced their ability to form colonies. Therefore, the data collected indicates an oncogenic function of endogenous FGF18 that encourages cancer progression via neo-angiogenesis and increased proliferative power with regards to colorectal cancer cells (Sonvilla et al., 2008).

In addition, results by (Chen et al., 2017), revealed knockdown of FGF18 in H460 cells, a non-small-cell lung carcinoma (NSCLC) cell line, led to suppressed proliferation and migration. Inhibition of the MAPK pathways, ERK and p38, resulted in decreased proliferation after FGF18 stimulation; thus, suggesting that these pathways are activated downstream of FGF18 activation (Chen et al., 2017). This was evidenced by a 48-hr siRNA-FGF18 treatment of the cells which resulted in the inhibition of ERK and p38 pathways (Chen et al., 2017). An equivalent role regarding cancer progression/growth was demonstrated with FGFR3 expression by Arai et al. (2015) in lung cancer cells and Shimokawa et al. (2003) in colon cancer cells. As it has been shown previously that FGF18 is capable of binding to FGFR3 isoforms, such as IIIc-FGFR3 (Liu et al., 2002), then perhaps there may be a link between FGF18, FGFR3 and the results observed.

3.3. Results

3.3.1. FGFs in TC7 cells

To determine what FGFs were present in TC7 cells, an RT-PCR analysis was carried out. TC7 cells were grown on glass coverslips until 90% confluent. RNA was extracted and purified using the Quick-RNA™ Miniprep Kit according to manufacturer's instructions. Dr. Mohammad K. Hajihosseini performed the RT-PCR. Due to its abundance in cells, β -actin primers were used in an initial test reaction, in order to confirm the reaction's efficiency and RNA quality. As expected, β -actin showed as a doublet at approximately 450 bp (Appendix 1A). Next, a test was run for FGF2, FGF8, FGF17, FGF18, FGF1, FGF9 and β -actin as a control. However, the gel image only showed FGF8 to be present (Appendix 1B). Unfortunately, due to lack of time, primarily because of the COVID-19 pandemic, not all FGFs were tested.

3.3.2. EB2 and FGFR3's localisation in TC7 cells

Human colorectal adenocarcinoma (TC7) cells were grown for 48-hrs until approximately 80% confluent and then immunolabelled for EB2 (Absea, 010614A11) and α -tubulin (Abcam, AB15246) or FGFR3 (Abcam, AB180908) and Tyrosinated-Tubulin (YL1/2, Abcam, AB11316) (Figure 12). Images showed MTs were organised in a dense radial array in TC7 cells (Figure 12B). There was some evidence that EB2 associated along MTs (Figure 12A). Immunolabelling confirmed junctional localisation of FGFR3 (Figure 12B).

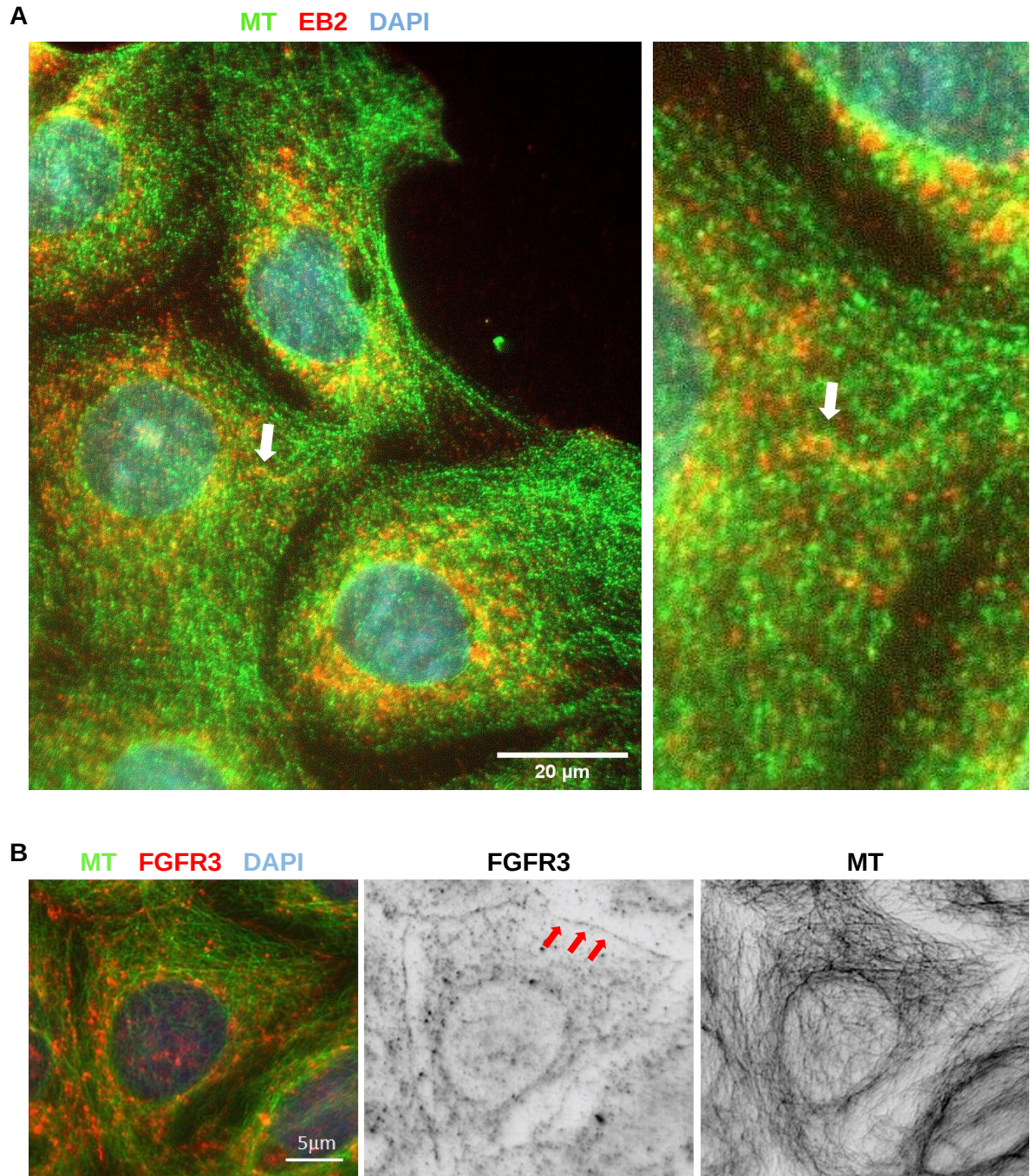


Figure 12. EB2 and FGFR3's localisation in TC7 cells. (A) TC7 cells were grown on glass coverslips and immunolabelled for EB2, shown in red (Absea, 010614A11), MT via α -tubulin, shown in green (Abcam, AB15246) and stained for DAPI (blue) to reveal EB2 expression dispersed throughout the cytoplasm. Images were taken on a widefield fluorescence microscope with a x63 oil objective. EB2's localisation along MT is indicated via the white arrows. (B) TC7 cells were immunolabelled for FGFR3, shown in red (Abcam, AB180908), MTs via YL1/2 (Tyrosinated-Tubulin), shown in green (Abcam, AB11316) and stained for DAPI. FGFR3 expression was apparent in the cell cytoplasm with localisation along the cell borders as indicated by the red arrows. Scale bars = 20 μ m (A) and 5 μ m (B).

3.3.3 Effect of FGFR Inhibition on EB2 expression in TC7 cells

In order to determine a potential link between EB2 expression and FGFR activity, TC7 cells were subject to the first generation FGFR TKI (tyrosine kinase inhibitor), SU5402, that targets the ATP-binding site of FGFR1-4 (Mohammadi et al., 1997), therefore inhibiting the activity of all FGFRs. 1 µl of SU5402 (100 mM suspended in DMSO) was diluted into 10,000 µl media, producing 10 µM working concentration (0.01%). The SU5402 solution (10 µM) was then diluted further in media to produce 5 µM (0.005%) and 2 µM (0.002%). The same method was repeated using DMSO to produce the negative control. First, a concentration range of SU5402, 2 µM, 5 µM and 10 µM was tested in TC7 cells (data not shown). A concentration of 10 µM proved detrimental to cell survival; thus, the lower concentration of 5 µM was adopted in subsequent analysis. A similar effect was demonstrated by Gudernova et al. (2016) who concluded SU5402 (10 µM) was toxic to rat chondrosarcoma (RCS) chondrocytes compared to other FGFR TKIs, PD173074, AZD1480, AZD4547 and BGJ398, for example. Post-incubation, the cells were methanol-fixed, immuno-labelled for EB2, stained for DAPI and mounted on slides. The slides were visualised by a widefield upright Zeiss Axiovert 200M microscope. Images were taken at a set exposure using the Axiovision software (Zeiss) to produce multichannel immuno-fluorescence images using 20x (air) and 63x (oil) objectives. Then, CellProfiler™ was used to analyse the fluorescence intensity of EB2 (Appendix 2 and 3). The images were calibrated by identifying the 'primary objects' (nuclei) and 'secondary objects' (cell borders), in order to measure EB2 fluorescence intensity within cytoplasm of each cell (Appendix 3).

The results showed that statistically significantly more EB2 fluorescence intensity, on average, was observed when cells were treated with DMSO than media only (Figure 13), demonstrating a slight 1.367 percentage increase (Table 4). Therefore, suggesting that the presence of DMSO significantly contributed to the mean increased fluorescence intensity of EB2 in TC7 cells. Cells treated with SU5402 revealed a statistically significant increase in EB2 fluorescence intensity ($P < 0.001$) compared to the DMSO control ($P < 0.01$) (Figure 13A- B), demonstrating a percentage increase of 6.85 (Table 4).

Table 4. Comparison of EB2 expression in TC7 cells when subject to media only, DMSO in media (0.005%) and SU5402 (5 uM, 0.005%). The table shows the percentage increase (%) in mean fluorescence intensity (arbitrary units) from media only treated TC7 cells.

Repeat	Percentage Increase (%)	
	DMSO	SU5402 (5 uM)
no.1 (FBS 10%)	0.932	6.850
no.2 (FBS FREE)	1.367	6.205
no.3 (FBS FREE)	1.276	3.931

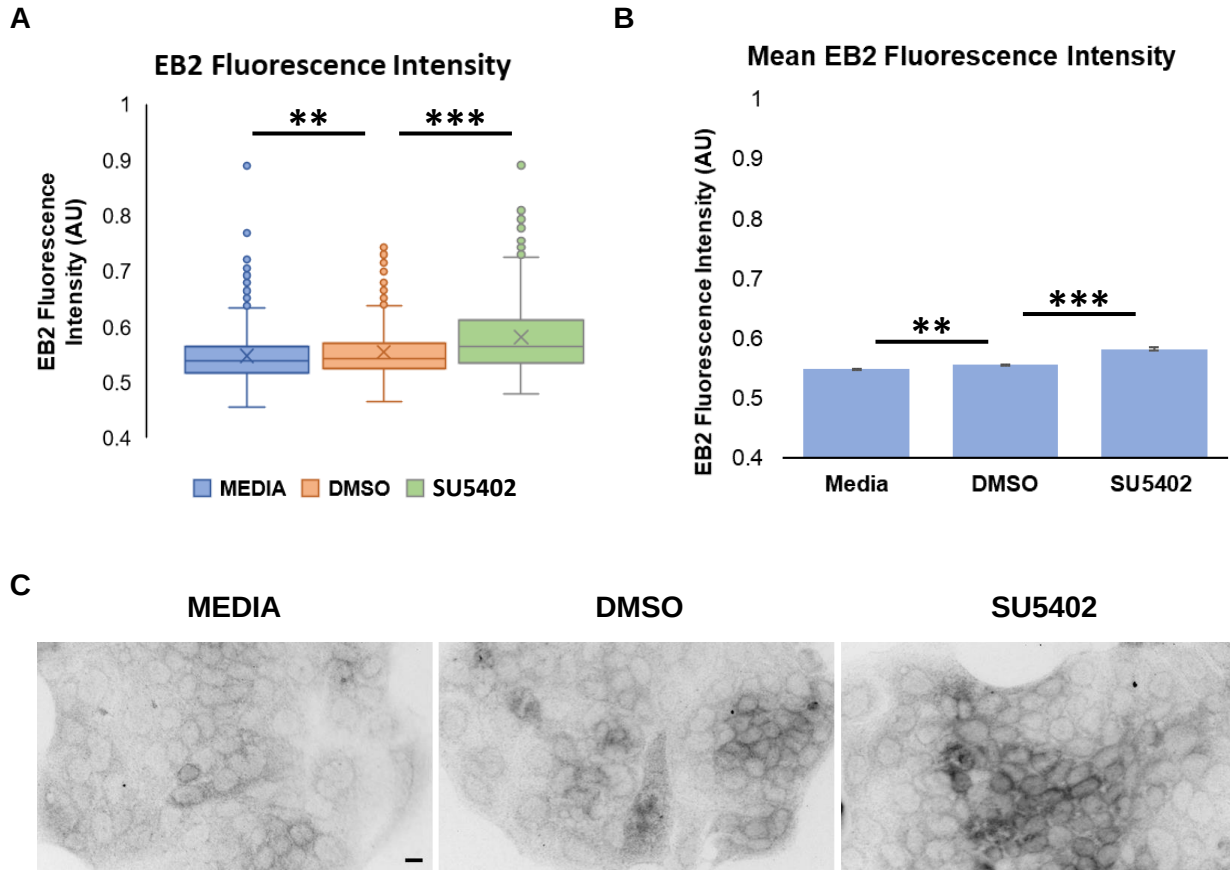


Figure 13. FGFR inhibition leads to increased EB2 expression. TC7 cells were grown on glass coverslips in 35 mm dishes until confluent. Cells were subject to an 18.5 hr incubation with SU5402 (5 μm, 0.005%), DMSO (0.005%) or media only. Next, cells were immunolabelled for EB2 (Absea, 010614A11) and stained for DAPI, then imaged on a widefield fluorescence microscope with a x63 oil objective. The data set is presented as both a box and whisker plot and bar chart. (A) Box and whisker plot demonstrating the distribution of mean fluorescence intensity (arbitrary units) of EB2 in TC7 cells post incubation with media only (blue) (N=1532), DMSO in media at 5uM (orange) (N=1259) and SU5402 in media only (green) (N=1285). (B) Bar chart representation of the mean fluorescence intensity of EB2 in TC7 cells ±1SE. (C) Example images taken on a widefield fluorescence microscope with a x20 air objective at fixed exposure of 6,500 ms showing EB2 labelling post incubation. Scale bar = 20 μm. (Asterisks indicate ** = P<0.01 and *** = P<0.001; Games-Howell test Non-Parametric Post Hoc Test). Analysed on CellProfiler™ N=11,080, three experiments, each with three replicas.

3. 4. Discussion.

3.4.1. FGFR inhibition with SU5402 leads to increased EB2 expression

The level of EB2 expression varies considerably and seems to be linked to the stage of cell development, MT organisation and dynamics (Goldspink et al., 2013). In addition, EB2 upregulation has been associated with highly invasive cancers such as certain pancreatic and liver carcinomas (Abiatari et al., 2009, Zhong et al., 2021). How EB2 is regulated is not known but evidence suggests that FGF signalling may be involved with, for example, loss of FGFR3 leading to EB2 upregulation (Szarama et al., 2012). In this study, immuno-labelling showed FGFR3 to be prominent at cell junctions in TC7 cells. This fits with previous studies which indicated that FGFR3 expression was evident in the human colorectal cancer cell line, Caco-2 from which TC7 cells are derived (Kanai et al., 1997).

Analysis of EB2 in TC7 cells revealed association along MTs as well as cytoplasmic presence. A similar distribution was previously reported by Komarova et al. (2009) and Goldspink et al. (2013) who demonstrated EB2 localised predominantly along the MT lattice, with some plus-end localisation of the epithelial derived cell lines CHO-K1 and ARPE-19, respectively. This is in contrast to EB1 and EB3 which strongly associate with the plus-end of MTs due to their high affinity for GTP-tubulin, therefore outcompeting EB2 for the plus-end location (Maurer et al., 2011). However, in many epithelial cell lines (ARPE-19, TC7, U2OS and HCT116) it has been demonstrated that EB2 depletion results in lattice localisation of EB1/3 (Goldspink et al., 2013). This suggests that while EB1/3 outcompetes EB2 for the plus-end position, EB2 likewise seems to outcompete EB1/3 for the lattice position.

SU5402 was dissolved in DMSO and TC7 cells were treated with this FGFR inhibitor or 0.005% DMSO. The effect of 0.005% DMSO on the fluorescence intensity of EB2 compared to media alone was therefore assessed. The results revealed that cells treated with 0.005% DMSO had a significantly higher EB2 fluorescence intensity than those treated with media only. One possible explanation for this may be that DMSO had a stabilising effect on MTs. For tubulin polymerisation and the formation of MTs to occur, a critical concentration of tubulin must be surpassed. The tubulin critical concentration can be dependent on several factors with DMSO being one of them (Himes et al., 1977, Robinson and Engelborghs, 1982, Algaier and Himes, 1988). The presence of DMSO has been shown to lower this critical concentration of tubulin up to 10-fold at a 10% concentration (Algaier and Himes, 1988). More recently, a concentration of 4% has resulted in a significant decrease in the critical concentration of tubulin-GTP (Sharma et al., 2007). However, in this study a concentration of 0.005% DMSO was utilised. An upregulation of EB2 has previously been associated with increased MT

dynamics (Goldspink et al., 2013); thus, it could be speculated that the stabilising effect of DMSO could mediate an upregulation of EB2 protein to counter-act the sudden increase in MT stability via a negative feedback mechanism. Further investigations will be needed in order to explore whether DMSO triggers EB2 upregulation through an increase in MT stability. One possibility is to treat TC7 cells with DMSO and media only, serving as a negative control, then subject the cells to ice-shock, followed by fixation and immunolabelling of the MT. From this, the mean number of MTs still intact can be estimated to indicate the MT stability of cells subject to DMSO compared to the control.

FGFR inhibition revealed a significant increase in EB2 fluorescence intensity compared to the DMSO control. The percentage increase in EB2 fluorescence intensity post FGFR inhibition was greater than that of the DMSO control. This suggests perhaps that FGFRs play a role in the regulation of EB2 expression in TC7 cells. Further experiments, such as a western blot analysis, would be needed to verify EB2 upregulation. A similar association between FGFRs and EB2 was also revealed by Szarama et al. (2012). Mouse cochlear explants were treated with the same FGFR inhibitor, SU5402, and displayed up to a 3-fold increase in MAPRE2 gene expression, compared to the controls. The microtubule affinity-regulating kinase-2 (MARK2), similarly demonstrated a significant increase following SU5402 treatment (Szarama et al., 2012). It is noteworthy that MARK2, like EB2, is involved in MT dynamics. MARK2 is a serine/threonine kinase that governs the binding of MAPs to MT which consequently effects MT dynamics and cell polarity (Drewes et al., 1997). Cyfip1, Cytoplasmic FMR1 Interacting Protein-1, was likewise upregulated and is involved in the cytoskeletal dynamics and protein translation (Schenck et al., 2001).

Since SU5402 inhibits all FGFRs, in order to investigate which were mediating the results shown, Szarama et al. (2012) explored FGFR3^{-/-} mice. Transmission electron microscopy (TEM) analysis of the non-sensory pillar cells suggested a significant reduction in MT number and an altered morphology compared to the control cells, suggesting that FGFR3 is a component in the signalling for MT polymerisation and/or stability. (Szarama et al., 2012). This perhaps implies that in the absence of FGFR3, MAPRE2 is upregulated while FGFR3 activation leads to a down-regulation in EB2. As a result, MTs become more dynamic leading to a significant reduction in stable MT bundles in the pillar/supporting cells of the inner ear.

A similar mechanism is indicated by Kanai et al. (1997) and Goldspink et al. (2013) in *in vitro* cultured cells. In Caco-2 cells, FGFR3 expression gradually increased with increasing confluency up to 7 days, while further differentiation led to a decrease in FGFR3 (Kanai et al., 1997). Goldspink et al. (2013) found a converse relationship whereby EB2 expression decreased with increasing cell confluence during epithelial differentiation. Perhaps suggesting

that FGFR upregulation leads to EB2 downregulation, and thus decreased MT dynamics that subsequently cause the formation of stable MT bundles, allowing cells to maintain a differentiated state. To supplement this investigation, a reverse-transcription PCR analysis was done to explore what FGFRs and FGF ligands are expressed in TC7 cells. Primers for several FGFs were used, FGF2, FGF8, FGF17, FGF18, FGF1 and FGF9, while β -actin primers were used as a control. The gel image revealed a faint band for FGF8 only, β -actin was verified as a doublet (Appendix 1). Further studies would be required to test the remaining FGFRs and FGFs and to determine whether the mechanism indicated between EB2 and FGFR3 specifically, is apparent in the gut *in vivo*.

However, the effectiveness of SU5402 must be considered. Gudernova et al. (2016), concluded that SU5402 (10 μ M) inhibited the activity of FGFR1-4 unequally. The results demonstrated that FGFR1 and FGFR2 were relatively suppressed to comparable degrees, FGFR3 and FGFR4 were also suppressed to similar levels. Though, relative suppression levels of FGFR1 and FGFR2 were higher than that of FGFR3 and FGFR4 (Gudernova et al., 2016). Thus, raising questions such as, does the 5 μ M concentration of SU5402 used in this study sufficiently inhibit all activity of the FGFRs. In addition, off-target activity should also be a consideration. Gudernova et al. (2016) concluded that SU5402 was one of the TKIs that demonstrated significant levels of off-target activity. SU5402 was subject to 14 unrelated tyrosine kinases and inhibited 7 (DDR2, IGF1R, FLT3, TRKA, FLT4, ABL and JAK3) to an alike or greater extent than that of FGFRs (Gudernova et al., 2016). Therefore, not only could the 5 μ M concentration of SU5402 be inhibiting individual FGFRs to different extents, but it could also be inhibiting other, potentially unrelated, TKIs that may be having an effect on EB2 expression. Future work should therefore also include depletion studies of individual FGFRs.

Chapter 4.

Role of EB2 in Unjamming

4.1. Overview

Here, MDCKII cells were used as a model for differentiated, partially polarised epithelial monolayers to study the effects of EB2 overexpression on tissue unjamming. Specifically, MDCKII cell clones overexpressing mCherry-EB2 (MDCKII^{mChEB2Hi}) or the empty mCherry control vector (MDCKII^{mChEMPTY}) as well as wildtype MDCKII cells (MDCKII^{WT}) were used. A previous study by Ben Rix in the Mogensen lab suggested that EB2 overexpression in MDCKII cells promotes unjamming. Therefore, the aim of this investigation was to provide further evidence for this and to explore the possible mechanisms responsible for high expression of EB2 leading to tissue unjamming and loss of normal architecture. In particular, the effects of EB2 overexpression on MT and actin organisation, as well as localisation of Par3, ZO-1, mDia-1 and mDia-2, were investigated. In addition, the effects of EB2 overexpression on adherens junctions were investigated by exploring the localisation and expression levels of E-cadherin, phosphorylated myosin light chain (pMLC), the MT associated Rho GEF GEF-H1 and dynamin-2 as well as dynamin inhibition. Finally, the effects of Rho activation on pMLC, mDia-1 and the cell shape index were studied.

4.2. Introduction

In epithelial tissue, junction function is dependent on adhesive contacts at adherens junctions. These contacts are made by actin filament mediated recruitment and organisation of E-cadherin, β -catenin and α -catenin (Levayer et al., 2011). E-cadherin is an integral player at such cell contacts as it governs both cohesion and remodelling of cell junctions. Junction components are under continuous turnover through endocytosis and exocytosis. Exocytosis describes the delivery of new proteins and lipids by fusion of vesicles to the plasma membrane for junction regeneration. Endocytosis is a process in which the areas of the cell plasma membrane are internalised. The distribution of E-cadherin at junctions is governed by clathrin- and dynamin-mediated endocytosis. Dynamin-2 is a GTPase protein, encoded by the DNMT2 gene in humans. During endocytosis, dynamin-2 accumulates at the stalk of a budding vesicle, assembles into a helical polymer and undergoes a GTP hydrolysis-dependent conformational change which then severs the stalk, releasing a vesicle from the membrane (Ferguson and De Camilli, 2012).

Actin fibres crosslinked with NMII make up the actomyosin network which is physically linked to apical cell junctions. Contraction of the actomyosin network, particularly at the cell apex, is required for epithelial cell shape and function (Marivin et al., 2022). This contraction is stimulated by GTPase activity such as RhoA which leads to modification of junction lengths (Acharya et al., 2017). GEFs are a group of proteins that are responsible for activating

GTPases, such as Rhos, by catalysing the exchange of GDP for GTP (Joo and Olson, 2021). GEF-H1, encoded by the *ARHGEF2* gene, is a guanine exchange factor (GEF). Phosphorylation of GEF-H1 causes localisation along MTs. GEF-H1 is inactive when bound to MTs but during MT depolymerisation, it is released from MTs and is activated which then leads to its activation of Rho (Krendel et al., 2002). GEF-H1 has also been suggested to be important in mediating MT stability. In addition to MT localisation, GEF-H1 has been revealed to localise at tight junctions of polarised epithelial cells and inactivated by binding to cingulin, which subsequently causes RhoA inactivation and cell arrest (Aijaz et al., 2005).

During contraction, Rho-signalling leads to activation of the Rho-associated protein kinase ROCK which then goes on to phosphorylate NMII light chain (Terry et al., 2011). Subsequently, NMII is activated and engages with actin filaments to induce actin filament sliding and contraction (Vicente-Manzanares et al., 2009). Thus, deeming apical junctions as vital players in contractility and cell-cell adhesion. Cell polarity is also largely governed by apical junctions. However, the molecular mechanism in which cells establish an apicobasal polarity and coordinate the cytoskeleton for cell shape and functions is not entirely understood.

Partitioning defective-3 (Par3) is a well evidenced polarity marker that mediates its effect via the PAR complex (Par3/Par6/aPKC). Par3 also plays a role in cell proliferation, migration and tumour development. Zonula occludens-1 (ZO-1) is a membrane phosphoprotein with a molecular mass of 220 kDa and is commonly used as a tight junction marker (Stevenson et al., 1986). ZO-1 exists in two isoforms, ZO-1 α - and ZO-1 α +, due to alternative RNA splicing and is expressed in all epithelial and endothelial cells (Howarth et al., 1992, Willott et al., 1992). Par-3 and ZO-1 were used in this investigation to explore the effects of EB2 overexpression, on cell polarity, junction integrity and as a marker for cell peripheries.

Previous studies have revealed that the FH1/2 domains of formins can increase MT stability and co-alignment with actin filaments (Bartolini and Gundersen, 2010). In addition, Goldspink et al. (2013) subjected EB2-depleted ARPE-19 cells to a formin inhibitor (SMIFH2) that specifically inhibited the formin's FH2 domain, as a result, MTs were less organised and no longer co-aligned with actin filaments or associated with ACF7. Also, there were fewer actin filaments perpendicular to the cortex and more stress fibres. The results indicated that inhibition of formins in EB2 depleted cells rescued the phenotype of the control scramble siRNA cells, suggesting that formins are a key player in the EB2-depleted phenotype in epithelial cells. Formins such as mDia-1 also play a role in linear actin filament nucleation at epithelial adherens junctions which then aids -cadherin stabilisation at the cell junctions (Rao and Zaidel-Bar, 2016). Therefore, this study explored the localisation of formins, mDia-1 and

mDia-2, in MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells to investigate any further link between formins and EB2.

EB2 is the most divergent in structure, localisation and often function compared to its other two family members EB1 and EB3. Interestingly, EB2 possesses a larger CH domain, also called the globular calponin homology domain, which is essential to MT binding at the N-terminus (Figure 6) (Nehlig et al., 2017). Perhaps this mediates EB2's higher affinity for GDP-bound tubulin and thus association along the MT lattice, while its family members, EB1 and EB3 associate more strongly to the plus-end of MTs which are high in GTP-tubulin (Maurer et al., 2011, Roth et al., 2018). Results by Roth et al (2019) suggest that EB1/3 cause a conformational change in tubulin which leads to a shift in EB binding closer to the MT plus-end. However, the shift does not appear to occur upon EB2 binding (Roth et al., 2019). Perhaps, this is attributed to differences in the CH domains, however, this is just speculation. In addition to MT lattice association, EB2 has been revealed to have an effect on MT dynamics although this may be indirect. Analysis of the mouse cochlear, which contains terminally differentiated sensory hair and pillar cells, revealed marked differences in EB2 expression. Relatively high EB2 expression was evident in the hair cells which contain dynamic unbundled MTs while the pillar cells with their large apico-basal bundles of stable MTs, expressed little EB2 (Goldspink et al., 2013). Additionally, investigations into EB2 in the gut have shown that EB2 is downregulated as epithelial cells differentiate and gradually move up in crypts. Stem cells at the base of the crypt, which are undifferentiated and highly proliferative, had the highest EB2 expression. (Goldspink et al., 2013). EB2 expression has also been linked to other cellular processes such as migration, proliferation, genomic stability and diseases such as cancer (Yue et al., 2014, Imori et al., 2016, Zhong et al., 2021).

Similarly, in cells *in vitro*, the expression of EB2 has been shown to decrease with increasing confluency as the cells elongate and become partially polarised. This is also associated with a dramatic reorganisation of the MTs from a radial array of relatively dynamic MTs to an apico-basal array of more stable MTs. The apical surface becomes smaller due to actomyosin contraction within the zonular adherence belt. It has been established that EB2 is required at early stages of differentiation to maintain a population of dynamic MTs and enable assembly of the apico-basal array and facilitate epithelial elongation (Goldspink et al., 2013). However, it is not known if continuous relatively high expression of EB2 would interfere with apical constriction, epithelial differentiation and maintenance of a jammed tissue layer. In other words, is low expression of EB2 required to maintain the differentiated state and normal tissue architecture?

When cells are jammed, they present as closely compacted monolayers. Individual cells in a jammed system move very little and only rarely swap positions with their neighbours. A jammed state is also described as a solid state. Layers of homeostatic, polarised and differentiated epithelial cells have been characterised as a jammed system. Cells may shift from a jammed to unjammed state, this process is called the unjamming transition and is mediated by several factors including, cortical tension, cell-cell adhesion and cellular crowding (Farhadifar et al., 2007, Bi et al., 2015, Park et al., 2015). Generally, when a system is unjammed, the cells are larger, more variable in shape and more motile. These cells are also described as being in a fluid state, allowing collective migration and mediate changes in tissue morphology. Scenarios of tissue formation, remodelling such as in wound healing and cancer have been described as unjammed systems (Farhadifar et al., 2007, Tambe et al., 2011, Park et al., 2015, Atia et al., 2018, Kim et al., 2020). This investigation uses the shape index equation to indicate where cells are in the UJT.

The involvement of another mechanism of collective cell migration, pEMT, has been questioned, as some similarities between the two are evident, including a loss of specialized apical features and an altered transcriptional profile (Ghosh et al., 2022). Although, it is not fully understood whether these processes work in isolation or collaboration. EMT is suggested to be a fluid process and therefore has a spectrum of pEMT phenotypes (Nieto et al., 2016). During EMT, degradation of cell-cell junctions, loss of apico-basal polarity (and gain of front-back polarity), impaired monolayer integrity and expression of mesenchymal markers are key steps for the loss of the epithelial phenotype and gain of a mesenchymal one. However, pEMT allows cell migration without commitment to a full mesenchyme phenotype (Revenu and Gilmour, 2009, Jolly et al., 2015). Cells undergoing pEMT have varying degrees of junctional integrity and coordination with neighbouring cells. In addition, apico-basal polarity is not completely lost, and front-back polarity is not completely gained (Campbell and Casanova, 2016).

However, during UJT the key stages include a characteristic change in cell shape that enables increased motility, and cells appear to maintain their epithelial phenotype despite becoming increasingly migratory; in contrast to pEMT, where the epithelial phenotype is progressively lost (Park et al., 2015, Mitchel et al., 2020). Moreover, Mitchel et al. (2020) investigated EMT and UJT by pEMT, by TGF- β 1 exposure, or UJT, by mechanical stress, in differentiated primary human bronchial epithelial cells. The results concluded that inducing UJT resulted in cell elongation and cooperative migration. The cell-cell junctions, apico-basal polarity, and barrier function were maintained, and no mesenchymal markers were apparent (Mitchel et al., 2020). However, the inverse was apparent when pEMT was induced, perhaps suggesting that the mechanisms work independently to one another. Also, pEMT appears to allow single cell

or small groups of cells to detach from other cells and the ECM to migrate. The UJT however, demonstrated a collective movement comprising most of the monolayer (Mitchel et al., 2020). Further research into the UJT and pEMT mechanisms is needed to distinguish the driving factors of each, and any possible relationship they may have to one another. However, this study is focused on the effect of EB2 overexpression on UJT.

4.3. Results

4.3.1. Fluorescence intensity analysis revealed that MDCKII^{mChEB2Hi} cells express six times more EB2 than MDCKII^{mChEMPTY} cells

Confluent, partially polarised cell layers of MDCKII cells overexpressing EB2, or the empty vector, as well as wildtype MDCKII were used as models for studying tissue unjamming. The two subclones were generated by Jonathan Gadsby (Mogensen Lab) by transfecting wildtype MDCKII (MDCKII^{WT}) cells with a vector containing the cDNA for the mouse MAPRE2 gene encoding the EB2 protein and generating a stable EB2 overexpressing clone (MDCKII^{mChEB2Hi}) or the empty vector containing mCherry (MDCKII^{mChEMPTY}). The MDCKII^{mChEMPTY} cells function as a negative control to verify that any effects observed in MDCKII^{mChEB2Hi} cells would be due to EB2 overexpression rather than the transfected mCherry vector. MDCKII^{WT} cells were also used as a negative control for the MDCKII^{mChEMPTY} cells.

A fluorescence intensity analysis of EB2 on the three MDCKII clones was performed to confirm a significant overexpression of EB2 in MDCKII^{mChEB2Hi} cells compared to the two controls, MDCKII^{WT} and MDCKII^{mChEMPTY} cells. MDCKII^{WT}, MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} cells were grown for 10 days to reach confluency and EB2 expression was determined based on fluorescence intensity analysis using CellProfiler™ (see section 2.5.). Cells were double immuno-labelled with antibodies against EB2, to label both endogenous EB2 and mCherryEB2 and β -catenin, to define cell borders. Analysis revealed that MDCKII^{mChEB2Hi} cells showed significantly higher EB2 fluorescence intensity compared to the controls ($P < 0.001$) (Figure 14). MDCKII^{mChEB2Hi} cells revealed approximately a 6.5-fold increase in EB2 fluorescence intensity compared to MDCKII^{WT} cells and a 5.8-fold increase compared to MDCKII^{mChEMPTY} cells (Table 5). In addition, MDCKII^{mChEMPTY} cells showed significantly higher EB2 fluorescence intensity compared to MDCKII^{WT} cells ($P < 0.001$) with a 1.13-fold (Figure 14 and Table 5). The data is presented both as a box plot to show the spread of data and as a bar chart to focus on the mean fluorescence intensity to better highlight the differences (Figure 14).

Table 5. EB2 Fluorescence Intensity of MDCKII cells. The table shows the mean EB2 Fluorescence Intensity ± 1 SE of Day 10 MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells, the percentage increase from the MDCKII^{WT} fluorescence intensity and the statistical significance (***) = P<0.001). Analysed in CellProfiler™ (N=473 per group).

	Mean EB2 Fluorescence Intensity ± 1 SE	Percentage increase (%)	Significance
Wildtype	0.0345 $\pm$ 0.0001	-	***
mChEMPTY	0.0392 $\pm$ 0.0002	13.6232%	***
mChEB2	0.2279 $\pm$ 0.0019	560.58%	***

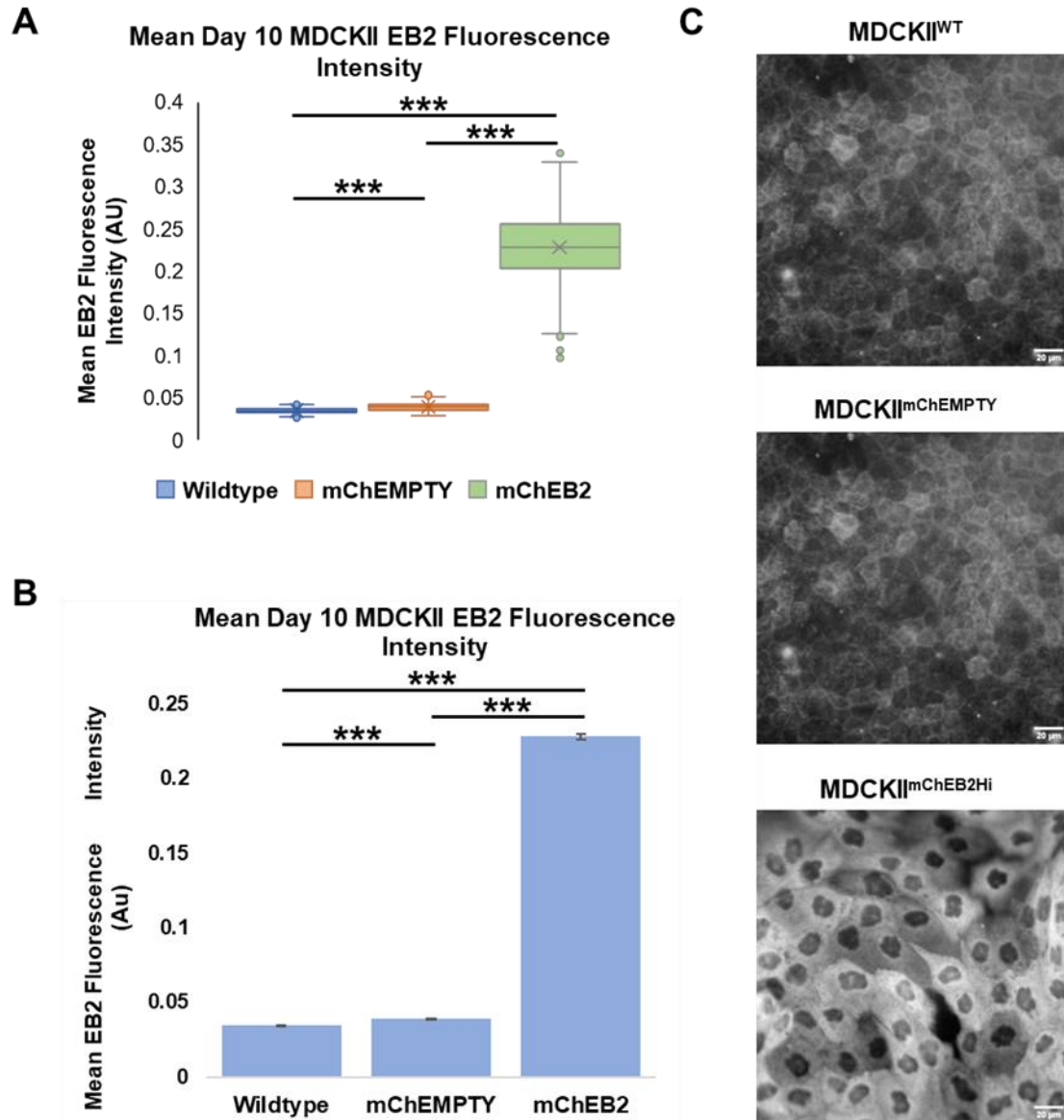


Figure 14. MDCKII^{mChEB2Hi} cells reveal a significantly higher mean EB2 fluorescence intensity compared to MDCKII^{WT} and MDCKII^{mChEMPTY} cells (P<0.001). The mean EB2 fluorescence intensity was significantly different between all MDCKII cell lines (P<0.001). MDCKII cells were grown on glass coverslips in a 24-well plate for 10 days, immuno-labelled for EB2 (Absea, 010614A11), stained with DAPI and imaged on a widefield fluorescence microscope with a x63 oil objective. Alexa488 fluorochrome was used for fluorescence intensity analysis. A) Box and Whisker plot demonstrating the spread and mean EB2 fluorescence intensity in arbitrary units (AU) in MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells (N=473 for each clone). B) Bar chart showing mean EB2 fluorescence intensity the MDCKII cell clones $\pm$ 1SE. C) Example images indicating relative EB2 fluorescence intensity, at same fixed exposure. MDCKII^{mChEB2Hi} cells showed a 6.5-fold increase in EB2 fluorescence intensity compared to MDCKII^{WT} cells and 5.8-fold increase compared to MDCKII^{mChEMPTY} cells. (Asterisks *** indicate P<0.001; Games-Howell test Non-Parametric Post Hoc Test). Analysed on CellProfilerTM. Scale bar = 20 μ m. One experiment with three replicas.

4.3.2. MDCKII^{mChEB2Hi} cells have a larger cell shape index compared to MDCKII^{mChEMPTY} cells

To explore the effects of EB2 overexpression on UJT, the shape index of each cell clone was calculated. Confluent monolayers of MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells were grown for 8, 10 or 14 days and immuno-labelled for E-cadherin or β -catenin to highlight the cell peripheries and then analysed initially using CellProfiler™. Unfortunately, CellProfiler™ was often not accurate enough in defining the cell borders (Appendix 4). Therefore, ImageJ was mainly used, though some border definitions by CellProfiler™ were deemed acceptable and included in the analysis. The cell borders were manually traced in ImageJ to measure the cell perimeter and area. The mean shape index was calculated using the formula: $\text{shape index} = \text{perimeter} / \sqrt{\text{area}}$ to determine the state of the monolayer, jammed ($P_0 < 3.81$) or unjammed ($P_0 > 3.81$). All MDCKII cells were classed as unjammed at all time points with a shape index above the critical threshold ($P_0 > 3.81$) (Figure 16, 18 and 21).

MDCKII^{mChEB2Hi} cells revealed a statistically significantly larger mean perimeter, area and shape index at Day 8, 10 and 14 than MDCKII^{WT} and MDCKII^{mChEMPTY} cells ($P < 0.001$) (Figure 15-18 and 20-21). It is important to note that no data was collected from MDCKII^{mChEMPTY} cells on Day 14 as the cells detached from the coverslips during the immunolabeling process (Figure 20-21). On Day 8, MDCKII^{mChEMPTY} cells had the lowest mean perimeter and area compared to MDCKII^{WT} and MDCKII^{mChEB2Hi} ($P < 0.001$) (Figure 15). No significant difference was seen in the mean cell shape index between MDCKII^{WT} and MDCKII^{mChEMPTY} cells (Figure 16). However, MDCKII^{mChEMPTY} cells had the lowest mean perimeter, area and shape index compared to MDCKII^{WT} and MDCKII^{mChEB2Hi} on Day 10 (Figure 17-18). Data for Day 10 was gathered from three experimental repeats, whereas data from Day 8 and 14 was gathered from one experiment. Each experimental repeat consisted of three replicas. Surprisingly, the cell shape index was significantly higher in MDCKII^{WT} compared to MDCKII^{mChEMPTY} cells at Days 10 and 14 although not at Day 8 (Figure 16, 18 and 21).

Circularity, also known as formfactor in CellProfiler™, is calculated as $4 \cdot \pi \cdot \text{Area} / \text{Perimeter}^2$. The closer to 1 the formfactor value is, the more circular an object is, with 1 equalling a perfect circle. Eccentricity is the ratio of the distance between the foci of the ellipse and its major axis length, giving a value between 0 and 1 in arbitrary units. A value of 0 specifies a perfect circle, while 1 specifies a line segment. Therefore, circularity and eccentricity can be indicators for changes in cell shape. MDCKII^{mChEB2Hi} cells had significantly lower mean circularity ($P < 0.001$) and higher mean eccentricity than controls ($P < 0.001$) (Figure 19). The MDCKII^{mChEB2Hi} cells were elongated and irregularly shaped consistent with a significantly higher cell shape index, thus indicating more unjammed cells on average (Figure 18). MDCKII^{mChEMPTY} cells, however,

had significantly higher mean circularity and the lowest mean eccentricity ($P < 0.001$), these cells were more circular and compact (Figure 19). This was consistent with a significantly lower cell shape index and therefore indicated that the cells were less unjammed than MDCKII^{WT} and MDCKII^{mChEB2Hi} cells (Figure 18).

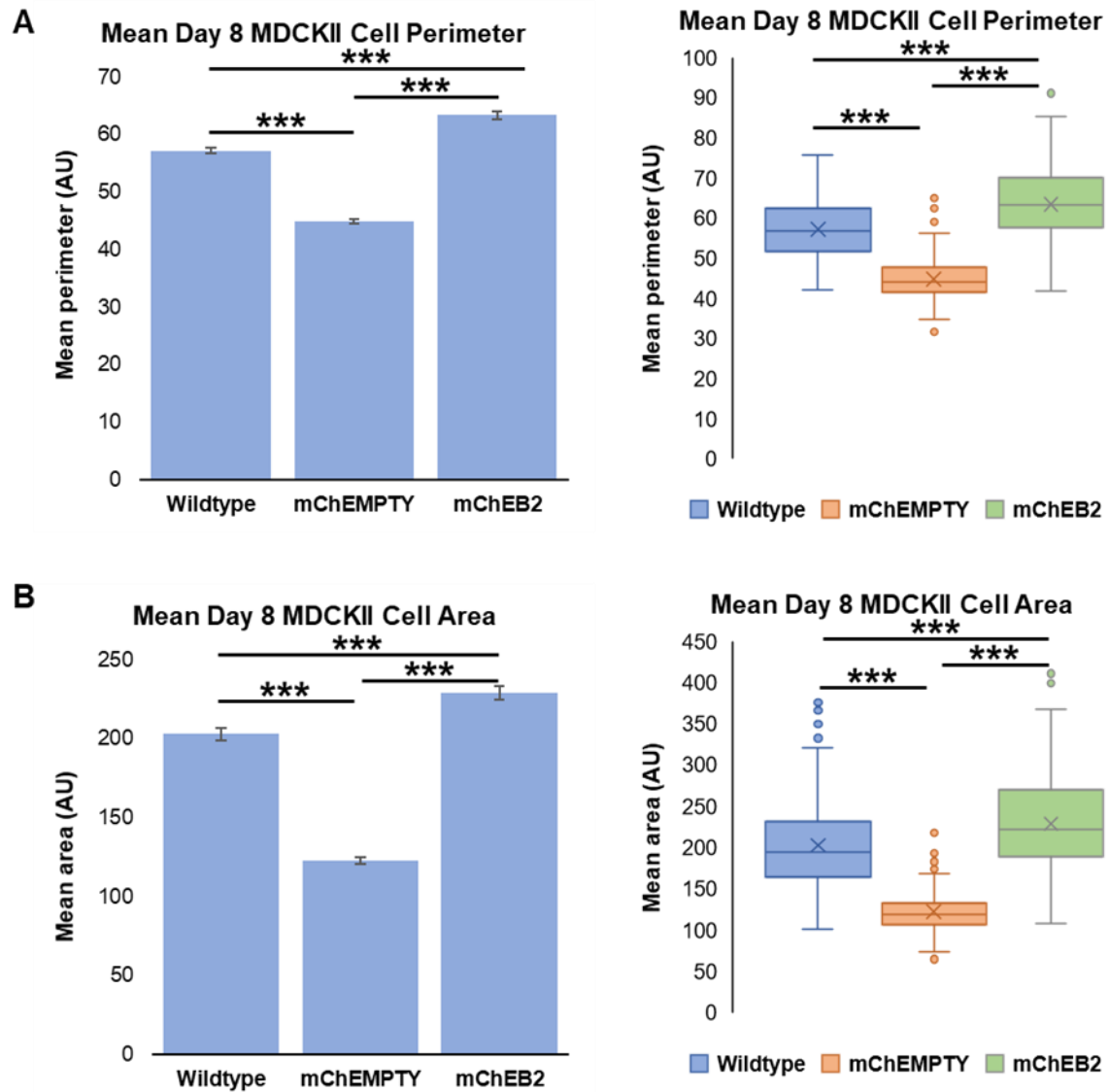


Figure 15. MDCKII^{mChEB2Hi} cells reveal a significantly greater mean cell perimeter and area than MDCKII^{WT} and MDCKII^{mChEMPTY} cells on day 8 ($P < 0.001$). MDCKII cells were grown on glass coverslips in 35 mm dishes for 8 days, immuno-labelled for E-cadherin (BD, 610181), stained with DAPI and imaged on a widefield fluorescence microscope with a x63 oil objective. The E-cadherin labelling allowed the mean cell perimeter ± 1 SE (A) and area ± 1 SE (B) to be determined using ImageJ. $N=200$ per cell clone. The Box and Whisker Plots highlight the spread of data with X indicating the mean of each group while the bar charts show the mean. MDCKII^{mChEMPTY} revealed a lower mean cell perimeter and area compared to MDCKII^{WT} ($P < 0.001$). (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test). Analysed with ImageJ. One experiment with three replicas.

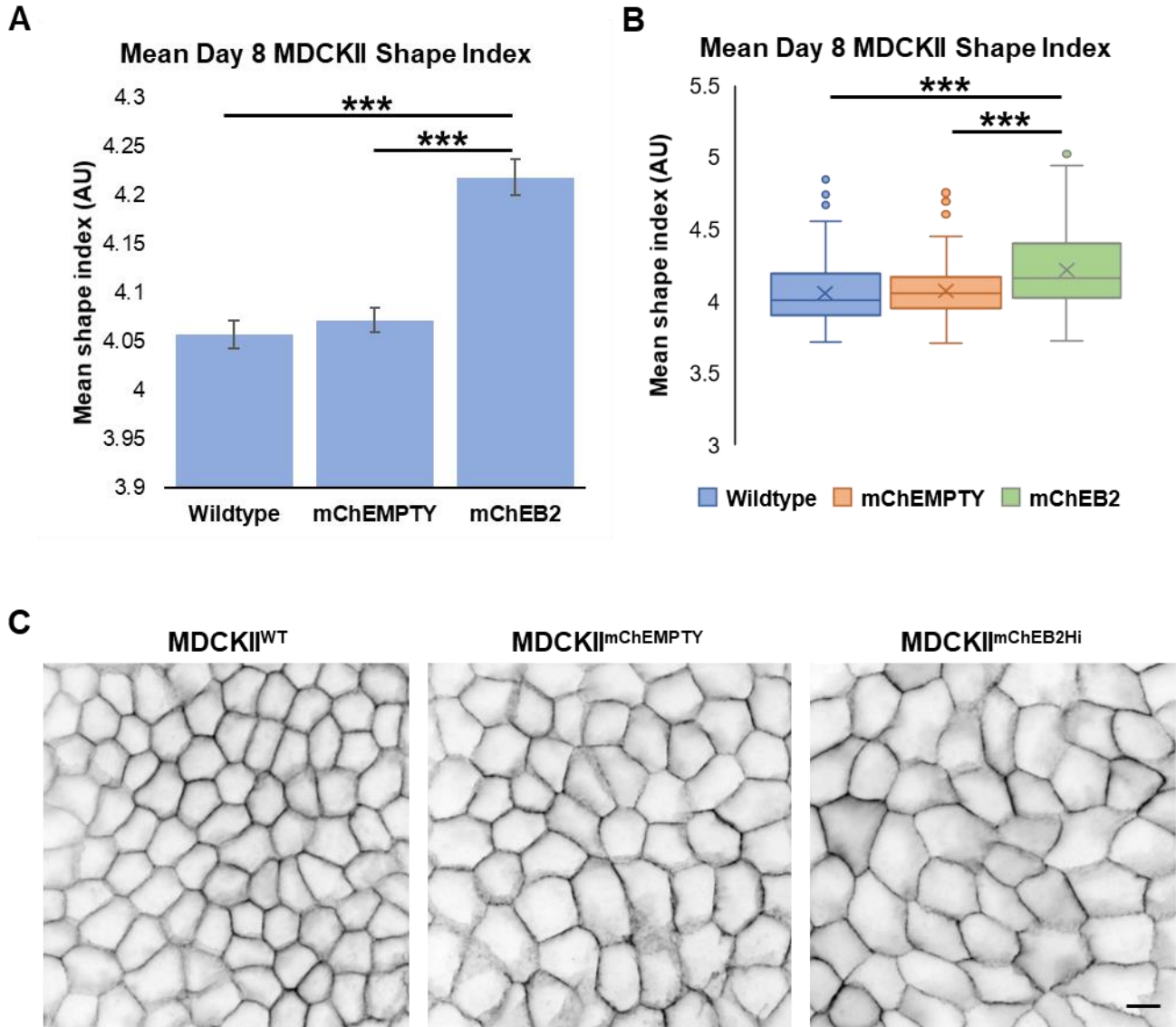


Figure 16. MDCKII^{mChEB2Hi} cells reveal a significantly larger mean cell shape index compared to MDCKII^{WT} and MDCKII^{mChEMPTY} at day 8 ($P < 0.001$). MDCKII cells were grown on glass coverslips in 35 mm dishes for 8 days, immuno-labelled for E-cadherin (BD, 610181), stained with DAPI and imaged on a widefield fluorescence microscope with a x63 oil objective. The E-cadherin labelling allowed the mean cell perimeter and area to be determined and thus the cell shape index to be calculated. No significant difference in mean cell shape index was evident between MDCKII^{WT} and MDCKII^{mChEMPTY} cells at day 8 ($P = 0.734$). (A) The mean cell shape index $\pm 1SE$ was calculated in arbitrary units (AU) using the formula: shape index = perimeter/ $\sqrt{\text{area}}$. $N = 200$ per cell clone. (B) The Box and Whisker Plots highlight the spread of data with X indicating the mean of each group. (C) Example images (inverted) indicating E-cadherin labelling in MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test). Scale bar = 20 μm . Analysed with ImageJ. One experiment with three replicas.

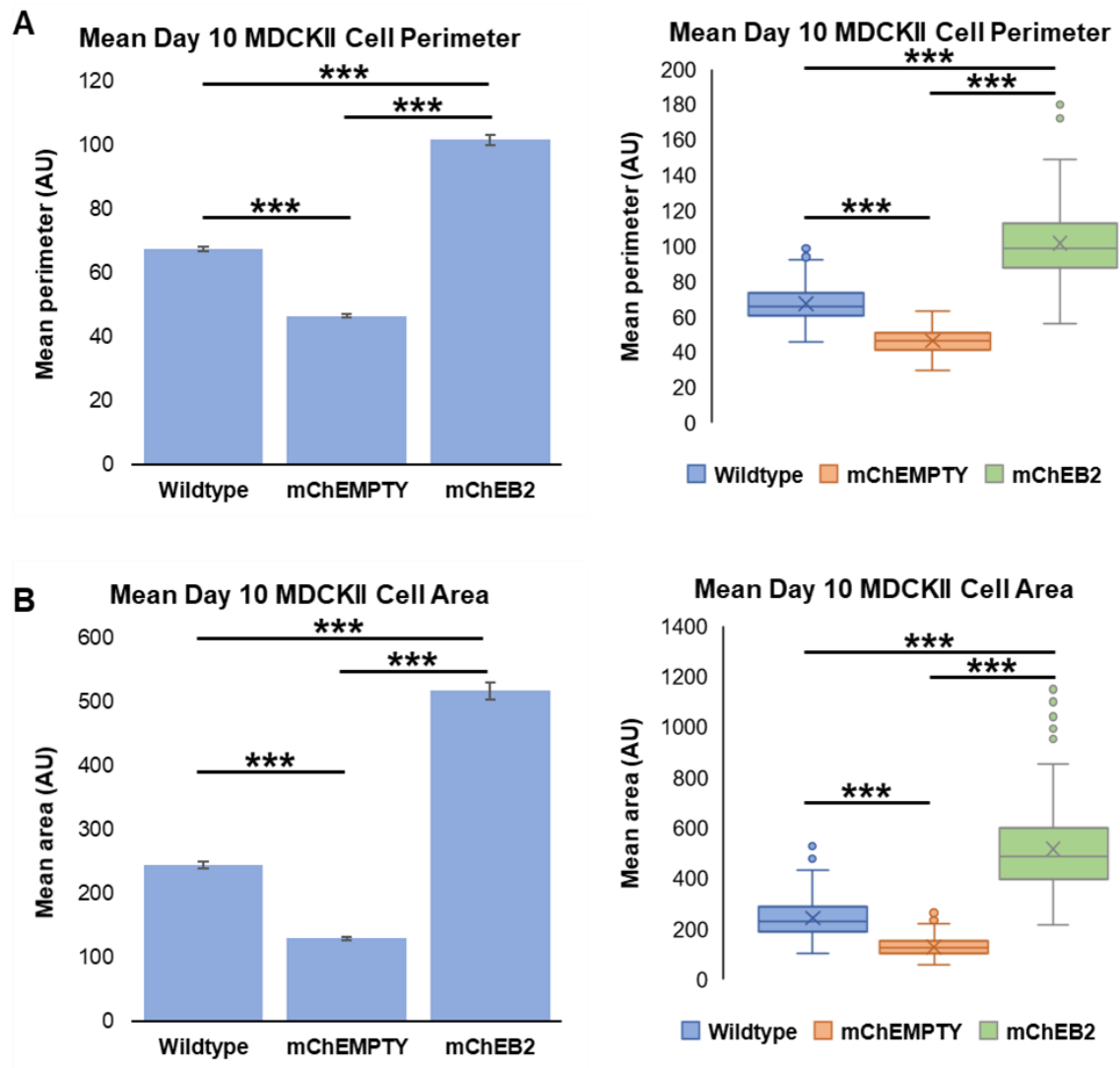


Figure 17. MDCKII^{mChEB2^{Hi}} cells reveal a significantly larger mean cell perimeter and area compared to MDCKII^{WT} and MDCKII^{mChEMPTY} at day 10 (P<0.001). MDCKII cells were grown on glass coverslips in a 24-well plate for 10 days, immuno-labelled for E-cadherin (BD, 610181), stained for DAPI and imaged on a widefield fluorescence microscope with a x63 oil objective. The E-cadherin labelling allowed the mean cell perimeter ± 1 SE (A) and area ± 1 SE (B) to be determined. N=200 per cell clone. MDCKII^{mChEMPTY} cells showed a significantly lower mean cell perimeter and areas compared to MDCKII^{WT} (P<0.001). The Box and Whisker Plots highlight the spread of data with X indicating the Mean of each group while the bar charts show the mean. (Asterisks *** indicate P<0.001; Games-Howell test Non-Parametric Post Hoc Test). Analysed with ImageJ. Three experiments each with three replicas.

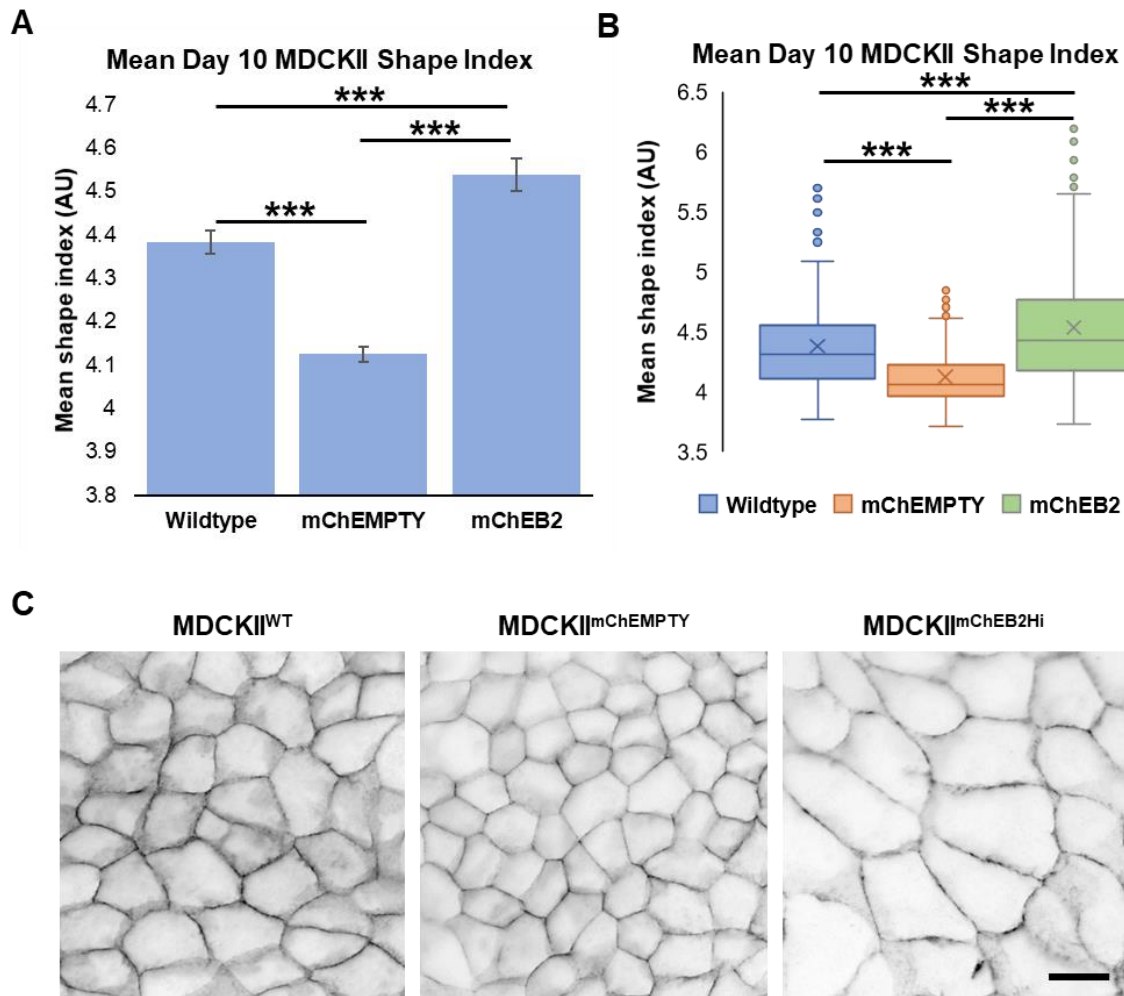


Figure 18. MDCKII^{mChEB2Hi} cells reveal a significantly larger mean cell shape Index compared to MDCKII^{WT} and MDCKII^{mChEMPTY} at day 10 (P<0.001). MDCKII cells were grown on glass coverslips in a 24-well plate for 10 days, immuno-labelled for E-cadherin (BD, 610181), stained with DAPI and imaged on a widefield fluorescence microscope with a x63 oil objective. The E-cadherin labelling allowed the mean cell perimeter and area to be determined. (A) The mean shape index ± 1 SE was calculated in arbitrary units (AU) using the formula: shape index = perimeter/ $\sqrt{\text{area}}$. N=200 per cell clone. MDCKII^{mChEMPTY} revealed a smaller mean cell shape index compared to MDCKII^{WT} (P<0.001). The bar chart shows the mean cell shape index (B) The Box and Whisker Plots highlight the spread of data with X indicating the mean of each group. (C) Example images showing E-cadherin (inverted) labelling in MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells (Asterisks *** indicate P<0.001; Games-Howell test Non-Parametric Post Hoc Test). Analysed using ImageJ. This analysis was originally done in CellProfiler™ using β -catenin (Sigma, C2206), (See Appendix 5-6), however, ImageJ was the method chosen for analysis. Scale bar = 20 μ m. Three experiments each with three replicas.

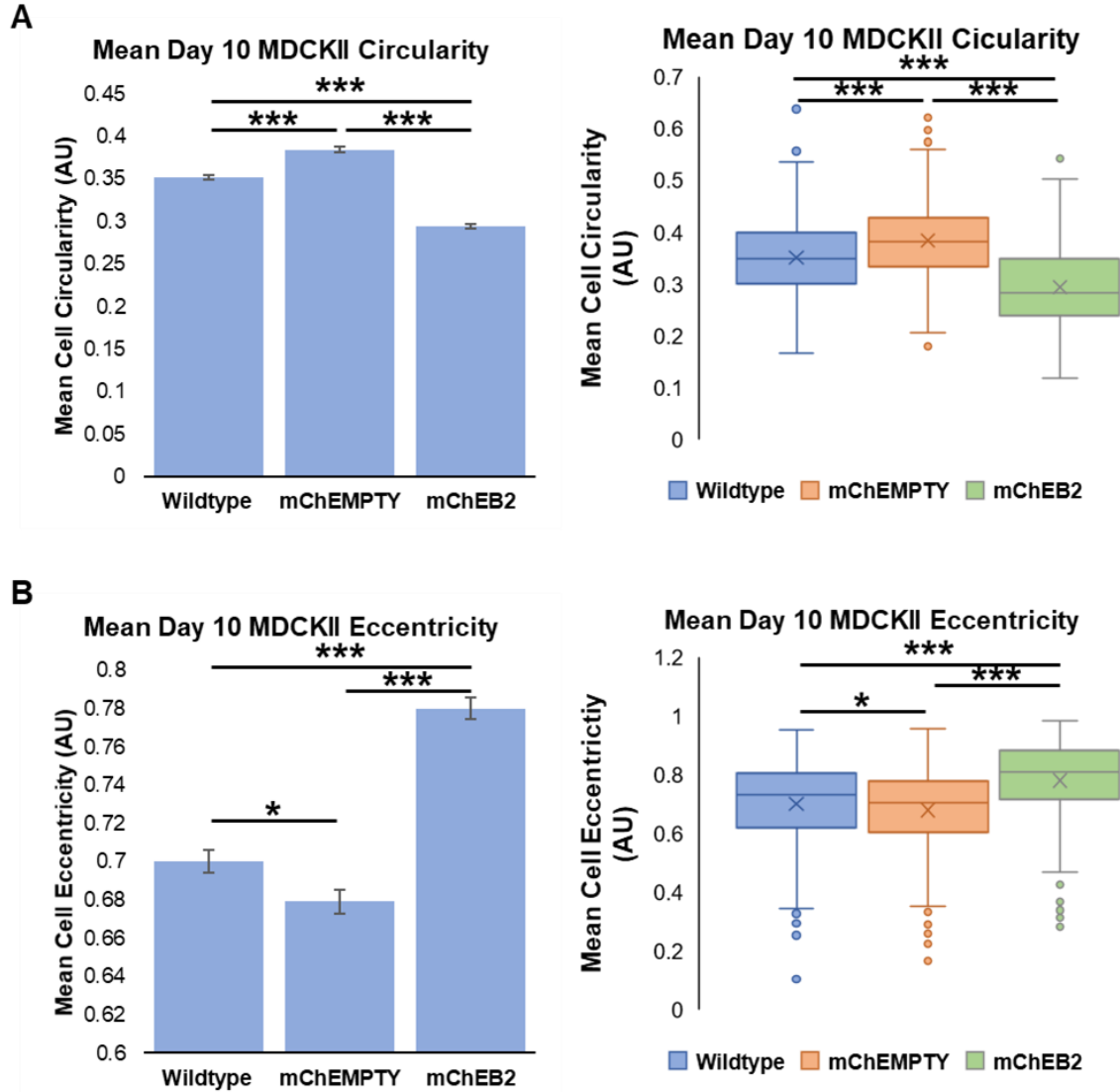


Figure 19. MDCKII^{mChEB2Hi} cells reveal a significantly lower mean cell circularity and higher mean cell eccentricity compared to MDCKII^{WT} and MDCKII^{mChEMPTY} at day 10 (P<0.001). MDCKII cells were grown on glass coverslips in a 24-well plate for 10 days, immuno-labelled for β -catenin (Sigma, C2206), stained for DAPI and imaged on a widefield fluorescence microscope with a x63 oil objective. The β -catenin labelling allowed the mean circularity ± 1 SE (A) and eccentricity ± 1 SE (B) to be determined. MDCKII^{mChEMPTY} cells showed a significantly higher mean cell circularity compared to MDCKII^{mChEB2Hi} and MDCKII^{WT} cells (P<0.001). While MDCKII^{mChEMPTY} cells showed a significantly lower mean cell eccentricity to MDCKII^{mChEB2Hi} and MDCKII^{WT} cells (P<0.05). The Box charts show the mean while the Whisker Plots highlight the spread of data with X indicating the mean for each group. (Asterisks * indicates P<0.05 and *** indicates P<0.001; Games-Howell test Non-Parametric Post Hoc Test). Analysed using CellProfiler™. One experiment with three replicas.

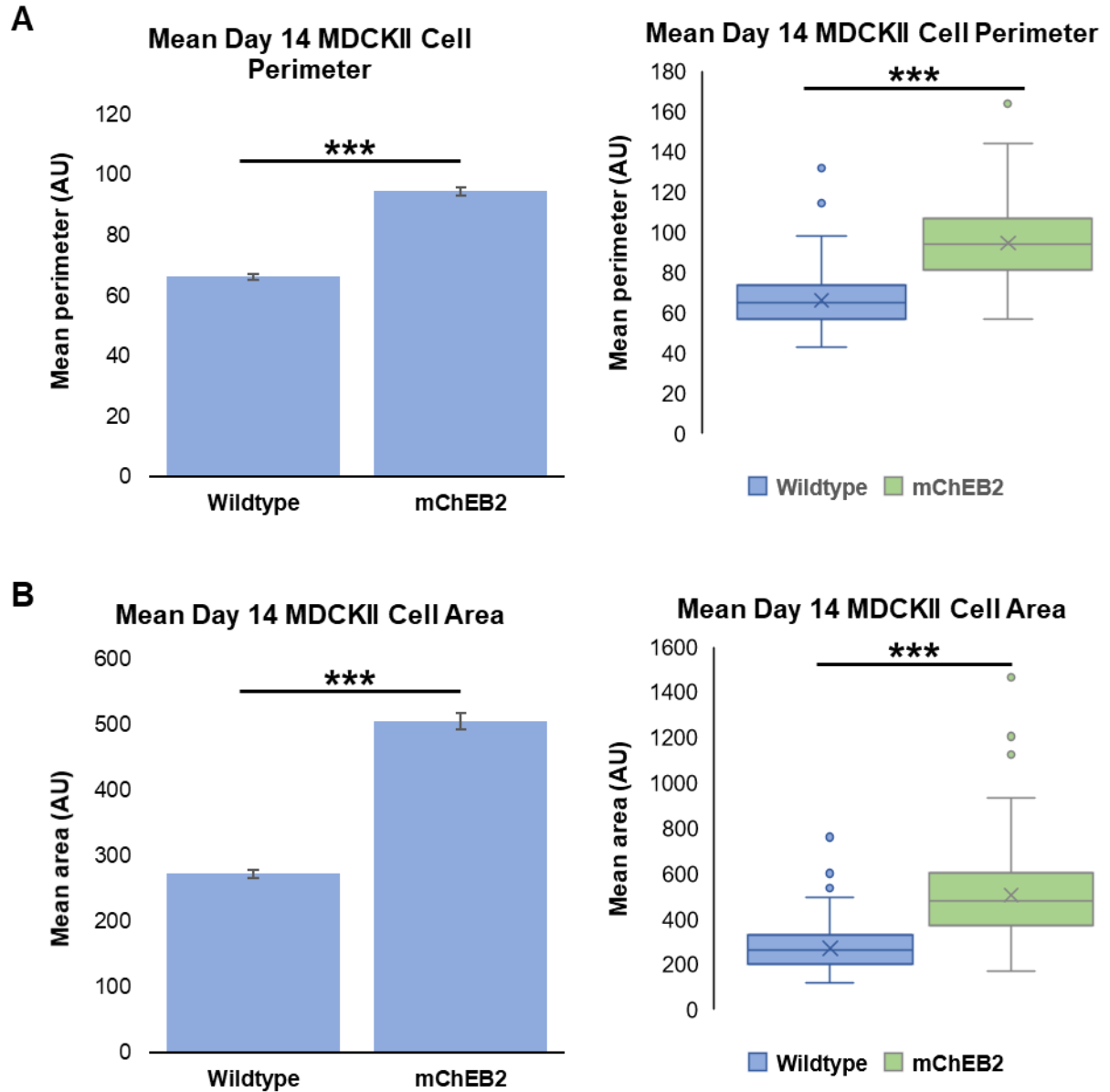


Figure 20. MDCKII^{mChEB2Hi} cells reveal a significantly larger mean cell perimeter and area compared to MDCKII^{WT} on day 14 ($P < 0.001$). MDCKII cells were grown on glass coverslips in a 24-well plate for 14 days, immuno-labelled for E-cadherin (BD, 610181), stained for DAPI and imaged on a widefield fluorescence microscope with a x63 oil objective. The E-cadherin labelling allowed the mean cell perimeter ± 1 SE (A) and area ± 1 SE (B) to be determined. $N=200$ per cell clone. MDCKII^{mChEB2Hi} reveal a significantly higher mean cell perimeter and area than MDCKII^{mChEMPTY} cells. The Box charts show the mean while the Whisker Plots highlight the spread of data with X indicating the mean of each group. (Asterisks *** indicate $P < 0.001$; Mann Whitney-U test Non-Parametric Test). Analysed using ImageJ. One experiment with three replicas.

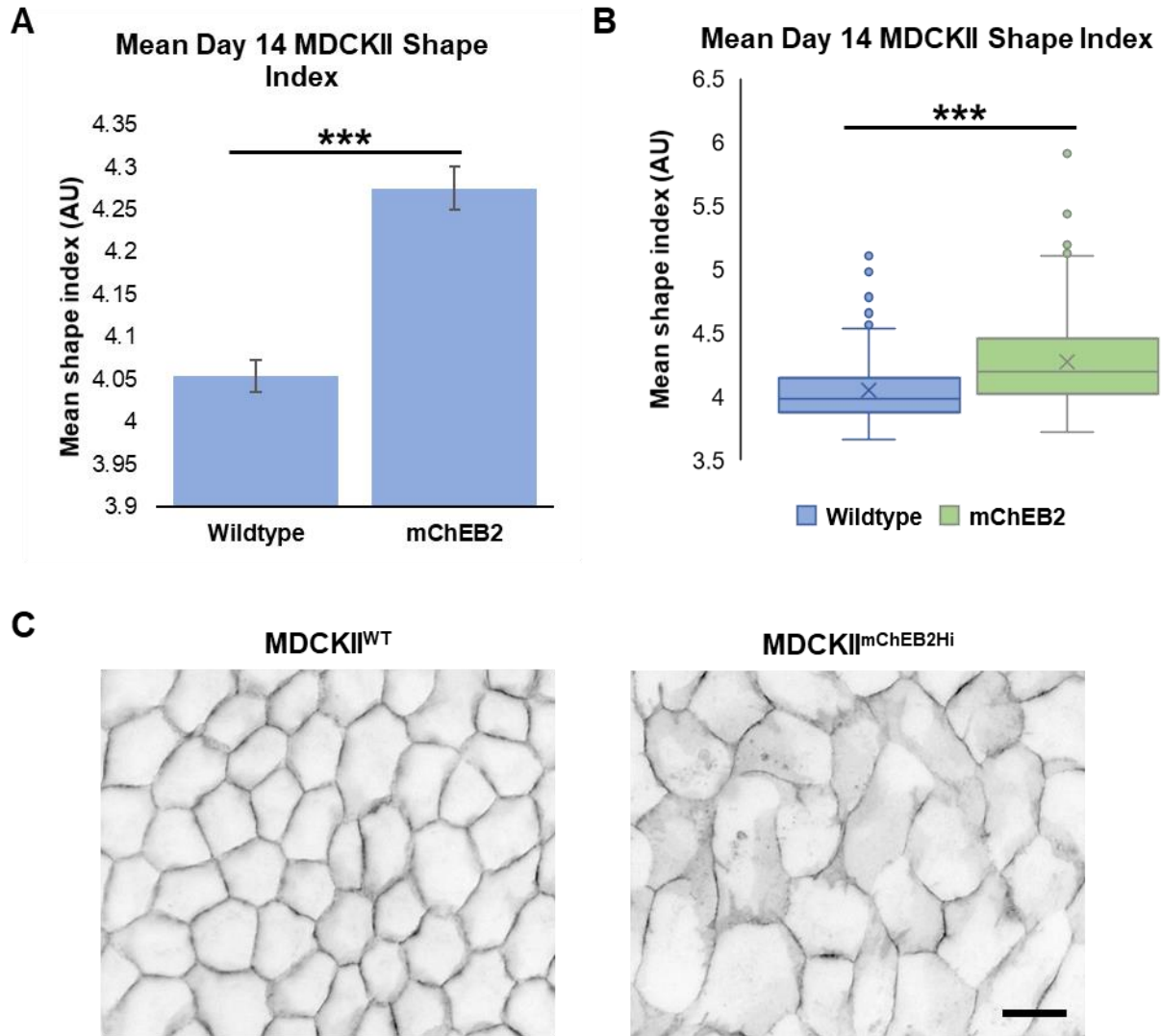


Figure 21. MDCKII^{mChEB2Hi} reveal a significantly larger mean cell shape Index compared to MDCKII^{WT} cells at day 14 (P<0.001). MDCKII cells were grown on glass coverslips in a 24-well plate for 14 days, immuno-labelled for E-cadherin (BD, 610181), stained with DAPI and imaged on a widefield fluorescence microscope with a x63 objective. The E-cadherin labelling allowed the mean cell perimeter and area to be determined. (A) The mean shape index $\pm 1SE$ was calculated in arbitrary units (AU) using the formula: shape index = perimeter/ $\sqrt{\text{area}}$. N=200 per cell line. The box charts show that the mean cell shape index for the two clones. (B) The Box and Whisker Plots highlight the spread of data with X indicating the mean of each group. (C) Example images showing E-cadherin (inverted) labelling in MDCKII^{WT} and MDCKII^{mChEB2Hi} cells at day 14. MDCKII^{mChEB2Hi} cells show a significantly higher mean shape index than MDCKII^{WT}. (Asterisks *** indicate P<0.001; Games-Howell test Non-Parametric Post Hoc Test). Analysed using ImageJ. Scale bar = 20 μm . One experiment with three replicas.

4.3.3. MDCKII^{mChEB2Hi} exhibit more undulating tight junctions and a denser apical MT array than MDCKII^{mChEMPTY} cells.

Since cells overexpressing EB2 (MDCKII^{mChEB2Hi}) had a higher shape index and thus were more unjammed than the controls, this raised the question as to whether apico-basal polarisation and differentiation could be affected. Previous studies have suggested a link between EB2 apico-basal polarisation and differentiation. For example, Goldspink et al. (2013) revealed that EB2 expression is critical during early stages of differentiation and elongation, while it decreases as epithelial cells increase in confluency and become partially polarised. However, whether elevated expression of EB2 affects polarisation, elongation and apical constriction is not known.

MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells were grown on glass coverslips for 10 days and double immuno-labelled for Par3 and β -tubulin or ZO-1 and β -actin and stained with DAPI. Immuno-labelling of Par3 revealed it to be most concentrated at the cell junctions with some cytoplasmic labelling apparent. In MDCKII^{mChEB2Hi} cells, Par3's localisation appeared slightly diminished at both cell junctions and in cytoplasm compared to the control (Figure 22). ZO-1 localisation was mainly junctional in both clones (Figure 23). Both markers, Par-3 and ZO-1, highlighted the junctions to be more undulated/ruffled in MDCKII^{mChEB2Hi} compared to the MDCKII^{mChEMPTY} control (Figure 22-23). A slight reduction in ZO-1 fluorescence intensity in MDCKII^{mChEB2Hi} compared to MDCKII^{mChEMPTY} cells may have been the case, however, fluorescence intensity and statistical analysis would be required to confirm this (Figure 23).

Furthermore, in previous studies, EB2 has been shown to play a role in MT and actin filament organisation. Goldspink et al. (2013), for example, revealed that EB2 knockdown resulted in straighter, bundled MTs with lattice associated EB1 and ACF7 which in turn, mediated MTs co-alignment with actin filaments. Immuno-labelling revealed β -actin concentrated at the cell junctions with a slight potential increase compared to MDCKII^{mChEMPTY} cells (Figure 24A). Also, actin puncta were evident in the apical regions in both EB2 overexpressing and control MDCKII cells. (Figure 24A). These puncta are likely to represent actin filaments associated with the apical cortex and they appeared less numerous in EB2 overexpressing than control cells. However, confocal optical sectioning would be needed to verify this.

Cells immuno-labelled with the anti- β -tubulin antibody and imaged on a widefield fluorescence microscope showed a typical radial apical MT array in MDCKII^{mChEMPTY}, whereas a denser and more disorganised apical array was apparent in MDCKII^{mChEB2Hi} cells. Both EB2 overexpressing and control MDCKII cells also showed β -tubulin labelling at the cell junctions, which may suggest apico-basal MT arrangement, but confocal optical sectioning would be needed to resolve the MT organisation in these partially polarised cells (Figure 24B).

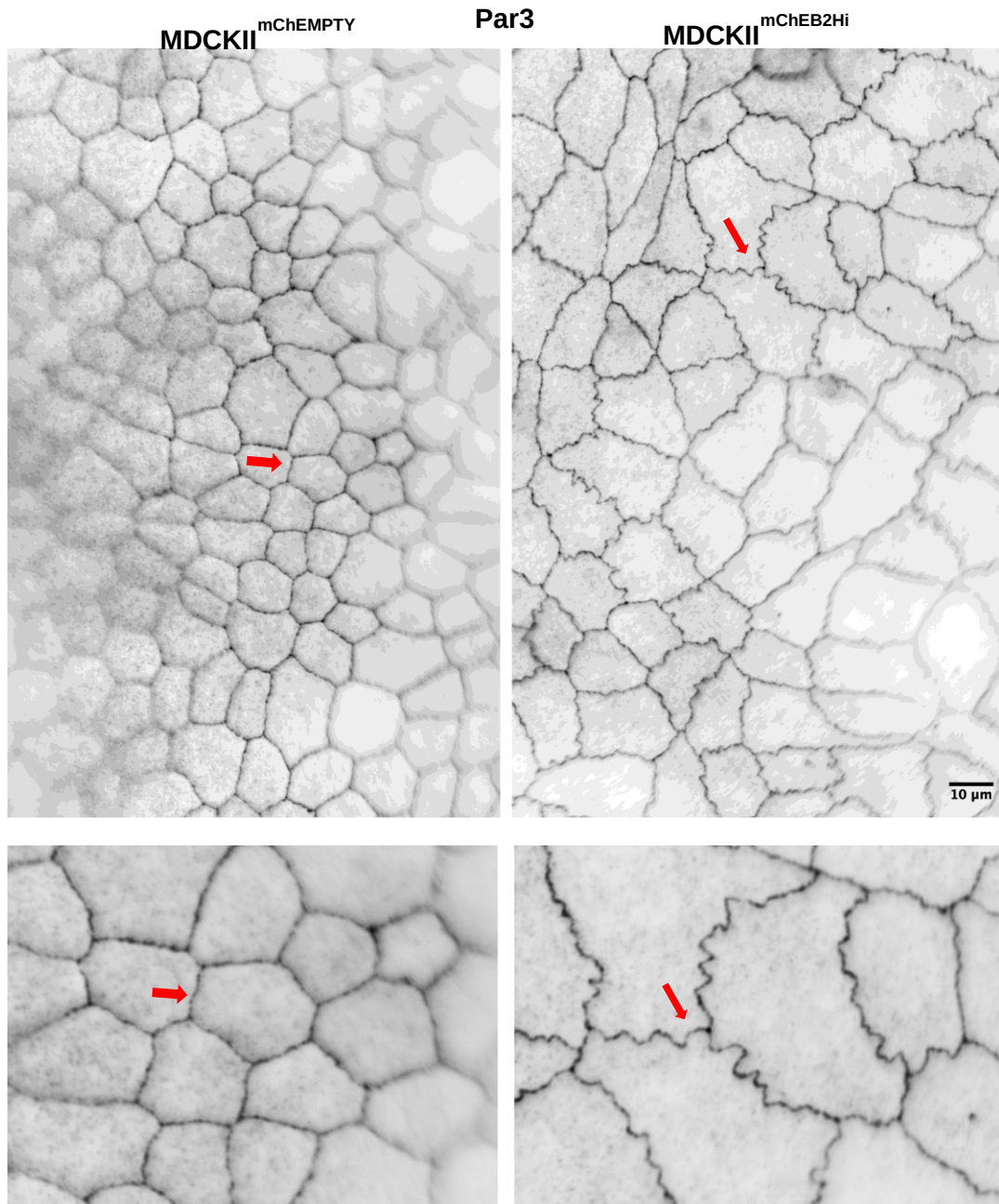


Figure 22. Effects of EB2 overexpression on Par3 localisation and tight junction organisation. MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells were grown on glass coverslips for 10 days, immuno-labelled as for figure 23 and imaged on a widefield fluorescence microscope with a x63 oil objective. Par3 labelling is evident at the junctions with apparent similar fluorescence intensity in both cell clones. However, Par3 labelling reveal distinct tight junction undulations in MDCKII^{mChEB2Hi} unlike in MDCKII^{mChEMPTY} cells where the junctions have a straight morphology (red arrows). These differences in tight junction morphology are highlighted in the enlarged images below. Scale bars = 10 μ m.

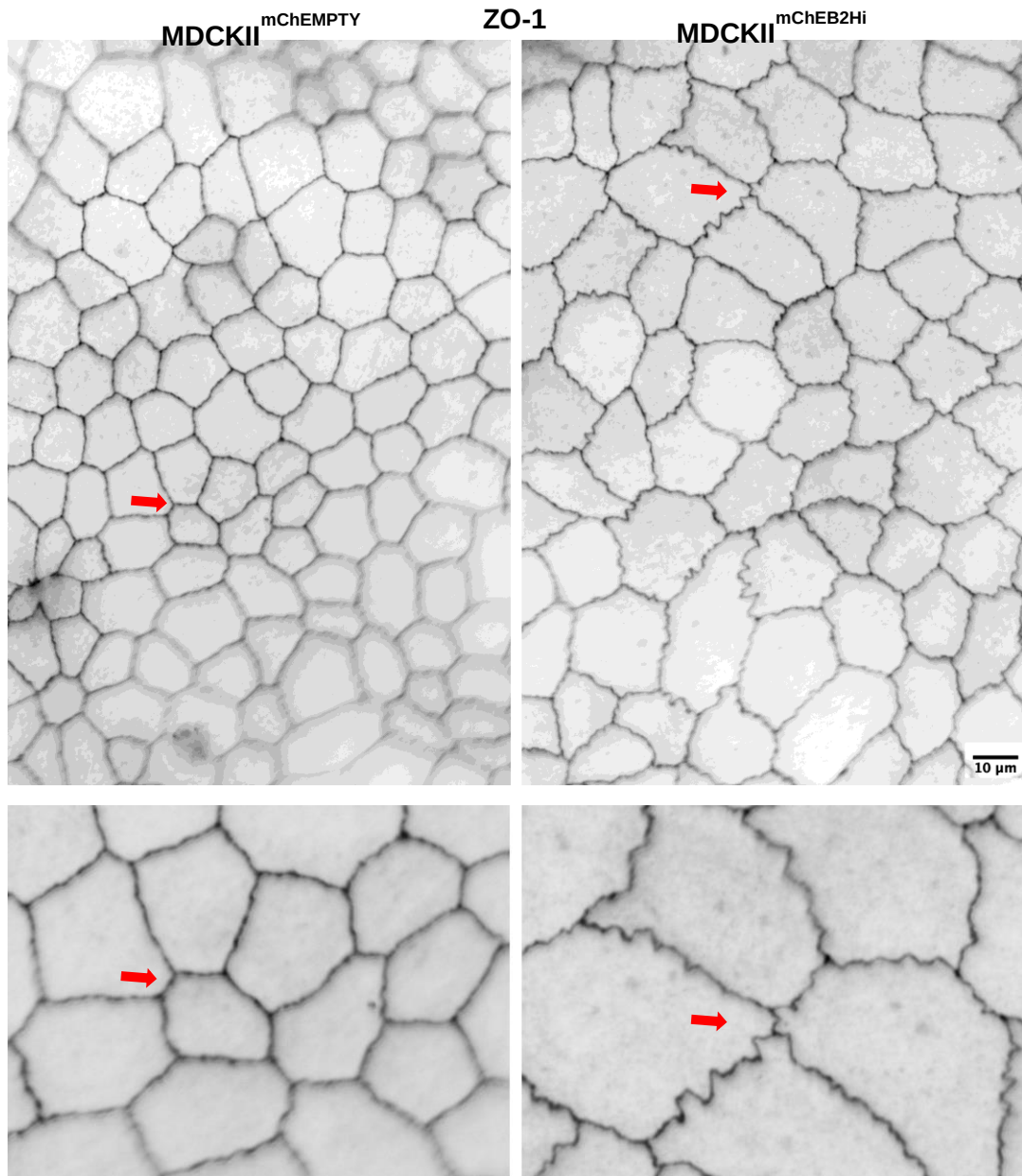


Figure 23. Effects of EB2 overexpression on ZO-1 localisation and tight junction organisation. MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells were grown on glass coverslips for 10 days, immuno-labelled as for figure 23 and imaged on a widefield fluorescence microscope with a x63 oil objective. ZO-1 labelling show distinct undulation of the tight junctions in MDCKII^{mChEB2Hi} compared with the straighter junctions in MDCKII^{mChEMPTY} cells (red arrows). These differences in tight junction morphology are highlighted in the enlarged images below. Second row of images is enlarged by a factor of 2. Scale bars = 10 μm.

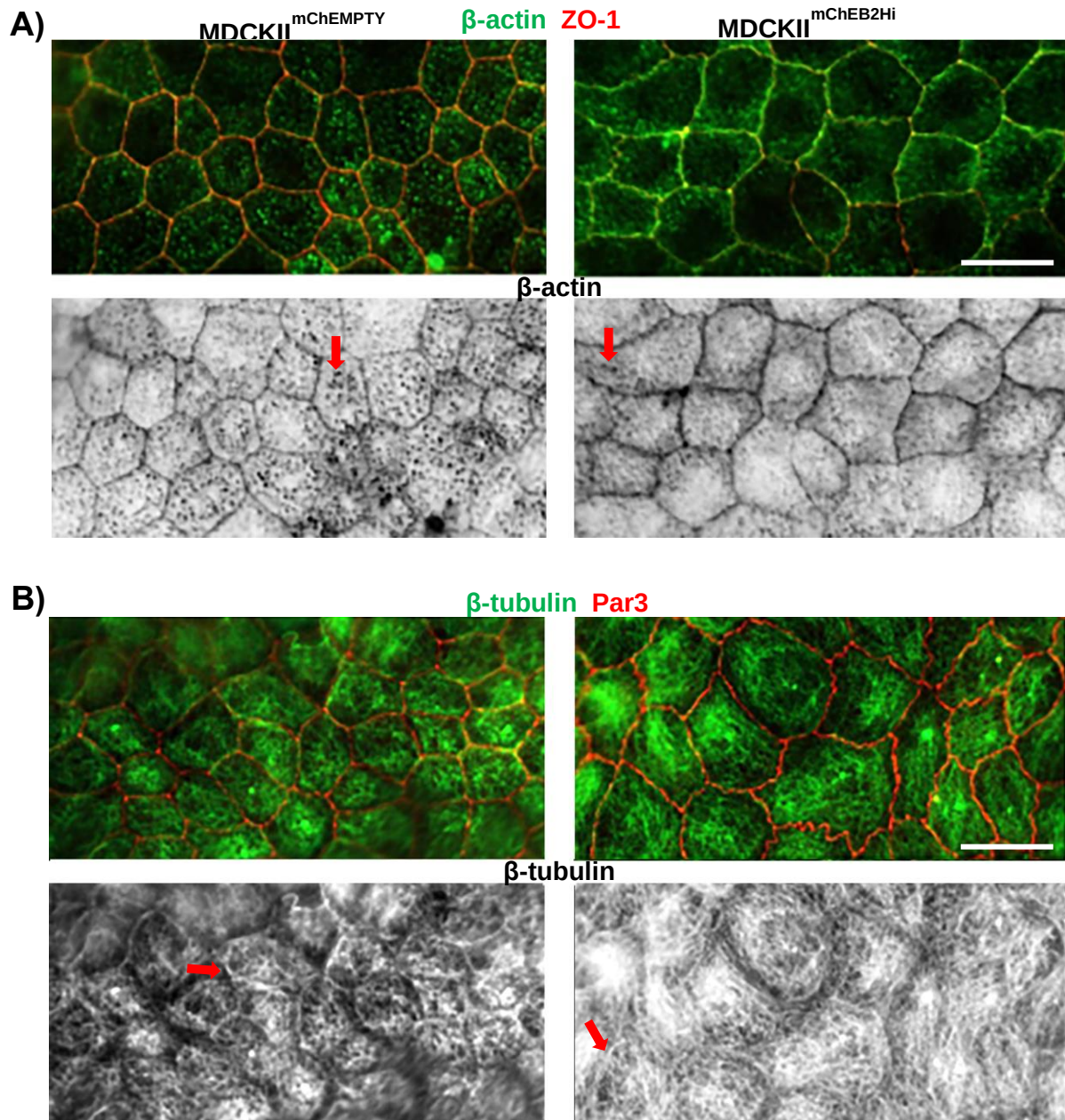


Figure 24. Effects of EB2 overexpression on MTs and actin organisation. were grown MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells on glass coverslips for 10 days, immuno-labelled for β-actin (Mouse; sigma cat no. A1978; 1:2000) and ZO-1 (Rabbit; Thermo Fisher cat no.61-7300; 1:200) or β-tubulin (Mouse, Sigma cat no. T4026; 1:300) or Par3 (Rabbit; Millipore cat no. 07-33D; 1:500) and imaged on a widefield fluorescence microscope with a x63 oil objective. A) Images showing possible increase in actin at cell junctions in MDCKII^{mChEB2Hi} compared to MDCKII^{mChEMPTY} cells. Distinct apical actin puncta are also evident (red arrows). B) Images suggesting cortical MT concentration (red arrows) in both clones but a denser apical MT network in MDCKII^{mChEB2Hi} compared to MDCKII^{mChEMPTY} cells. Scale bars = 10 μm.

4.3.4. Junctional mDia-1 is more diffuse in MDCKII^{mChEB2Hi} than in MDCKII^{mChEMPTY} cells.

Formins have been previously linked to MT stability, actin nucleation at adherens junctions and EB2 expression (Bartolini and Gundersen, 2010, Goldspink et al., 2013). mDia-1 and mDia-2 are actin-nucleating formins of the Diaphanous family that are activated downstream of Rho GTPases. Immuno-labelling of mDia-1 revealed localisation predominantly at the cell junctions with some diffuse labelling throughout the cytoplasm. MDCKII^{mChEB2Hi} cells revealed a more punctate, broader and less dense pattern of mDia-1 at the junctions compared to MDCKII^{mChEMPTY} cells (Figure 25A). Junctional labelling for mDia-2 was less apparent in both EB2 overexpressing and control cells. Furthermore, puncta of mDia-2 were apparent in both EB2 overexpressing and control cells. In addition, mDia-2 labelling perhaps suggests an association with mitochondria, which has also been suggested by Cangkrama et al. (2022). However, this would have to be investigated further using mitochondrial stains in combination with mDia-2 immuno-labelling, for example. No obvious difference was apparent in mDia-2 localisation in MDCKII^{mChEB2Hi} cells and MDCKII^{mChEMPTY} cells (Figure 25B).

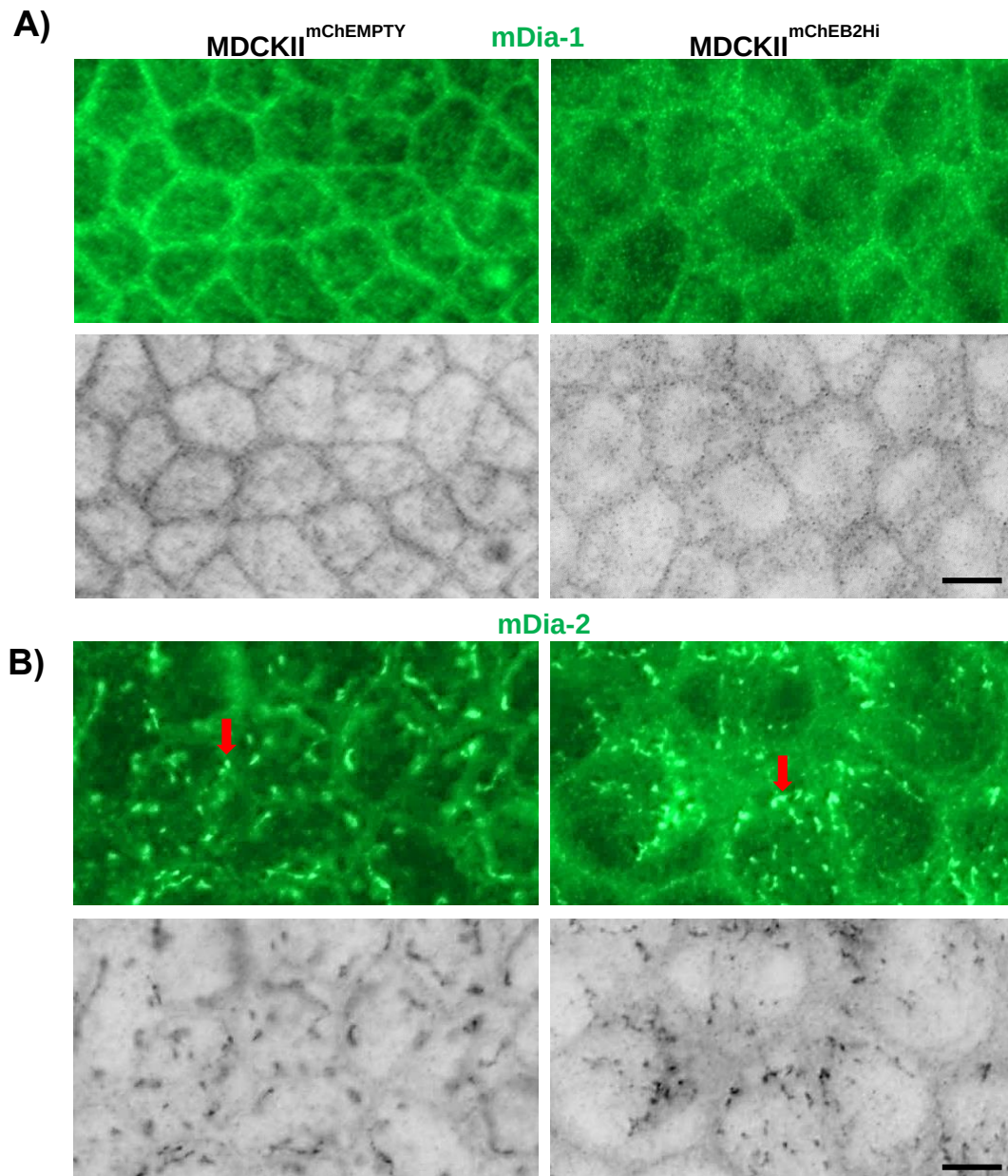


Figure 25. Localisation of mDia-1 and mDia-2 in MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells. MDCKII cells were grown on glass coverslips for 10 days, immuno-labelled for mDia-1 (Mouse, BD cat no. 610849; 1:200) or mDia-2 (Rabbit; Abcam cat no. AB12319; 1:200). Cells were imaged on a widefield fluorescence microscope with a x63 oil objective at a fixed exposure (2500 m/s and 800 m/s, respectively). A) Images highlighting mDia-1 localisation in MDCKII cells. MDCKII^{mChEB2Hi} cells reveal more diffuse junctional mDia-1 compared to MDCKII^{mChEMPTY} cells. B) Images highlighting mDia-2 localisation in MDCKII cells. No obvious difference is apparent in mDia-2 localisation in MDCKII^{mChEB2Hi} cells and MDCKII^{mChEMPTY} cells. Scale bars = 10 μ m.

4.3.5 MDCKII^{mChEB2Hi} show no significant difference in pMLC fluorescence intensity at cell junctions to MDCKII^{mChEMPTY} cells.

One theory is that the transition of epithelial cells between jammed and unjammed states is mediated by the balance between the oppositional forces, tension and adhesion, along the cell-cell junction length. E-cadherin is an example of an adhesion molecule that contributes to the lengthening of the cell-cell junctions. Under normal homeostatic conditions, when epithelial tissues are in a mature and stable state, cells are jammed, and tensile forces dominate over adhesion. Therefore, in order for cells to go through the UJT and become unjammed, adhesion forces need to supersede tensile. Actomyosin contraction is a tensile force that induces sliding, shortening the cell-cell junctions and, as a result, contributes to jamming. As such, in an unjamming situation it is suspected that actomyosin contraction is reduced. Phosphorylation of myosin light chain is an important step in actomyosin activation and contraction. Therefore, this investigation explored the effects of EB2 overexpression on actomyosin contraction through the mean fluorescence intensity of phosphorylated myosin light chain (pMLC) at the cell junctions (Appendix 8). The higher the fluorescence intensity of pMLC, the greater the actomyosin contraction is expected to be.

MDCKII^{WT}, MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} cells were grown for 10 days to reach confluency and double immuno-labelled for pMLC and E-cadherin. E-cadherin labelling was used to highlight cell junctions. Bars were placed in the E-cadherin channel to measure junctional PMLC (Appendix 8). Immuno-labelling showed pMLC to be localised at the cell junctions with some dispersed in the cytoplasm. Analysis revealed that MDCKII^{mChEB2Hi} cells mean pMLC fluorescence intensity at cell junctions was not significantly different compared to the MDCKII^{mChEMPTY} ($P>0.05$, $P=0.263$) but was significantly higher than MDCKII^{WT} controls ($P<0.001$), with a 13.85% increase. (Table 6, Figure 26).

Table 6. The table highlights the mean $\pm 1SE$ values for junctional pMLC fluorescence intensity in MDCKII cells with the percentage (%) increase from MDCKII^{WT} cells.

	Mean $\pm 1SE$	% Increase
MDCKII ^{WT}	7053.82 $\pm$ 155.95	-
MDCKII ^{mChEMPTY}	8325.61 $\pm$ 159.16	18.02%
MDCKII ^{mChEB2Hi}	8031.20 $\pm$ 99.73	13.85%

4.3.6. Localisation of GEFH1 in MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells

Activation of Rho signalling via GEF is a key step in cell contraction. Previous studies have identified GEF-H1 at tight junctions in polarised epithelial cells (Aijaz et al., 2005). Thus, to explore the mechanism by which cells change shape during UJT, MDCKII cells were immuno-labelled for GEF-H1. Day 10, MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} cells were double immuno-labelled for GEF-H1 and E-cadherin. Immuno-labelling of GEF-H1 in MDCKII cells showed a mainly cytoplasmic distribution with some junctional localisation in MDCKII^{mChEMPTY} but very little junctional location in MDCKII^{mChEB2Hi} cells suggesting that there is more GEFH1 at the junctions in MDCKII^{mChEMPTY} than MDCKII^{mChEB2Hi} cells (Figure 27). However, fluorescence intensity analysis across junctions would be needed to confirm this. In both MDCKII^{mChEMPTY} cells and MDCKII^{mChEB2Hi} cells, relatively large clusters of GEF-H1 were present in the cytoplasm. In addition, GEF-H1 indicated a centrosomal localisation. However, further testing would be needed to verify this, co-immunolabelling for GEF-H1 and pericentrin, for example.

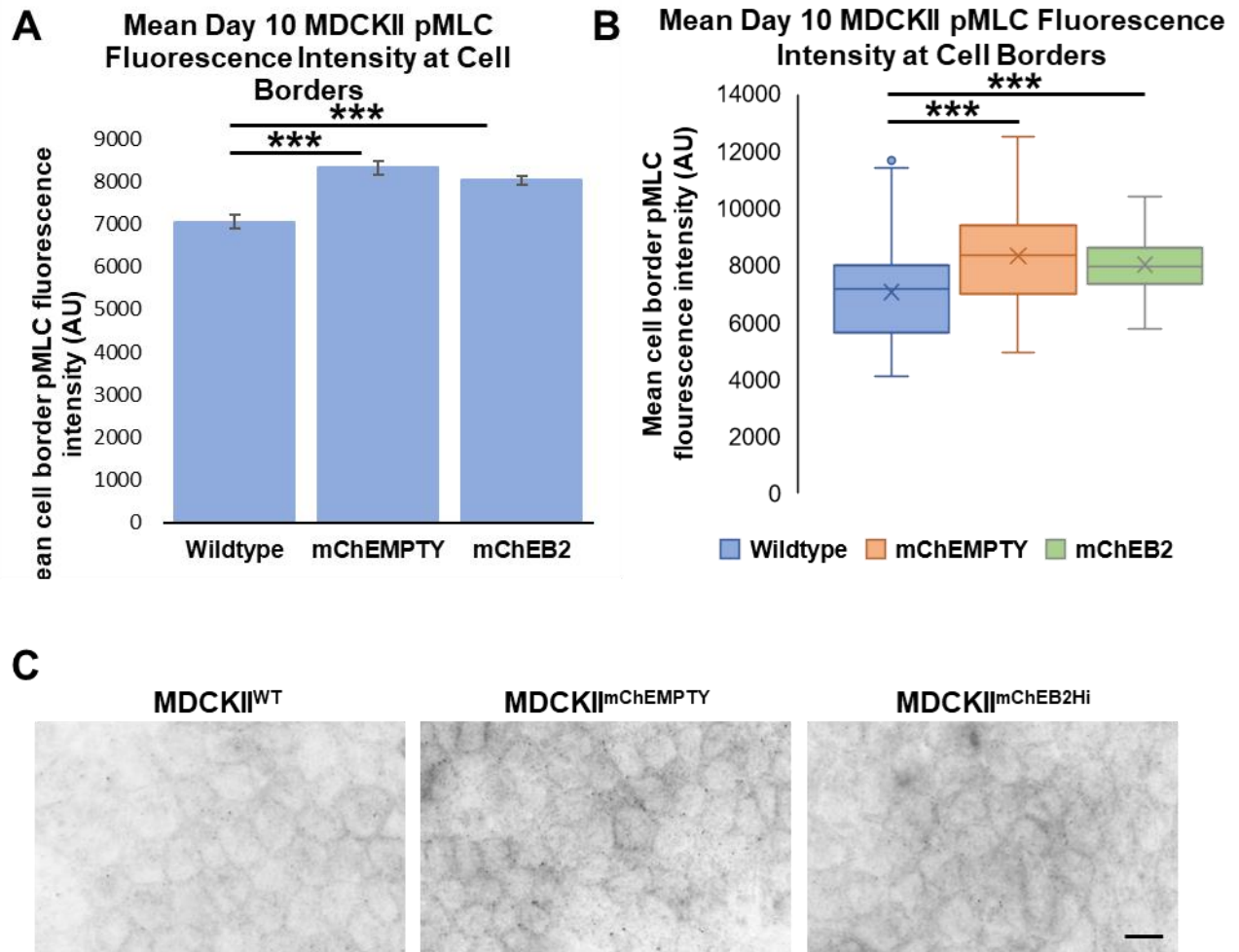


Figure 26. MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells reveal; no significant difference in mean fluorescence intensity of phosphorylated myosin light chain (pMLC) (P=0.263). MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells were grown on glass coverslips for 10 days, double immuno-labelled for pMLC (Abcam, AB2480) and E-cadherin (Mouse; BD cat no. 610181; 1:500), then imaged on a widefield fluorescence microscope with a x63 objective. E-cadherin was used to highlight cell junctions. (A) Bar chart representation of the mean fluorescence intensity of pMLC $\pm 1SE$ at cell borders indicating no significant difference between MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells but significantly higher than MDCKII^{WT} (B) Box and whisker plot demonstrating the distribution of mean pMLC fluorescence intensity in arbitrary units (AU) in day 10 cells. (N=100 per cell clone). (C) Example images taken at fixed exposure of 1000 showing pMLC localisation at junctions. Fluorescence intensity at junctions was measured in ImageJ using ROI (Appendix 8). Scale bar = 20 μm . (Asterisks *** indicate P<0.001; Games-Howell test Non-Parametric Post Hoc Test). Analysed in ImageJ. One experiment with three replicas.

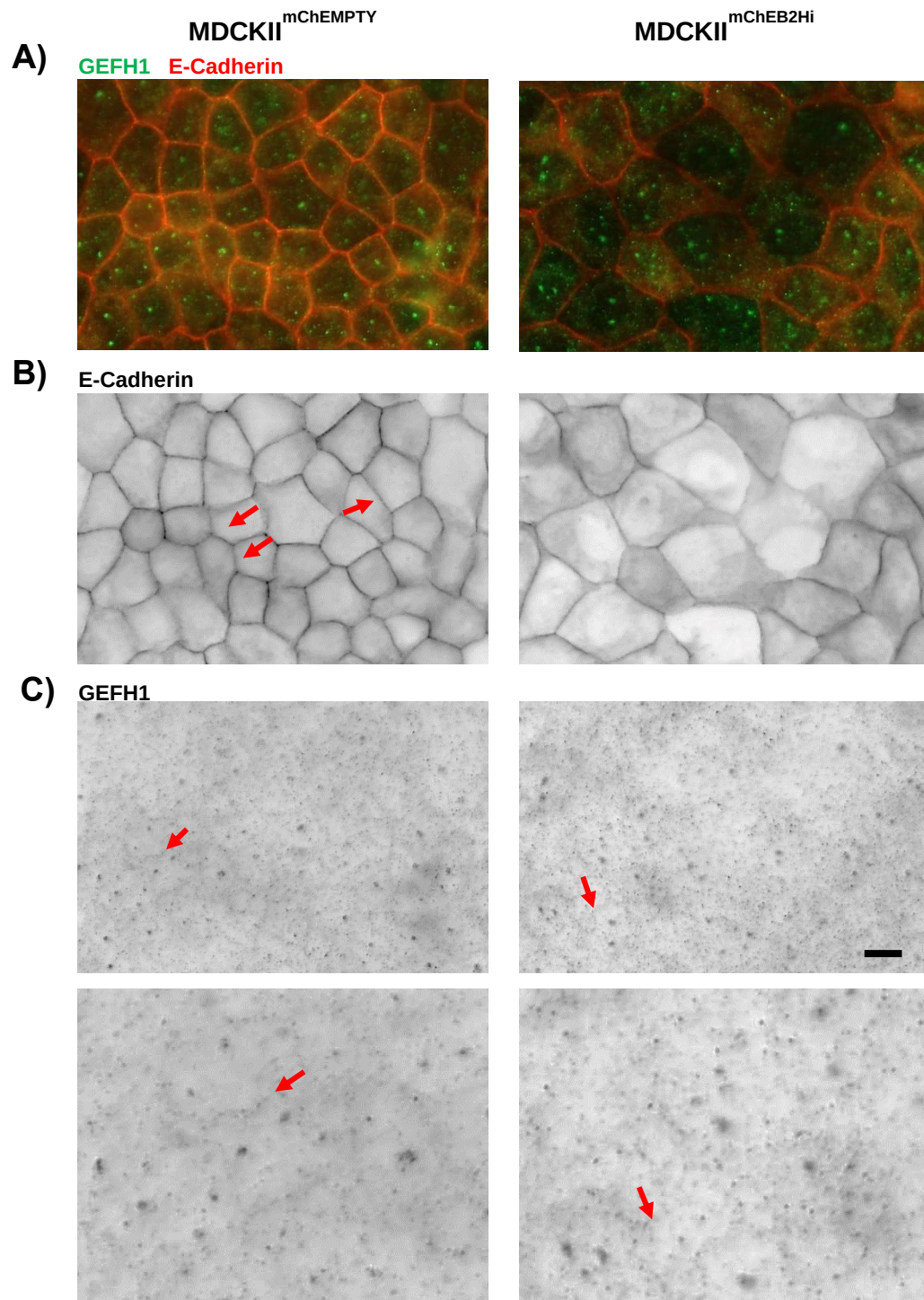


Figure 27. Localisation of GEFH1 in MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells. MDCKII cells were grown on glass coverslips for 10 days, immuno-labelled for GEF-H1 (Rabbit; Abcam cat no. AB201687; 1:500) and E-cadherin (Mouse; BD cat no. 610181; 1:500). Cells were then imaged on a widefield fluorescence microscope **with a x63 oil objective**. **Example** images **showing** GEF-H1 and E-cadherin localisation in MDCKII cells with merged images in (A), E-cadherin channel in (B) and GEF-H1 channel in (C) including enlargement of selected cells (red arrow). E-cadherin labelling appears more diffuse in MDCKII^{mChEB2Hi} compared to MDCKII^{mChEMPTY} cells (red arrows in B). GEF-H1 labelling revealed localisation throughout the cell with some accumulation at cell junctions in MDCKII^{mChEMPTY} cells but to a lesser extent in MDCKII^{mChEB2Hi} cells (red arrows in C). Scale bar = 10 μ m.

4.4. Rho activation significantly decreases the shape index in MDCKII^{mChEB2Hi} cells

Rho GTPase signalling is an important step in actomyosin contractility. Therefore, this investigation explored the effects of Rho activation on the shape index and in particular whether Rho activation could partially rescue the shape index in EB2 overexpressing cells (Figure 28-29). Day 10, MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} cells were treated with the Rho activator (1 unit/ml, 0.5%, G-switch™, Cat no. CN01 from Cytoskeleton) or DMSO (0.5%) control then double immuno-labelled for pMLC and E-cadherin. A preliminary experiment using the Rho activator at a concentration of 2 units/ml caused the cell monolayer to detach from the glass coverslips during the immuno-labelling process. Perhaps this occurred due to the increased Rho activations stimulating the cells to contract to a degree whereby they detach from the glass coverslip.

MDCKII^{mChEB2Hi} cell's mean perimeter, area and shape index decreased when treated with Rho activator compared to the DMSO control ($P < 0.001$). MDCKII^{mChEB2Hi} cell's mean shape index decreased 2.17%, from 4.13 ± 0.013 (DMSO control) to 4.04 ± 0.011 (Rho activator). However, no significant difference in mean perimeter, area and shape index was seen in MDCKII^{mChEMPTY} cells when treated with the Rho activator (mean shape index, 3.94 ± 0.008) compared to the DMSO control ($P < 0.05$) (mean shape index, 3.95 ± 0.008) (Figure 28-29).

Despite MDCKII^{mChEB2Hi} cells showing a significantly higher mean shape index compared to MDCKII^{mChEMPTY} cells when treated with either Rho activator or the DMSO control ($P < 0.001$) (Figure 28-29), however, the percentage difference between the two cell clones decreases when Rho is activated. For instance, DMSO treated MDCKII^{mChEB2Hi} cells have a 4.55% larger mean shape index than MDCKII^{mChEMPTY} cells. However, when MDCKII^{mChEB2Hi} cells are treated with the Rho activator, the mean shape index is only 2.27% larger than DMSO control MDCKII^{mChEMPTY} cells. Therefore, indicating that Rho activation leads to a partial rescue of the shape index in MDCKII^{mChEB2Hi} cells.

The experiment was repeated with 3 replicas, and similar results regarding shape index were found. Here, MDCKII^{mChEB2Hi} cells showed a significantly lower shape index when treated with the Rho activator, even more so than the DMSO control ($P > 0.001$) (Appendix 10). Although, when the data was combined and analysed the result revealed the same as the first repeat (Figure 28-29).

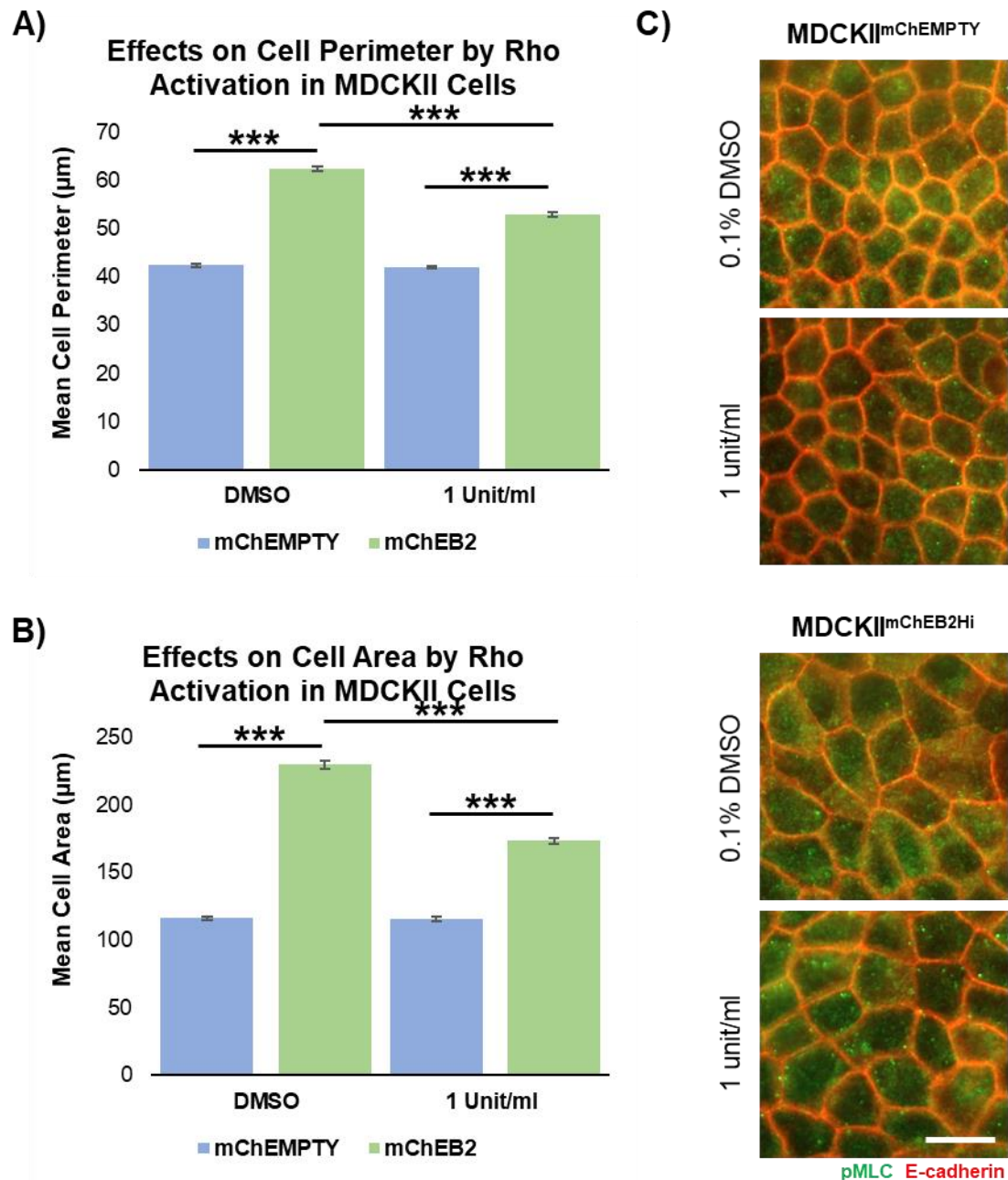
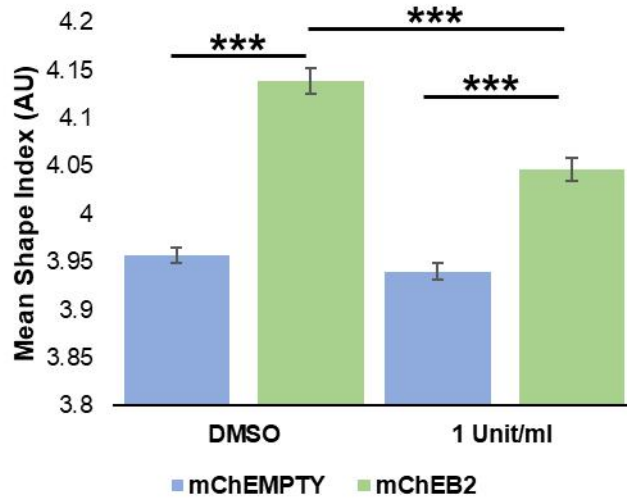


Figure 28. Rho activation resulted in a decrease in MDCKII^{mChEB2Hi} cells' mean perimeter and area in compared to DMSO control while MDCKII^{mChEMPTY} showed no significant difference. MDCKII cells were grown on glass coverslips for 10 days then treated with Rho activator (1 unit/ml, 0.5%) or a DMSO (0.5%) control for 30 minutes. Then cells were double immuno-labelled for pMLC (Phospo S20, Rabbit; Abcam cat no. AB2480; 1:300) and E-cadherin (Mouse; BD cat no. 610181; 1:500) and stained for DAPI. Cells were imaged on a widefield fluorescence microscope with a x63 oil objective. The E-cadherin labelling revealed the cell perimeter. A) and area B). The mean shape index $\pm 1SE$ was calculated in arbitrary units (AU) using the formula: shape index = perimeter/ $\sqrt{\text{area}}$ C) Examples images at fixed exposure indicate typical cell sizes. N=300 per cell clone, per condition. (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test). Analysed on ImageJ. Scale bar = 20 μm . One experiment with three replicas.

A) Effects on Shape Index with Rho Activation in MDCKII Cells



B) E-Cadherin

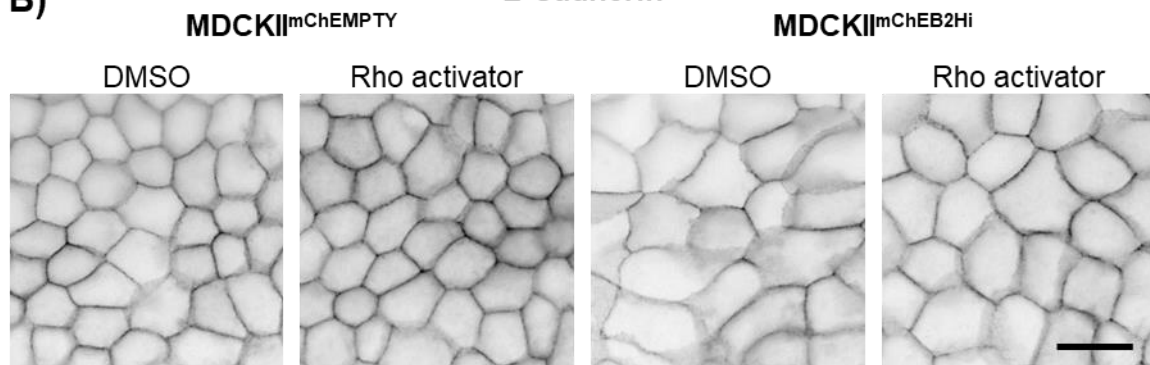


Figure 29. Rho activation resulted in a decrease in the shape index in MDCKII^{mChEB2Hi} cells compared to DMSO control while MDCKII^{mChEMPTY} showed no significant difference. MDCKII cells were grown on glass coverslips for 10 days then treated with Rho activator (1 unit/ml, 0.5%) or a DMSO (0.5%) control for 30 minutes. Then cells were double immunolabelled for pMLC (Phospho S20, Rabbit; Abcam cat no. AB2480; 1:300) and E-cadherin (Mouse; BD cat no. 610181; 1:500) and stained for DAPI. Coverslips were imaged on a widefield fluorescence microscope with a x63 oil objective. The E-cadherin labelling allowed the mean cell perimeter and area to be determined (Figure 27). The mean shape index $\pm 1SE$ was calculated in arbitrary units (AU) using the formula: $\text{shape index} = \text{perimeter} / \sqrt{\text{area}}$ (A). B) Examples of inverted images of E-cadherin labelling at fixed exposure indicating cell sizes following DMSO or Rho activator treatment.. N=300 per cell clone, per condition. (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test). Analysed on ImageJ. Scale bar = 20 μm . One experiment with three replicas.

4.4.1. Rho activation increases junctional pMLC in MDCKII^{mChEB2Hi} cells

To elucidate the relationship between EB2 overexpression and junctional contractility, the effect of Rho activation on pMLC was explored in cells overexpressing EB2. Junctional fluorescence intensity was measured in ImageJ (Appendix 8). No significant difference was observed in total pMLC fluorescence intensity when either MDCKII^{mChEB2Hi} or MDCKII^{mChEMPTY} cells were treated with Rho activator, compared to the DMSO controls (Appendix 11). MDCKII^{mChEB2Hi} cells subjected to Rho activation displayed a significant increase in mean pMLC fluorescence intensity at the junctions compared to the DMSO control ($P < 0.001$) (Figure 30, Table 7). MDCKII^{mChEMPTY} cells revealed no significant difference in mean pMLC fluorescence intensity at the junctions upon Rho activation, compared to the control ($P > 0.05$). There was no significant difference in junctional pMLC fluorescence intensity between the MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} DMSO controls. However, MDCKII^{mChEB2Hi} cells displayed a higher junctional pMLC fluorescence intensity than MDCKII^{mChEMPTY} cells when treated with Rho activator ($P < 0.001$) (Figure 30, Table 7).

Table 7. The table highlights the mean ± 1 SE values for junctional pMLC fluorescence intensity in MDCKII cells after Rho activation. $\uparrow$ indicates percentage increase and $\downarrow$ indicates percentage decrease.

	0.1% DMSO Control	1 unit/ml Rho activator	% Change	Significance
MDCKII^{mChEMPTY}	13937.96 $\pm$ 145.5	12387.13 $\pm$ 175.7	11.12% $\downarrow$	N/A
MDCKII^{mChEB2Hi}	11072.42 $\pm$ 101.0	13645.43 $\pm$ 122.1	23.23% $\uparrow$	$P < 0.001$

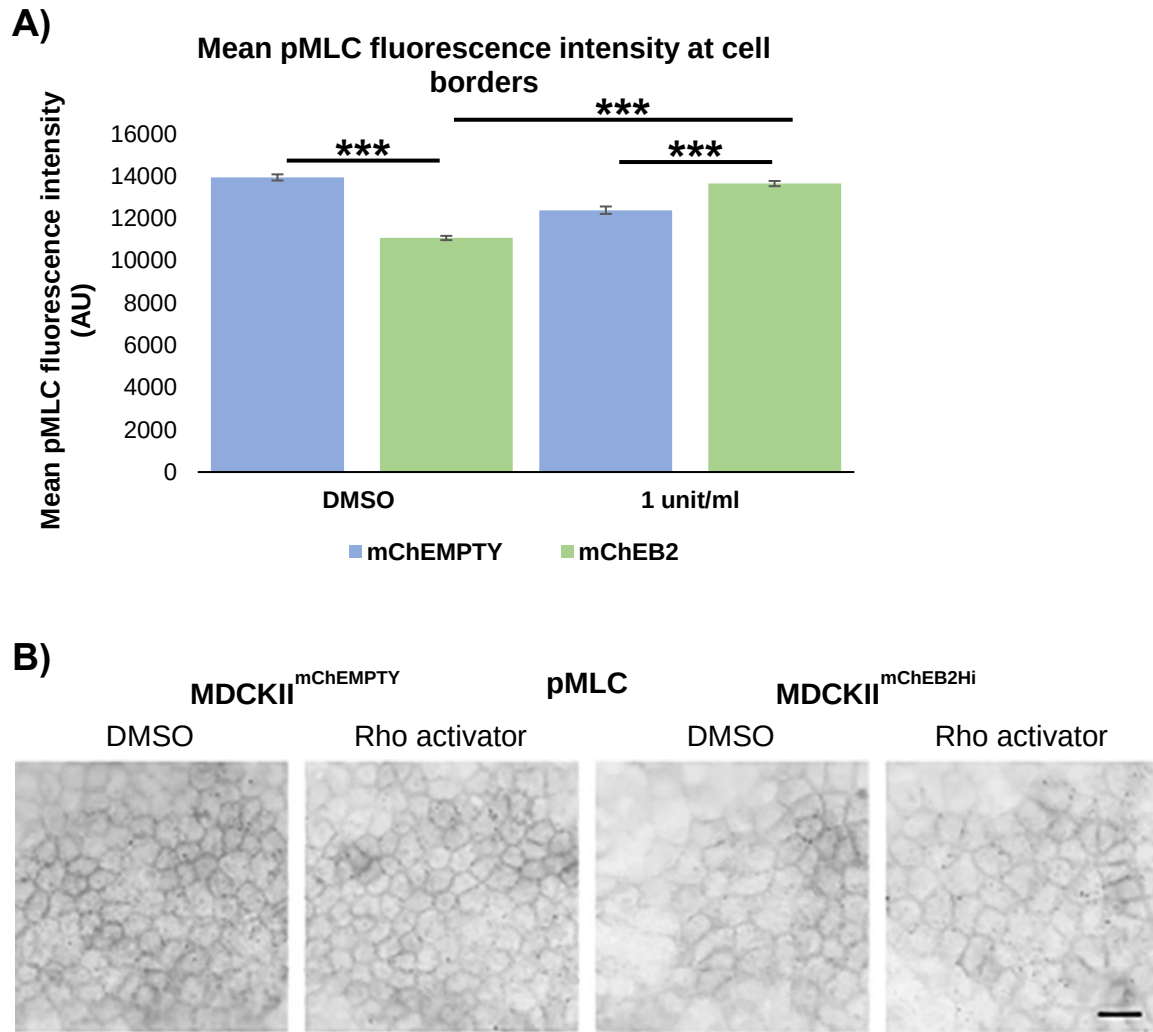


Figure 30. Rho activation increases junctional pMLC in MDCKII^{mChEB2Hi} cells. MDCKII cells were grown on glass coverslips for 10 days then treated with Rho activator (1 unit/ml, 0.5%) or a DMSO (0.5%) control for 30 minutes. Then cells were double immuno-labelled for pMLC (Phospo S20, Rabbit; Abcam cat no. AB2480; 1:300) and E-cadherin (Mouse; BD cat no. 610181; 1:500) and stained for DAPI. Cells were imaged on a widefield fluorescence microscope with a x63 oil objective. Junctional pMLC fluorescence intensity was Analysed in ImageJ (A) Rho activation led to an increase in junctional pMLC in MDCKII^{mChEB2Hi} cells while MDCKII^{mChEMPTY} cells showed no significant difference. When treated with the Rho activator, MDCKII^{mChEB2Hi} cells showed more junctional pMLC than MDCKII^{mChEMPTY} cells. B) Examples inverted images at fixed exposure indicating relative pMLC labelling only. N=300 per cell clone, per condition. Fluorescence intensity at junctions was measured in ImageJ using ROI (Appendix 8). (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test). Scale bar = 20 μm . One experiment with three replicas.

4.4.2. Rho activation leads to a decrease in total cell and junctional mDia-1 fluorescence intensity

One way Rho signalling may affect cell contractility is via formin activation; thus, mDia-1 distribution and fluorescence intensity were investigated in response to Rho activation. Immuno-labelling revealed m-Dia-1 to be more diffuse and cytoplasmic in MDCKII^{mChEB2Hi} than MDCKII^{mChEMPTY} cells (Figure 31B). Fluorescence intensity analysis of mDia-1 at the junctions was done in Image J (Appendix 8). MDCKII^{mChEB2Hi} cells showed a significantly higher total mDia-1 fluorescence intensity than MDCKII^{mChEMPTY} cells when treated with Rho activator or DMSO control ($P < 0.001$) (Appendix 12). Although, Rho activation led to a decrease in total mDia-1 and junctional fluorescence intensity in both MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} cells ($P < 0.001$) (Figure 31, Appendix 12). Thus, indicating that Rho activation only leads to a partial rescue of 'normal' junctional mDia-1 fluorescence intensity.

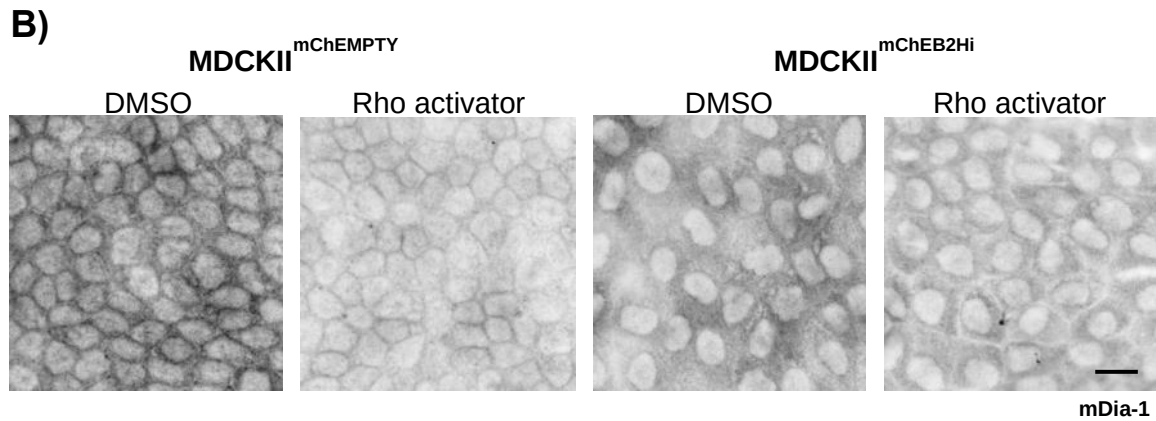
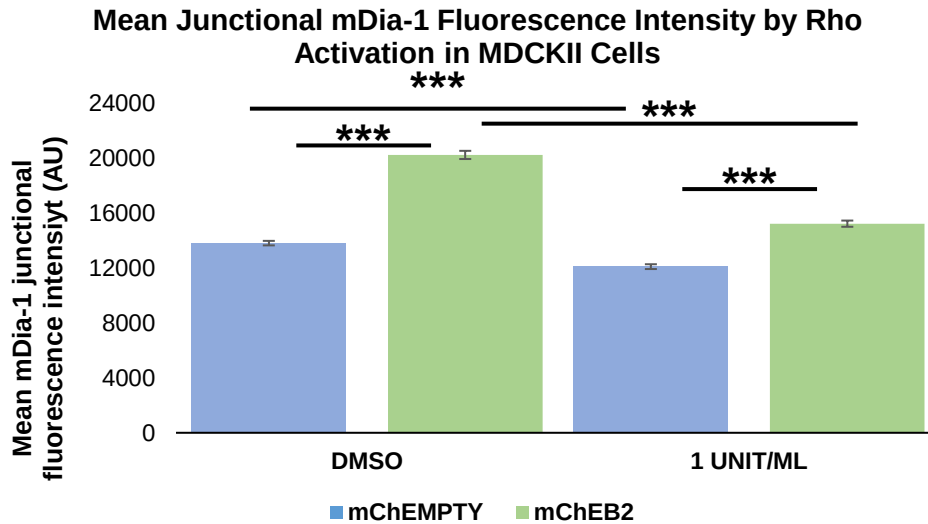


Figure 31. Rho activation significantly decreases junctional mDia-1 in MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} cells. MDCKII cells were grown on glass coverslips for 10 days then treated with Rho activator (1 unit/ml, 0.5%) or a DMSO (0.5%) control for 30 minutes. Then cells were double immuno-labelled for pMLC (Phospho S20, Rabbit; Abcam cat no. AB2480; 1:300) and E-cadherin (Mouse; BD cat no. 610181; 1:500) and stained for DAPI. Coverslips were imaged on a widefield fluorescence microscope with a x63 oil objective. (A) MDCKII^{mChEB2Hi} cells showed a significantly higher junctional mDia-1 fluorescence intensity when treated with Rho activator or DMSO control compared to MDCKII^{mChEMPTY} cells. Junctional pMLC fluorescence intensity was Analysed on ImageJ. B) Examples inverted images at fixed exposure indicating relative pMLC labelling only. Fluorescence intensity at junctions was measured in ImageJ using ROI (Appendix 8). N=300 per cell clone, per condition. (Asterisks *** indicate P<0.001; Games-Howell test Non-Parametric Post Hoc Test). Scale bar = 20 μ m. One experiment with three replicas.

4.5. Dynamin-2 fluorescence intensity is significantly increased in MDCKII^{mChEB2Hi} cells

Previous data by Ben Rix explored the effect of EB2 overexpression on E-cadherin localisation at junctions (Appendix 13). The fluorescence intensity analysis across junctions using line profiles (Appendix 9) showed that MDCKII^{mChEB2Hi} cells had a larger bell curve than the control MDCKII^{mChEMPTY} cells (Appendix 13). These findings by Ben Rix suggests that MDCKII^{mChEB2Hi} cells have a greater spread of E-cadherin across the junctions. However, no significant difference was found in peak fluorescence intensity (Appendix 13). This study explored this further by investigating the role of dynamin-2 which plays an important role in E-cadherin endocytosis.

This investigation explored whether EB2 overexpression had an effect on dynamin-2 distribution and its fluorescence intensity at the junctions and across junctions (Appendix 8 and 9). Day 10, MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} cells were double immuno-labelled for Dynamin-2 and E-cadherin. MDCKII^{mChEMPTY} cells presented diffuse dynamin-2 localisation throughout the cytoplasm with some accumulation at the cell junctions, while MDCKII^{mChEB2Hi} cells showed considerably more junctional dynamin-2 (Figure 32). MDCKII^{mChEB2Hi} cells showed a significantly higher mean Dynamin-2 fluorescence intensity at the cell junctions compared to MDCKII^{mChEMPTY} control cells ($P < 0.001$) (Figure 32). MDCKII^{mChEMPTY} cells revealed a mean Dynamin-2 junctional fluorescence intensity of 13515.5 ± 93.02 (AU), while MDCKII^{mChEB2Hi} cells had 18332.8 ± 140.89 (AU), therefore displaying an approximate 35.6% increase.

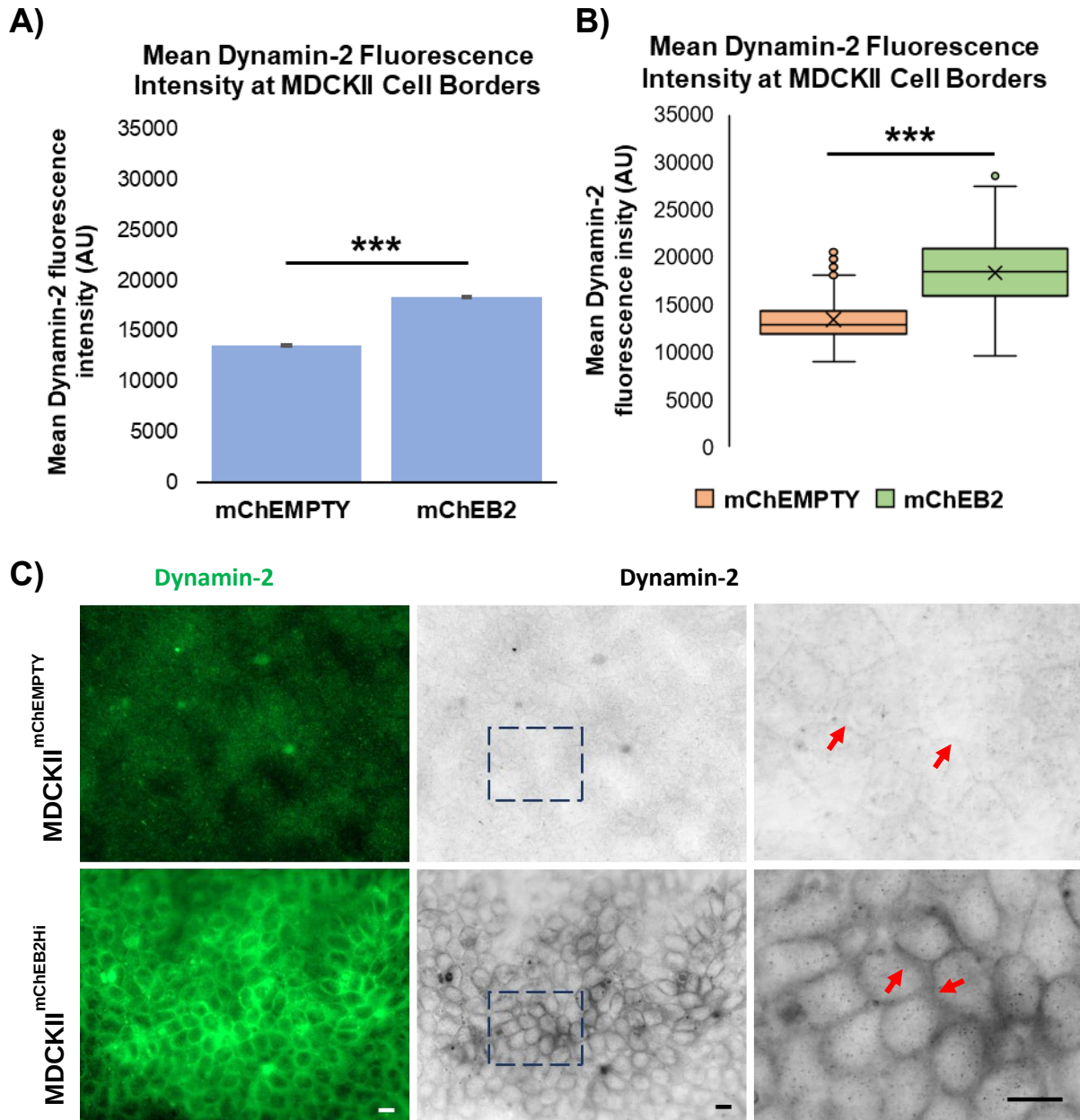


Figure 32. More dynamin-2 is evident at junctions in MDCKII^{mChEB2Hi} than in MDCKII^{mChEMPTY} cells. MDCKII cells were grown on glass coverslips for 10 days, immuno-labelled for Dynamin-2 (Rabbit; Abcam cat no. AB3457 1:300) and E-cadherin (Mouse; BD cat no. 610181; 1:500). Cells were then imaged on a widefield fluorescence microscope with a x63 oil objective. A) Bar chart representation of the mean fluorescence intensity of Dynamin-2 $\pm$ 1SE at cell junctions in arbitrary units (AU). B) Box and whisker plot demonstrating the distribution of mean Dynamin-2 fluorescence intensity at the cell junctions in arbitrary units (AU). (N=600 per cell clone). (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test). Analysed in ImageJ. C) Example images highlighting Dynamin-2 (green/invert) localisation. The black thatched boxes indicating the enlarged area in image to the right. MDCKII^{mChEB2Hi} cells revealed a significantly higher mean Dynamin-2 fluorescence intensity at the cell junctions compared to MDCKII^{mChEMPTY} cells (see red arrows in C). Dynamin-2 appeared to localise diffusely throughout the cytoplasm in MDCKII^{mChEMPTY} cells with minimal junction localisation. Fluorescence intensity at junctions was measured in ImageJ using ROI (Appendix 8). Scale bars = 10 μ m. One experiment with three replicas.

4.5.1. Dynasore treatment leads to an increase in junctional E-cadherin fluorescence intensity and shape index in MDCKII^{mChEB2Hi} cells

To further investigate the role of Dynamin-2, EB2 and the UJT, Dynasore was used to suppress dynamin activity in MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells by inhibiting GTP hydrolysis. The effect on E-cadherin distribution was explored by analysing the mean E-cadherin fluorescence intensity at the cell junctions (Appendix 8). Fluorescence microscope analysis suggested that E-cadherin is more dispersed and cytoplasmic when cells are treated with Dynasore, especially in MDCKII^{mChEB2Hi} cells (Figure 33). This is expected as inhibition of dynamin with Dynasore would impair dynamin-mediated E-cadherin turnover with less E-cadherin localising to junctions. In MDCKII^{mChEB2Hi} cells, E-cadherin fluorescence intensity at the junctions significantly decreased when treated with Dynasore compared to the DMSO control ($P < 0.001$); whereas, in MDCKII^{mChEMPTY} cells, a significant increase was observed ($P < 0.05$, $P = 0.041$). When cells were treated with DMSO, MDCKII^{mChEB2Hi} cells showed a significantly higher mean E-cadherin fluorescence intensity at the junctions compared to MDCKII^{mChEMPTY} cells ($P < 0.001$). However, when cells were treated with Dynasore, the opposite effect was seen: MDCKII^{mChEB2Hi} cells revealed significantly lower mean E-cadherin fluorescence intensity at the junctions than MDCKII^{mChEMPTY} cells ($P < 0.01$) (Figure 33-34). Therefore, indicating that inhibition of dynamin in cells overexpressing EB2 causes junctional E-cadherin to become dispersed in the cytoplasm (Figure 34).

Next, the effects on dynamin inhibition on the cell shape index was explored. MDCKII^{mChEB2Hi} cells reveal a larger mean perimeter, area and shape index when treated with either DMSO or Dynasore compared to MDCKII^{mChEMPTY} cells ($P < 0.001$) (Figure 35). MDCKII^{mChEB2Hi} cells' mean perimeter ($P < 0.001$), area ($P < 0.01$) and shape index ($P < 0.001$) increased when treated with Dynasore compared to the DMSO control (Table 8). However, no significance difference in mean perimeter, area and shape index was seen in MDCKII^{mChEMPTY} cells when treated with Dynasore compared to the DMSO control ($P > 0.05$, $P = 0.069$) (Table 8).

Table 8. The table highlights the mean $\pm 1SE$ shape index of MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells when treated with Dynasore or a DMSO control. $\uparrow$ indicates percentage increase and $\downarrow$ indicates percentage decrease. Note the percentage increase seen in MDCKII^{mChEB2Hi} cells when treated with Dynasore is smaller, yet significant, than that of the percentage decrease seen in MDCKII^{mChEMPTY} cells which was found to be not significant. Perhaps one reason for this is the wider variation seen in MDCKII^{mChEB2Hi} cells which is reflected by the larger standard error.

	Mean Shape Index $\pm 1SE$			
	DMSO	Dynasore	Percentage change (%)	Significance
MDCKII^{mChEMPTY}	3.93 $\pm$ 0.008	3.90 $\pm$ 0.006	0.69% $\downarrow$	P>0.05
MDCKII^{mChEB2Hi}	4.07 $\pm$ 0.120	4.09 $\pm$ 0.146	0.52% $\uparrow$	P<0.001

in junctional E-cadherin in Dynasore treated MDCKII^{mChEB2Hi} compared to DMSO control treated MDCKII^{mChEMPTY} cells. Fluorescence intensity at junctions was measured in ImageJ using ROI (Appendix 8). (N=300 per cell clone, per condition). One experiment with three replicas.

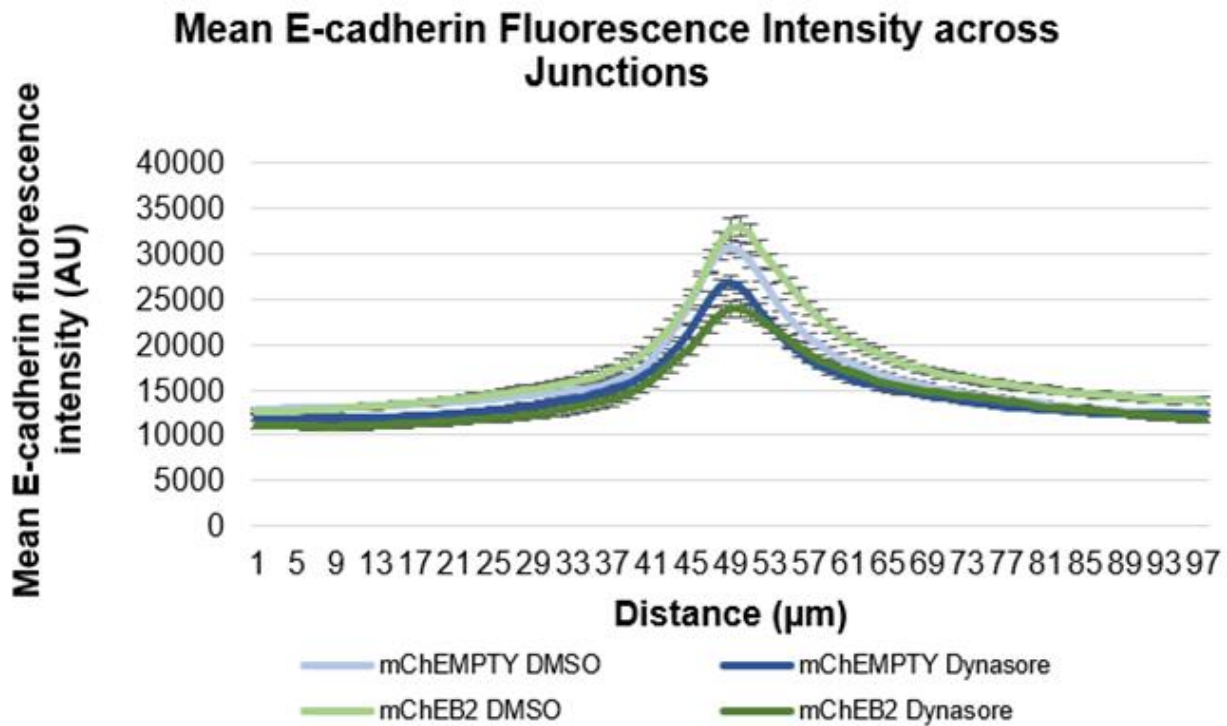


Figure 34. Effects of Dynamin inhibition, with Dynasore, on E-cadherin distribution across junctions in MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells. MDCKII cells were grown on glass coverslips for 10 days, double immuno-labelled for Dynamin-2 (Rabbit; Abcam cat no. AB3457 1:300) and E-cadherin (Mouse; BD cat no. 610181; 1:500). Cells were treated with Dynasore (80 μM, 0.1%) or DMSO (0.1%) control for 1 hour then imaged on a widefield fluorescence microscope at x63 oil objective. A marked decrease in peak fluorescence intensity is evident in EB2 overexpressing cells. Fluorescence intensity across junctions was measured in ImageJ using ROI (Appendix 9). N=50 per cell clone, per condition.. One experiment with three replicas.

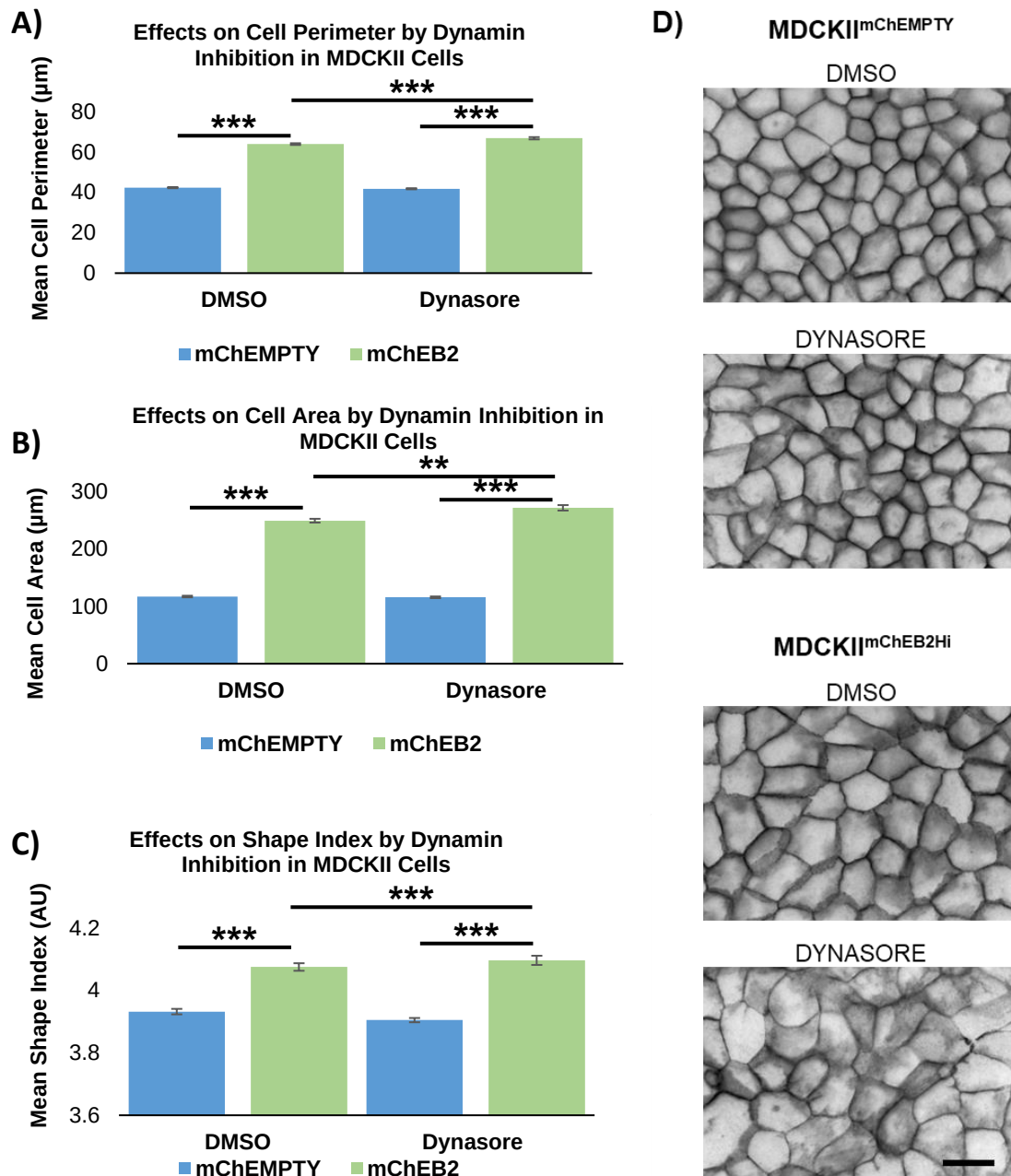


Figure 35. MDCKII^{mChEB2Hi} cells revealed a significantly higher mean perimeter ($P < 0.001$), area ($P < 0.01$), and shape index ($P < 0.001$) than when treated with Dynasore compared to the DMSO control. MDCKII cells were grown on glass coverslips for 10 days, immuno-labelled for Dynamin-2 (Rabbit; Abcam cat no. AB3457 1:300) and E-cadherin (Mouse; BD cat no. 610181; 1:500). Cells were treated with Dynasore (80 μ M, 0.1%) or a DMSO (0.1%) control for 1 hour then imaged on a widefield fluorescence microscope with a x63 oil objective. The E-cadherin labelling allowed the mean cell perimeter A) and area B) to be determined. The mean shape index ± 1 SE was calculated in arbitrary units (AU) using the formula: shape index = perimeter/ $\sqrt{\text{area}}$ C). N=300 per cell clone, per condition. D) Enlarged inverted images highlighting the cell junctions with E-cadherin labelling. Dynamin inhibition significantly increases the mean perimeter, area and shape index in MDCKII^{mChEB2Hi} cells. (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test). Analysed using ImageJ. Scale bar = 20 μ m. One experiment with three replicas.

4.6. Discussion.

4.6.1. EB2 overexpression leads to increased cell shape index and unjamming

The jamming-unjamming transition was investigated using the cell shape index as an indicator. The cell shape index is calculated using the formula $\text{shape index} = \text{perimeter} / \sqrt{\text{area}}$. This transition is marked by a critical change in the preferred cell shape (P_0), in arbitrary units, with a threshold factor of $P_0 = 3.813$. According to previous research by Park et al. (2016), cells below this threshold are jammed while those above are unjammed. In this investigation, MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells were grown for 8, 10 and 14 days to determine the shape index at increasing confluency. At every time point, the cells revealed a mean shape index above the UJT threshold factor, $P_0 = 3.813$, therefore indicating that all cells were unjammed and thus in a fluid-like state. This suggests that, at the very least, the wildtype cells had not reached partial polarisation.

MDCKII^{mChEB2Hi} cells revealed the highest mean cell perimeter, area and shape index while MDCKII^{mChEMPTY} cells had the lowest. This indicates that EB2 overexpression resulted in greater unjamming. Surprisingly, MDCKII^{WT} showed significantly higher mean cell perimeter, area and shape index compared to MDCKII^{mChEMPTY} cells at days 10 and 14. It is possible that transfection and expression of the Empty mCherry vector contributed to a significantly smaller cell perimeter and area. MDCKII^{WT} cells were of a lower passage (P4) compared to MDCKII^{mChEMPTY} (P10) and MDCKII^{mChEB2Hi} cells (P12). Due to MDCKII^{mChEMPTY}'s higher passage number than MDCKII^{WT}, the cells may have reached 100% confluency before MDCKII^{WT} cells indicating that MDCKII^{mChEMPTY} cells proliferated faster. In addition, the MDCKII^{WT} cells used in this study were not the parental clone, this may have contributed to the differences seen. Another factor to consider is that the transfected cells, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi}, were grown in supplemented media containing the selective marker Geneticin (G418) which blocks polypeptide synthesis, except in transfected cells that contain the neo gene. It is possible that the antibiotic gave the cells a selective advantage for growth due to reduced competition from bacteria.

MDCKII^{mChEB2Hi} cells revealed to have a significantly higher EB2 fluorescence intensity compared to the negative controls and were used as a model system for epithelial cells overexpressing EB2. Additionally, MDCKII^{mChEMPTY} cells showed significantly higher EB2 fluorescence intensity compared to MDCKII^{WT} cells. MDCKII^{mChEMPTY} cells showed a significantly higher EB2 fluorescence intensity compared to MDCKII^{WT} cells. This suggests that transfection of the vector, although unattached, leads to an increase in EB2 expression. However, there are other possible contributing factors to this result. For example, as explained above, MDCKII^{mChEMPTY} cells were of a higher passage number than the MDCKII^{WT} cells. It is

commonly evidenced that cells of a higher passage number proliferate at a faster rate, and EB2 expression has been shown to positively correlate with increasing confluency (Goldspink et al. 2013). Perhaps suggesting that since MDCKII^{mChEMPTY} cells were more confluent than MDCKII^{WT} cells, their EB2 expression was increased. MDCKII^{mChEMPTY} cells were also treated with supplemented media containing the selective marker Geneticin (G418). Thus, it may be possible that the antibiotic gave the MDCKII^{mChEMPTY} cells a selective advantage for growth due to reduced competition from bacteria.

It is hypothesised that tensile forces dominate over adhesive forces in jammed cells, while adhesive dominate over tensile in unjammed states (Farhadifar et al., 2007, Bi et al., 2015, Park et al., 2016). The data therefore suggests that MDCKII^{mChEMPTY} cells possess stronger tensile forces than MDCKII^{mChEB2Hi}, while their adhesion forces are likely to be weaker.

The cell perimeter, apical surface area and shape index in MDCKII^{mChEB2Hi} cells were significantly larger, showing an almost 10% increase in mean shape index at day 10, than control cells. Goldspink et al. (2013) found that endogenous EB2 expression was varied among cells within confluent monolayers of mouse kidney epithelial cells. Similarly, in this study, variations in EB2 expression were observed in MDCKII^{mChEB2Hi} cells. This suggests that within epithelial layers and tissues there are heterogenous populations of differential EB2 expressing cells.

Cell circularity and eccentricity are two other ways to investigate cell shape, and both were analysed using CellProfiler™ on Day 10 in MDCKII cells. All MDCKII cell clones revealed a significantly different circularity ($P < 0.001$). MDCKII^{mChEMPTY} cells were most circular with a value of 0.38 ± 0.003 , while MDCKII^{mChEB2Hi} revealed the least with 0.29 ± 0.003 , and MDCKII^{WT} an intermediate at 0.35 ± 0.003 . Similarly, all MDCKII cell lines revealed a significantly different eccentricity ($P < 0.001$). MDCKII^{mChEMPTY} cells revealed the lowest mean eccentricity value of 0.68 ± 0.006 . Thus, suggesting the MDCKII^{mChEMPTY} cells to be the most circular. MDCKII^{mChEB2Hi} cells had the highest eccentricity value of 0.78 ± 0.005 , therefore indicating them to be the most elongated cells. MDCKII^{WT} cells' mean eccentricity was 0.70 ± 0.006 . In summary, from an apical view, MDCKII^{mChEMPTY} cells were the most compact and circular, mimicking a more jammed state while MDCKII^{mChEB2Hi} cells were more elongated and irregular, resembling that of an unjammed system (Park et al., 2015, Kim et al., 2020).

In conclusion, the data collected here suggests that EB2 overexpression in MDCKII cells, correlates with a significant increase in cell perimeter, area and shape index compared to the controls. This suggests that EB2 overexpression contributes to unjamming in epithelial cells.

4.6.2. EB2 overexpression does not affect Par3 and ZO-1 localisation but reveals altered tight junction morphology.

Par3 is essential for tight junction formation and is part of the Par complex that coordinates with the Crumbs complex to establish an apical domain during apico-basal polarisation. Therefore, deeming Par3 to be a suitable polarity marker, in this investigation, to explore the effects of EB2 overexpression on cell polarity. The localisation of Par3 suggests that EB2 overexpression does not affect apico-basal polarity. This is distinct from EMT/pEMT whereby the apico-basal polarity is replaced by a front-back polarity phenotype (Nieto et al., 2016, Oswald et al., 2017, Mitchel et al., 2020).

Previous papers have suggested that the UJT is a precursor to EMT, specifically pEMT (Tambe et al., 2011). Meanwhile, results from other investigations suggest that the UJT can occur independently (Trepatt et al., 2009, Serra-Picamal et al., 2012, Park et al., 2015). Both pEMT and unjamming have been shown to occur in situations of development, wound healing and cancer. In addition, both mechanisms show a change in cell shape and gain in motility. However, distinct differences have been recognised. For example, during pEMT, TGF- β expression promoted the downregulation of E-cadherin and gain of mesenchymal markers such as N-cadherin and vimentin (Pickup et al., 2013). Whereas E-cadherin expression is relatively constant throughout the UJT (Mitchel et al., 2020). Other key indicators of pEMT, is the loss of an apico-basal polarity and gain of front-back polarity (Campbell and Casanova, 2016). Therefore, suggesting that unjammed cells maintain their epithelial phenotype, unlike cells undergoing pEMT (Park et al., 2015, Mitchel et al., 2020). This investigation did intend to explore the similarities and differences between pEMT and jamming/unjamming further, however time restrictions did not allow this. Future works could include testing for a wide range of EMT/pEMT markers in unjammed cells.

ZO-1 is a transmembrane protein localised at the tight junctions that mediates a crosslink between claudins and the actin cytoskeleton (Fanning and Anderson, 2009, Tornavaca et al., 2015). ZO-1 labelling served as a qualitative indicator for tight junction integrity in this investigation. The results revealed that ZO-1 showed a mainly junctional localisation in the control cells as expected. When EB2 was overexpressed, ZO-1 localisation did not appear to change however, it did highlight more undulating or ruffled tight junctions. Tight junction ruffling occurs via the interaction between claudins, ZO-1 and the actin network. Ruffling has been found to be mediated by factors including mechanical stimulation, hypoxia inducing factor and integrin mediated signalling (Lynn et al., 2020). Currently, only tight junctions have been shown to demonstrate ruffling, however it is hypothesised that ruffling is possible in other

structures such as adherens junctions (Lynn et al., 2020). However, labelling for E-cadherin in this investigation did not indicate any undulations in adherens junctions.

Claudins are a group of transmembrane proteins that interact with other transmembrane proteins, scaffold proteins and the actin cytoskeleton to sustain a barrier function in epithelial cell layers (Lynn et al., 2020). Claudin binding to ZO-1, a key scaffolding protein, mediates a cross-link with the actin cytoskeleton. Changes to this cross-link lead to a change in tight junction structure from linear to ruffled which effects barrier function (Krause et al., 2015, Samak et al., 2014). This suggests that EB2 overexpression may induce tight junction ruffling resulting in compromised junction integrity and barrier function.

Tokuda et al. (2014) showed that when ZO-1 was depleted in MDCKII cells, tight junctions were linear and therefore unruffled, whereas high ZO-1 expression resulted in junction ruffling. Depletion of ZO-2 or ZO-3 did not significantly affect tight junction structure (Tokuda et al., 2014). Thus, suggesting that the ZO-1 crosslink between claudins, and actin is required for tight junction ruffling. Lohmann et al. (2020) likewise used ZO-1 distribution as an indicator for tight junction integrity in epithelial monolayers. The results demonstrated that as a monolayer of MDCK^{WT} cells matured (Day 1 to 6), ZO-1 labelling at the cell junctions became more complete (Lohmann et al., 2020). The ZO-1 labelling on Day 6 resembled the labelling present in day 10 MDCKII^{mChEMPTY} in this investigation, where ZO-1 lined the cell junctions with minimal-to-no cytoplasmic labelling (Lohmann et al., 2020).

Mitchel et al. (2020) investigated the similarities and differences between the UJT and pEMT. Using confluent layers of primary human bronchial epithelial (HBE) cells as a model system, the team induced the UJT by mechanical stress for 3 hours, while pEMT was induced by TGF- β 1 exposure. In contrast to the results seen in this investigation, Mitchel et al. (2020) found that during the UJT, ZO-1 labelling was continuous and predominantly at the tight junctions. The same distribution was apparent in control cells. In addition, no change in total E-cadherin was apparent (Mitchel et al., 2020). Consequently, UJT appears to maintain junctional integrity and apico-basal polarity unlike pEMT which led to ZO-1 disruption and decreased E-cadherin. Thus, indicating that apico-basal polarity and tight junction integrity is affected by pEMT but not UJT.

4.6.3. High EB2 expression appears to lead to increased MT density

EB2 knockdown in partially polarised epithelial cells led to increased MT stability and decreased MT dynamics (Goldspink et al., 2013) suggesting perhaps, that EB2 overexpression would result in more dynamic MTs. In this study, Day 10 MDCKII^{mChEB2Hi} cells

were used as a model for apico-basally differentiating epithelial cells overexpressing EB2. Immuno-labelling of β -tubulin in control cells revealed junctional β -tubulin suggestive of an apico-basal arrangement with some lateral MTs visible in the apical cytoplasm. However, in EB2 overexpressing cells, the apical cytoplasmic MT network was considerably denser with less junctional labelling. Zhong et al. (2021) found that EB2 overexpression was associated with an increase in HCC cell proliferation rate, cell migration and invasion ability; while EB2 knockdown resulted in the opposite effects, *in vitro* (Zhong et al., 2021). This may suggest that MDCKII^{mChEB2Hi} cells have less stable apico-basal MTs and more dynamic centrosomally anchored MTs. However, confocal imaging as well as live time-lapse analysis of GFP-tagged MTs would be needed to confirm this.

Junctional β -actin labelling did not appear to change when EB2 was overexpressed in this study. However, higher resolution and optical sectioning would be needed to confirm this. An increase in polymerized actin was apparent in unjammed airway epithelium by Ghosh et al. (2022). The epithelium was unjammed due to cigarette smoke exposure or was derived from chronic obstructive pulmonary disease (COPD) patients. The results indicated that the increase in actin was due to a decrease in cofilin-1, an actin-severing protein (Ghosh et al., 2022). Inhibition of actin polymerisation reverted the cigarette smoke exposed and COPD epithelial layer to a jammed state. Thus, proposing the amount of polymerised actin to play a role in the UJT (Ghosh et al., 2022). Perhaps it could be investigated whether inhibition of actin polymerisation stimulates a partial rescue of shape index in MDCKII^{mChEB2Hi} cells.

Rho GTPases are key regulators of the MT and actin cytoskeleton and therefore, play a significant role in MT and actin crosstalk, dynamics and reorganisation. One of the downstream effectors of Rho GTPases are formins, which encourage actin polymerisation at junctions. Immuno-labelling for mDia-1 in this study suggested that MDCKII cells overexpressing EB2 have less, but more punctate junctional mDia-1 compared to the control. However, fluorescence intensity analysis would be needed to confirm this. EB2 overexpression appeared to have no effect on mDia-2 distribution. Therefore, suggesting that EB2 overexpression affects junctional localisation of select formins.

Acharya et al. (2017) investigated the role of mDia-1 at cell junctions using Caco-2 cells as a model. They found that knockdown of mDia-1 caused a reduction in junctional mDia-2, whereas levels of mDia-1 remained constant in mDia-2 knockdowns. Therefore, suggesting mDia-1 is dominant over mDia-2 regarding junctional localisation, perhaps suggesting mDia-1 is required for mDia-2 localisation (Acharya et al., 2017). Interestingly, in MDCKII^{mChEB2Hi} cells, where mDia-1 was more diffuse at cell junctions, mDia-2 was unaffected. Perhaps suggesting that the mDia-1 displacement was not enough to have an effect on mDia-2

distribution. Moreover, Acharya et al. (2017) showed that mDia-1 localised along the cell junctions in between actin filament bundles in polarised Caco-2 cells. E-cadherin was also shown localising along the apical ZA, with actomyosin bundles. E-cadherin knockdown disrupted mDia-1 labelling, indicating that E-cadherin plays an important role in the recruitment of mDia-1 to the junctions (Acharya et al., 2017). Furthermore, E-cadherin knockdown indicated that mDia-1 facilitated E-cadherin-based contractility and so integrity via cell-cell adhesion (Acharya et al., 2017). Previous data by Ben Rix showed that E-cadherin had a greater spread at the junctions in EB2 overexpressing cells. Perhaps this change in junctional E-cadherin localisation leads to the more diffuse mDia-1 labelling seen here.

This raises the question – does EB2 overexpression interfere with mDia-1 delivery to the junctions? Since both mDia-1 and the Arp2/3 complex are involved in actin filament nucleation at the junctions, this may suggest that when EB2 is overexpressed there is less mDia-1 induced actin nucleation, which may trigger increased Arp2/3 nucleation. Additional experiments are required to determine whether the actin filament organisation and Arp2/3 expression is affected by EB2 overexpression in order to reveal the mechanism responsible for EB2 overexpression which causes changes in cell shape.

4.6.4. Rho activation partially rescues the cell shape index in MDCKII^{mChEB2Hi} cells

The data presented here reveals that EB2 overexpression results in a larger perimeter, area and cell shape index suggesting that contraction at the apex is reduced compared to control cells. The fluorescence intensity of pMLC was analysed as an indicator of cortical actomyosin contraction. However, the results revealed that MDCKII^{mChEB2Hi} cells showed no significant difference in junctional pMLC compared to MDCKII^{mChEMPTY} cells.

Rho GEFs mediate the exchange of GDP for GTP on RhoA, leading to RhoA activation. GEF-H1 is a Rho GEF found associated along the lattice of stable MTs. Destabilisation and depolymerisation of MTs leads to phosphorylation of GEF-H1 which results in its detachment from MTs (Joo and Olson, 2021). GEF-H1 triggers RhoA activation leading to Rho-associated coiled-coil containing protein kinases 1 and 2 (ROCK1/2) activation. ROCK1/2 are significant drivers in actin cytoskeleton dynamics and key players in actomyosin contraction, cell shape and migration (Joo and Olson, 2021). Immuno-labelling suggested slightly less junctional and more cytoplasmic GEF-H1 in MDCKII^{mChEB2Hi} cells compared to MDCKII^{mChEMPTY}. However, fluorescence intensity analysis is required to confirm this.

EB2 overexpression is suggested to lead to an increase in MT dynamics which, in turn, is likely to increase the release of GEF-H1 from MTs resulting in more cytoplasmic and less

junctional GEF-H1. As more dynamic MTs are rapidly polymerising and depolymerising, GEF-H1 may be released from MTs before it can be delivered to the cortex. The knock-on effect would be less RhoA and ROCK activation and thus less actomyosin contraction resulting in an increase in the cell shape index. However, further investigations into MT dynamics and GEF-H1 localisation would be needed to resolve this.

To elucidate further the relationship between EB2 overexpression of cell contractility, the effect of Rho activation was explored. Rho GTPase signalling is a key player in actomyosin contractility and cell shape. Therefore, this investigation explored the effects of Rho activation on shape index. Rho activation led to partial rescue of the shape index in EB2 overexpressing cells, suggesting that overexpression of EB2 suppresses Rho activation. Similarly, Cavanaugh et al. (2020) used Rho inhibition and activation to investigate the cell shape index and morphological changes in Caco-2 epithelial. The control cells revealed a shape index value of 3.9, therefore indicating an unjammed scenario. Global Rho inhibition with C3 transferase resulted in an increase in junction length and the shape index to 4.5, thus further unjammed. In addition, cells were more elongated and motile. However, Rho inhibition did not appear to have an effect on membrane internalisation (Cavanaugh et al., 2020). Removal of C3 transferase recovered the shape index and junction length and caused an increase in membrane internalisation (Cavanaugh et al., 2020). The higher shape index and the longer and less circular morphology are all characteristics of unjammed EB2 overexpressing cells (Park et al., 2015, Kim et al., 2020). Therefore, potentially supporting the hypothesis that EB2 overexpression leads to reduced Rho activation and a more unjammed phenotype, mimicking the Rho inhibition experiments by (Cavanaugh et al., 2020). Though, in this investigation, no significant difference was seen in pMLC fluorescence intensity when EB2 was overexpressed. Therefore, suggesting that there was no difference in the amount of activated myosin. Perhaps implying that, instead, the actomyosin interaction is less efficient which then results in less efficient contraction.

Furthermore, Cavanaugh et al. (2020) demonstrated that dynamic RhoA activity at junctions leads to changes in Caco-2 cell morphology. For example, relatively short pulses of active RhoA led to rapid contraction and reversible shortening of cell-cell junctions. Whereas a single prolonged RhoA pulse led to irreversible shortening of junctions. The team concluded that, during contraction, when the strain threshold (physical force deforming an object) is not reached, the junction can recover to its original length. However, the prolonged pulse was sufficient to surpass the critical threshold leading to a permanent increase in tension at the junctions and therefore irreversible shortening (Cavanaugh et al., 2020). A similar principle is relevant for the UJT since the transition relies on a balance of tensile forces. Perhaps in unjammed cells, when Rho activity is suppressed, the threshold is not surpassed and thus

junction lengths are longer, and so contribute to a larger cell shape index. However, further exploratory studies would need to be undertaken as pMLC fluorescence intensity did not indicate this.

Another way Rho signalling mediates an effect on cell contractility is via formin activation. It was hypothesised that Rho activation would lead to an increased mDia-1 fluorescence intensity at the junctions. However, this was not the case, and Rho activation resulted in a decrease in junctional mDia-1 fluorescence intensity in both MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} cells. In contrast, Acharya et al. (2017) showed that suppression of RhoA activity with C3-transferase or RNAi resulted in a decrease in junction mDia-1. Perhaps repeat experiments and C3-transferase treatment would elucidate the mechanism in which mDia-1 is responding in control and EB2 overexpressing cells.

Moreover, live cell imaging revealed that during Rho activation and junction shortening, E-cadherin was internalised (Cavanaugh et al., 2020). Rho activation initiates junction contraction in distinct contractile regions along the membrane, causing it to buckle and E-cadherin puncta to coalesce (Cavanaugh et al., 2020). Endocytosis of junctional E-cadherin is required for irreversible junctional shortening. Thus, raising the question as to whether E-cadherin endocytosis is affected by EB2 overexpression and contributes to the cell shape changes seen in the UJT.

4.6.5. Epithelial cells overexpressing EB2 have increased junctional dynamin suggesting increased dynamin-mediated endocytosis

In order to investigate further the mechanism in which EB2 overexpression contributes to the UJT, this study explored the possible effects that may be occurring at the cell junctions, specifically involving dynamin-2. During endocytosis, dynamin-2 is important for severing the stalk to release a vesicle from the membrane (Chua et al., 2009). Previous data from the Mogensen lab indicated an interaction between EB2 and dynamin-2. Whether the interaction between EB2 and dynamin-2 was direct or indirect, however, was not determined. The results here indicate that there was significantly more dynamin-2 at the junctions in MDCKII^{mChEB2Hi} cells compared to the control. Therefore, suggesting that EB2 overexpression may be associated with dynamin-2 upregulation and increased E-cadherin endocytosis. Interestingly, previous data by Ben Rix showed that in EB2 overexpressing epithelial cells, the spread of E-cadherin across junctions was greater. This was confirmed in this study suggesting that E-cadherin internalisation is increased in EB2 overexpressing cells. This is further supported by the increased presence of dynamin-2 at junctions in MDCKII^{mChEB2Hi} cells.

Dynasore was used in this study to inhibit dynamin-mediated vesicle scission, a key step in endocytosis (Ferguson and De Camilli, 2012). Dynamin inhibition resulted in a significant decrease in E-cadherin at the junctions in MDCKII^{mChEB2Hi} cells, suggesting an increase in E-cadherin internalisation as seen by the greater spread across the junctions. Cavanaugh et al. (2020) similarly used Dynasore on epithelial monolayers in order to determine whether E-cadherin internalisation by dynamin-mediated endocytosis played an important role in permanent changes in junction length following Rho activation. They found that epithelial monolayers treated with Dynasore were slower in remodelling their junctions while control cells were not (Cavanaugh et al., 2020). This suggests that dynamins are essential for the permanent remodelling and shortening of junctions. It seems therefore that permanent junction shortening requires prolonged Rho activation, formin dependent E-cadherin clustering, and E-cadherin internalisation via dynamin-mediated endocytosis (Cavanaugh et al., 2020).

Dynamins also play a role in regulating F-actin and F-actin rich structures. The mechanisms in which Dynamins regulate F-actin is unclear, however, one hypothesis is that dynamins mediate an effect through proteins that directly or indirectly interact with F-actin. Mooren et al. (2009) investigated how Dynamin-2 and cortactin influenced F-actin. The results showed that the level of actin bundling increased as Dynamin-2 concentration increased (Mooren et al., 2009). Perhaps suggesting that in EB2 overexpressing cells, where there is significantly more dynamin-2, there is also increased F-actin bundling. Immuno-labelling of β -actin did not suggest increased bundling, however further investigations are needed to comprehensively explore the effects of EB2 overexpression of the actin network. Also, Cortactin was revealed to enhance GTPases activity of Dynamin-2, in addition to stabilising the Dynamin-2 and F-actin interaction, in the presence of GTP (Mooren et al., 2009). It would be interesting to compare Cortactin expression levels and distribution in EB2 overexpressing epithelial cells to deduce any relationship.

Furthermore, Mooren et al. (2009) showed that addition of GTP resulted in actin filaments unravelling and fragmenting. Dynasore inhibits GTP hydrolysis: forcing Dynamin-2 to be in a GTP-bound state. Although, treatment of Dynasore had no significant effect on F-actin organisation (Mooren et al., 2009). In this study, inhibition of Dynamins, with Dynasore, caused an increase in cell perimeter, area and shape index in EB2 overexpressing cells. Possibly indicating that the Dynasore treatment inhibited dynamins, allowing F-actin to unravel and become more dynamic to facilitate junction lengthening and cell shape change.

Results by Chua et al. (2009) also suggested a link between dynamin-2 and actin. They found that endogenous dynamin-2 localised mainly at the apical junctions co-localised with ZO-1.

Depletion of dynamin in fully polarised MDCK cells resulted in an irregular actin organisation, severely fragmented or no ZO-1 labelling, indicating junctional complexes did not assemble and impaired polarity as a result (Chua et al., 2009). This suggests that dynamin-2 is important in the maintenance of junctional complexes through actin and junctional proteins such as ZO-1. In addition, the team found that expression of a dynamin-2 mutant (Dyn2K44A), which was unable to bind GTP, led to considerable apical constriction in polarised mutant MDCK cells, compared to the control cells (Chua et al., 2009). Inhibition of myosin II ATPase activity and ROCK, via blebbistatin and Y27632 respectively, reversed the constrictive state of the dynamin-2 mutant cell. Indicating that the apical constriction was governed by Rho activation signalling to myosin II for actomyosin contraction (Chua et al., 2009).

Mooren et al. (2009) previously reported that Dynamin-2 and cortactin associate with one another to cross link F-actin, *in vivo*, by stimulating dynamin-2's intrinsic GTPase activity. Cortactin has also been seen to bind to ZO-1 (Ammer and Weed, 2008). Chua et al. (2009) found that when cells expressing the mutant dynamin-2 (Dyn2K44A) also expressed a mutant cortactin, that was unable to bind dynamin, apical constriction was impaired. Thus, supporting the theory that dynamin and cortactin bind, working as a complex in order to bind F-actin and form crosslinks during apical contraction.

Since dynamin inhibition would impair dynamin-mediated endocytosis of E-cadherin, it would be expected that there is reduced E-cadherin turnover and therefore an effect on junction adhesion, integrity and remodelling. Also, dynamin-2 has been seen to play a role in actomyosin contraction via Rho signalling (Chua et al., 2009). When EB2 is overexpressed, inhibition of dynamins leads to further unjamming, indicated by the larger shape index. Perhaps suggesting that dynamin inhibition via Dynasore could also affect shape index via actomyosin contraction and/or a decrease in junction adhesion, allowing looser junctions. Perhaps suggesting that in untreated MDCKII^{mChEB2Hi} cells, dynamin-mediated endocytosis of E-cadherin is working to some extent but not to the capacity that it does in MDCKII^{mChEMPTY} cells presenting with a smaller shape index.

Recent studies have indicated that apical actomyosin assembly, needed for cell shape, is governed by the PAR complex and DAPLE (Dishevelled-associated protein with a high frequency of leucine residue) (Marivin et al., 2022). DAPLE is a GEF found at apical cell junctions. Depletion of DAPLE in MDCKII cells resulted in a change in cell morphology indicated by more diffuse E-cadherin labelling and disrupted ZO-1 labelling (Marivin et al., 2022). E-cadherin and ZO-1 junctional labelling in DAPLE-depleted MDCK cells resembled that of EB2 overexpressing cells in this study; perhaps indicating that when EB2 is overexpressed, DAPLE expression is reduced and therefore contributes to changes in E-

cadherin and ZO-1. Co-immunoprecipitation experiments found that Par3 was required for recruitment of DAPLE to the apical junctions, while the rest of the PAR complex components were not essential. It was subsequently shown that DAPLE governs actomyosin contraction by increasing RhoA activation (Marivin et al., 2022). This study found that Rho activation partially rescued shape index in MDCKII cells overexpressing EB2, suggesting that when EB2 is overexpressed, DAPLE expression is reduced, and Rho activation decreased. The team then explored how DAPLE effects the actomyosin network. MDCK cells with depleted DAPLE had less F-actin and myosin II in the apical region (Marivin et al., 2022). Therefore, concluding that at apical cell junctions, the Par complex recruits DAPLE, which then recruits the actin-stabilizing protein CD2AP. From here, G protein signalling is activated to stimulate RhoA-myosin activation (Marivin et al., 2022). Therefore, suggesting DAPLE to be a molecular link between polarity complexes and apical cytoskeletal assembly. These findings may suggest that when EB2 is overexpressed, DAPLE expression and Rho activation is reduced, thus the actomyosin network is affected. However, the results in this investigation show that pMLC fluorescence intensity does not significantly change when EB2 is overexpressed. Thus, suggesting that phosphorylation of myosin is not affected and perhaps indicating that actomyosin contraction is being affected in another way. For example, the interaction between myosin and actin may be disturbed, when EB2 is overexpressed, which then leads to less efficient contraction.

Chapter 5. General discussion

5.1. Introduction

For the development of novel therapies, and even preventative measures for diseases such as cancer, we must first understand the driving mechanisms. Epithelial cancers are one of the most common, however the mechanisms behind epithelial tissue dysregulation, a preliminary step in such cancers, is poorly understood. To understand the mechanism in which an epithelial tissue changes prior to the onset of the cancer itself could be vital in early detection and development of preventative therapies. In previous studies, a link between EB2 expression and Fibroblast growth factors (FGFs) and their receptors (FGFRs) was demonstrated during cancer progression (Halaban, 1991, Wilkinson et al., 1993, Anandappa et al., 1994). This was investigated further in collaboration with Dr. Mohammad Hajihosseini.

Recently, the unjamming transition has been identified as a potential mechanism that stimulates changes in tissue architecture which may then result in pathologies such as cancer. In addition, research suggests that high expression of the MT end binding protein EB2 is associated with early stages of epithelial differentiation, MT dynamics, cell migration and increased invasiveness in cancers. Since cytoskeletal reorganisation is important for changes in cell shape, and thus tissue architecture, it was hypothesised that dysregulation of EB2 expression may be a contributing factor to unjamming.

In this study, MDCKII cells were used as a model for studying the effects of EB2 overexpression on the cell shape index, an indicator of unjamming, and elements of the cytoskeleton such as actin and MTs. In addition, apico-basal polarisation and junction integrity, and therefore tissue architecture, were analysed. To explore the effects of EB2 overexpression on cell polarity and junction integrity the localisation of markers Par-3, ZO-1 and E-cadherin were investigated. E-cadherin is a major adhesion molecule required for epithelial tissue integrity. E-cadherin is stabilised at the junctions by F-actin. Formins such as those in the mDia family and RhoA signalling have previously been shown to be linked to actin polymerisation at junctions and cell-cell adhesions. Therefore, the effects of EB2 overexpression on formins mDia-1 and mDia-2 were also explored. To study the possible effects on junctions, E-cadherin was assessed, as well as inhibition of dynamin mediated endocytosis. Rho activation was used in an attempt to stimulate apical contraction in order to rescue the shape index of cells overexpressing EB2.

5.2. FGFR Inhibition suggests an association between FGF signalling and EB2 expression

The plus-tip protein EB2 plays a central role in several cellular processes including epithelial differentiation, MT dynamics and migration. More recently, abnormal expression of EB2 has

been linked to FGFs and FGFRs. Similar to previous studies, this investigation found that EB2 associated along the MT lattice in TC7 cells (Komarova et al., 2009). FGFR3 was also shown to be present at the cell peripheries. In order to explore any potential link between EB2 and FGFRs, TC7 cells were subject to an inhibition of all FGFRs with SU5402. FGFR inhibition led to a significant increase in EB2 fluorescence intensity, indicating an increase in EB2 expression, compared to the controls. Therefore, the data suggests that FGF signalling may play a role in EB2 expression. Previous studies suggest that FGFR3 expression increases with confluency until 100% and then is followed by a decrease (Kanai et al., 1997). Goldspink et al. (2013) showed that EB2 expression decreases with cell confluency. Perhaps suggesting that the expression of FGFRs is associated with a decrease in EB2 expression in confluent cells. Here, FGFR inhibition resulted in EB2 upregulation suggesting that signalling via FGFR3 suppresses EB2 expression, perhaps exhibiting a negative feedback mechanism.

5.3. EB2 overexpression promotes unjamming and loss of normal tissue architecture

EB2 has also been associated with cancer progression. In relation to this, the UJT has been hypothesised to be one mechanism by which cells adopt a more motile phenotype, similar to that of cancer cells. Thus, EB2 expression and the UJT was explored. An unjammed system has been characterised as cells in a more fluid-like state, whereby tensile forces such as contraction are reduced, adhesion forces are increased, cells are more motile and mesenchymal in cell shape (Farhadifar et al., 2007, Tambe et al., 2011, Park et al., 2015, Atia et al., 2018, Kim et al., 2020). All cells in this study were classified as unjammed, however, cells overexpressing EB2 were significantly more unjammed (presenting a higher shape index) than the controls at every time point. EB2 overexpressing cells also differed in shape, being longer and less circular. Therefore, the data suggests that overexpression of EB2 is a contributing factor to the UJT, though the mechanism by which EB2 does this is not completely understood.

5.4. Elevated levels of EB2 expression induce morphological changes in tight junctions

Labelling for tight junctions with antibodies against Par3 and ZO-1 revealed that EB2 overexpressing cells showed a loss of linear, and gain of ruffled, undulating morphology. Previous studies have associated ruffling of tight junctions with compromised barrier function (Samak et al., 2014, Krause et al., 2015). Therefore, suggesting that high EB2 expression affects junction integrity and barrier function. Ruffled junctions can be quantified using the zig-

zag index. The zig-zag index is calculated by A/B: with A referring to the path length between two tricellular junctions and B the minimum path length (Tokuda et al., 2014). A zig-zag index of 1 indicates an unruffled (linear) tight junction, whereas a value higher than 1 suggests the junction is ruffled (Tokuda et al., 2014). Future research could explore the ruffling seen in this investigation using the zig-zag index and investigate junction leakiness by exploring permeability between cells. One way to do this is via live-cell imaging of the passage of fluorescently tagged macromolecules. Another method to analyse barrier integrity is by investigating the transepithelial electrical resistance (TEER) between a cell junction using electrodes.

MDia-1 expression appeared to be reduced at the junctions when EB2 was overexpressed, but fluorescence intensity analysis would be needed to confirm this. This may indicate that formin-mediated actin nucleation at adherens junctions is compromised, although, β -actin labelling did not highlight any differences in actin organisation. However, confocal optical sectioning and higher resolution imaging would be needed to verify this. Interestingly, fluorescence intensity analysis of DMSO treated cells (as part of the Rho activation experiments) indicated increased junctional mDia-1 in MDCKII^{mChEB2Hi} compared to MDCKII^{mChEMPTY} cells. The MT organisation appeared denser in MDCKII^{mChEB2Hi} compared to MDCKII^{mChEMPTY} cells, suggesting that EB2 overexpression promoted tubulin upregulation and/or increased MT dynamics. This should be explored in future experiments (Goldspink et al., 2013, Zhong et al., 2021).

5.5. Rho activation partially recues the cell shape index in EB2 overexpressing cells

It was hypothesised that the unjamming effects seen by EB2 overexpression were attributed to a change in apical contraction. A common GEF that influences Rho signalling, and is involved in contraction, is GEF-H1. Immuno-labelling indicated reduced GEF-H1 at the junctions in MDCKII^{mChEB2Hi} cells, possibly suggesting a decrease in Rho activation. However, this needs to be confirmed by fluorescence intensity analysis. It is also likely that other GEFs, especially p114RhoGEF, contribute to NMII activation (Haas et al., 2020). An antibody against p114RhoGEF was tested in MDCKII cells but, unfortunately, it did not work.

Next, junctional pMLC abundance, as a marker for activated NMII and therefore Rho activation and contraction, was analysed using fluorescence intensity. It was expected that MDCKII^{mChEB2Hi} cells would have significantly less junctional pMLC, therefore indicating reduced cortical actomyosin contraction. Unexpectedly, no significant difference in junctional pMLC was observed between cells overexpressing EB2 and the Empty vector control. This

suggests that Rho activation had triggered similar levels of NMII activation in EB2 overexpressing and control cells.

Under normal circumstances, Rho activation leads to actomyosin contraction and the maintenance of a smaller, jammed cell shape. MDCKII cells were therefore subjected to Rho activation in an attempt to determine whether the shape index of MDCKII^{mChEB2Hi} cells could be restored to that of MDCKII^{mChEMPTY} cells. A partial rescue of the shape index was observed, indicating that the cells were less unjammed. It would be expected that Rho activation would lead to an increase in junctional pMLC. A significant increase in junctional pMLC fluorescence intensity was observed in MDCKII^{mChEB2Hi} cells following Rho activation, while no significant differences were observed in MDCKII^{mChEMPTY} cells. Interestingly, unlike untreated cells, significantly less junctional pMLC fluorescence intensity was evident in DMSO treated MDCKII^{mChEB2Hi} compared to MDCKII^{mChEMPTY} cells. This suggests that when EB2 is overexpressed, the level of Rho activation is not sufficient to induce the required NMII activation needed for normal junction contraction. Instead, the junctions are looser, less contracted and thus longer in length, resulting in more unjammed cells. Alternatively, if junctional pMLC is unchanged in EB2 overexpressing cells, as indicated in untreated cells, then the actomyosin contraction mechanism may be less effective. Perhaps suggesting that cortical actomyosin contractions was being affected in another way. One possibility may be that through changes to the myosin or actin organisation, leading to impaired myosin head engagement and therefore less efficient contraction. Another consideration would be that the data here is based on one experiment with three repeats of a relatively small sample size. Thus, repeat experiments would be needed to verify this result. Therefore, cells require increased Rho activation to partially rescue the cell shape index. However, further verification of junctional pMLC levels would be required. It is important to bear in mind that the amount of pMLC present may vary. For example, MLC would be phosphorylated to initiate contraction and then de-phosphorylated later as junctions are remodelled, their lengths change, and the mechanism would then reset. If time permitted, several experiments at varying Rho activator concentrations and time points would be undertaken to determine the best circumstance to capture an accurate representation of MLC phosphorylation.

Rho activation partially rescued junctional mDia-1 in cells overexpressing EB2, perhaps suggesting that when EB2 is overexpressed, junctional formin abundance is increased. This is contrast to what previous mDia-1 immuno-labelling suggested.

Cells in a jammed system are packed closely to one another and possess strong junction complexes which are maintained by the turnover of junctional proteins such as E-cadherin. Dynamin-2 is the only dynamin present in epithelial cells and mediates E-cadherin endocytosis

by severing the E-cadherin filled vesicles from the membrane for exportation (Chua et al., 2009). In EB2 overexpressing cells, more dynamin-2 was present at junctions, cells were more unjammed and previous data showed the spread of E-cadherin across junctions was greater than the controls. To explore if EB2 overexpression is affecting dynamin-mediated endocytosis of E-cadherin, and therefore its spread at the junctions, MDCKII cells were subjected to Dynamin inhibition. Dynamin inhibition resulted in a decrease in junctional E-cadherin in MDCKII^{mChEB2Hi} cells suggesting reduced E-cadherin trafficking to the junctions. The results showed that dynamin inhibition in cells overexpressing EB2 significantly increased the shape index. Therefore, suggesting that when EB2 is overexpressed dynamin-mediated endocytosis is compromised, perhaps indicating dynamin-2 is sequestered by EB2.

In addition, results by Chua et al. (2009) indicate that dynamin-2 can influence the actin network. Dynamin-depletion in polarised MDCK cells led to disruption of actin organisation and junctional protein localisation (Chua et al., 2009). Furthermore, cells expressing a mutant dynamin experienced significant apical constriction. Therefore, indicating that dynamin-2 plays a role in the global actomyosin cytoskeleton for epithelial junction maintenance and apical constriction. Additionally, previous studies have suggested that dynamin-2 expression increases MT dynamics (Tanabe and Takei, 2009). Goldspink et al. (2013) showed that EB2 depletion resulted in more stable MTs. Therefore, EB2 overexpression would be hypothesised to increase MT dynamic instability. This study found cells overexpressing EB2 had significantly more junctional dynamin-2 than the controls, perhaps suggesting both EB2 and dynamin-2 play a role in mediating MTs dynamic instability.

5.6. Conclusion

Table 9. Key findings

Key finding	Implication	Supporting publications
FGFR3 evident at cell cortex and in cytoplasm in TC7 cells.	Makes FGFR3 a potential FGF receptor involved in regulation of EB2 expression.	FGFR3 shown to present in Caco-2 cells (Kanai et al., 1997).
FGFR inhibition with SU5402 results in increased EB2 expression in TC7 cells.	Suggests FGF signaling influences EB2 expression.	Cochlear explant treated with SU5402 resulted in MAPRE2 upregulation (Szarama et al., 2012)
EB2 overexpression in MDCKII cells leads to increased cell perimeter, area, and shape index.	Suggests high EB2 expression leads to unjamming and loss of normal tissue architecture.	Shape index indicating $P_0 < 3.813$ suggest cells in an unjammed state (Park et al., 2016).

MDCKII cells overexpressing EB2 are more elongated and less circular than control cells.	Suggests high EB2 expression alters cell morphology and tissue architecture.	As cells undergo the UJT, they change from a compacted 'solid-like state' to a more dynamic and varied morphology, mimicking a 'fluid-like' state (Park et al., 2015, Kim et al., 2020).
Par3 and ZO-1 labelling reveal undulating/ruffled tight junctions in MDCKII cells overexpressing EB2.	Indicates that high EB2 expression affects tight junction integrity and function.	Ruffling in tight junctions affects barrier function (Samak et al., 2014, Krause et al., 2015). ZO-1 depletion in MDCKII cells led to linear 'unruffled' junctions (Tokuda et al., 2014).
MDCKII cells overexpressing EB2 show no apparent change in actin but reveal denser apical MT network.	Further investigations needed but may suggest high EB2 expression increasing MT polymerization and dynamics.	EB2 knockdown resulted in less stable, more dynamic MTs (Goldspink et al., 2013). EB2 overexpression is associated with increased proliferation, migration and invasiveness, <i>in vivo</i> , while EB2 knockdown contributes to decreased proliferation and migration, <i>in vitro</i> (Zhong et al., 2021).
MDCKII cells overexpressing EB2 show more diffuse junctional location of mDia1 than control cells.	Suggests that high EB2 expression may interfere with junctional location of mDia1, perhaps via disrupted E-cadherin.	E-cadherin knockdown disrupts junctional mDia-1 localisation (Acharya et al., 2017). Preliminary data by Ben Rix showed that EB2 overexpression resulted in disrupted E-cadherin labelling.
MDCKII cells overexpressing EB2 cause no significant change in junctional pMLC fluorescence intensity.	Suggests that high EB2 expression does not affect the level of activated myosin but actomyosin contraction mechanism is less effective.	-
MDCKII cells overexpressing EB2 appear to have less junctional GEF-H1.	High EB2 expression may reduce RhoA activation at junctions resulting in weaker or less effective actomyosin contraction.	GEF-H1 triggers RhoA activation required for actomyosin contraction (Joo and Olson, 2021).
Rho activation partially rescues the cell shape index in EB2 overexpressing MDCKII cells.	Suggests that high EB2 expression affects normal Rho activation and actomyosin contraction. Alternatively, higher level of Rho activation is	Rho inhibition contributed to further unjamming, more elongated and motile cells (Cavanaugh et al., 2020). Prolonged Rho activation is

	needed due to less effective actomyosin contraction mechanism.	required for sustained junction shortening and actomyosin contraction (Cavanaugh et al., 2020).
Rho activation partially rescues pMLC fluorescent intensity at cell junctions in EB2 overexpressing MDCKII cells.	Suggests that Rho activation is reduced in high EB2 expressing cells leading to reduced actomyosin contraction and unjamming. Alternatively, the actomyosin contraction mechanism is less effective.	-
Rho activation partially rescues mDia1 fluorescent intensity at cell junctions in EB2 overexpressing MDCKII cells.	Suggests when EB2 is overexpressed, Rho activation is decreased, and junctional mDia-1 abundance is reduced.	Suppression of RhoA leads to a decrease in junctional mDia-1 (Acharya et al., 2017).
Dynamin-2 fluorescence intensity is increased at cell junctions in EB2 overexpressing MDCKII cells.	Suggests that excess EB2 may lead to increased dynamin2 delivery to junctions resulting in increased E-cadherin internalization.	Unpublished CO-IP data from Mogensen lab suggests that EB2 and dynamin-2 interact. Preliminary data by Ben Rix showed that EB2 overexpression resulted in disrupted E-cadherin labelling.
Dynamin-2 inhibition with Dynasore decreases peak and spread of junctional E-cadherin in EB2 overexpressing MDCKII cells.	Suggests that high levels of EB2 expression interferes with normal dynamin-2 localization and E-cadherin turnover.	Dynasore treatment led to slower junctions remodelling in epithelial cells (Cavanaugh et al., 2020). Dynamin-2 encourages actin bundling (Mooren et al., 2009). Dynamin depletion in MDCK cells disrupted actin organisation and junctional ZO-1 localisation (Chua et al., 2009).

In conclusion, this study has shown that FGFR activity influences EB2 expression, as hypothesised. In addition, MDCKII^{mChEB2Hi} cells showed a larger shape index than the controls, therefore suggesting that EB2 overexpression is a driver of UJT. The mechanism in which this occurs is unclear. However, the result in this investigation supports the hypothesis that EB2 overexpression contributes to unjamming via changes to cell contraction. For example, there is a decrease in GEF expression such as GEF-H1. As a consequence, Rho activation is decreased, despite no change in pMLC, and apical contraction is reduced. Due to this, the junctions remodel and lengthen which is mediated by E-cadherin turnover. E-cadherin turnover appeared to be compromised and this is possibly due to less mDia1 and more dynamin2 at the junctions. MDia-1 is an important formin required for actin polymerisation at

junctions. Dynamin-2 may play a role in the junction remodelling directly through dynamin-mediated endocytosis and/or via the actin cytoskeleton. Further investigations into this mechanism would be required.

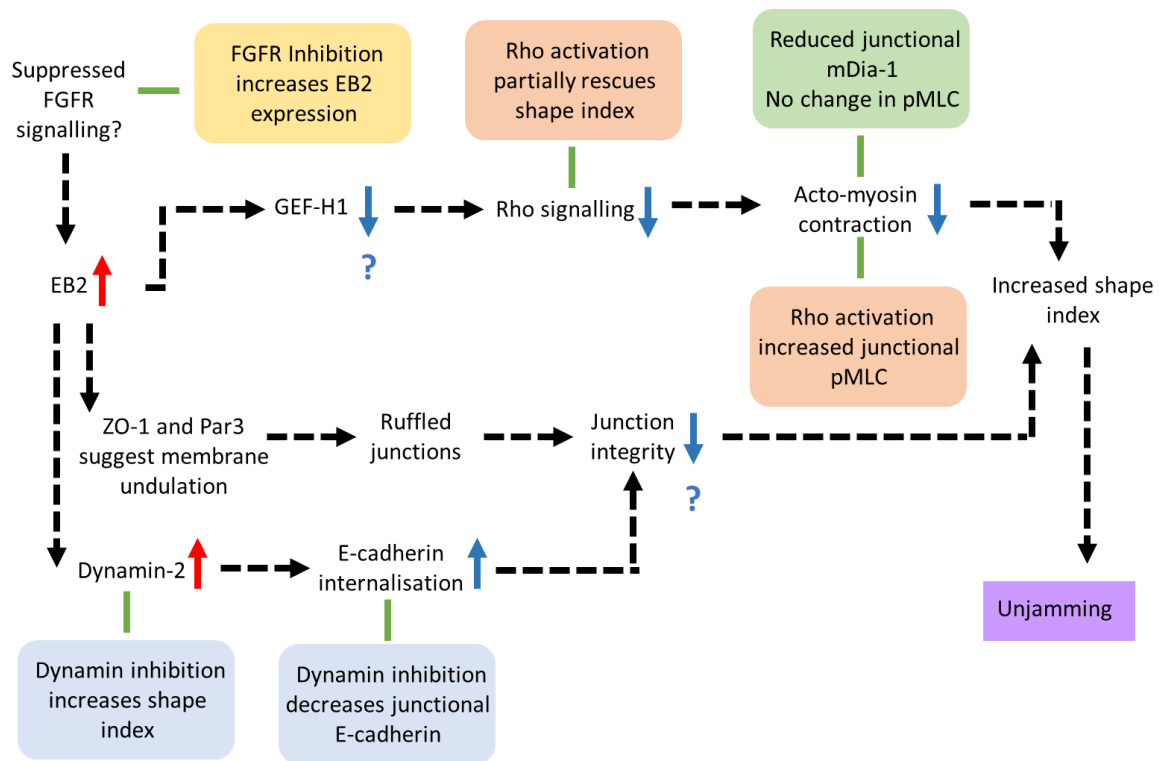


Figure 36. Summary model. The diagram presents one possible model for the results found in this investigation.

5.7. Future Work

In this investigation, it was decided that protein abundance, rather than RNA level, was investigated as it gives a more accurate insight to protein expression within cells. One reason for this is that RNA levels do not always directly correlate to protein abundance. In this investigation fluorescence intensity analysis was used to analyse protein abundance. Complementary experiments such as western blot analysis would be beneficial in verifying the changes in protein content seen in this investigation. Due to limited laboratory access in the beginning of this investigation, as a result of the Covid-19 pandemic, there were time limitations, and such experiments were not carried out.

FGFR inhibition significantly increased EB2 fluorescence intensity, suggesting that EB2 expression is linked to FGFs and their receptors in TC7 cells. Immuno-labelling revealed junctional FGFR3 in TC7 cells. Whereas a RT-PCR analysis indicated the presence of FGF8 and absence of FGF2, FGF17, FGF18, FGF1 and FGF9. Future works need to determine the presence or absence of the remaining FGFs and FGFRs via immuno-labelling, RT-PCR and western blot analysis; this would give a full profile of which members are at play in TC7 cells. Another option would be to expand into other colorectal cancer cell types and even *in situ* immuno-labelling of human and mouse gut sections from both healthy and colorectal cancer patients to compare expression of EB2, FGFs and FGFRs. Moreover, subjecting cells to recombinant FGF18, a ligand for FGFR3, would be useful in determining if FGF18-FGFR3 binding influenced EB2 expression. This could then be analysed via a fluorescence intensity analysis and western blotting.

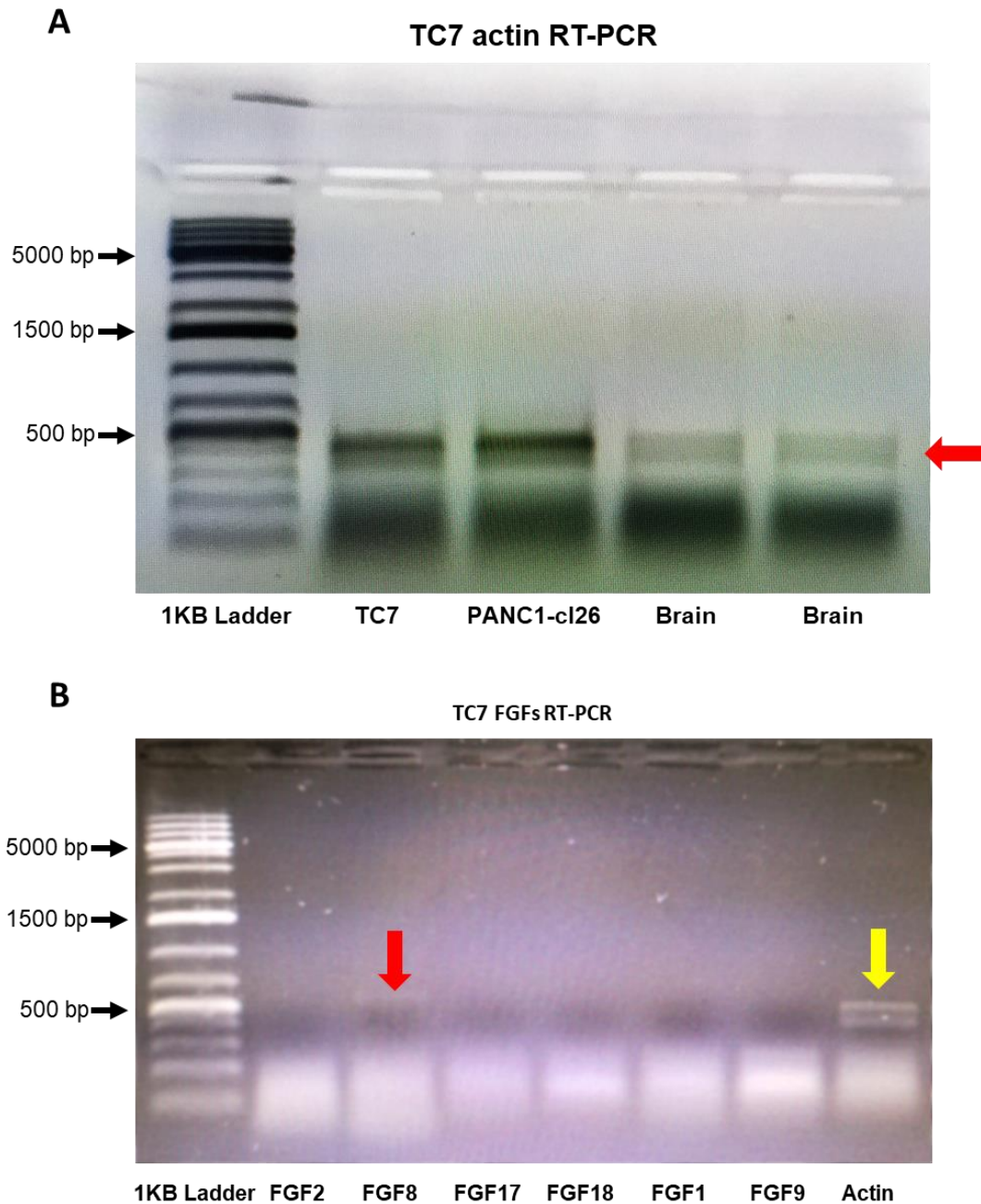
The results showed that MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} monolayers were consistently unjammed at every time point (Day 8, 10 and 14). Thus, it would be beneficial for this study to create a jammed system as a model for normal physiological conditions *in vivo*. This could be achieved by seeding the cells at a higher density or allowing more time for cells to become fully polarised. However, it was shown that MDCKII^{mChEMPTY} cells on Day 14 detached from the coverslips during the immunolabeling process, therefore, cells would need to be seeded onto an ECM to encourage adherence to the glass coverslips. This was trialled with pre-coated Matrigel slides and poly-d-lysine coated glass coverslips, but it encouraged cyst formation. Other ECM components, such as collagen, would have been tested if time had permitted. Further testing would be valuable in developing conditions that resembled the *in vivo* environment to give the most accurate results. If successful, the way in which unjamming occurs in EB2 overexpressing cells compared to controls could then be investigated.

Furthermore, as MDCKII cells were partially polarised, there was the question whether taking images with a widefield fluorescence microscope was sufficient to analyse the effects of EB2 overexpression on the cytoskeleton. In other words, using the widefield fluorescence microscope only allowed images to be taken at one focused layer at a time. Confocal optical sectioning would be one way to remedy this and give an extensive image of the cytoskeleton network, specifically highlighting MTs and actin. In addition, images taken on a widefield microscope would also pick up background fluorescence, whereas confocal images would be able to distinguish fluorescence of individual optical sections. This may allow a more accurate quantitative analysis of fluorescence intensity.

In addition, further experiments to explore the effect of EB2 overexpression on MT dynamics would be advantageous. Live time-lapse imaging of MTs in GFP-tubulin expressing cells would be critical for determining whether EB2 overexpression influences MT growth, shrinkage and pausing. In addition, comparing the abundance of detyrosinated tubulin as an indicator for longer-lived, more stable MTs, would indicate MT dynamics. Another experiment to consider would be to investigate the recovery time and capability of MTs after cold treatment and/or Nocodazole to evaluate if cells overexpressing EB2 are more dynamic or more stable than that of the control cells. Future work should also further explore the effects of EB2 overexpression on E-cadherin turnover. One way to do this is via Fluorescence recovery after photobleaching (FRAP) analyses using fluorescently tagged E-cadherin to track the E-cadherin turnover at the junctions. This would determine how efficiently E-cadherin is delivered and inserted into the junctions.

It would also be interesting to investigate Rho activity. One way to measure Rho activity is via a Pull-down assay. The assay works by incubating cells with a 'bait protein' that will bind to known downstream effectors of specific GTP-bound (active) Rho GTPases which are being tested for. The amount of active Rho GTPase is then visualised via a western blot analysis. Another option would be to use Förster resonance energy transfer (FRET)-based biosensors, specific to the Rho GTPase of interest, to visualise active Rho GTPases.

Appendices



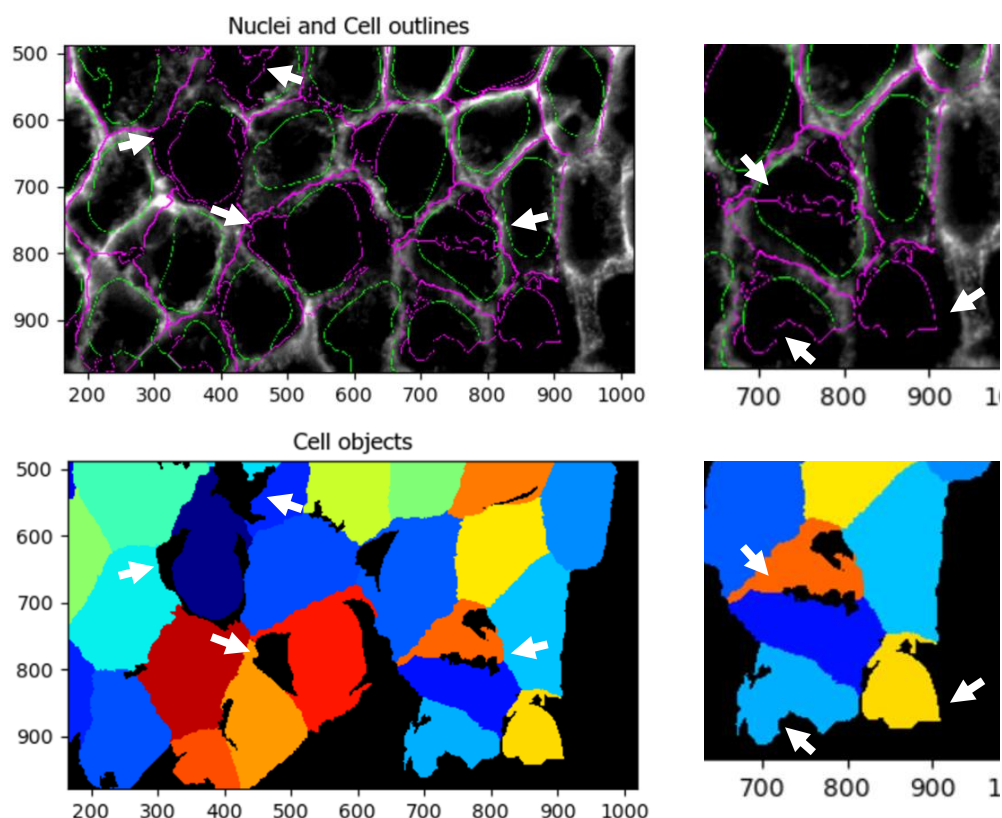
Appendix 1. RT-PCR for FGFs. TC7 cells were grown until confluent and then their RNA extracted and purified using the Quick-RNA™ Miniprep Kit. **A)** RT-PCR product on 1% agarose gel from the test reaction using β -actin primers as these are abundant in all cells. Lanes left to right indicate the 1 KB ladder for reference, TC7 cells, PANC1 = clone 26 and two mouse brain RNA. 1 μ g of RNA were used per reaction. The band sizes were as expected at approximately 450 bp. **B)** Indicates the gel electrophoresis image from testing TC7 cells against several FGF primers and actin as a control. Lanes left to right highlight, 1KB ladder for reference, FGF2, FGF8, FGF17, FGF18, FGF1, FGF9 and actin. Only a faint band was seen for FGF8 (red arrow). Actin was also seen as a doublet (yellow arrow) as expected, confirming the reactions efficiency and RNA quality. The RT-PCR was carried out by Dr. Mohammad Hajhosseini.

Appendix 2. Total number of cells analysed in CellProfiler™. The table indicates the total number of cells analysed for the EB2 fluorescence intensity analysis at each condition in each repeat using CellProfiler™; then, the total number of cells overall.

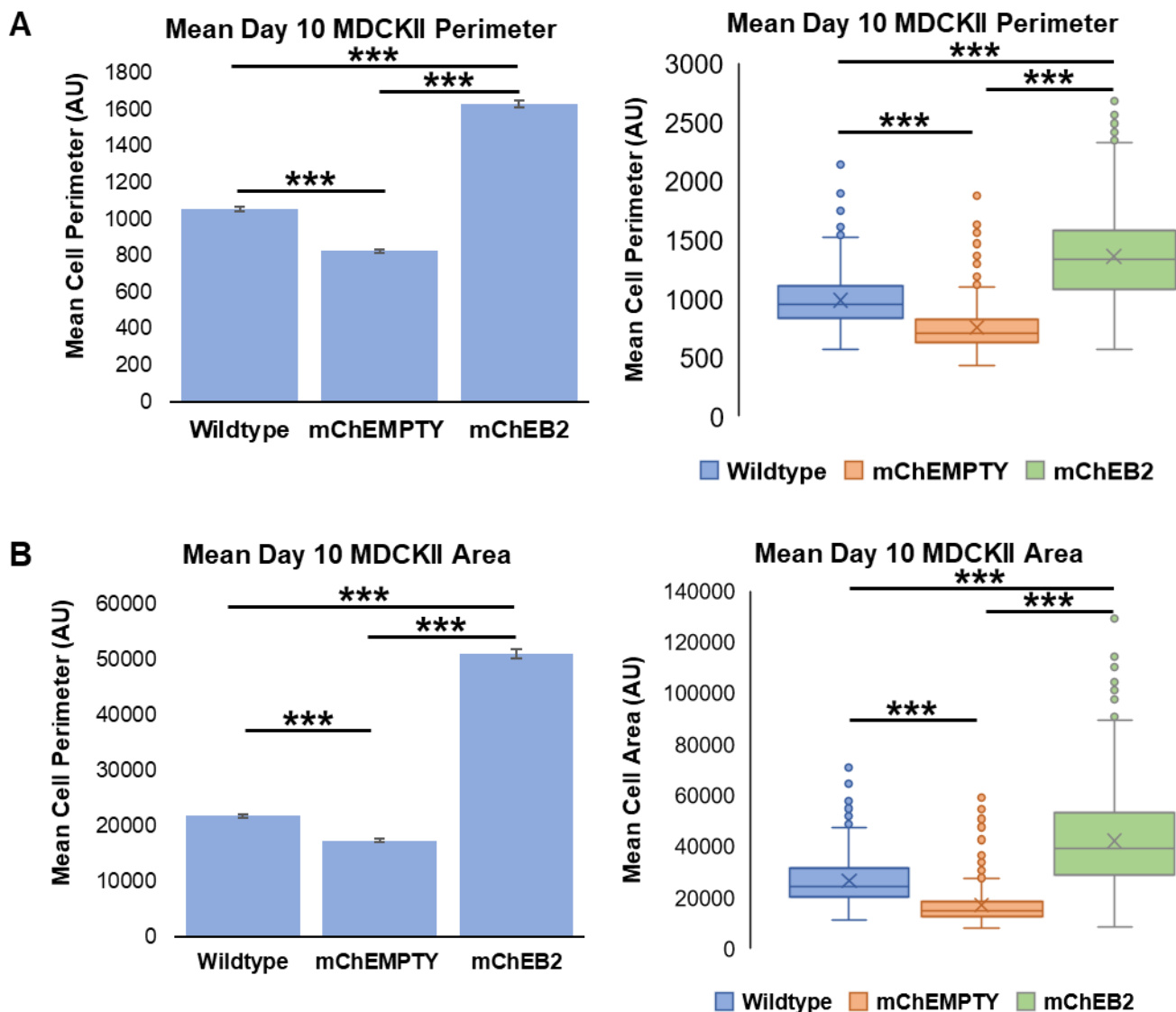
	R1	R2	R3	Total
Media	1641	838	1532	4011
DMSO	1056	957	1259	3272
SU5402	1817	695	1285	3797
			Total	11,080

Appendix 3. EB2 Fluorescence Intensity Analysis. Comparison of EB2 expression in TC7 cells when subject to media only, DMSO in media (5 uM) and SU5402 (5 uM). The table indicates the mean fluorescence intensity (arbitrary units) of EB2 $\pm 1SE$.

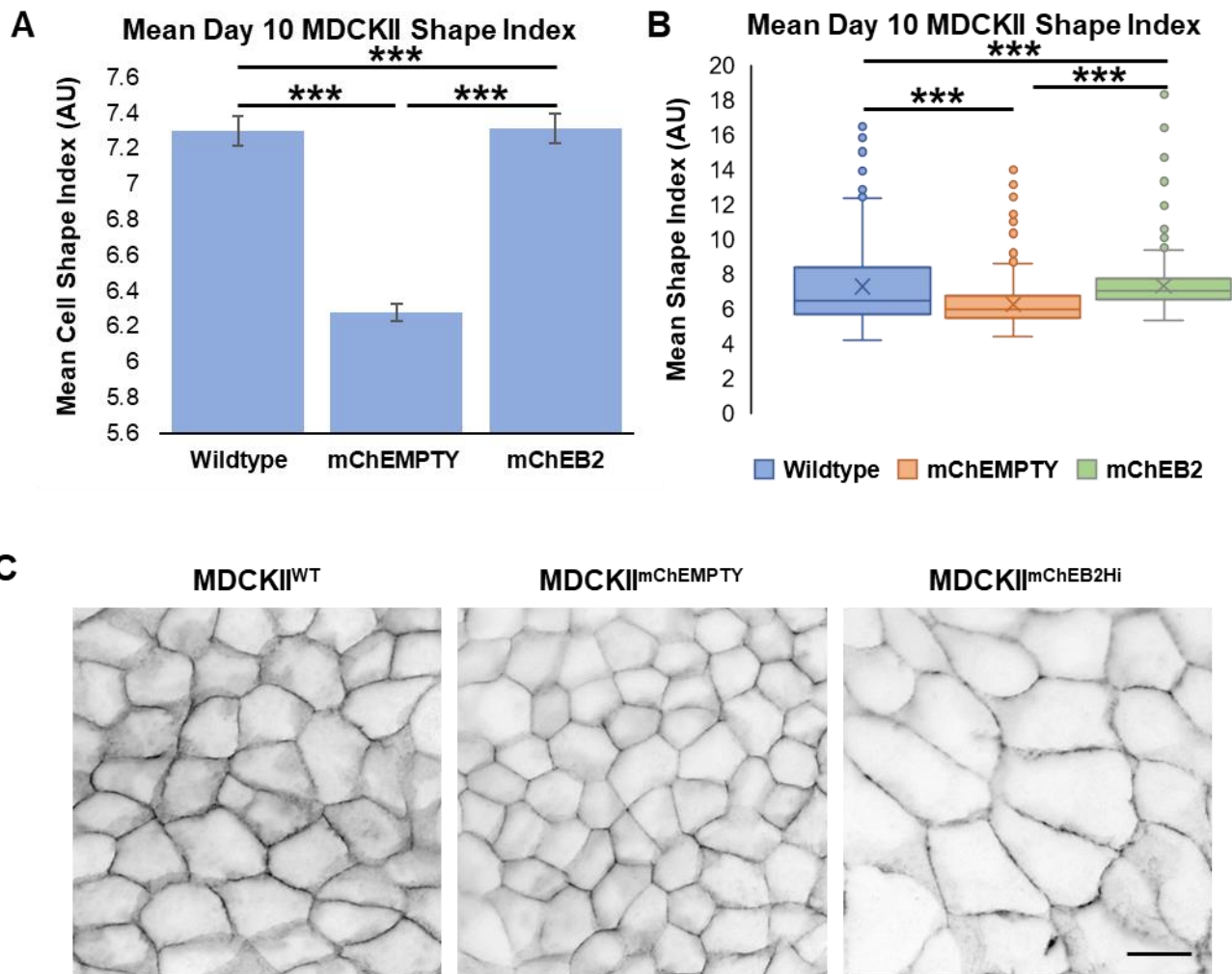
Mean EB2 Fluorescence Intensity (Arbitrary Units) $\pm 1SE$			
	Media Only	DMSO	SU5402 (5 uM)
Repeat no.1	0.393142427	0.396806087	0.420074011
(FBS 10%)	± 0.000803523	± 0.000899772	± 0.000959856
Repeat no.2	0.54793376	0.555421406	0.581931178
(FBS FREE)	± 0.001527531	± 0.001417327	± 0.00238541
Repeat no.3	0.539591822	0.546478302	0.560800893
(FBS FREE)	± 0.00082581	± 0.000918675	± 0.001055563



Appendix 4. Example output images from CellProfiler™ showing inaccuracies of cell border tracing. MDCKII cells were immuno-labelled for E-cadherin and stained with DAPI. The top row highlights the tracing of an analysed image, the green lines outline the primary object (nuclei) and purple lines outline the cell border. The bottom row images show each individual cell detected as a different colour. The white arrows highlight tracing inaccuracies. Often, large portions of cells were discounted, therefore, suggesting that there were gaps in the cell monolayer where there were none. In addition, one of the parameter set in CellProfiler™ was that cells out of frame (incomplete) are to be discarded from the analysis. However, often the program would include half cells in the analysis, therefore skewing results for cell perimeter, area and shape index (see bottom right image).



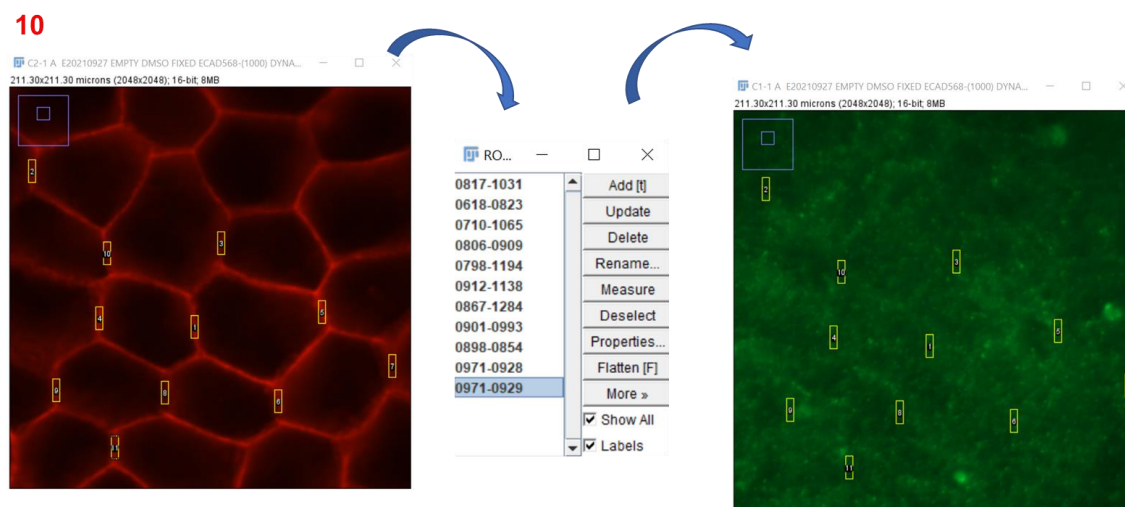
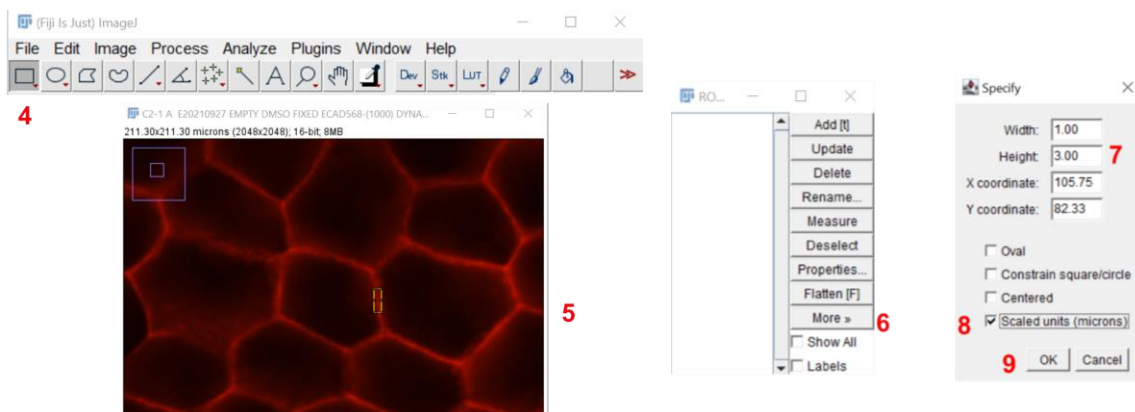
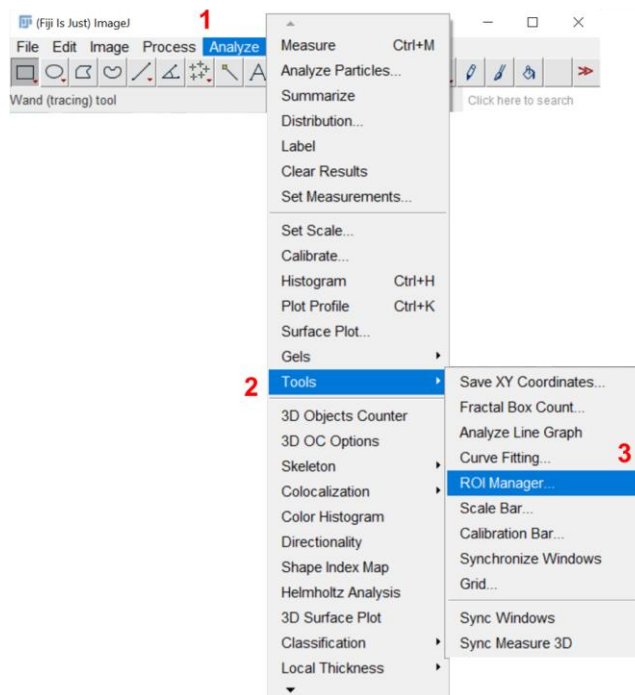
Appendix 5. MDCKII^{mChEB2Hi} cells reveal significant larger mean cell perimeter and area compared to MDCKII^{WT} and MDCKII^{mChEMPTY} at day 10 (P<0.001). MDCKII cells were grown on glass coverslips in a 24-well plate for 10 days, immuno-labelled for β -catenin (Sigma, C2206), stained for DAPI and imaged on a widefield fluorescence microscope. The β -catenin labelling allowed the mean cell perimeter ± 1 SE (A) and area ± 1 SE (B) to be determined. N=546 per cell clone. MDCKII^{mChEMPTY} revealed a smaller mean cell shape index compared to MDCKII^{WT} (P<0.001). The Box and Whisker Plots highlight the spread of data with X indicating the mean of each group. (Asterisks *** indicate P<0.001; Games-Howell test Non-Parametric Post Hoc Test). Analysed using CellProfiler™. This analysis was repeated in ImageJ using E-cadherin labelling (Figure 17-18).



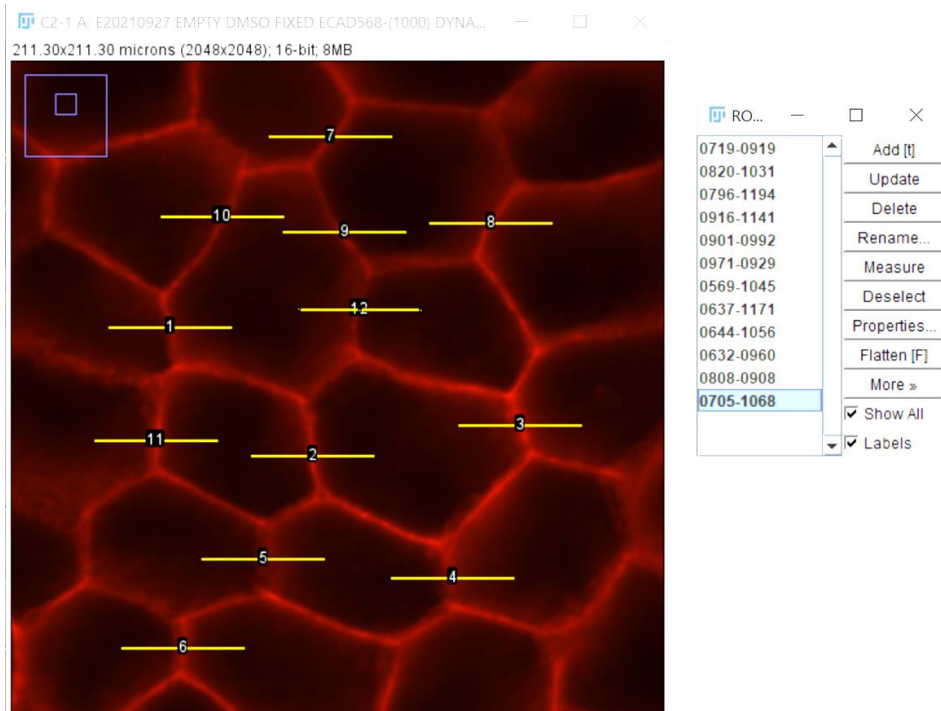
Appendix 6. MDCKII^{mChEB2Hi} reveal a statistically significant larger mean cell shape Index compared to MDCKII^{WT} and MDCKII^{mChEMPTY} cells at day 10 ($P < 0.001$). MDCKII cells were grown on glass coverslips in a 24-well plate for 10 days, immuno-labelled for β -catenin (Sigma, C2206), stained for DAPI and imaged on a widefield fluorescence microscope using a x63 oil objective. The β -catenin labelling allowed the mean cell perimeter and area to be determined. $N=546$ per cell clone. MDCKII^{mChEMPTY} revealed a smaller mean cell shape index compared to MDCKII^{WT} ($P < 0.001$). (A) The mean shape index ± 1 SE was calculated in arbitrary units (AU) using the formula: shape index = perimeter/ $\sqrt{\text{area}}$. $N=546$ per cell line. (B) The Box and Whisker Plots highlight the spread of data with X indicating the mean of each group (C) Example images showing E-cadherin (inverted) labelling in MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells (left to right). Note markedly larger cells present in the MDCKII^{mChEB2Hi} culture. (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test). Analysed on CellProfiler™. This analysis was repeated in ImageJ using E-cadherin labelling and a parallel result was determined. The scale bars indicate 20 μm .

Appendix 7. MDCKII perimeter, area and shape index. The table indicates the mean perimeter, area and shape index (arbitrary units $\pm 1SE$) of MDCKII^{WT}, MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells at several time points. Note data was not collected for MDCKII^{mChEMPTY} cells on day 14 as the cell monolayer detached from the glass coverslips during the immunolabelling process. The blue row highlights the day 10 data set when analysed using CellProfiler™. Investigation into the reliability of CellProfiler™ led to the day 10 data, and the other data sets, being analysed using ImageJ. For more information please see.

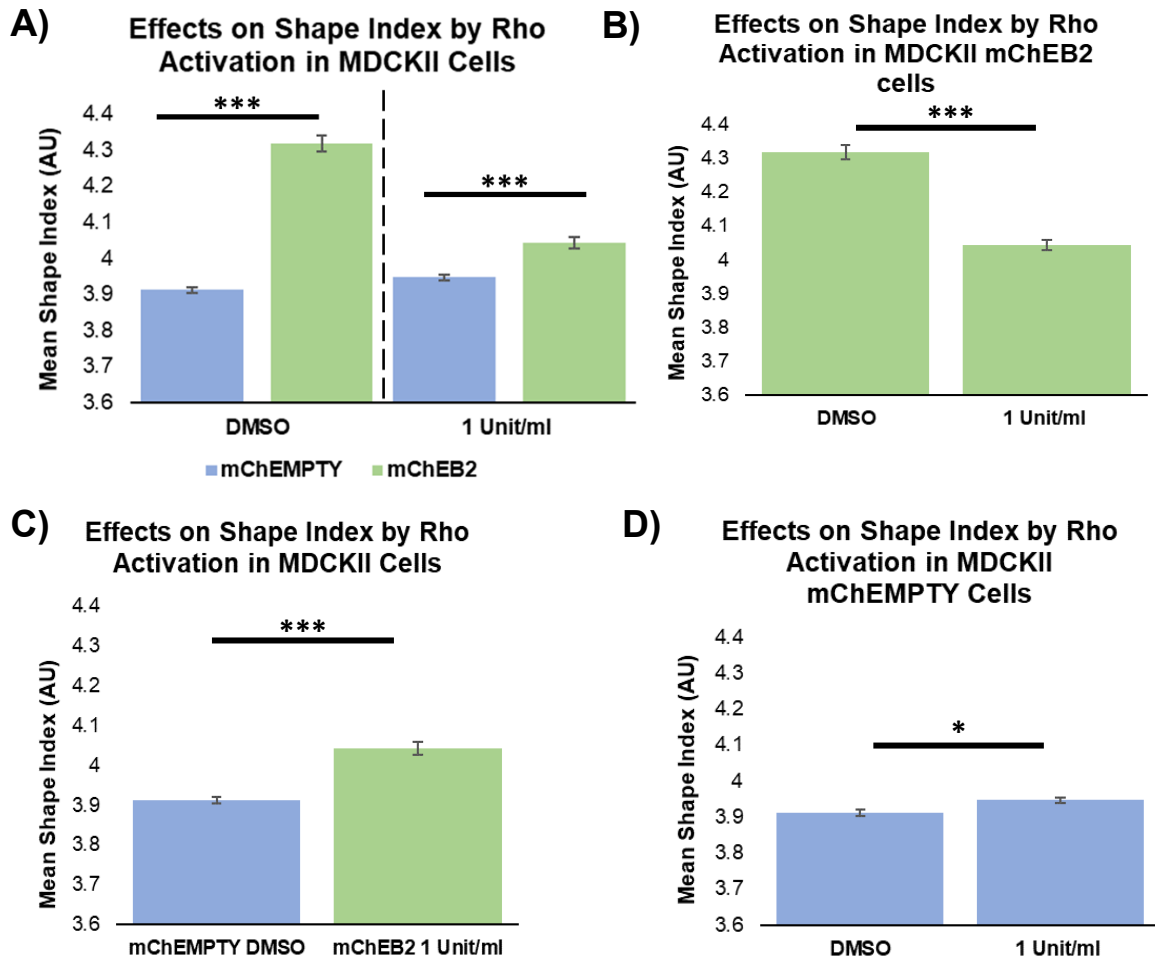
MDCKII line	Arbitrary Units $\pm 1SE$		
	Perimeter	Area	Shape index
<u>Day 8</u>			
MDCKII ^{WT}	57.2 ± 0.51	202.7 ± 3.83	4.05 ± 0.01
MDCKII ^{mChEMPTY}	44.8 ± 0.35	122.6 ± 1.84	4.07 ± 0.01
MDCKII ^{mChEB2Hi}	63.3 ± 0.66	229.0 ± 4.22	4.21 ± 0.01
<u>Day 10</u>			
MDCKII ^{WT}	1055.8 ± 12.60	21785.9 ± 272.88	7.30 ± 0.08
MDCKII ^{mChEMPTY}	824.5 ± 11.82	17305.6 ± 296.03	6.27 ± 0.04
MDCKII ^{mChEB2Hi}	1628.9 ± 19.82	51014.6 ± 866.70	7.31 ± 0.08
<u>Day 10</u>			
MDCKII ^{WT}	67.6 ± 0.75	245.2 ± 5.48	4.38 ± 0.026
MDCKII ^{mChEMPTY}	46.6 ± 0.46	130.8 ± 2.58	4.12 ± 0.017
MDCKII ^{mChEB2Hi}	101.7 ± 1.47	517.5 ± 13.03	4.53 ± 0.036
<u>Day 14</u>			
MDCKII ^{WT}	66.3 ± 0.92	272.8 ± 6.49	4.05 ± 0.01
MDCKII ^{mChEB2Hi}	94.5 ± 1.31	505.6 ± 13.21	4.27 ± 0.02



Appendix 8. Analysis of fluorescence intensity at the junctions using ImageJ. All images were taken at a fixed exposure to allow fluorescence intensity comparisons. **(1-3)** The Region of Interest (ROI) Manager was opened (Analyze → Tools → ROI Manager). **(4-5)** A small rectangle was manually drawn using the 'rectangle select function'. **(6-9)** This rectangle was then formatted to the desired size, in this investigation a 1 x 3 μm bar was sufficient (ROI Manager → More → Specify). **(10)** To avoid bias the ROI bars were placed in a second antibody channel against a protein not being investigated. For example, when Dynamin-2 fluorescence intensity at the junction was investigated, bars were placed in the E-cadherin channel. Note, if the second antibody labelling was not sufficient to do this, bars were placed in every possible junction systematically throughout the entire image of the channel being measured. For example, when investigating E-cadherin fluorescence intensity at the junctions, in some images Dynamin-2 labelling did not highlight the cells junctions clearly, therefore bars had to be placed in the E-cadherin channel. Once a bar was placed, Ctrl+M allowed the sample to be measured. Analyze → Set Measurements, on the ImageJ home bar, allowed the desired parameters to be selected. For this investigation 'Mean gray value' was selected to measure the mean fluorescence intensity of the ROI within the junction. Note, the bar's position was occasionally altered to ensure the entire bar is within the junctional labelling to avoid a skew created by measuring the fluorescence intensity within the cytoplasm.



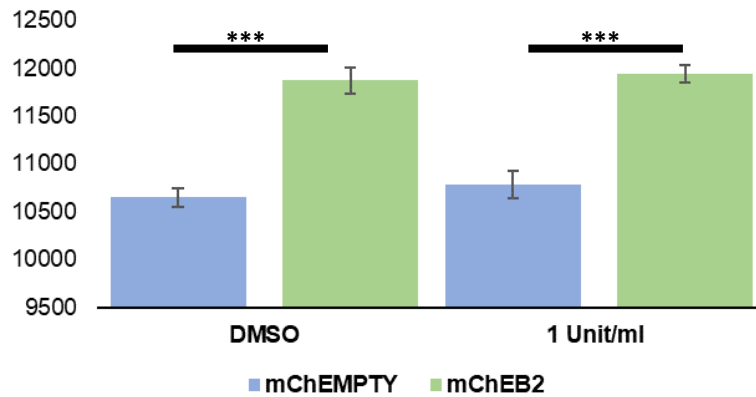
Appendix 9. Analysis of fluorescence intensity across junctions. Analysis of junction profiles was completed in the same way as analysis at cell junctions however instead of a rectangle bar, 10 μm straight lines were manually drawn perpendicular across a cell-cell junction. As before, to avoid bias this was done in a second antibody channel against a protein not being investigated. However, the position of the lines may have been slightly altered to ensure the centre of the line resides in the centre of the junction. This was done in order to measure the spread of E-cadherin across junctions and the peak fluorescence intensity at the centre of the line. The intensity was measured every 0.1 μm and plotted on a graph to give a continual measurement across the junction. The raw values of each plot profile were then used for statistical analysis.



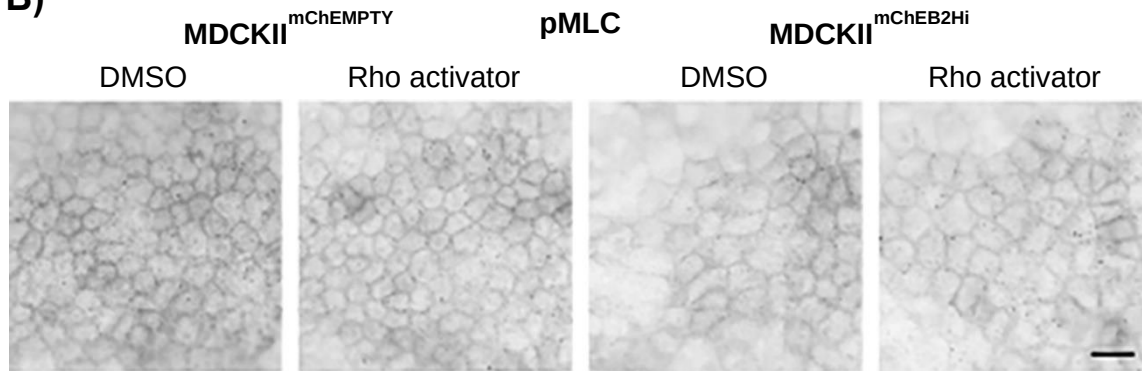
Appendix 10. Experimental repeat of Rho activation. Activation of Rho resulted in a decrease in the shape index in MDCKII^{mChEB2Hi} cells compared to DMSO control while MDCKII^{mChEMPTY} showed no significant difference. MDCKII cells were grown on glass coverslips for 10 days then treated with Rho activator (1 unit/ml) or a DMSO control for 30 minutes. Then cells were double immuno-labelled for pMLC (Phospho S20, Rabbit; Abcam cat no. AB2480; 1:300) and E-cadherin (Mouse; BD cat no. 610181; 1:500) and stained for DAPI. Coverslips were imaged on a widefield fluorescence microscope with a x63 oil objective. The E-cadherin labelling allowed the mean cell perimeter and area to be determined. The mean shape index $\pm 1SE$ was calculated in arbitrary units (AU) using the formula: $\text{shape index} = \text{perimeter} / \sqrt{\text{area}}$ (A-D). N=300 per cell clone, per condition. (Asterisks *** indicate $P < 0.001$, * indicates $P < 0.05$; Games-Howell test Non-Parametric Post Hoc Test). Analysed on ImageJ.

A)

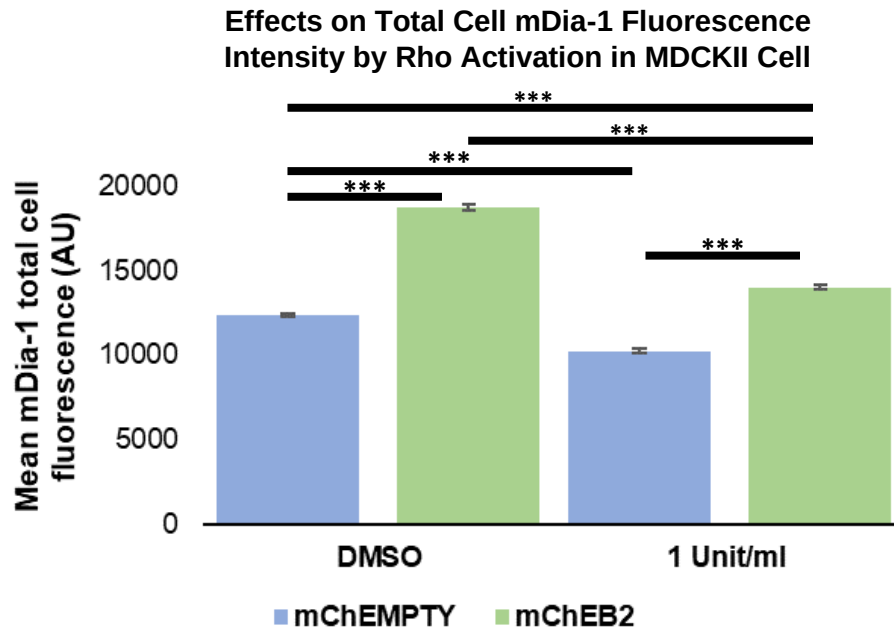
Effects on Total Cell pMLC Fluorescence Intensity by Rho Activation in MDCKII Cells



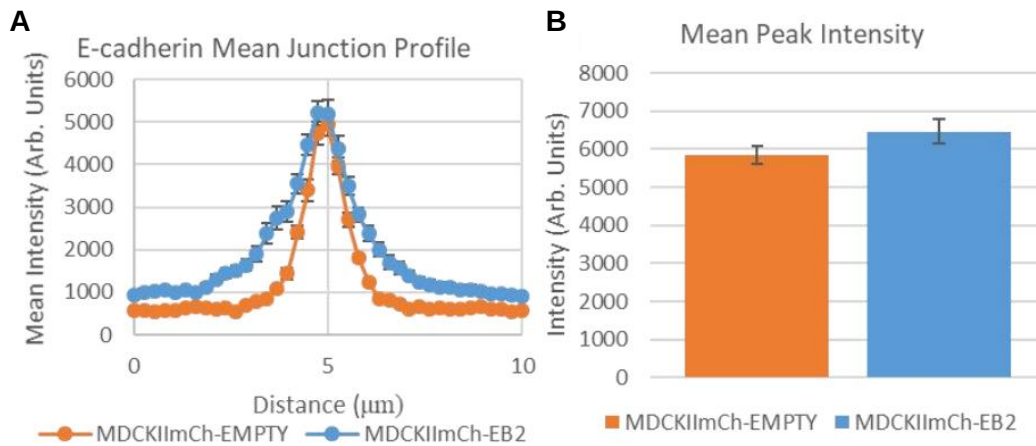
B)



Appendix 11. Rho activation has no effect on total cell pMLC fluorescence intensity in MDCKII^{mChEB2Hi} cells or MDCKII^{mChEMPTY} cells. MDCKII cells were grown on glass coverslips for 10 days then treated with Rho activator (1 unit/ml, 0.5%) or a DMSO (0.5%) control for 30 minutes. Then cells were double immuno-labelled for pMLC (Phospo S20, Rabbit; Abcam cat no. AB2480; 1:300) and E-cadherin (Mouse; BD cat no. 610181; 1:500) and stained for DAPI. Cells were imaged on a widefield fluorescence microscope with a x63 oil objective. Total cell pMLC fluorescence intensity was Analysed in ImageJ A). MDCKII^{mChEB2Hi} cells showed a higher total cell pMLC fluorescence intensity than MDCKII^{mChEMPTY} cells when treated with the Rho activator or DMSO control ($P < 0.001$). **B)** Examples inverted images at fixed exposure indicating relative pMLC labelling only. N=300 per cell clone, per condition. (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test). Scale bar = 20 μ m. One experiment with three replicas.



Appendix 12. Total cell mDia-1 fluorescence intensity decreased in MDCKII^{mChEB2Hi} and MDCKII^{mChEMPTY} cells when treated with a Rho activator (1 unit/ml, 0.5%). MDCKII^{mChEB2Hi} cells have a significantly higher total cell mDia-1 fluorescence intensity when treated with either the Rho activator or DMSO compared to MDCKII^{mChEMPTY} cells ($P < 0.001$). However, MDCKII^{mChEB2Hi} cells decreased to 14008.3 ± 169.1 upon Rho activation compared to the DMSO control 18729.2 ± 213.0 . MDCKII^{mChEMPTY} cells decreased to 10255.8 ± 144.3 upon Rho activation compared to the DMSO control 12363.1 ± 129.1 . Therefore, indicating activation of Rho resulted in a partial rescue of total cell mDia-1 fluorescence intensity. (Asterisks *** indicate $P < 0.001$; Games-Howell test Non-Parametric Post Hoc Test).



Appendix 13.- Ben Rix E-cadherin across junctions' analysis. MDCKII cells were grown on glass coverslips for 7 days, immuno-labelled for β -actin and E-cadherin and then imaged with a widefield fluorescence microscope. Fluorescence intensity across junctions was measured in ImageJ using ROI. A) Fluorescence intensity analysis showing the mean E-cadherin intensity at set points across the junction, giving a fluorescence profile $\pm 1\text{SE}$. B) N=20-line profiles. 1 experiment. MDCKII^{mChEB2Hi} cells B) Mean peak E-cadherin fluorescence intensity at points across the junctions. Not clear need to describe results No significant difference in peak junction intensity between MDCKII^{mChEMPTY} and MDCKII^{mChEB2Hi} cells. N=55-line profiles. 1 experiment.

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