# Extracellular trafficking of Wnt signals in gastric cancer

Submitted by Daniel Routledge

to the University of Exeter as a thesis for the degree of

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I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

**Abstract**

Wnt proteins are secreted glycoproteins which signal in a tissue to regulate multiple cellular processes, such as cell differentiation, migration, and proliferation. However, post-translational modifications result in Wnt ligands being hydrophobic in nature. Thus, their ability to freely diffuse in the aqueous extracellular environment is restricted, and alternative mechanisms of transport have been proposed. In this thesis, I investigate and characterise the use of signalling filopodia – termed cytonemes – in the intercellular transport of Wnt3 ligands by gastric cancer cells, which display overactivated Wnt/β-catenin signalling. Additionally, I identify the membrane scaffolding protein Flotillin-2 (Flot2), which is overexpressed in gastric cancers, as a novel positive regulator of Wnt cytoneme formation and consequently proliferation. Mechanistically, I show that Flot2 is required for the intracellular transport, membrane localisation and thus signalling of the Wnt co-receptor Ror2; a known regulator of Wnt cytonemes.

In parallel, I show that Flot2 also has a function in transducing signals in the Wnt-receiving cell. Here, Flot2 co-localises with the Wnt co-receptor Lrp6 and is involved in its endocytic uptake. Additionally, Flot2 knockdown results in the perinuclear accumulation of Lrp6 and its absence from recycling endosomes. Therefore, I suggest Flot2 may also be involved in the endosomal transport of Lrp6 following internalisation.

Finally, following my observed co-localisations of both Ror2 and Lrp6 with Flot2, I found that these Wnt co-receptors co-localise with one another, as well as the cognate Wnt receptor Frizzled 7, in Flot2 microdomains. Expression of a mutant Ror2 missing its cysteine-rich domain, however, causes loss of co-localisation with Lrp6 and perturbed Wnt/β-catenin signalling. Together, these findings led me to propose a model whereby Frizzled 7, Ror2 and Lrp6 all interact and form one large complex, which I have termed the Wnt Receptor Supercomplex (WRS). I hypothesise that these receptors may interact, even in the absence of Wnt ligands, to regulate one another’s binding affinities for either Wnt/β-catenin or Wnt/PCP ligands. Here, I propose that flotillin microdomains provide the scaffold necessary for these interactions.

**Acknowledgements**

First and foremost, I would like to thank Dr. Steffen Scholpp for his invaluable support and guidance throughout my PhD. His willingness to make time to help and giving me the creative freedom to pursue my own scientific curiosities within the project, have made this journey both more enjoyable and rewarding.

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Finally, I would like to thank my friends at Exeter University Trampoline Club (EUTC). Being a part of this club throughout my PhD has helped me keep my sanity and formed the core of my memories outside of my academic work. I have made some of my closest friends at EUTC and their emotional support has been invaluable, and I wouldn’t have been able to do it without them.

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**COVID-19 Impact Statement**

Whilst the COVID-19 pandemic and associated lockdowns did disrupt and slow the progress of my research, I was able to adapt my plans accordingly. Alongside this, I received a 3-month funded extension in lieu of this and was given the opportunity to mitigate against this lost time.

There was, however, a significant adaptation of my *in vivo* experimental plans due to restrictions on international travel. Following characterisation of Wnt cytonemes *in vitro*, the plan was to undergo a secondment with Prof. Maria Mione's lab at the University of Trento, Italy. I had received funding from the GW4 Biomed DTP to undergo training here in xenografting gastric cancer cells into zebrafish embryos, which would be used as a 3D microenvironment to monitor the growth, migration and invasion of the cancer cells *in vivo*. This secondment was to include training in xenografting techniques, imaging and data analysis for monitoring the effects of Wnt cytoneme inhibition on these parameters.

Since I was unable to travel to Italy to complete this training, my experimental plan was adapted to use zebrafish embryos as a tool for validating my findings *in vitro*, since there were the facilities and training available for this at Exeter. In this thesis, this was to validate the function of Flotillin-2 in Wnt signalling using microinjections, confocal imaging and *in situ* hybridisation.

## Contents

**List of figures**

**Author's declaration**

I declare that all of the work in this thesis is my own work, unless clearly stated or cited in text. For clarity, sections of the introduction include work from my published review article (Routledge and Scholpp, 2019). Similarly, chapters 1 and 2 are based on the findings of a paper which will shortly be published (Routledge *et al.,* 2022):

**Routledge, D**. *et al*., Flotillin-2 regulates Wnt3 dissemination via cytonemes in gastric cancer. *In revision, eLife.*

**Routledge, D**. & Scholpp, S., Mechanisms of intercellular Wnt transport. *Development* 146, dev176073 (2019).

**List of abbreviations**

AGS – Primary gastric adenocarcinoma

APC – Adenomatous polyposis coli

APP – Amyloid precursor protein

Arp2/3 – Actin-related proteins 2/3

BrdU - Bromodeoxyuridine

Cdc42 – Cell division control protein 42 homologue

CE – Convergent extension

CIE – Clathrin-independent endocytosis

CK1 – Casein kinase 1

CME – Clathrin-mediated endocytosis

CNS – Central nervous system

CRD – Cysteine-rich domain

CTB – Cholera toxin B

DAAM1 - Dishevelled associated activator of morphogenesis 1

Dkk1 – Dickkopf-related protein 1

DMSO – Dimethyl sulfoxide

Dvl – Dishevelled

ECM – Extracellular matrix

EEA1 – Early endosome antigen 1

EGF – Epidermal growth factor

EMT – Epithelial-mesenchymal transition

ER – Endoplasmic reticulum

ESA – Epidermal surface antigen

ESCRT - Endosomal sorting complexes required for transport

Evi/Evi – Evenness interrupted / Wntless

FB - Forebrain

FGF – Fibroblast growth factor

Flot2 – Flotillin-2

Fzd – Frizzled

GC – Gastric cancer

GPCR – G-protein coupled receptor

GPI – Glycosylphosphatidylinositol

GSK3 – Glycogen synthase kinase-3

HB - Hindbrain

HEK293 - Human embryonic kidney 293

HFE-145 – normal gastric epithelial cells

Hh – Hedgehog

HSPG – Heparan sulphate proteoglycan

IHC - Immunohistochemistry

IRSp53 – Insulin receptor tyrosine kinase substrate p53

ISH – In situ hybridisation

IWP2 - Inhibitor of Wnt production-2

JNK – C-jun N-terminal kinase

KTR – Kinase translocation reporter

LDL – Low-density lipoproteins

Lef – Lymphoid enhancer factor

Lrp – Low-density lipoprotein receptor related protein

MB - Midbrain

miRNA – micro ribonucleic acid

MKN - Gastric tubular adenocarcinoma

mRNA – messenger ribonucleic acid

MVB – Multivesicular body

MyoX – Myosin X

PCC – Pearson’s correlation co-efficient

PCP – Planar cell polarity

PCR – Polymerase chain reaction

PFA – Paraformaldehyde

PM – Plasma membrane

Porcn – Porcupine

PTM – Post-translational modification

Rac – Ras-related C3 botulinum toxin substrate

Rho – Ras homologue gene family

Ror2 – Receptor tyrosine kinase-like orphan receptor 2

RTK – Receptor tyrosine kinase

RT-qPCR – Reverse transcriptase quantitative polymerase chain reaction

sFRP – secreted frizzled-related protein

Shh – Sonic hedgehog

SPFH - stomatin, prohibitin, flotillin, HflK/C

STF – Super TOPflash

TCF – T-cell factor

TIR – Total internal reflection

TSG101 - Tumour susceptibility gene 101

Vangl2 - Vangl planar cell polarity protein 2

Wg – Wingless

WISP-1 - WNT1-inducible-signaling pathway protein 1

Wnt – Wingless-integrated

WRS – Wnt receptor supercomplex

# 1. Introduction

## 1.1. Cell-cell communication

Multicellular organisms are highly complex and are comprised of neatly organised and structured organs, tissues, and cells. The ability of a single cell to divide, migrate and acquire numerous cell fates to fulfil defined functions is a complicated process and this level of co-ordination cannot be achieved on a single cell level. In this respect, cells must work together and communicate with one another in order to be spatially, temporally and functionally determined. A primary mechanism by which this is achieved is through chemical signalling, whereby cells release proteins or chemicals into the surrounding environment, and other cells expressing the necessary receptors can respond to these cues (Goryachev and Mallo, 2020). These proteins, termed morphogens, result in altered cellular behaviours or identities as determined by changes in cellular signalling or gene expression, for example (Perrimon *et al.*, 2012). In developmental processes, chemical signalling is required for co-ordinating cell proliferation, differentiation and migration, which permits the formation of discrete and functional organs and tissues, and sub-structures within these. Post-development, in adult tissues, chemical signalling is important in maintaining tissue homeostasis by providing means for cells, tissues and organs to communicate with each other, over short and long distances, to co-ordinate proper and timely responses (Valls and Esposito, 2022). Details of these functions and how they are mechanistically achieved will be introduced in the context of Wnt signalling; a signalling network which exemplifies the importance of cell-cell communication.

## 1.2. The Wnt Signalling Network

The Wnt signalling network comprises several signalling pathways, which are genetically and functionally conserved throughout metazoans (Niehrs, 2012; Loh *et al*., 2016). To date, 13 Wnt gene sub-families have been described: Wnt1-11, 16 and WntA, although the number of Wnt genes in individual species varies greatly, from 6 in insects to 27 in zebrafish; whilst humans have 19 Wnt genes (Miller, 2001; Duncan *et al.*, 2015).

Wnt signalling regulates multiple cellular processes, including cell polarity, migration and proliferation (Logan and Nusse, 2004). Aberrations in Wnt signalling can therefore lead to dysregulation of homeostatic processes, which control tissue size, organisation and function (Nusse and Clevers, 2017). Thus, dysregulation of Wnt signalling is implicated in a multitude of diseases, ranging from developmental disorders, such as Williams Syndrome, to several types of cancer, including colorectal, gastric and pancreatic cancers (Zhao, 2005; Chiurillo, 2015; Flanagan et al., 2017; Zhan et al., 2017). Therefore, the Wnt signalling network has been extensively studied for over 40 years, and here I summarise some of the key findings and mechanisms of Wnt signalling.

### 1.3. Wnt ligand structure and processing

Wnt proteins are a family of highly conserved glycoproteins which share a conserved run of 22 cysteine residues and an N-terminal signal sequence that targets them for secretion (Willert and Nusse, 2012). The cysteine residues are thought to be integral to the secondary structure of Wnts through the formation of disulfide bridges (Janda *et al.*, 2012). The tertiary structure of Wnts are commonly referred to as the “hand” structure, with the N-terminal domain “index finger”, linker “palm” region and C-terminal “thumb” (Fig. 1). The latter is particularly important as this is the main domain involved in the binding of Wnt to its cognate receptor, Frizzled (Fzd) (Janda *et al.*, 2012).

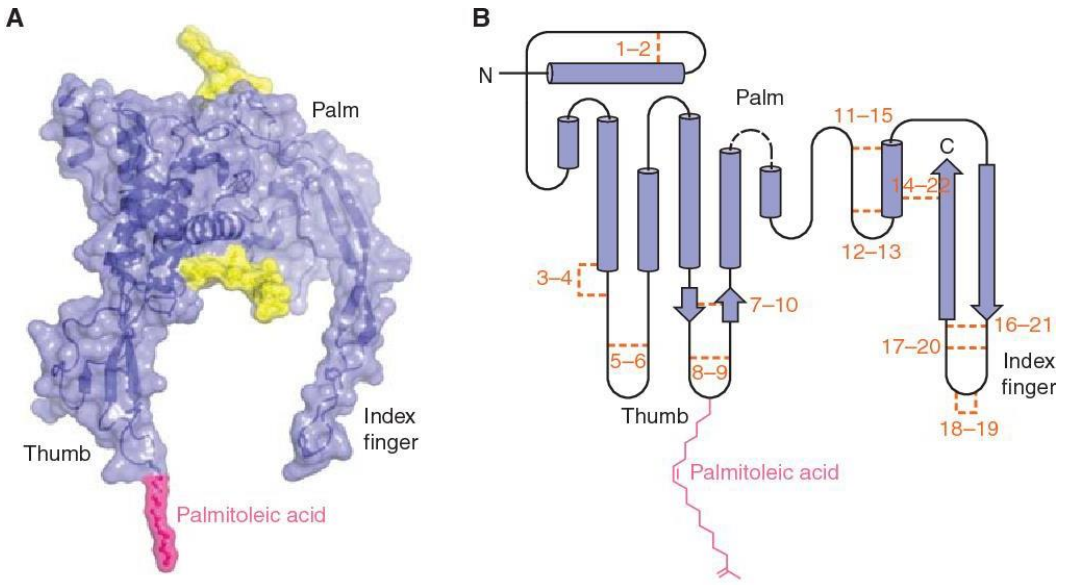


**Figure 1 – Structure of Wnt ligands. (A)** Space-filling model of XWnt8 bound to Fzd, with the Fzd CRD structure removed. Key structures of the Wnt ligand are highlighted. **(B)** Secondary structure of XWnt8, with key residues highlighted (disulphide bridges in orange). Adapted from Janda *et al.,* (2012).

Despite the amino acid sequence of Wnts suggesting a soluble protein, Wnts are actually highly hydrophobic and aggregate in the ECM unless stabilised by detergents or serum (Fuerer *et al.,* 2010). This can be attributed to the post-translational mono-palmitoylation of a conserved serine residue (Fig. 1)(Willert *et al.*, 2003). This post-translational modification (PTM) is seen on every discovered Wnt besides one, *Drosophila* WntD, which lacks this otherwise conserved site of lipidation (Ching *et al.,* 2008). The attachment of this palmitoleic acid to Wnt proteins occurs during its processing in the endoplasmic reticulum (ER) and is achieved by the enzyme Porcupine (PORCN) (Kadowaki *et al.*, 1996). PORCN is an O-acyl transferase which was discovered to regulate the processing of the *Drosophila* Wnt homologue Wingless (Wg) in 1996, but it was not until 2004 it was shown that PORCN acylates Wnt proteins (Kadowaki *et al.*, 1996; Zhai *et al.,* 2004). This PTM is integral to Wnt secretion and function, as several studies have reported that deletion or inhibition of PORCN results in the aberration of Wnt signalling and retention of Wnt in the ER (Barrott *et al.*, 2011; Biechele *et al.,* 2011). Analogous results have been observed using an S209A mutant of Wnt3a, which prevents PORCN-mediated acylation at this site and also results in its retention in the ER (Takada *et al.*, 2006). Varying patterns of other PTMs, such as glycosylation, distinguish Wnt proteins and

their concomitant signalling properties. For example, Wnt11 harbours four N-linked glycosylations, whereas Wnt3a only has two, and these discrepancies regulate their secretion in polarised epithelial cells (Yamamoto *et al.*, 2013).

Following processing in the ER, Wnt proteins are transported to the Golgi apparatus for packaging into vesicles and targeting to the plasma membrane (PM) for secretion (Fig. 2). In 2006, two groups simultaneously discovered this process requires the multipass transmembrane protein Wntless (Wls/Evi) (Bänziger *et al.*, 2006; Bartscherer *et al.*, 2006). Evi binds to Wnt proteins and acts as an intracellular chaperone: depletion of Evi disrupts Wnt signalling in HEK293T cells by preventing Wnt3a reaching the cell surface or being secreted into the culture medium (Bänziger *et al.*, 2006). Since Evi was found to predominantly localise to the Golgi, it was originally thought that this is where Evi-Wnt interactions began (Bänziger *et al.*, 2006). Additionally, Evi was found to act downstream of PORCN, since Evi binds to the palmitoleic acid moiety of Wnts (Yu *et al.*, 2014). However, it was also discovered that Evi contains an ER-localisation sequence and binds to Wnts in the ER, cycles them to the PM and is recycled in a retrograde manner to the ER via the Golgi (Fig. 2) (Yu *et al.*, 2014). This concurs with the observation that Wnts are retained in the ER when PORCN-mediated palmitoylation is blocked, which may be due to lack of binding to and concomitant transport from the ER via Evi.

In addition to Evi, it has been suggested that the *Drosophila* p24 cargo adaptor protein Opossum (Opm) shuttles proteins, including the *Drosophila* Wnt orthologue, Wingless (Wg), across the ER-Golgi interface (Buechling *et al.*, 2011). Thus, the chaperone-like proteins Evi and Opm have both been proposed to mediate ER-to-Golgi transport of Wnt proteins.

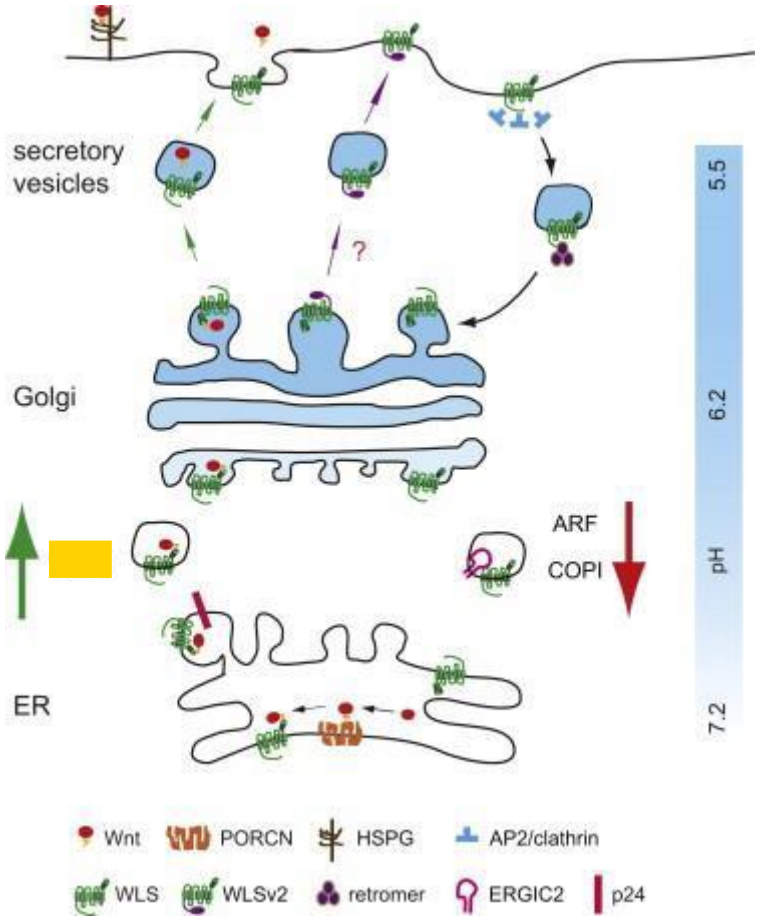


**Figure 2 – Wnt secretion and Evi recycling.** Evi binds to palmitoylated Wnts in the ER and transports them through the Golgi secretory pathway to the membrane. After pH-dependent Wnt release, Evi is retrogradely recycled back to the Golgi and then ER in a retromer-dependent manner, where it can then bind newly synthesised Wnts. Taken from Yu *et al*., 2014.

Following delivery of Wnt to the plasma membrane, Evi is thought to be endocytosed and recycled in the Wnt-producing cell via the retromer complex: a multi-protein complex that redirects Evi away from the lysosomal degradative pathway and back to the ER (Yang *et al.*, 2008). Here, it can bind to newly synthesised Wnt proteins and traffic them back to the membrane (Belenkaya *et al.*, 2008; Yu *et al.*, 2014). This model explains how inhibiting retromer function in Wnt-producing cells attenuates Wnt secretion, by preventing the recycling of Evi and thus trafficking of Wnt to the cell surface (Franch-Marro *et al.*, 2008). Interestingly, Wnt proteins also stabilise Evi levels, as Wnt3a expression in HEK293T cells results in increased levels of Evi protein . This accumulation is not accompanied by an increase in Evi mRNA levels,

suggesting that Wnt signalling does not transcriptionally regulate Evi. As treatment with proteasome inhibitors increases Evi protein levels in the absence of Wnt3a, and levels of poly-ubiquitylated Evi decreases in the presence of Wnt3a, it has been suggested that Wnt proteins aid stabilisation of Evi by preventing its proteasome-dependent degradation (Glaeser *et al.*, 2018).

## 1.4. Mechanisms of Wnt Signalling

### 1.4.1 Transduction of Wnt Signals

Transduction of Wnt signals begins when Wnt ligands bind receptors, including their cognate receptor, Fzd, at the cell membrane. Fzd receptors are seven-pass-transmembrane receptors with an extracellular cysteine-rich domain (CRD) and an intracellular PDZ-binding domain . The CRD of Fzd receptors is critical to Wnt binding, as this hosts a hydrophobic groove in which the Wnt lipid moiety inserts (Janda *et al.*, 2012). There are ten known Fzd paralogues in humans (Bhanot *et al.*, 1996; Strutt *et al.*, 2012). Along with the 19 Wnt proteins in humans, there are a multitude of possible combinations of Wnt-Fzd interactions and thus signalling events. These are discerned by differential binding affinities of Wnts for particular Fzd receptors, which have been quantitatively predicted using known Wnt-Fzd structural interactions (Agostino *et al.*, 2017). Fzd and Wnt expression profiles are also tissue- and context-specific, as well as being spatiotemporally regulated, adding further layers of control over Wnt-Fzd interactions (Wu *et al.*, 2017; Wang *et al.*, 2018). In fact, functional Wnt-Fzd interactions have been mapped using a multiplex CRISPR-Cas9 screening approach to identify the specificity of particular Wnt ligands for varying Fzd receptors (Voloshanenko *et al.*, 2017). For example, Wnt3a displays affinity for a broad spectrum of Fzd receptors, whereas Wnt8a acts through a more specific subset of receptors.

Alongside Fzd receptors, Wnt proteins also bind with co-receptors, which form a complex at the cell surface. The most well-studied co-receptors are Receptor tyrosine kinase like Orphan Receptor 2 (Ror2), a pseudokinase, and LDL Receptor related Protein 5/6 (Lrp5/6) (Tamai *et al.*, 2000; Oishi *et al.*, 2003). These two co-receptors are commonly thought to bind to different sets of Wnt proteins and are involved in the activation of two distinct branches of the Wnt signalling network.

### 1.4.2. Wnt/β-catenin Signalling

The Wnt/β-catenin pathway (or “canonical” pathway) is the most well-studied and understood pathway in the Wnt signalling network. It regulates multiple cellular processes, including proliferation, differentiation and cell fate acquisition (Komiya and Habas, 2008). Typically “canonical” Wnt proteins include Wnt1, Wnt3, Wnt3a, Wnt8a, Wnt8b and Wnt10 (Ackers and Malgor, 2018). Activation of this pathway centres around the levels of the protein β-catenin in the cytosol. In the absence of a Wnt signal, β-catenin undergoes continuous turnover by the destruction complex. This complex comprises of the scaffolding proteins Axin and adenomatous polyposis coli (APC), and the kinases glycogen synthase 3 kinase (GSK3β) and casein kinase 1α (CK1α). In this Wnt OFF state, GSK3β and CK1α phosphorylate β-catenin. This promotes ubiquitination of β-catenin by β-transducin repeats containing protein (β-TrCP) and subsequent proteasomal degradation (Fig. 3). In this state, TCF/LEF transcription factors in the nucleus are associated with Groucho and repress the expression of Wnt target genes (Komiya and Habas, 2008).

In the presence of Wnt ligands, Wnt binding to Fzd receptors and the co-receptor Lrp5/6 results in formation of a ligand-receptor complex. This causes a conformational change which promotes the recruitment of the intracellular protein Dishevelled (Dvl) to the C-terminal tail of Fzd via its DEP domain. Here, Dvl promotes the recruitment of components of the destruction complex to the cell membrane, forming a complex known as the signalosome (DeBruine *et al.*, 2017). Formation of the signalosome promotes phosphorylation of Lrp5/6 by GSK3β and CK1α (Gao and Chen, 2010a). Recruitment of these components to the PM inhibits the formation of a functional destruction complex and thus prevents the degradation of β-catenin, permitting its cytosolic accumulation. β-catenin subsequently translocates to the nucleus, where it displaces Groucho to bind with TCF/LEF transcription factors and inhibit their DNA binding, thus relieving their inhibition of Wnt target genes. These include genes which promote proliferation and cell cycle progression, such as c-Myc and cyclin D1, as well as components of the Wnt signalling pathway, such as Axin2, as part of a negative feedback loop (Lecarpentier *et al.*, 2019).

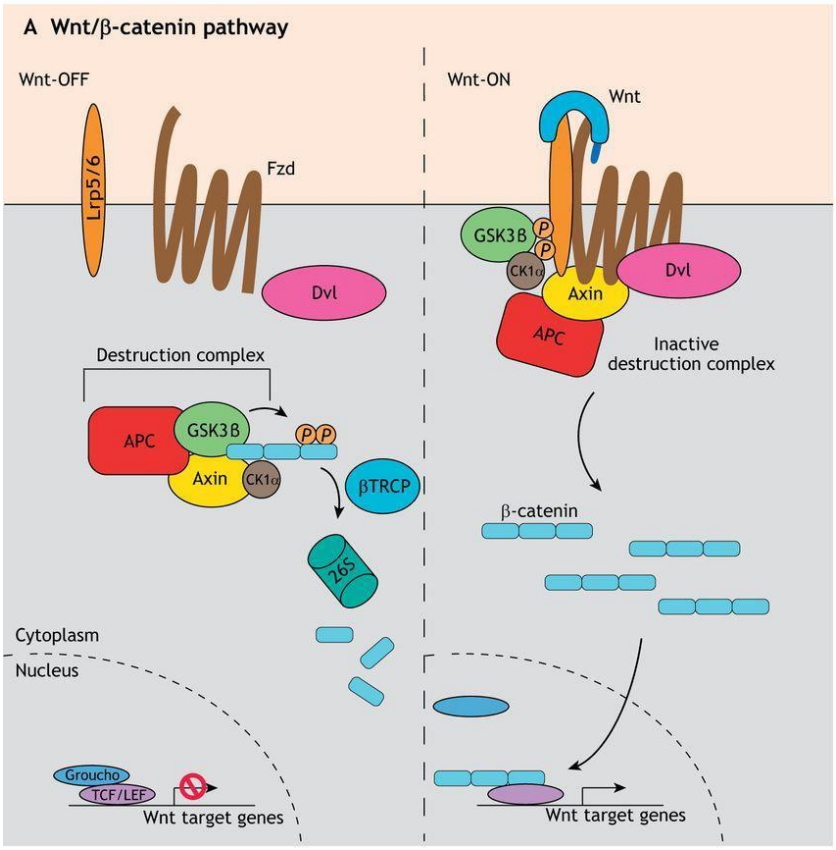


**Figure 3 – The Wnt/β-catenin Pathway.** In the β-catenin-dependent pathway, β-catenin undergoes continuous turnover in the absence of Wnt signals by the destruction complex (Wnt-OFF). In this state, Wnt target genes are suppressed by Groucho and TCF/LEF transcription factors. Upon Wnt binding to canonical Fzd receptors and the co-receptor Lrp5/6, a ligand-receptor complex called the 'signalosome' is formed. This causes the intracellular recruitment of Dvl and components of the destruction complex. Recruitment to the plasma membrane inhibits the formation of a functional destruction complex and thus prevents the degradation of β-catenin, permitting its cytosolic accumulation. β-Catenin subsequently translocates to the nucleus, where it binds with TCF/LEF transcription factors to inhibit their DNA binding. Wnt target genes, such as cyclin D1 and *Myc*, are disinhibited to control cell fate acquisition and proliferation

### 1.4.3. Wnt/PCP Signalling

The Wnt/Planar Cell Polarity (PCP) pathway (or "non-canonical" pathway) is an important branch of the Wnt signalling network, as it regulates actin cytoskeletal elements and thus cell migration and polarity. Typically non-canonical Wnt ligands include Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b and Wnt11 (Ackers and Malgor, 2018). Despite also initiating signalling through Wnt-Fzd bindings, it differs from the Wnt/β-catenin pathway as it is independent from β-catenin and requires the co-receptor Ror2 for activation (Fig. 4) (Komiya and Habas, 2008). Following binding of

Wnt proteins to Fzd and Ror2, Dvl is recruited to this complex at the PM, and this in turn recruits Dishevelled associated activator of morphogenesis 1 (Daam1). Daam1 is an activator of Rho-GTPases, which promote actin cytoskeleton remodelling through activation of ROCK and Cdc42 (Habas *et al.,* 2001). Independently from Daam1, the Wnt-Fzd-Dvl complex simultaneously activates Rac GTPases, which in turn promotes Jun N-terminal kinase (JNK) activity and subsequent phosphorylation of c-Jun (Habas, *et al*., 2003). Phospho-c-Jun then translocates to the nucleus, where it upregulates genes associated with cell migration and polarity (Schambony and Wedlich, 2007; Zhang *et al.*, 2020). Wnt5a-induced recruitment of the transmembrane protein Vangl2 in complex with Fzd and Ror2, and its subsequent phosphorylation by CK1ε, also promote JNK signalling (Brunt *et al.*, 2021).

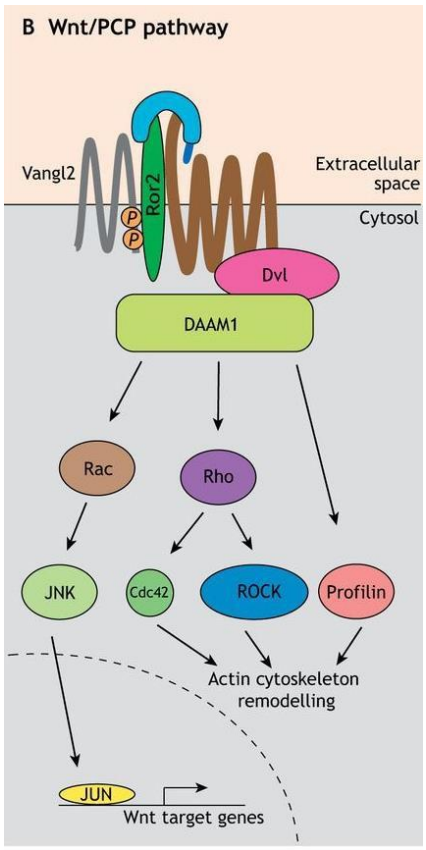


**Figure 4 – The Wnt/PCP Pathway.** In the β-catenin-independent/PCP pathway, Wnt binding to non-canonical Fzd receptors, along with co-receptors such as Ror2, induces actin polymerisation through activation of cytoskeletal regulators. These include the small GTPases Rho, Rac and Cdc42, which promote elongation or branching of actin filaments. These drive extension of the cell membrane in the form of lamellipodia and filopodia to regulate cell polarity and migration. Activation of JNK signalling promotes expression of PCP target genes, which further promote cell migration and polarity. Adapted from Routledge and Scholpp, 2019.

### 1.4.4. Integration of Wnt Signalling Pathways

The two Wnt signalling pathways described above are primarily thought to act in a mutually repressive manner because both compete for common proteins, such as the scaffolding proteins Dvl1-3 (Gao and Chen, 2010). For example, Wnt5a-mediated activation of the Wnt/PCP pathway has been shown to inhibit Wnt/β-catenin signalling (Topol *et al.*, 2003; Nemeth *et al.*, 2007). Expression of the non-canonical co-receptor Ror2 also has the same effect (Yuan *et al.*, 2011). Conversely, Lrp6 over-expression has been shown to inhibit Wnt5a-mediated Wnt/PCP signalling and thus regulate convergent extension (CE) movements in *Xenopus* embryos (Bryja *et al.*, 2009).

Additionally, several Fzd receptors are capable of binding both “canonical” and “non-canonical” Wnt ligands, and some Wnt ligands, such as Wnt8a, are capable of activating either pathway depending on the Fzd and co-receptor(s) they bind to (Voloshanenko *et al.*, 2017; Mattes *et al.*, 2018). The prevalence of a particular pathway therefore depends not only on the expression levels of specific Wnt ligands, but also on the availability of Fzd receptors, co-receptors and intracellular binding proteins in a given cell or tissue at a given time point.

The complexity of the Wnt signalling pathway is furthered by the existence of multiple other co-receptors and regulators; for some of which it is still unclear whether they are positive or negative regulators of particular pathways. For example, Protein Tyrosine Kinase 7 (PTK7) is a transmembrane protein which was first identified as an interactor of Fzd7, where it co-precipitates with canonical Wnt3a and Wnt8, but negatively regulates their signalling (Peradziryi *et al.*, 2011). Contradicting these findings, PTK7 has since been shown to interact with and stabilise Lrp6 in the *Xenopus* neural plate (Bin-Nun *et al.*, 2014). Consequently, PTK7 promoted activation of the Wnt/β-catenin pathway and strongly inhibited Wnt/PCP signalling. Following this, PTK7 was shown to interact with and form heterodimeric complexes with Ror2 to promote Wnt/PCP signalling (Martinez *et al.*, 2015). These conflicting reports on the role of PTK7 in Wnt signalling highlight the complexity of the Wnt signalling network and how integration of receptors and signals is a multifactorial process (Niehrs, 2012).

Other known Wnt co-receptors include Ryk, a receptor tyrosine kinase which can bind to non-canonical Wnts to modulate Wnt/PCP signalling (Macheda *et al.*, 2012), and Muscle-specific tyrosine kinase (MuSK), which can bind Wnt11r to regulate PCP-mediated neural crest cell migration in zebrafish (Banerjee *et al.*, 2011). Most recently, Cachd1, a type I transmembrane protein, has been identified as an interactor of Fzd receptors and Lrp6, where it simultaneously binds to both receptors to modulate Wnt/β-catenin signalling during neurogenesis (Powell *et al.*, 2022).

## 1.5. Wnt Signalling in Development and Disease

### 1.5.1. Wnt Proteins as morphogens

Morphogens control the form or shape of tissues and organs and consequently the patterning and organisation of the entire body. Here, they are able to regulate the formation of tissue boundaries through the formation of morphogen gradients. In early development, only a subset of specialised cells express and secrete morphogens, known as the source cells (or “organisers”) (Spemann and Mangold, 1923). Naturally, the spreading of these morphogens away from the source cells produces a gradient as the ligands become more diffuse. As groups of cells experience different concentrations of multiple morphogen gradients, these act as thresholds for determining cell fate (Gurdon and Bourillot, 2001). This concept was developed from pioneering work by Alan Turing, who proposed the idea that morphogenesis could be co-ordinated by diffusion of two different substances with antagonistic effects and opposing gradients (Turing, 1952). Refining this idea, Lewis Wolpert proposed the ‘French flag’ model, depicting how embryonic cells can acquire different fates along a morphogen concentration gradient depending on levels of exposure and reception (Wolpert, 1969).

During embryonic development, Wnt proteins are one of the key morphogens which co-ordinate the timing and directionality of growth and tissue patterning (Yang, 2012). Morphogens act as cues to guide cells on when and where to divide and migrate, and which cell fates to acquire. For example, during vertebrate skeletogenesis Wnt/β-catenin signalling regulates the differentiation of mesenchymal progenitors into osteoblasts and chondrocytes (Day *et al.*, 2005). In zebrafish embryogenesis, Wnt/PCP signalling is crucial for CE movements during gastrulation, brain boundary

formation and pigment cell specification (Gray *et al.*, 2011; Sepich *et al.*, 2011; Mattes *et al.*, 2018; Sutton *et al.*, 2021).

### 1.5.2 Wnt Signalling in Stem Cell Renewal

The importance of Wnt signalling gradients is also evident in its function in adult homeostasis. In multiple organs, including the intestine, stomach, skin and liver, Wnt signals regulate the proliferation and differentiation of stem cells (Barker *et al.*, 2010; Lim *et al.*, 2013; Wang *et al.*, 2015; Mah *et al.*, 2016). Here, Wnt signalling promotes the maintenance of stem-cell like properties to provide a stem cell niche from which new cells can proliferate and differentiate (Nusse, 2008). For example, in the epithelium, continual renewal of epithelial cells is required to replace old or dysfunctional cells and thus maintain healthy and functional tissues. For example, in the intestinal epithelium of mice, loss of the Wnt/β-catenin transcription factor *TCF4* results in loss of intestinal crypts (Korinek *et al.*, 1998). Ectopic expression of the Wnt inhibitor Dickkopf1 (Dkk1) results in the same loss of intestinal crypts and alters differentiation patterns (Pinto *et al.*, 2003). In the following section, I will outline how Wnt signal gradients contribute to stem cell renewal and differentiation in the context of gastric homeostasis.

### 1.5.3 Wnt Signalling in Gastric Homeostasis

The anatomy of the stomach is divided into two main sections. The main body of the stomach, the corpus, is where a variety of acids and enzymes are secreted to mediate the majority of digestion. At the base of the stomach, the antrum, hormones and mucus are secreted by a variety of cell types found in the gastric epithelium. Within the antral epithelium are gastric crypts/pits; invaginations in the stomach wall which give way to gastric glands (Engevik *et al.*, 2020).

Gastric crypts are not static structures and they represent highly proliferative and dynamic structures. Two types of stem cells defined by their expression of Axin2 and Lgr5 (both Wnt target genes); Axin2+/Lgr5+ and Axin2+/Lgr5-; reside in the base of the crypt (Fig. 5). Both are proliferative and provide a source of nascent epithelial cells, which differentiate as they migrate up the wall of the crypt. This migration continues and cells are eventually shed into the gastric lumen. This continual turnover results in the periodic renewal of the gastric epithelium. Both Lgr5+ and Lgr5- stem cells are capable of repopulating the gastric epithelium upon

deletion of the other, with Lgr5- cells being more proliferative and permitting epithelial turnover in as short as 7 days, and Lgr5+ cells in 10-14 days (Sigal *et al.*, 2017; Hata *et al.,* 2018).

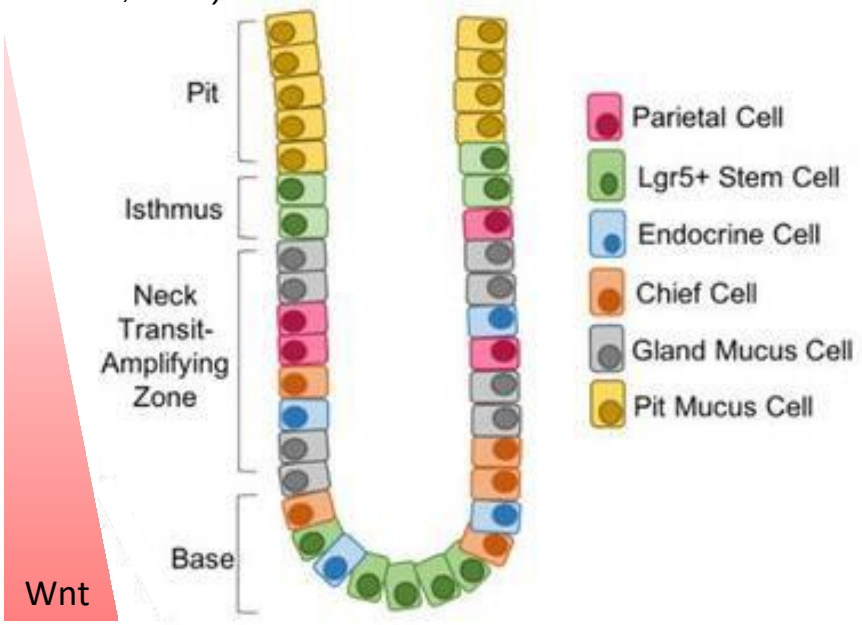


**Figure 5 – Structure and signalling in gastric crypts.** Architecture of the gastric crypt with the Wnt gradient depicted. Lgr5+ stem cells residing at the base of the crypt are a source of nascent epithelial cells. Adapted from Stanland and Luftig, 2020.

The proliferation, differentiation and migration of gastric epithelial cells is, in concert with other signals, controlled by a Wnt signalling gradient; where the concentration of Wnt is higher at the base and gradually declines as the cells travel upwards (Fig. 5)(Reya and Clevers, 2005; Flanagan *et al.*, 2018). *In situ* hybridisation (ISH) stainings revealed that a number of other Wnt ligands are expressed in gastric crypts, including Wnt3a, Wnt4, Wnt5a and Wnt11 (Sigal *et al.*, 2017). Indeed, Lgr5+ stem cells alone are capable of forming functional gastric organoids, but these deteriorate in the absence of Wnt3a-conditioned media (Barker *et al.*, 2010). The reception of Wnt signals by Lgr5+ cells in murine gastric epithelia was found to rely on Fzd7. Here, Fzd7 is highly expressed by Lgr5+ cells and deletion of Fzd7 is deleterious, triggering rapid repopulation (Flanagan *et al.*, 2017). Lgr5+ stem cells are also supported by underlying myofibroblasts, found in the surrounding stroma. These cells secrete R-spondin 3 (R-spo3), which regulates Wnt signalling by preventing the ubiquitination and subsequent degradation of Fzd receptors. Expression of R-spo3 by the myofibroblasts was found to promote proliferation of Lgr5+ stem cells and enhance organoid formation (Sigal *et al.*, 2017). These findings

highlight the importance of Wnt signals in the regulation of stem cell renewal in gastric crypts. Therefore, over-expression of Wnt proteins or dysregulation of the signalling pathway disrupts the Wnt gradient and promotes maintenance of stem cell-like properties and hyperproliferation. This can lead to the formation of tumours in the gastric epithelium (Chiurillo, 2015).

### 1.5.4. Wnt Signalling in Gastric Cancer

Gastric cancer (GC) is the fourth most common cancer worldwide but is the second-leading cause of cancer-related deaths (Sitarz *et al.*, 2018). This is partly due to diagnosis during advanced stages, which often requires invasive surgery and is associated with poor prognosis (Takahashi et al., 2013). Understanding GC in the earlier stages, on a molecular and cellular level, is critical to advancing our understanding of its progression and thus our ability to treat it.

In recent years, the role of Wnt signalling in GC tumourigenesis has become increasingly prevalent. Genomic analysis shows that deregulation of the Wnt/β-catenin pathway is found in 46% of gastric tumours (Oo*i et al*., 2009). Multiple Wnt ligands are frequently over-expressed in gastric cancers, including Wnt1, Wnt2b, Wnt3, Wnt5a, Wnt6 and Wnt10a (Katoh *et al.*, 2001; Kirikoshi, Sekihara and Katoh, 2001; Saitoh, Mine and Katoh, 2002; Yuan *et al.*, 2013; Mao *et al.*, 2014; Wang *et al.*, 2016). Over-expression of Wnt1 in the gastric cancer cell line, AGS, enhances their proliferative rate and spheroid formation. Indeed, there is a strong correlation between Wnt1 expression and cancer grade in gastric cancer patient tissues (Mao *et al.*, 2014). Knockdown of Wnt3 in GC cell lines reduces their proliferation, migration and invasion (Wang *et al.*, 2016).

Deregulation of Wnt signalling can also occur through mutations in proteins involved in the intracellular cascade. For example, mutations of *APC*, a component of the destruction complex and therefore a tumour suppressor gene, are common in gastric cancers. One particular study found that 22% of patients with sporadic gastric carcinoma had mutations in the *APC* gene (Fang *et al.*, 2002). Another study of GC patients found that 26% had mutations in β-catenin, most of which were phosphorylation sites or in the adjacent region, thus allowing escape from degradation (Clements *et al.*, 2002).

These findings highlight the importance of tight control over the Wnt signalling pathway, as its over-activation, regardless of the cause, is associated with poor prognosis (Koushyar *et al.*, 2020). In recent years, the Wnt signalling pathway has taken centre stage in research for novel therapeutic targets to combat several types of cancer, including breast, gastric and pancreatic cancers (Zhang and Wang, 2020). For example, OMP-18R5 (vantictumab) is a monoclonal antibody which binds to and blocks the activity of Fzd1, 2, 5, 7 and 8. OMP-18R5 showed promising results, blocking tumour growth in xenograft mouse models of multiple cancers. However, phase I clinical trials were halted over fears of detrimental effects to healthy tissues, particularly the bone marrow, where Wnt signalling regulates bone homeostasis (Houschyar *et al.*, 2019; Davis *et al.*, 2020). This is a common issue with Wnt-targeted therapeutics, since the Wnt signalling pathway is necessary for healthy adult tissue homeostasis and stem cell renewal in multiple organs. Hence, there are currently no approved cancer drugs which target the Wnt signalling pathways.

### 1.6 Intercellular Wnt Transport

Once Wnt has reached the plasma membrane, the question of how Wnt is released from Evi and secreted to fulfil its paracrine function remains highly debated. Long-range free diffusion of the lipid-modified Wnt proteins in the aqueous extracellular space seems unlikely, because of their hydrophobic nature. Indeed, Wnt proteins form aggregates in the ECM unless stabilised by detergents or serum (Fuerer *et al.,* 2010). Thus, without assistance, Wnt signalling is restricted to autocrine and probably juxtacrine signalling. However, it has been suggested that short-range signalling is sufficient in several tissues. A report highlights the short-range transport of Wnt proteins in the intestinal crypt, where non-essential Wnt protein can be detected travelling away from its source in a cell-bound manner through cell division, suggesting Wnt transport may not necessitate secretion in all cases (Farin *et al.*, 2016).

In *Drosophila*, a membrane-tethered form of the Wnt orthologue, Wingless (Wg), is viable, despite attenuated Wg gradients. However, membrane tethered Wg mutants develop slightly smaller wings with a delay. Thus, it has been suggested that early *Wg* expression is sufficient to induce persistent target gene expression, and long-

range signalling supports, but is not critical to, later stages of wing growth and development by promoting cell proliferation (Alexandre *et al.*, 2014). Conversely, in *Drosophila*, extracellular Wg protein has been detected up to 11-cell diameters from the producing cells, and target genes are expressed up to 20-cell diameters away (Zecca *et al.*, 1996; Neumann and Cohen, 1997; Chaudhary and Boutros, 2018). Wg has been shown to control wing growth through long-range activation of target genes, such as *Distal-less* (*Dll*) and *Vestigial (Vg)*, and ectopic expression of Wg causes ectopic expression of these genes and overgrowth of the wing pouch (Neumann and Cohen, 1997). Thus, the suggestion that long-range Wg signalling is dispensable in tissue patterning has questioned our understanding of Wg as a morphogen and the reasons for these discrepancies are yet to be clarified.

But what determines whether a Wnt protein is destined for short- or long-range dispersal? In *Drosophila*, this is thought to be regulated in a polarised manner, since apical and basolateral secretion of Wnt proteins can produce short- and long-range gradients, respectively (Bartscherer and Boutros, 2008, Chaudhary and Boutros, 2018). The extracellular Wg gradients form on the basolateral surface of the wing disc (Strigini and Cohen, 2000). In polarised human epithelial cells, Wnt3a is secreted basolaterally in a Evi-dependent manner and secretion of Wnt3a is also attenuated by depletion of clathrin, a protein that forms a major role in vesicle formation, suggesting that endocytosis in involved in Wnt3a secretion (Yamamoto *et al.*, 2013). Concurrent with this notion, *Drosophila* expressing a *shibire* mutant of dynamin, which is required for endocytosis, accumulate Wg protein in the Wg-producing cells (Strigini and Cohen, 2000). Conversely, Wnt11 is secreted apically, and its secretion is not affected by Evi or clathrin depletion, suggesting a different mechanism is at play (Yamamoto et al., 2013). Differences in post-translational glycosylation of Wnt3a and Wnt11 are proposed to determine their secretory routes (Yamamoto *et al.*, 2013). A proposed explanation for these polarised phenotypes is Wnt transcytosis, whereby Wnt ligands are first presented at the apical membrane to mediate short-range signalling, before being re-endocytosed, packaged into endosomes and transported to the basolateral membrane for secretion (Yamazaki *et al.*, 2016). Indeed, Wg has been observed on the apical membrane before being re-endocytosed in the secreting cells (Pfeiffer *et al.*, 2002). From here, Wnt secretion is thought to mediate long-range signalling and gradient formation. Several

mechanisms to explain this long-range spreading of Wnt have been proposed, including Wnt-binding chaperone proteins, lipoproteins, exosomes, and cytonemes (Port and Basler, 2010, Stanganello and Scholpp, 2016).

### 1.6.1 Chaperone Proteins

Wnt proteins are transported over long distances to fulfil their function in signalling in many tissues. A common mechanism utilised by cells to shield hydrophobic structures or proteins from the aqueous environment is through binding to other proteins, which protect these hydrophobic regions, aid their stabilisation and improve solubility. This is exemplified by intracellular binding proteins, such as fatty acid-binding proteins (FABPs) and retinol-binding protein (RBP), which help the solubilisation, transport and secretion of fatty acids and retinol, respectively (Ronne *et al.*, 1983; Storch *et al.*, 1996).

Given the hydrophobic nature of Wnts, it is therefore conceivable that Wnt proteins could be transported through a similar mechanism. A recent study reports free extracellular dispersal of Wnt/EGL-20 in *C. elegans*, however, how diffusion is achieved is unclear. For example, this may be achieved through stabilisation by Wnt/EGL-20-binding proteins or ECM components, which would not be considered free diffusion (Pani and Goldstein, 2018)**.**

One family of proteins known to bind to Wnts are Secreted Frizzled-related proteins (sFRPs) (Hoang *et al.*, 1996). While sFRPs are known to modulate Wnt signalling, this is thought to be through interacting with Wnt receptors or sequestration of Wnt proteins (Üren *et al.*, 2000; Galli *et al.*, 2006). Additionally, the role of sFRPs in modulating Wnt signalling is unclear; sFRPs were first reported as Wnt inhibitors (Leyns *et al.*, 1997), however increased expression of sFRPs can both inhibit and augment Wnt signals in context- and concentration-dependent manners (Üren *et al.*, 2000; Xavier *et al.*, 2014). In *Xenopus* embryos, sFRPs have been shown to enhance the diffusion of Wnt8 and Wnt11 by forming a complex (Mii and Taira, 2009). Therefore, sFRPs might aid the transport of Wnt, but at high concentrations, sFRPs could also outcompete Wnt receptors to inhibit Wnt signalling. How exactly sFRPs differentially modulate Wnt signalling is yet to be determined.

Recently, a secreted protein in *Drosophila*, termed Secreted Wg-interacting Molecule (Swim), has been suggested to facilitate long-range Wg transport, (Mulligan *et al.*, 2012). Swim is a member of the lipocalin superfamily of extracellular transport proteins. These proteins harbour a conserved and characteristic lipocalin fold, which forms a hydrophobic binding pocket to accommodate a variety of hydrophobic structures (Flower, 1996). For Swim, this structure was discovered to be the C93 palmitate moiety on Wg, as free palmitate could compete with Wg for Swim binding, and a C93A mutation (which abolishes acylation) resulted in the loss of signalling capabilities. While $Wg^{C93A}$ could still be secreted, it quickly aggregated in the extracellular space. Furthermore, *in vivo* knockdown of Swim decreased extracellular Wg distribution and impaired long-range signalling. Together, these findings suggest that Swim could mediate long-distance Wg signalling by maintaining its solubility in the extracellular matrix and thus aiding its transport to Wg-receiving cells (Mulligan *et al.*, 2012) However, no vertebrate homologue of Swim has been identified, thus follow-up genetic studies would be necessary to assess any evolutionary function of Swim.

In vertebrates, a human Wnt-binding glycoprotein, afamin, has been reported. Afamin is a member of the serum albumin family group of binding proteins that display an affinity for a wide variety of poorly soluble molecules, notably lipid-modified proteins via a hydrophobic binding pocket. While afamin is renowned for its vitamin E-binding capabilities, it has been unexpectedly identified to also bind to Wnt following its co-purification with Wnt3a from HEK293 cells (Dieplinger and Dieplinger, 2015). Alongside its ability to enhance Wnt3a secretion in a dose-dependent manner, afamin has also been shown to form a complex with Wnt3a and thus improve its solubility. Crucial to its function as a paracrine signalling factor, Wnt3a maintains its biological activity when in complex with afamin (Mihara *et al.*, 2016). Afamin has been subsequently shown to associate with and enhance the secretion of 12 different Wnt proteins *in vitro* and was thus suspected to bind to palmitoleic acid via its hydrophobic pocket (Mihara *et al.*, 2016). Furthermore, Naschberger and colleagues have computationally modelled the Wnt3a-afamin complex, based on the crystal structure of *Xenopus* Wnt8 bound to the CRD of Frizzled 8 (XWnt8-Fzd8-CRD). Here, the hydrophobic cavity of Fzd8-CRD accommodates the S187 palmitoleic acid of XWnt8 (Janda *et al.*, 2012b). Indeed, the resulting model describes the S209

palmitoleic acid of Wnt3a to be central to its binding to afamin (Naschberger *et al.*, 2017). Together, these findings highlight a novel role for afamin in extracellular Wnt transport. However, *afamin* is primarily expressed in the liver and transported in the blood in vertebrates. Although its role in the context of *in vivo* Wnt signalling is yet to be elucidated, it is unlikely to facilitate the evolutionarily conserved functions of Wnt because invertebrates do not express albumin family proteins.

### 1.6.2 Lipoproteins

Lipoproteins are a class of extracellular membrane vesicle, which function as a critical intercellular communication mediator regulating the exchange of proteins and genetic materials between donor and surrounding cells. The first evidence that Wnts may be transported via lipoproteins came from the co-localisation of membrane-tethered, GPI-anchored GFP with Wg-containing vesicles. These structures, initially called argosomes, have been thought to derive from the basolateral membrane of Wg-producing cells (Greco *et al.*, 2001). More recently, these structures have been identified as lipoproteins; globular vesicles typically used for transporting hydrophobic lipids and proteins. Lipoprotein particles are of interest in Wnt signalling because Wg co-purifies with lipophorins—the *Drosophila* homologue of lipoproteins. In addition, Wg co-localises with lipophorins in the developing wing epithelium. Furthermore, RNAi knockdown of *lipophorins* shortens Wg gradients, as measured by expression of target genes in Wg-receiving cells (Panáková *et al.*, 2005). Analogous results were seen for Hedgehog (Hh) signalling, another lipid-modified morphogen, suggesting lipoprotein particles as a common mechanism for long-range morphogen signalling. This concept has also been observed in a mammalian context, where Wnt3a associates with lipoproteins *in vitro* in the media of mouse fibroblasts (Neumann *et al.*, 2009). However, when grown in media containing delipidated foetal calf serum (DL-FCS), which lack lipoproteins, Wnt3a is not detected in the media. The addition of high-density lipoproteins (HDLs), but not low-density lipoproteins (LDLs), leads to the release and increased levels of Wnt3a in the media, suggesting overexpressed *Wnt3a* can be loaded onto exogenous HDLs (Neumann *et al.*, 2009). More recently, it has been shown that Wnt5a, produced by the developing murine choroid plexus, circulates in the cerebrospinal fluid (CSF) on lipoproteins (Kaiser *et al.*, 2019). However, a mechanism explaining how Wnt may be loaded onto lipoproteins is poorly understood. In *Drosophila*, Wg localises to Flotillin-2-positive microdomains at

the plasma membrane. Flotillin-2 (Flot2) is an acylated, membrane-bound scaffolding protein, which can localise and oligomerise at sphingolipid-rich lipid rafts (Langhorst *et al.*, 2007). While the exact function of Flot2 remains to be clarified, in the context of Wg signalling it is suggested to aid the secretion of Wg. Indeed, *Flot2* overexpression and knockdown expands and reduces extracellular Wg gradients, respectively (Katanaev *et al.*, 2008). In addition, Wg has been observed to partially co-localise with Flot2. One hypothesis is that Flot2 microdomains serve as 'dating points' to which lipoprotein receptors and Wnt/Wg co-localise; permitting the loading of Wnt/Wg onto exogenous lipoproteins (Solis *et al.*, 2013).

Alternatively, some cell types are capable of lipoprotein synthesis. For example, Wnt3a-secretion via endogenous lipoprotein particles is observed *in vitro* in intestinal epithelial cells. Here, Wnt3a-Myc co-precipitates with newly-synthesised apoB100—a poorly-lipidated apolipoprotein associated with LDLs (Neumann *et al.*, 2009). Concurrent with original reports that argosomes were basolaterally-derived, these endogenous lipoproteins are also observed on the basolateral side, whereas exogenous HDLs and SR-BI/II (a lipoprotein receptor) localisation is predominantly at the apical surface of polarised epithelial cells (Reboul *et al.*, 2006; Neumann *et al.*, 2009). This could suggest two different lipoprotein-based mechanisms for Wnt secretion. This concept is supported by the observation that Wnt3a and Wnt11 are secreted basolaterally and apically, respectively, and that they are both differentially regulated (Yamamoto *et al.*, 2013). However, whether Wnts maintain biological activity when lipoprotein-bound has not been clarified, and a role for lipoproteins in Wnt transport *in vivo* is yet to be examined.

### 1.6.3 Exosomes

Supporting the concept of Wnt transport via extracellular vesicles, exosomes are also proposed to mediate extracellular Wnt transport. While mechanistically comparable in its concept (shielding hydrophobic proteins in a membranous vesicle) exosomes differ strongly from lipoproteins in their composition and biosynthesis. Exosomes are cell-derived vesicles that form during the maturation of early endosomes into multivesicular bodies (MVBs), in which they are contained. As they are trafficked through the endosomal compartments, exosomes are loaded with

cargo proteins and secreted from cells through the fusion of MVBs with the plasma membrane (Hessvik and Llorente, 2018). Exosomes then traffic through the ECM to deliver proteins to other cells; probably mediating intercellular communication.

A role for exosomes in transporting Wnt proteins was first reported in the *Drosophila* neuromuscular junction (Korkut *et al.*, 2009). Here, Wg is carried across the synaptic cleft by Evi-containing exosomes to influence synaptic growth, function and plasticity. This observation is supported by a study showing that Wnt3a can also be localised with exosomes from HEK293 cells (Gross *et al.*, 2012). Using TSG101, one of the three proteins forming the ESCRT-I complex, as an exosomal marker, immunoblot analysis of lysates of Wnt-expressing cells revealed the presence of Wnt3a and Wnt5a in the exosomal fractions. Furthermore, *in vivo* staining of *Drosophila* wing disc revealed co-localisation of Wg and the exosomal marker CD63-GFP in both intracellular MVBs and the extracellular space, although this was only a fraction of the total Wg staining (Gross *et al.*, 2012). The significance of exosome-mediated transport is becoming more evident in a variety of contexts. In CNS injury, fibroblast-derived exosomes promote axonal regeneration by inducing re-localisation of neuronal Wnt10b to lipid rafts and downstream activation of the mTOR pathway, which promotes CNS repair (Tassew *et al.*, 2017). Conversely, the presence of exosomes in cancer often correlates with poor prognosis, as there is evidence of stromal cells utilising exosomes to transport pro-tumourigenic factors, such as growth factors, micro RNAs (miRNAs) and Wnt proteins (Halvaei *et al.*, 2018; Hu *et al.*, 2018).

Interestingly, the Wnt chaperone Evi has also been found in MVBs, where it co-localises with Wnt and exosomal/MVB markers, CD81 and TSG101 (Gross *et al.*, 2012). An essential maturation step of MVBs is endosomal acidification, which can be blocked by the V-ATPase inhibitor bafilomycin A1 (Clague *et al.*, 1994). Inhibiting endosomal acidification (and thus MVB maturation) causes intracellular accumulation of the Evi-Wnt3a complex (Coombs *et al.*, 2010). Additionally, the v-SNARE protein Ykt6, which mediates vesicular fusion events, is crucial for correct sorting and secretion of Wnts on exosomes (Linnemannstöns *et al.*, 2018, 2020). However, the persistence of the Evi-Wnt complex in exosomes is unclear; Evi and Wnt are separated in MVBs, and suggested to be secreted on different exosomes because only 10% of total Evi and Wg protein co-localises extracellularly (Gross *et al.*, 2012).

Furthermore in *Drosophila* embryos, Wg remains tightly associated with producing cells and is endocytosed from the plasma membrane (Pfeiffer *et al.*, 2002). These findings are consistent with the proposed retromer-dependent recycling of Evi in Wg-producing cells, which requires its endocytosis (Port *et al.*, 2008). However, Evi has been observed on secreted exosomes *in vivo* in the neuromuscular junction of *Drosophila*, where bi-directional Wg signalling modulates synaptic structure and function (Ataman *et al.*, 2008; Koles *et al.*, 2012). This bi-directional signalling breaks from the classical 'Wnt-producing and Wnt-receiving' model and could be explained by defining the synaptic cleft as a confined space where directionality of the signal is less important; both pre- and post-synaptic cells can secrete and respond to Wg. While the point at which Evi and Wnt/Wg dissociate is disputed, there is substantial evidence that Wnt is transported on exosomes. However, the low levels of co-localisation between Wnt proteins and exosomal markers may indicate that this may not be the primary mechanism for Wnt dispersal (Saha *et al.*, 2016).

### 1.6.4 Cytonemes

First described in *Drosophila* wing imaginal disc, cytonemes represent a subset of specialised filopodia capable of transporting signalling components to neighbouring cells (Ramírez-Weber and Kornberg, 1999). Here, cytonemes are primarily associated with the transport of morphogens, such as fibroblast growth factor (FGF), Hh and Decapentaplegic (Dpp), suggested to aid formation of gradients pivotal to correct tissue patterning during development (Roy *et al.*, 2014; González-Méndez *et al.*, 2017; Zhang and Scholpp, 2019). Wg is also a key morphogen in *Drosophila* development; particularly in wing imaginal disc formation, as loss of Wg signalling results in loss of wing structures (Sharma and Chopra, 1976). Whilst transport of Wg on cytonemes has not been directly observed, its receptor Fzd is present on the cytonemes of wing disc myoblasts, which orient towards Wg-expressing cells in the air sac primordium. Here, Wg forms a complex with Fzd and the cytoneme retracts towards the Wg-receiving cell in a retrograde manner (Huang and Kornberg, 2015). Experiments in *Drosophila* and cell culture revealed that signalling molecules could be disseminated by cell protrusions (Mattes and Scholpp, 2018). Indeed, high-resolution imaging experiments in zebrafish confirmed such a novel and unexpected mechanism for the extracellular transport of Wnt. In particular, specialised signalling

filopodia, termed cytonemes, were demonstrated to be fundamental in Wnt trafficking in vertebrates (Stanganello *et al.*, 2015).

Cytoneme-mediated transport of Wnt is most extensively studied in vertebrate organisms where, unlike in *Drosophila*, the ligand (Wnt), rather than the receptor (Fzd), is transported via cytonemes to the target cells (Stanganello *et al.*, 2015). For example, Wnt2b-EGFP and Wnt8a-GFP have been visualised on cell protrusions in *Xenopus* and zebrafish embryos, respectively (Holzer *et al.*, 2012; Luz *et al.*, 2014). In the latter, Wnt8a is transported on the tips of Cdc42/N-Wasp-positive cytonemes to influence tissue patterning in the neural plate by inducing Wnt signalling in receiving cells (Stanganello *et al.*, 2015).

The formation of Wnt-positive cytonemes is driven by the expression of *Wnt* itself. Akin to the models as mentioned earlier, Wnt is proposed to traffic from the ER to the plasma membrane with its chaperone Evi (Gradilla *et al.*, 2018). Cytoneme formation is driven by activation of cytoskeletal regulators—such as the small GTPases Rho, Rac1 and Cdc42—which drive actin polymerisation (Spiering and Hodgson, 2011). In the context of Wnt signalling, activation of the Wnt/PCP pathway causes downstream activation of these components and thus drives filopodia extension (Fig. 6) (Schlessinger, Hall and Tolwinski, 2009). Binding of Wnt8a to Ror2 is thought to drive *de novo* biogenesis of filopodia by inducing actin polymerisation via the PCP pathway (Mattes et al., 2018). Wnt proteins are thought to regulate their dissemination from producing cells in this way, as both *Ror2* and *Wnt8a* expression correlate with the number of filopodia. Concordantly, expression of the dominant-negative mutant *Ror2*$^{3l}$ in zebrafish embryos reduces the number of filopodia; corresponding with a significant reduction of target gene expression in neighbouring cells and suggesting Ror2-dependent cytonemes are capable of transporting and delivering Wnt to target cells (Mattes *et al.*, 2018). Regulation of Wnt8a cytonemes has since been shown to involve Van-Gogh-like 2 (Vangl2), a four-pass transmembrane protein which forms a complex with Ror2 to promote Wnt/PCP signalling. Indeed, Wnt5a-Ror2 binding and concomitant Dvl recruitment promote the phosphorylation of Vangl2 by CK1δ/ε and activate downstream JNK signalling to promote actin cytoskeletal remodelling. Over-expression of Vangl2 increases length and stability of cytonemes and regulates anteroposterior patterning of the zebrafish neural plate (Brunt *et al.*, 2021). Most recently, a novel mechanism of Wnt handover has been proposed, whereby active

Wnt5b/Ror2 complexes on the cytonemes of Wnt-producing cells are handed over to recipient cells, where they can induce Wnt/PCP signalling (Zhang *et al.*, 2022). This mechanism overcomes the issue of the 'handover problem', however, it is unclear whether a similar means of Wnt handover can be achieved for inducing Wnt/β-catenin signalling.

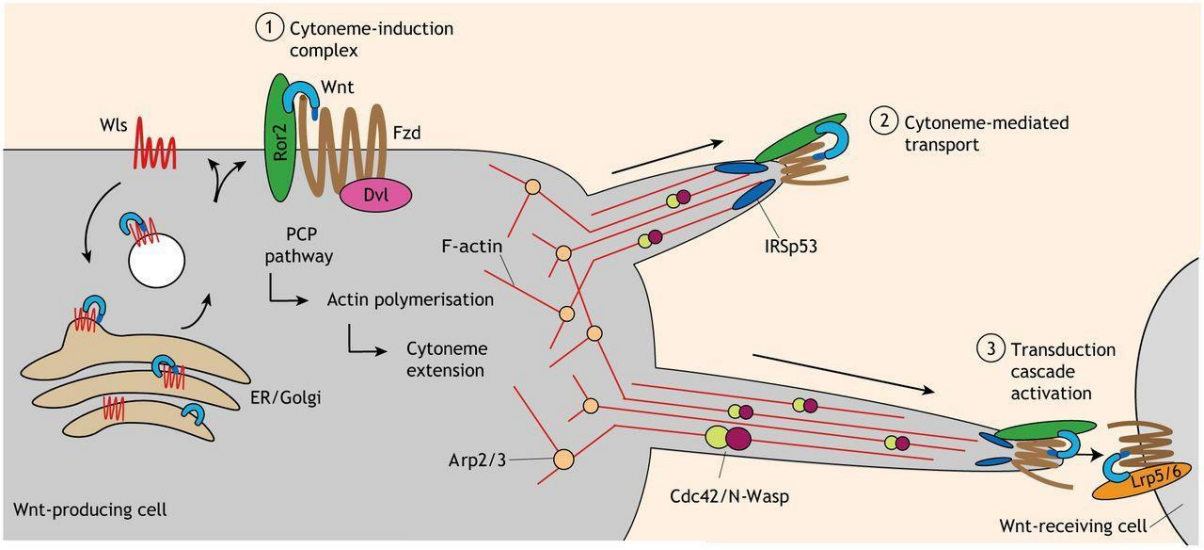


**Figure 6 - Cytoneme-mediated Wnt transport**. Wnt binds to non-canonical Fzd receptors, such as Fzd7, and Ror2, to activate the Wnt/PCP pathway (1). Clustering of Wnt-Fzd receptor complexes causes downstream activation of cytoskeletal regulators, such as Cdc42/N-Wasp, and thus actin polymerisation, which drives the extension of Wnt-bearing cytonemes from the Wnt-producing cell (2). At the Wnt-receiving cell, Wnt binds to Fzd and the co-receptor Lrp5/6 to induce β-catenin-dependent signalling and thus the expression of Wnt target genes (3). Taken from Routledge and Scholpp, 2019.

A similar Ror2-dependent mechanism for the regulation of Wnt cytonemes was described *in vitro* in gastric cancer (GC) cells, which display upregulated Wnt signalling activity (Chiurillo, 2015; Flanagan, Vincan and Phesse, 2017; Mattes *et al.*, 2018). Modulation of Wnt cytonemes also influences Wnt-mediated proliferation of GC cells (Mattes *et al.*, 2018). Furthermore, there is emerging evidence that cytonemes are used to deliver Wnt protein in the intestinal crypt in the mucosa of the small intestine in the mouse (Snyder *et al.*, 2015). The intestinal crypt cells need high Wnt activity to regulate the fast cell proliferation required to continually replenish the intestinal epithelium as it migrates up the crypt/villus axis and is shed into the gut lumen. The stromal cells localised around the crypt have been identified as essential Wnt sources (Greicius *et al.*, 2018; Shoshkes-Carmel *et al.*, 2018). Similarly, epithelial Paneth cells in the small intestine and Reg4+ cells in the colon also secrete

Wnts to contribute to the intestinal stem cell niche (Sato *et al.*, 2011; Sasaki *et al.*, 2016). Intestinal myofibroblasts co-cultivated with *Porcn*$^{-/-}$ crypt cells, which generate Wnt-deficient cells, leads to the induction and maintenance of intestinal crypt organoids. Knockdown of *Ror2* in these myofibroblasts prior to co-culture not only reduced the number of filopodia but also attenuated organoid formation (Mattes *et al.*, 2018). Indeed, cancer-associated fibroblasts (CAFs) have since been shown to activate Wnt/PCP signalling in receiving GC cells via handover of active Wnt5a/Ror2 complexes, in a similar fashion to Wnt5b/Ror2 in zebrafish (Rogers *et al.*, 2022). Together, these results highlight a role for cytonemes in transporting Wnt in several vertebrate tissues to regulate stem cells and tissue homeostasis.

Cytonemes have also been suggested to act as conduits in a system where exosomes act as the carrier. In *Drosophila*, the localisation of Hh to cytonemes appears to occur in a punctate fashion, where they move along the cytoneme. Due to their size and the observed co-localisation of Hh and its co-receptor Ihog with the exosomal marker CD63-GFP, these were suspected to be exosomes (Gradilla *et al.*, 2014). While inhibiting MVB biosynthesis has been shown to reduce Hh secretion and gradient formation, this was not evaluated in the context of Hh localising to cytonemes. It would be interesting to assess perturbations in the localisation of Hh puncta to cytonemes upon inhibition of exosome synthesis, as a reduction could suggest a role for exosomes in transporting Hh on cytonemes. Since Wnt also co-localises with exosomal markers, and Wnt puncta have been detected on cytonemes, this begs the question of whether a similar mechanism is utilised in transporting Wnt (Stanganello et al., 2015). More recently, an interaction between exosomes and filopodia has been reported in the delivery of exosomes to target cells. Here, exosomes are seen to ‘surf' along the filopodia before being endocytosed at their base, which appears to be endocytic hotspots (Heusermann *et al.*, 2016). It may be speculated that morphogen-containing exosomes could also interact with cytonemes at target cells. While these mechanisms have not been studied in the context of Wnt, interactions between exosomes and cytonemes cannot be ruled out and may offer a viable, synergistic view for Wnt trafficking.

### 1.6.5. The role of heparin sulphate proteoglycans

While the free diffusion of Wnt proteins is largely disputed, Wnt proteins can be stabilised to prevent aggregation and thus facilitate spreading through the ECM. One mechanism proposes interactions of Wnt proteins with heparan sulfate proteoglycans (HSPGs), a component of the ECM.

HSPGs bind to a plethora of ligands and are traditionally thought to serve as a co-receptor to promote binding of ligands to their receptors. In addition, HSPGs have also been shown to interact with many morphogens (Kirkpatrick and Selleck, 2007). Indeed, HSPGs are thought to enhance Wnt spreading through ligand stabilisation. For example, in *Drosophila*, over-expression of the glypican *Dally-like* (*Dlp*) leads to sequestration of Wg at the cell surface (Baeg *et al.*, 2001). Conversely, Wg is not observed on the surface of cells expressing sugar-deficient HSPGs. Indeed, it has recently been shown that Dlp is capable of binding and shielding the palmitoleate moiety of Wnt in a hydrophobic groove, thus stabilising extracellular Wnt and enhancing its long-distance spreading (McGough *et al.*, 2020).

HSPGs are also suggested to facilitate binding of Wg to its receptor, because overexpression of *Wg* can rescue the phenotypes of *sugarless* mutants; an enzyme involved with proteoglycan synthesis (Hacker *et al.*, 1997). This function of HSPGs appears to be conserved outside of *Drosophila*. In vertebrates, the zebrafish HSPG encoding gene, *knypek* (*XGly4*), regulates gastrulation events through potentiation of Wnt11 signalling (Topczewski *et al.*, 2001). Furthermore, in *Xenopus* embryos, glypican4 interacts with Wnt11 and the glycosyltransferase, XEXT1—involved with HS synthesis—is necessary for Wnt11-induced axis formation (Ohkawara, 2003; Tao *et al.*, 2005). Together, these findings implicate HSPGs as mediators of Wnt signalling and presumably also influencing spreading.

HSPGs may also aid the delivery of Wnt-bearing structures through interactions with transport machinery. For example, HSPGs are thought to act as bulk endocytosis receptors and thus help the delivery of lipoproteins and exosomes to target cells (Christianson and Belting, 2014). In *Drosophila*, the HSPGs Dally and Dlp aid the recruitment of Hh-positive lipoproteins to wing disc cells through direct interactions with lipophorins (Eugster *et al.*, 2007). These interactions are thought to be mediated through HSPG sugar moieties, as altering HS GAGs attenuates their affinity for and

clearance of lipoproteins in hepatocytes (Olsson *et al.*, 2001; Stanford *et al.*, 2010). HSPGs may also interact with LRPs on the surface of lipoproteins, which have been shown to co-immunoprecipitate in mouse embryonic fibroblasts (MEFs) (Wilsie and Orlando, 2003). Similarly, endocytic uptake of exosomes is thought to be dependent on HSPGs, as inhibition of proteoglycan synthesis attenuates exosome uptake in glioblastoma cells (Christianson *et al.*, 2013). Analogous results were achieved through treatment with free HS chains, which compete with HSPGs for exosome binding, although binding interactions here are not known (Christianson *et al.*, 2013). Together, these findings highlight a potential role for HSPGs as endocytic receptors for extracellular vesicles. In this manner, HSPGs may aid the internalisation of Wnt-bearing lipoproteins and exosomes in target cells.

The function of HSPGs may also allow the formation of long-range gradients of Wnt proteins without the need of ligand mobilisation. In chick development, Wnt ligands can be loaded onto migrating neural crest cells that deliver their message at a distance (Serralbo and Marcelle, 2014). To improve the delivery process, neural crest cells express *Glypican4* that acts in trans to deliver the Wnt ligand to the receiving cells in the somites. Therefore, by mobilising the source cells, one can achieve a long-range signalling gradient in some tissues. To further demonstrate their multi-functionality, HSPGs can also modulate cytoneme extension. In *Drosophila*, depletion of the glypicans Dally or Dlp significantly reduces the expansion of cytonemes and cytonemes are rarely detected in *dally/dlp* double mutants (González-Méndez, Seijo-Barandiarán and Guerrero, 2017). In the context of Hh signalling, contacts between cytonemes from anterior and posterior compartment cells are thought to be stabilised by trans interactions between glypicans and Ihog, a co-receptor of Hh; over-expression of either stabilises the cytonemes and contact points (González-Méndez, Seijo-Barandiarán and Guerrero, 2017). As Hh is another lipid-modified morphogen, a similar mechanism may be conceivable in cytoneme-mediated delivery of Wnt, although the effects of perturbing HSPG function on Wnt-positive cytonemes has not yet been evaluated.

## 1.7. Flotillin-2

### 1.7.1 Discovery of Flotillins

Flotillin-2 (Flot2) was first described in 1994 as a predicted antigen of the monoclonal antibody ECS-1 (based on its novel cDNA sequence) and was hence named epidermal surface antigen (ESA) (Schroeder et al., 1994). In 1997 two proteins homologous to ESA, termed reggie-1 and reggie-2, were identified to be upregulated during axonal regeneration in retinal ganglion cells following optic nerve lesion in goldfish (Schulte et al., 1997). Only 4 months later, a separate group identified two proteins associated with the 'floating' detergent-resistant membrane fractions from mouse lung tissue and were hence termed flotillin-1 (reggie-2) and flotillin-2 (reggie-1) (Bickel et al., 1997). Once again, these proteins were described as homologues of ESA. However, it was later shown that ESA was an N-terminally truncated form of Flot2 and the full-length protein was not the true antigen of ECS-1 (Lang et al., 1998; Hazarika et al., 1999). Hence, this name became obsolete and the term flotillin was more commonly adopted.

### 1.7.2 Structure of Flotillins

Flotillin-1 and -2 are 47kDa proteins belonging to the SPFH (Stomatin/Prohibitin/Flotillin/HflK&C) protein superfamily; members of which all share an SPFH domain. Whilst most SPFH proteins possess an N-terminal single transmembrane domain, flotillins have an N-terminal short hydrophobic stretch which is post-translationally acylated, with Flot2 being myristoylated at Gly2 and palmitoylated at Cys4 and to some extent Cys19 and Cys20 (Fig. 7). Flot1 lacks a myristoylation site but is palmitoylated at Cys34 (Neumann-Giesen et al., 2004).

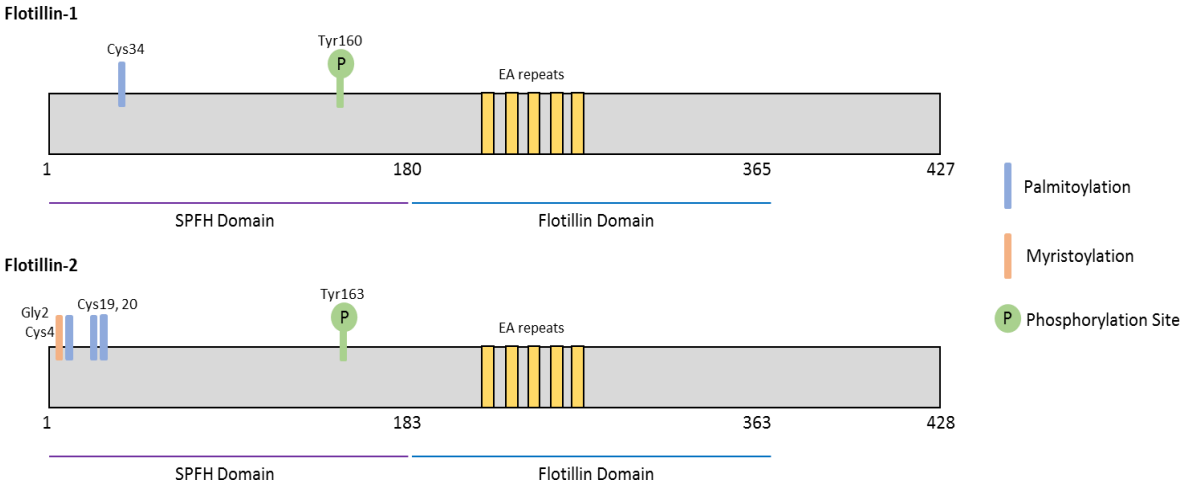


**Figure 7 – The structure of Flotillins**. A simplified overview of the structure of Flotillins 1 and 2. Flotillins possess an N-terminal SPFH domain, which characterises all SPFH proteins. This domain is responsible for membrane localisation due to post-translational modifications (PTMs). Flot2 is more heavily modified, with three myristoylation sites and one palmitoylation site, whilst Flot1 is only palmitoylated once. Y160 and Y163 represent conserved tyrosine residues which are required for endocytosis (through phosphorylation). The EA repeats in the C-terminal Flotillin domain are Gly-Ala-rich regions which mediate oligomerisation. All SPFH proteins have EA repeats, although this is extended in flotillins and defines this domain.

These acylations are crucial to the localisation and function of flotillins, which is discussed in greater detail in section 1.7.3. Another common feature of SPFH proteins is a short stretch of EA-repeats (repeats of Glu- and Ala-rich motifs) within the SPFH domain. In flotillins these EA repeats are extended and characterise the C-terminal flotillin domain, which is predicted to form a coiled-coil structure (Rivera-Milla *et al.*, 2006). These EA-repeats are thought to be important for oligomerisation, a common characteristic of SPFH proteins (Snyers *et al.*, 1998; Huber et al., 2003; Tatsuta *et al.*, 2005). Flot1 and Flot2 are capable of forming both homo- and hetero-oligomers as well as stable tetramers (Solis et al., 2007). Oligomerisation of flotillins is important for their stabilisation, as downregulation of one flotillin affects the levels and localisation of the other, with Flot1 more dependent on Flot2 (Solis et al., 2007). Site-directed mutagenesis of EA repeats (EA → GL) in bacterial flotillins FloT and FloA, orthologous to Flot1 and Flot2 respectively, abrogated oligomerisation and altered their cellular distribution (Schneider et al., 2015). Interestingly, their ability to homo- and hetero-oligomerise was affected differentially depending on which EA repeat site was mutated, suggesting these interactions require different residues.

### 1.7.3 Transport and Localisation of Flotillins

On a cellular level, flotillins are most notably localised at the plasma membrane but also display associations with intracellular compartments. Indeed, flotillins are reported to localise to a number of membranous structures, including the golgi apparatus, endosomes, lysosomes and multi-vesicular bodies (Otto and Nichols, 2011). The post-translational acylations of flotillins are responsible for their predominantly membrane-associated sub-cellular localisation; achieved by insertion of the palmitate (and in the case of Flot2 its myristoylate) moieties into the membrane; not via a transmembrane domain as originally suggested (Bickel et al., 1997). Here, flotillin hetero- and homo-oligomers/tetramers form clusters, termed flotillin microdomains, and are associated with detergent-resistant, cholesterol-rich regions of the membrane (lipid rafts) which can be visualised as distinct puncta along the plasma membrane (Frick et al., 2007; Solis et al., 2007). Flot2 mutants lacking acylation sites lose their ability to tether to membranes, display a mostly cytosolic distribution and are largely soluble (Neumann-Giesen et al., 2004).

Transport of flotillins to the plasma membrane is largely thought to occur in a Golgi-dependent manner. Whilst reversible myristoylation occurs co-translationally, post-translational palmitoylation is likely to require trafficking through the Golgi, where the majority of DHHC enzymes involved in palmitoylation reside (Bhatnagar et al., 2001; Ernst et al., 2018). Furthermore, flotillins are observed to co-localise with the Golgi marker GM130 and Flot2 mutants lacking the SPFH domain accumulate in the Golgi, suggesting this domain is necessary for Golgi exit (Langhorst, *et al*., 2008). From the Golgi, vesicular trafficking of Flot2 is the primary mechanism suggested since cycling of Flot2-positive vesicles to the plasma membrane, in a ‘kiss and run’ fashion, has been observed (Langhorst *et al.*, 2008). Flotillins are then thought to be continually recycled between the PM and endosomes (Neumann-Glesen *et al.*, 2007). These findings are conducive of the reported functions of Flot2 in intracellular trafficking, which are discussed in detail in section 1.8.4.

## 1.8. Functions of Flotillins

### 1.8.1. Flotillins as signalling platforms

One of the primary functions of flotillin microdomains is as a signalling platform, where Flot2 acts as a scaffolding protein to aid the formation and localisation of protein complexes necessary for signal transduction. This function was first suggested in insulin receptor signalling, where insulin stimulation causes dissociation of a complex (including Cbl and Cbl-associated protein (CAP)) from the insulin receptor and its translocation to flotillin microdomains (Baumann *et al.*, 2000). This localisation to flotillin lipid rafts is necessary for subsequent signal transduction and transport of the glucose transporter GLUT4 to the plasma membrane.

Flotillin microdomains were later shown to be important for signalling in immune cells. In lymphocytes, flotillins are asymmetrically localised to one side of the cell, and this polarised pre-assembled platform (PAP or “flotillin cap”) marks the location of the immunological synapse, where multiple signalling molecules, such as CD3 and Thy1, are recruited (Rajendran *et al.*, 2003). Flotillin platforms were also identified as the site of T cell receptor (TCR) activation. Here, following co-stimulation with anti-CD3 and anti-CD28 antibodies, TCR-associated signalling components, such as linker for activation of T cells (LAT) and the Src kinase Lck, can assemble in flotillin rafts to promote TCR signalling (Slaughter *et al.*, 2003).

Further diversifying the signalling capabilities of flotillins, they have also been implicated in G protein-coupled receptor (GPCR) signalling. Flotillins bind with Gαq, an effector of GPCRs, and promote the activation of p38 mitogen-activated protein kinase (MAPK). Interestingly, this activation is sensitive to the Src family tyrosine kinase inhibitor PP2, suggesting Src-mediated regulation of flotillins (Sugawara *et al.*, 2007). Indeed, it was later shown that both Flot1 and Flot2 can be phosphorylated by Src and Fyn kinases and that this regulates their localisation and endocytosis (Neumann-Glesen *et al.*, 2007; Riento *et al.*, 2009).

Phosphorylation of Flot1 and Flot2 by Src kinases can be induced by epidermal growth factor (EGF) signalling. Flotillins were originally suggested to regulate EGF signalling by acting as a platform for EGFRs and promoting their membrane localisation. However, it was later shown that flotillins are more directly involved in the transduction of EGFR signals. EGF stimulation promotes Src- or Fyn-mediated

phosphorylation of Flot1 and Flot2 at Y160 and Y163, respectively, promoting their internalisation (Neumann-Glesen *et al.*, 2007; Riento *et al.*, 2009). In Flot1-deficient HeLa cells, EGF-induced clustering of EGFRs and subsequent MAPK signalling is attenuated (Amaddii *et al.*, 2012). Flotillins have also been implicated in other growth factor signalling, such as fibroblast growth factor (FGF). Here, flotillins promote FGF signalling through recruitment of the signalling component FRS2$\alpha$ to FGFRs (Tomasovic *et al.*, 2012).

Together, these examples demonstrate the importance of flotillins in a multitude of signalling pathways due to their nature as multifunctional signalling platforms. However, more recently, it has become more evident that flotillins can also regulate signalling through endocytic events.

### 1.8.2. Flotillin-mediated endocytosis

Endocytosis is commonly referred to by two distinct mechanisms; clathrin-mediated endocytosis (CME) or caveolae-mediated endocytosis (or clathrin-independent endocytosis, CIE). Clathrin is comprised of three heavy and three light chains which form a triskelion structure. Clathrin monomers can interact to form a polyhedral ‘cage’ which surrounds plasma membrane invaginations (called clathrin-coated pits (CCPs))(Royle, 2006; Kaksonen and Roux, 2018). Attachment of clathrin to the membrane is achieved through adaptor proteins. The coated pits then bud from the surface in a dynamin-dependent manner, which mediates membrane scission. Once internalised, the clathrin coat disassembles and the uncoated vesicles fuse with early endosomes, where cargo is sorted into different endocytic routes, e.g., for degradation or recycling (Kaksonen and Roux, 2018).

Caveolae-mediated endocytosis was the first CIE mechanism described and is coordinated by caveolins 1-3 (Williams and Lisanti, 2004). Similarly to clathrin, caveolins homo-oligomerise and induce membrane curvature, producing caveolae (“little caves”) of 50-100 nm in diameter. Budding of caveolae from the membrane also requires the GTPase dynamin and vesicles subsequently fuse with early endosomes for sorting (Williams and Lisanti, 2004; Hansen and Nichols, 2009).

The key differences between CIE and CME are in their localisations and intracellular trafficking routes. Caveolae are found in cholesterol-rich membrane microdomains enriched in specific lipids; often called lipid rafts; and occupy distinct regions of the membrane from clathrin (Lajoie and Nabi, 2010). Following internalisation into early endosomes, cargo undergoing CIE is commonly sorted into a recycling endocytic route via Rab4- or Rab11-positive recycling endosomes, where cargo is shuttled back to the PM. Conversely, CME cargo is often associated with protein degradation, whereby cargo is trafficked to Rab7-positive late endosomes and subsequently the lysosome (Cullen and Steinberg, 2018). For example, Wnt3a induces internalisation of the co-receptor Lrp6 via CIE to trigger signal transduction, and Lrp6 is subsequently recycled back to the membrane (Yamamoto *et al.*, 2006). However, following binding of the Lrp6 inhibitor Dkk1, Lrp6 is internalised via CME and targeted for lysosomal degradation (Liu *et al.*, 2014).

More recently, a third type of endocytosis mediated by flotillins has been described. Flotillins were first associated with endocytosis upon the observation that Flot1-enriched portions of the membrane, as visualised by total internal reflection (TIR) and electron microscopy, bud into the cell and are distinct from clathrin or caveolae (Glebov et al., 2006). Additionally, internalisation of CD59, a GPI-anchored protein, was reduced upon siRNA knockdown of Flot1. Since then, numerous cargo for flotillin-mediated endocytosis have been identified, including cholera toxin B subunit (CTxB) and its receptor ganglioside GM1, and Niemann-Pick C1-like (NPC1L1), an important mediator of the uptake of dietary cholesterol (Saslowsky *et al.*, 2010; Ge *et al.*, 2011).

Flotillins were originally thought to be present in caveolae and form a hetero-oligomeric complex with caveolins to assist in vesicle formation and endocytosis (Bickel *et al.*, 1997; Volonté *et al.*, 1999). Flot1 was also reported to associate with and stabilise caveolin-1 in intestinal epithelial cells (Vassilieva *et al.*, 2009). However, it has since been shown that flotillins occupy distinct membrane microdomains and induce membrane curvature and caveolae-like structures in cell types lacking caveolin expression (Stuermer *et al.*, 2001; Frick *et al.*, 2007). Furthermore, altering caveolin expression does not perturb Flot2 localisation and Flot1 knockdown does not interfere with caveolin-mediated endocytosis (Glebov *et al.*, 2006; Fernow *et al.*, 2007). Interactions between caveolins and flotillins could also not be detected by

yeast two-hybrid or co-immunoprecipitation assays (Fernow *et al.*, 2007). Therefore, flotillins are considered to mediate a third type of endocytosis independent from clathrin or caveolin.

Despite these findings, the topic is still debated and another mechanism of action proposed, which involves co-operation of flotillins and clathrin, is flotillin-assisted endocytosis. In this model, flotillin is required for the pre-endocytic clustering of certain cargo at the membrane, such as EGFRs, dopamine transporter (DAT) and amyloid precursor protein (APP). However, the cargo then undergo clathrin-mediated endocytosis without direct involvement of flotillins (Meister and Tikkanen, 2014). This model arises from the following observations: (1) Depletion of Flot1 does not affect the endocytosis of EGFR, nor its ubiquitination (Amaddii *et al.*, 2012). (2) Flot2 promotes the clustering of APP at the membrane, but its internalisation is attenuated by inhibition of CME through AP-2 knockdown (Schneider *et al.*, 2008). (3) Similarly, flotillins are necessary for the clustering and reduced mobility of DAT prior to endocytosis, but its internalisation is also clathrin-dependent (Cremona *et al.*, 2011).

Whilst the mechanistic underpinnings are still disputed, it is clear from these examples that flotillins play an important role in the regulation of endocytic events and subsequent downstream signalling.

### 1.8.3. The role of Dynamin in Flotillin endocytosis and trafficking

Similarly to clathrin- and caveolae-mediated endocytosis, flotillin-mediated endocytosis relies on the membrane scission protein dynamin. Dynamin is a GTPase which mediates vesicle fission during endocytosis and is required for successful internalisation of cargo (Singh *et al.*, 2017). Flot1 endocytosis was originally shown to be dynamin-independent, as expression of a dominant-negative K44A dynamin mutant, which is deficient in GTP binding and hydrolysis, did not affect uptake of CTxB into Flot1-positive vesicles (Glebov *et al.*, 2006). Contradicting these findings, it was later shown that internalisation of GPI-anchored proteins and proteoglycan-bound ligands via flotillin-mediated endocytosis was dynamin-dependent (Payne *et al.*, 2007; Aït-Slimane *et al.*, 2009). Furthermore, internalisation of Flot1 and Flot2

following EGF stimulation requires dynamin, and inhibition of dynamin with the chemical inhibitor dynasore impaired their translocation from the PM to late endosomes (Meister and Tikkanen, 2014). The authors speculate that discrepancies in dynamin dependency could be due to differences in endocytosis stimulation, i.e., constitutive uptake vs active signalling. However, the general consensus is that dynamin is required, as no other papers corroborate the findings of Glebov *et al.,* (2006).

However, dynamin also has lesser known functions beyond endocytosis and its membrane scission is involved in intracellular trafficking, including tubular fission of early endosomes, budding of transport vesicles from the Golgi and recycling of endosomes to the *trans*-Golgi network (Jones *et al.*, 1998; Nicoziani *et al.*, 2000; Mesaki *et al.*, 2011). Consequently, inhibition or downregulation of dynamin also perturbs intracellular transport of a number of cargo. For example, expression of the K44A dynamin mutant causes redistribution of the cation-independent mannose 6-phosphate receptor (CI-MPR) from late endosomes into LAMP1-positive vesicles, suggesting defective recycling to the Golgi (Nicoziani *et al.*, 2000). In MDCK cells, this impairs post-Golgi transport of the neurotrophin receptor, p75, and thus it accumulates in the Golgi (Kreitzer *et al.*, 2000).

More recently, dynamin has been implicated in the intracellular transport of Flot2. Over-expression of $Dyn2^{K44A}$ causes accumulation of Flot2 in the perinuclear region, where it colocalises with the late endosomal marker LAMP3 (Meister *et al.*, 2014). Interestingly, treatment of HeLa cells with the chemical inhibitor dynasore only resulted in partial co-localisation of Flot2 and LAMP3. The authors speculate this could be inhibiting Flot2 transport at a different point in its transport, however, it has been suggested that off-target effects of dynasore cause disruptions in lipid rafts and actin cytoskeletal regulation (Preta *et al.*, 2015). Thus, the discrepancies could be due to perturbation of other processes which involve Flot2 function.

### 1.8.4 Flotillin-2 and endosomal cargo sorting

More recent research has revealed Flot2 is not only involved in the uptake of some cargo through endocytosis, but also intracellular trafficking and recycling through endosomal compartments (Meister and Tikkanen, 2014). Whilst Flot1 knockdown

was shown to attenuate CTxB uptake via endocytosis (Glebov *et al.,* 2006), this work was later contradicted by Saslowsky et al., (2010) who showed that whilst depletion of either flotillins did render zebrafish resistant to CTB intoxication, this was due to attenuated transport of CTB-GM1 complexes from endosomes to the ER via the trans-Golgi network (TGN), not a defect in endocytosis (Saslowsky *et al.*, 2010). Supporting this, Flot1- and Flot2-depleted mammalian COS-1 cells were also resistant to CT and CTB localised to the ER in only 36% of cells lacking Flot1, compared to 80% in control cells. Therefore, the authors concluded flotillins are involved in the endosomal sorting of CTB-GM1 from the plasma membrane to the ER.

Flotillins are similarly important in the processing of the plant toxin ricin and bacterial Shiga toxin (Stx). Concurrent with Saslowsky et al., silencing of both flotillins showed no defect in endocytic uptake of the toxins, but inhibited their intracellular transport (Pust et al., 2010). Specifically, the retrograde transport of Stx and ricin to the Golgi were inhibited. Interestingly, whilst flotillin depletion reduces the toxicity of CT, the opposite occurs for Stx and ricin, where flotillin inhibition causes an accumulation of these toxins and thus increases their toxicity. However, both seem to be caused by defective retrograde transport of these toxins.

A function for Flot2 in trafficking cargo to and/or from the Golgi is conceivable given that Flot2 has been shown to localise to the Golgi, as seen by immunogold labelling and co-localisation with the Golgi marker GM130 (Langhorst et al., 2008). The N-terminal SPFH domain of Flot2 is necessary for Golgi exit, as a mutant lacking this domain accumulates in the Golgi. Furthermore, trafficking of newly synthesised Flot1 and Flot2 is sensitive to brefeldin A (BFA), an inhibitor of COPI vesicles responsible for anterograde ER-Golgi trafficking. This contradicts findings by Morrow et al., (2002), who showed that Flot1 traffics to the membrane in a BFA-resistant manner, suggesting a Golgi-independent pathway (Morrow et al., 2002). The reason for these discrepancies is unclear, although Langhorst et al., (2008) suggest potential differences in palmitoylation enzymes in the different cell types used in the respective studies. Differences in BFA treatments may also explain these discrepancies, although Langhorst et al., (2008) do not state the concentration of BFA used.

### 1.8.5. Flotillin-2, actin and filopodia

Several studies have shown that the membrane localisation and stability of Flot2 is dependent on interactions with the actin cytoskeleton, which occur via its SPFH domain (Langhorst *et al.*, 2007; Rossy *et al.*, 2009; Affentranger *et al.*, 2011). Indeed, stabilisation of F-actin by Jasplakinolide treatment decreases the lateral mobility of Flot2 microdomains (Langhorst *et al.*, 2007). Similarly, formation of flotillin “caps” in neutrophils is perturbed by disruption of the actin cytoskeleton by latrunculin and this attenuates uropod formation, which is required for neutrophil migration (Rossy *et al.*, 2009). However, the relationship between actin and Flot2 is bidirectional, as Flot2 also regulates actin cytoskeleton components. One of the most notable and observable phenotypes of Flot2 function is that modulation of its function and expression are associated with drastic changes in cell morphology due to changes in actin-based structures, such as filopodia and lamellipodia, which are involved with cell migration and spreading (Hazarika *et al.*, 1999; Rajendran *et al.*, 2003; Langhorst *et al.*, 2008).

In multiple cell lines over-expression of Flot2 induces a filopodial phenotype by increasing the number of filopodia (Hazarika *et al*., 1999; Rajendran *et al*., 2003; Neumann-Giesen, Falkenbach, Beicht, Claasen, Uers, *et al.*, 2004). In contrast, over-expression of a dominant-negative Flot2 mutant in N2a neuroblastoma cells decreases the length of filopodia (and number of neurites) whilst producing more lamellipodia-like (growth cone) structures (Langhorst, Jaeger, *et al.*, 2008). Similar phenotypes are observed upon siRNA-mediated knockdown of Flot2 (Munderloh *et al.*, 2009).

Flot2 is thought to alter the actin cytoskeleton by interacting with and/or activating a number of cytoskeletal regulators, including the small Rho GTPases RhoA, Rac1, and Cdc42, which are thought to promote formation of stress fibers, lamellipodia, and filopodia, respectively (Nobes and Hall, 1995; Raftopoulou and Hall, 2004). Indeed, Flot2 knockdown in N2a cells (which have fewer filopodia and abnormally large lamellipodia) was shown to perturb the activation of all three GTPases, with RhoA and Rac1 activation increasing and Cdc42 activation decreasing (Munderloh *et al.*, 2009). Alongside known functions of Cdc42 in promoting filopodia formation, the aforementioned effects of Flot2 on filopodia are suggested to arise through perturbed

activation of Cdc42 and thus its downstream effectors, such as cortactin, Arp2/3 and IRSp53 (Fig. 8) (Munderloh *et al.*, 2009).

Thus, flotillins have been implicated with regulating cell migration and polarity, which are actin-dependent processes. In Flot1-knockout mice, loss of the flotillin "cap" and thus cell polarity in neutrophils impairs their ability to migrate towards chemoattractant cues *in vivo* (Ludwig *et al.*, 2010). Mechanistically, Flot1 is suggested to interact with cytoskeletal proteins myosin II and spectrin. Similarly, Flot1-deficient T cells show altered cell morphology and impaired migratory capabilities, which are attributed to loss of myosin II-mediated contractility (Ficht *et al.*, 2019).

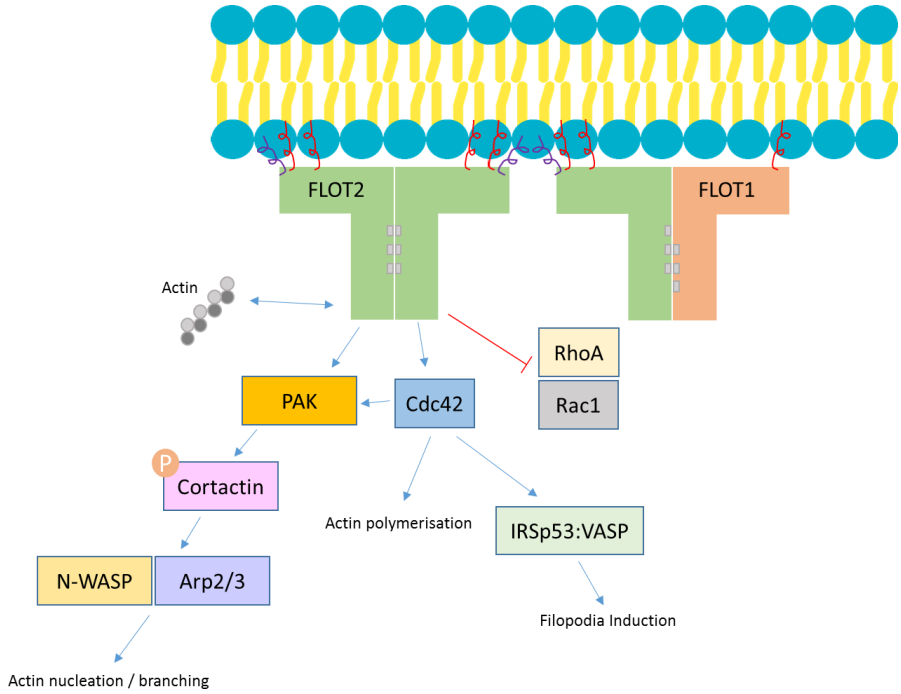


**Figure 8 – Flotillin-2 and the actin cytoskeleton.** Flotillin-2 (Flot2) regulates the actin cytoskeleton through activation of the Rho GTPase Cdc42 and inhibition of RhoA and Rac1. Inactivate Cdc42-GDP interacts with the SH3 domain of IRSp53, an adaptor protein, providing autoinhibition. Activated Cdc42 (Cdc42-GTP) promotes actin polymerisation and formation of filopodia through interactions with the CRIB motif of IRSp53, relieving inhibitory interactions with its SH3 domain. This permits the formation of IRSp53:VASP complexes, which promote filopodia induction. Flot2 also stimulates actin filament nucleation through activation of PAK and subsequent phosphorylation of cortactin. Cortactin interacts with N-WASP and Arp2/3 to form complexes required for actin nucleation and branching.

### 1.9. Flotillin-2 in Disease

As highlighted by this literature review, the multifunctionality and promiscuity of Flot2 as a scaffolding protein results in its involvement in a broad range of signalling pathways and cellular processes. Naturally, dysfunction or dysregulation of Flot2 is therefore implicated in a number of disease contexts, some of which are highlighted below.

#### 1.9.1. Flotillin-2 in Neurodegenerative Diseases

Alzheimer's disease (AD) is a type of dementia characterised by loss of hippocampal neurons and thus attenuation of memory function, among other symptoms. Pathologically, it is characterised by the accumulation of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFTs), which are detrimental to the health and function of hippocampal neurons (Deture and Dickson, 2019). Aβ is produced through the cleavage of amyloid precursor protein (APP) by β- and γ-secretases, including β-site APP-cleaving enzyme (BACE1) (Angelopoulou *et al.*, 2020). This cleavage of APP is thought to occur in lipid rafts and this process requires endocytosis of APP into early endosomes to be cleaved by BACE1 (Vetrivel and Thinakaran, 2006). These endocytic events are thought to be flotillin-mediated, since Flot2 enhances APP clustering and knockdown of Flot2 impairs APP endocytosis, reducing Aβ production in primary culture hippocampal neurons (Schneider *et al.*, 2008). It was later shown that Flot2 (with Flot1) interacts with BACE1 and mediates its intracellular trafficking through endosomes (John *et al.*, 2014). Thus, Flot2 has become a protein of interest in AD research, both as a clinical biomarker and potential therapeutic target (Angelopoulou *et al.*, 2020).

Flot2 is also implicated in the pathogenesis of Prion diseases. Prions are GPI-anchored proteins which can induce the misfolding of other wildtype Prion ($PrP^{c}$) proteins into an abnormal isoform ($PrP^{Sc}$), which causes them to accumulate and impair neuronal function. This effect is not limited to a specific brain region, as in AD and PD, and spreads throughout multiple brain regions. Thus, prion diseases have rapid progression and patients usually die within 1 year of diagnosis. This is seen in Creutzfeldt-Jakob disease (CJD), which is a human prion disease with a 95% mortality rate (Sun *et al.*, 2020). Mechanistically, Flot2 is thought to directly interact with $PrP^{c}$ proteins, which promote PrP-Flot2 clustering in flotillin microdomains and

subsequent activation of Fyn and MAP kinases, as well as recruitment of N-cadherin to cell adhesion sites (Bodrikov *et al.*, 2011). This association of $PrP^{c}$ to flotillin microdomains is supported by the observation that in scrapie-infected mice (another prion disease), $PrP^{sc}$ accumulates in Flot1-positive vesicles, akin to the accumulation of Aβ in flotillin-positive structures in AD (Pimpinelli *et al.*, 2005). Flotillin compartments may act as a general store for aggregated proteins in neurodegenerative disorders due to its role in protein clustering and endosomal trafficking.

### 1.9.2 Flotillin-2 in Cancer

Flot2 was first linked to cancer in 2004 when it was found to be upregulated in metastatic melanomas, where its upregulation is associated with increased migration rates *in vitro* and melanoma progression *in vivo*. (Hazarika *et al.*, 2004). Since then, Flot2 has been implicated as an oncogene in a number of cancers, including breast, colorectal and gastric cancer (Wang *et al.*, 2013; Zhu *et al.*, 2013; Li *et al.*, 2019). Flot2 expression often correlates with poor prognosis, and in some cancers it is used as a prognostic marker (Liu *et al*., 2018). IHC analysis of Flot2 expression in breast cancer specimens revealed 48% of patients showed high Flot2 expression, which significantly correlated with clinical stage (Wang *et al.*, 2013). Similarly, renal cell carcinoma (RCC) specimens showed upregulated Flot2 compared to adjacent matched tissue and Flot2 expression was an independent prognostic marker for overall RCC patient survival (Yan *et al.*, 2014).

In GC, over-expression of Flot2 is observed in both patient tissue samples and cell lines and is associated with increased cell proliferation, migration and invasion (Cao *et al.*, 2013; Liu *et al.*, 2018). siRNA-mediated knockdown of Flot2 abrogates these effects (Cao *et al.*, 2013). Additionally, miRNAs miR-449a and miR-133, which target and promote degradation of Flot2 mRNA, are frequently downregulated in GC cancer; correlating with increased levels of EMT markers and increased invasiveness (Cheng *et al.*, 2014; Li *et al.*, 2015; Wei *et al.*, 2018).

Mechanistically, the function of Flot2 in tumourigenesis is broadly related to its capability to modulate RTK signalling pathways. For example, in some breast and gastric cancers, Flot2 is associated with the upregulation of the RTK ErbB2/HER-2,

which signals to promote proliferation, among other functions (Moasser, 2007; Zhu *et al.*, 2013). Flot2 is thought to stabilise ErbB2 at the PM, as depletion of Flot1 and Flot2 leads to internalisation and degradation of ErbB2 (Pust *et al.*, 2013). Concurrently, another study found that silencing of Flot2 function in breast cancer cells decreased the activation of the PI3K/Akt signalling pathway, which acts downstream of ErbB2 (Xie *et al.*, 2015). Whereas in hepatocellular carcinoma cells, Flot2 promotes EMT and cell cycle progression by modulating Raf/MEK/ERK1/2 signalling, which is downstream of the EGFR (Wang *et al.*, 2017). However, these apparently discrete mechanisms are somewhat intertwined. Flot1 has been shown to regulate H-Ras activity in breast cancer cells (Koh *et al.*, 2016). H-Ras is a GTPase which is a central modulator of kinase signal cascades. Here, it is activated by EGF and ErbB2 signalling but acts upstream of PI3K/Akt and ERK1/2 signalling cascades (Gimple and Wang, 2019). Hence, despite apparent disparities between mechanistic underpinnings of Flot2 in these cancers, these pathways are linked and the common denominator is kinase signalling.

### 1.10. Flotillin-2 and Wnt Signalling

As well as kinase signalling, Flot2 has also been implicated in Wnt signalling. In *Drosophila* wing imaginal disc, Flot2 misexpression expands the expression domain of Wingless (Wg), the Drosophila Wnt homologue, and leads to formation of abnormal wings resembling a Wg overexpression phenotype (Hoehne et al., 2005). Concurrently, Flot2 over-expression or knockdown expands or shortens the gradient of Wg and enhances long-range Wg target gene expression (Katanaev et al., 2008). Wg and Flot2 also partially co-localise at the plasma membrane and in intracellular compartments; which may represent a portion of Wg which is secreted via a Flot2-dependent mechanism. Indeed, a portion of Wg activity has been shown to depend on lipid raft-dependent pathways (Zhai *et al.*, 2004) and tissue culture of *Drosophila* cell lines revealed that Flot2 over-expression increases secretion of Wg (Katanaev et al., 2008).

It is unclear how, mechanistically, Flot2 enhances Wg secretion. It is suggested Flot2 could enhance packaging of Wg into secretory vesicles, such as lipoproteins, since lipoprotein receptors localise to lipid rafts and knockdown of apolipophorin (the *Drosophila* homologue of apolipoproteins) reduces extracellular Wg in both apical

and basolateral epithelial regions (Panáková et al., 2005). Similarly, Flot2 has been suggested to aid loading of Wg into argosomes; membranous vesicles similar to exosomes, which can travel in a paracrine fashion, but are synthesised directly from the producing-cell membrane; a mechanism proposed to occur in lipid raft domains (Greco *et al.*, 2001; Vincent and Magee, 2002). However, there is no direct evidence for either of these hypotheses. These effects of Flot2 on Wnt secretion and signalling have not been assessed in vertebrates and so it is yet to be determined whether these are species- or Wg-specific effects, or whether other mechanisms may be at play.

### 1.11. Aims of this work

The role of cytonemes in Wnt transport and signalling is becoming increasingly evident. Previous work from our lab showed that in GC cells Wnt8a is transported intercellularly via cytonemes in a Ror2-dependent manner (Mattes *et al.*, 2018). My research aimed to continue to further decipher the mechanisms underlying cytoneme-mediated Wnt transport in GC cells and evaluate their importance in the transport of more physiologically relevant Wnt proteins, such as Wnt3, which is overexpressed in GC (Wang *et al.*, 2016).

On a molecular level, this work aimed to decipher the role of Flot2 in cytoneme-mediated Wnt transport. Flot2 was identified as a protein of interest due to the following: (1) Since Flot2 regulates cytoskeletal dynamics, including inducing and enhancing filopodial phenotypes, it follows that Flot2 may influence the formation of cytonemes; an actin-dependent process (Neumann-Giesen *et al.,* 2004) . (2) Flot2 has been associated with promoting Wg signalling in *Drosophila* wing imaginal disc, where Flot2 over-expression or knockdown expands or shortens the gradient of Wg and enhances long-range Wg target gene expression (Katanaev *et al.*, 2008). (3) Flot2 has been shown to enhance the Hh gradient and formation of Hh cytonemes in *Drosophila* (Bischoff *et al.*, 2013). (4) Flot2 is frequently over-expressed in GCs, where the Wnt signalling pathway is commonly over-activated (Cao *et al.*, 2013). I hypothesised that regulation of Wnt cytonemes by Flot2 may offer an explanation for

the above observations, by promoting filopodia (and therefore cytoneme) formation to enhance Wnt transport (and thus signalling).

Using *in vitro* techniques to quantify Wnt cytonemes and cytoneme-mediated signalling, I aimed to assess the ability of GC cells to transport fluorescently-tagged and endogenous Wnt proteins via cytonemes and the impact these have on cell proliferation. On a molecular level, using high-resolution microscopy and manipulating protein function(s) by knockdown or mutagenesis, I aimed to assess the impact of Flot2 on cytoneme formation and decipher a potential mechanism of action. Finally, I aimed to validate these findings *in vivo* using the zebrafish model organism and identify a potentially conserved function for Flot2 in Wnt cytoneme regulation.

## 2. Materials & Methods

### 2.1. Materials

#### 2.1.1. DNA Plasmids

| DNA Construct | Plasmid backbone | Source |
|---|---|---|
| Membrane-mCherry | pCS2+ | Scholpp *et al.,* 2009 |
| Membrane-GFP | pCAG-mGFP | Addgene #14757 |
| Flotillin-2-GFP | pEGFP-N1 | Neumann-Giesen *et al.,* 2004 |
| Flotillin-2-GFP | pCS2+ | Flotillin-2 CDS was cloned into pCS2+-GFP with ClaI and XbaI |
| ΔN-Flotillin-2-GFP | pEGFP-N1 | Neumann-Giesen *et al.,* 2004 |
| Y163F-Flotillin-2-GFP | pEGFP-N1 | Neumann-Giesen *et al.,* 2004 |
| Ror2-eBFP | pCS2+ | Cloned by inserting eBFP2 into pCS2+ Ror2 plasmid using XbaI and SnaBI |
| mRor2-ΔCRD-GFP | pEGFP-N3 | XhoI-XhoI mRor2 insert taken from pcDNA-mRor2, subcloned into SalI site of pEGFP-N3 vector |
| hRor2-mCh | pCS2+ | hRor2 cloned from AGS cells was inserted into pCS2+-mCh using ClaI and XbaI |
| hRor2-GFP | pCS2+ | hRor2 cloned from AGS cells was inserted into pCS2+-GFP using ClaI and XbaI |
| Wnt1 | pcDNA3.2 | Addgene #35905 |
| Wnt3 | pcDNA3.2 | Addgene #35909 |
| Wnt1-mCh | pCS2+ | Cloned into pCS2+-mCh backbone with ClaI and XbaI |
| Wnt3-mCh | pCS2+ | Cloned into pCS2+-mCh backbone with ClaI and XbaI |
| RFP-Dyn$^{K44A}$ | pEGFP | Addgene #128153 |

| | | |
|---|---|---|
| LifeAct-GFP | pCS2+ | Addgene #128428 |
| Lrp6-mCherry | pCS2+ | Chen *et al.,* 2014 |
| IRSp53$^{4K}$-GFP | pCS2+ | Mattes *et al.,* 2018 |
| 7xTCF-NLS-mCherry | pDEST | Moro *et al.,* 2012 |
| JNK-KTR-mCherry | pPBbsr | Addgene #115493 |
| Wnt8a-mCh | pCS2+ | Stanganello *et al.,* 2015 |
| mTurq2-Golgi | pmTurqoise2-N1 | Addgene #36205 |
| LAMP1-mTurqoise2 | pEGFP-N1 | Addgene #98828 |
| mCherry-Rab4 | pCS2+ | Hagemann *et al.,* 2009 |
| mCherry-Rab11 | pCS2+ | Hagemann *et al.,* 2009 |
| Rab5-GFP | pCS2+ | Zerial group |
| Rab7-eGFP | pCS2+ | Rudiger Rudolf group |
| Frizzled 7-GFP | pCS2+ | Fzd7 cloned into pCS2+-GFP plasmid from Fzd7-CFP (Mattes *et al.* 2018) |
| mRFP-Clathrin | pmRFP-C3 | Addgene #14435 |

### 2.1.2. Antibodies

| Antibody | Working Dilution | Source | Validation |
|---|---|---|---|
| Anti-Wnt3 | 1:100 | Abcam #116222 | In this thesis (Fig. 10) |
| Anti-Flotillin-2 | 1:100 (IF), 1:1000 (WB) | Abcam #96507 | In this thesis (Fig. A4) |
| Anti-Lrp6 | 1:100 | Abcam #24386 | See references in Abcam |
| Anti-Calnexin | 1:100 | Abcam #92573 | See references in Abcam |
| Anti-EEA1 | 1:100 | Abcam #2900 | See references in Abcam |
| Anti-beta-actin | 1:1000 | Proteintech® 60008-1-Ig | In this thesis (Fig.15, A4) |
| Anti-Caveolin-1 | 1:10 | Abcam #17052 | See references in Abcam |
| Anti-Myosin-X | 1:10 | Santa Cruz C-1 | See references on website |
| Anti-Evi | 1:50 | EMD Milipore YJ5 | See references on website |
| Anti-Ror2 | 1:10 | Santa Cruz H-1 | See references on website |
| Anti-Wnt1 | 1:100 | Abcam #63934 | See references in Abcam |
| Goat anti-rabbit AlexaFluor®488 | 1:1000 | Abcam #150077 | See references in Abcam |

| | | | |
|---|---|---|---|
| Goat anti-rabbit AlexaFluor®568 | 1:1000 | Abcam #175471 | See references in Abcam |
| Goat anti-mouse IRDye®800CW | 1:5000 | Abcam #216772 | See references in Abcam |
| Donkey anti-rabbit AlexaFluor®680 | 1:5000 | Abcam #175772 | See references in Abcam |
| Donkey anti-goat AlexaFluor®647 | 1:1000 | Abcam #150135 | See references in Abcam |
| Donkey anti-rabbit AlexFluor®488 | 1:1000 | Abcam #150073 | See references in Abcam |
| Donkey anti-mouse AlexaFluor®568 | 1:1000 | Abcam #175472 | See references in Abcam |
| Phalloidin iFluor405 | 1:1000 | Abcam #176752 | See references in Abcam |
| Phalloidin iFluor594 | 1:1000 | Abcam #176757 | See references in Abcam |

### 2.1.3. Primers

| Primer | Protocol | Sequence (5’ → 3’) |
|---|---|---|
| Flotillin-2 F | qPCR | GGCTTGTGAGCAGTTTCTGG |
| Flotillin-2 R | qPCR | AATCTGCTCCACTGTCAGGG |
| GAPDH F | qPCR | GTCTCCTCTGACTTCAACAGCG |
| GAPDH R | qPCR | ACCACCCTGTTGCTGTAGCCAA |
| Wnt1 F | Cloning | GGGGATCGATATGGGGCTCTGGGCGCT |
| Wnt1 R | Cloning | TGTGTCTAGACAGACACTCGTGCAGTAC |
| Wnt3 F | Cloning | TGTGATCGATATGGAGCCCCACCTGCTC |
| Wnt3 R | Cloning | TGTGTCTAGACTTGCAGGTGTGCACGTCGT |
| Flotillin-2 F | Cloning | TGTGATCGATATGGGCAATTGCCACACGGT |
| Flotillin-2 R | Cloning | CTGATCTAGACACCTGCGCACCAGTGGC |

## 2.2. Methods

### 2.2.1. Cell Culture Experiments

The following cell lines, kindly gifted from Dr. Toby Phesse (Cardiff University), were used: MKN7 (Gastric tubular adenocarcinoma, derived from metastatic site (lymph nodes), point mutation in TP53), MKN28 (Gastric tubular adenocarcinoma, derived from metastatic site (liver), point mutation in TP53) and AGS (Lgr5+ cancer stem cells derived from primary gastric adenocarcinoma, point mutation in CTNNB1) (Flanagan et al., 2019). HFE-145 (normal human gastric epithelial) cells were kindly provided by Hassan Ashktorab.

AGS, MKN7 and MKN28 cells were maintained in RPMI-1640 (Sigma-Aldrich), and HFE-145 in DMEM (Thermofisher) media, all antibiotic-free and supplemented with 10% FBS. Cells were routinely passaged (with 0.05% EDTA-free trypsin (Thermofisher)) at ~80% confluency.

Transient transfections of cells, either reverse or forward, were performed using FuGene HD (Promega) according to manufacturer's protocol using 3:1 FuGene:DNA ratio. siRNA transfections were performed using Lipofectamine RNAiMAX (Thermofisher) according to manufacturer's protocol, with a final siRNA concentration of 30 pmol. Control siRNA (MISSION® siRNA Universal Negative Control #1) and Flotillin-2 siRNA (Thermo Fisher 122408) were used.

For endocytosis studies, AGS cells were grown in serum-free media for 24 hrs prior to treatments. Cells were treated with 250ng/µl Wnt3a (Biotechne) or Dkk1 (Biotechne) for 30 mins.

### 2.2.2. 7xTCF-NLS- mCherry TOPFlash Assays

For autocrine signalling (single cell population), $1x10^6$ HFE or AGS cells were reverse transfected with 7xTRE Super TOPFlash (STF) reporter plasmid along with indicated plasmids. Cells were incubated for 48 hrs before image acquisition on a Leica DMI6000 SD with a 20x objective.

For co-cultivation assays, $2x10^6$ HFE-145 cells were reverse transfected with 7xTRE Super TOPFlash plasmid (7xTCF-NLS-mCherry) and $2x10^6$ AGS cells with indicated plasmids in 6 well plates. After 24 hrs incubation, both cell types were trypsinised,

counted and $1x10^6$ of each were co-cultivated in 6 well plates for a further 48 hrs before image acquisition.

For all assays, at least 5 images were taken in random locations for each biological repeat on a 20x objective. Fluorescence intensity of nuclei were measured on Fiji software by drawing around the nucleus of cells and subtracting background, which was taken as an average from circles on the image where no cells were present. The number of fluorescent nuclei per 20x image were also counted by eye (using LASX software) as a measure of cell number. The control with untransfected AGS cells was used as the baseline, and differences in cell number for other conditions were relative to this.

### 2.2.3. JNK-KTR-mCherry Assay

AGS cells stably expressing JNK-KTR-mCherry (AGS-JNK8) were reverse transfected with indicated plasmids and incubated for 48 hrs. Cells were imaged on a Leica DMI6000 SD with a 20x objective. At least 5 images were taken in random locations for each biological repeat. Fluorescence intensity of each cell's cytoplasm and nucleus was measured using Fiji software. A circle was drawn around the nucleus and measured, and the entire cytoplasm was then drawn around (minus the nucleus) and measured. In the case where the nucleus was not clearly visible due to differences in fluorescence, the brightfield image was used to decipher nucleus-cytoplasm boundaries. After background subtraction, cytoplasmic:nuclear ratio (C:N) was calculated.

### 2.2.4. Antibody Staining

Cells were plated onto glass coverslips and following indicated treatment/incubation, cells were washed in 1xPBS and fixed using 4% PFA (10 mins, RT) or modified MEM-Fix (4% formaldehyde, 0.25-0.5% glutaraldehyde, 0.1M Sorenson's phosphate buffer, pH7.4) (Bodeen et al., 2017; Rogers and Scholpp, 2021) for 7 mins at 4$^{o}$C. Cells were then incubated in permeabilisation solution (0.1% Triton-X-100, 5% serum, 0.1M glycine in 1xPBS) for 1hr at RT. Primary antibodies were diluted in incubation buffer (0.1% Tween20, 5% goat serum in 1xPBS) and coverslips incubated in 50 µl spots on parafilm overnight at 4$^{o}$C. Coverslips were then washed with 1xPBS 3x for 5 mins before incubation in 50 µl spots of secondary

antibodies
diluted in incubation buffer for 1hr at RT. Coverslips were then washed 3x for 15 mins with 1xPBS before mounting onto glass slides using ProLong Diamond anti-fade mountant (Invitrogen) and left to dry for 24hrs before imaging. Confocal microscopy for immunofluorescent antibody imaging was performed on an inverted Leica TCS SP8 X laser-scanning microscope using the 63x water or oil objectives.

### 2.2.5. RT-qPCR

RNA for qPCR was collected from cell pellets using QIAGEN RNeasy kit according to manufacturer's protocol. RT-qPCR was then performed using SensiFAST™ SYBR® Lo-ROX One-Step Kit with half volumes according to manufacturer's protocol and run using Applied Biosystems QuantStudio6 Flex. See Table 3 for primer sequences.

### 2.2.6. Western Blotting

Cell lysates were collected from cell pellets by resuspending in 100uL of ice cold TNT lysis buffer (20mM Tris pH8.0, 200mM NaCl, 0.5% Triton-X-100, 1X cOmplete™ EDTA-free protease inhibitor) (Sigma Aldrich) per $1x10^6$ cells. Cells were agitated in lysis buffer on ice for 30 mins before centrifuging at 12,000RPM, 4°C for 20 mins and removing the supernatant. Pierce™ BCA protein assay kit was then used (according to manufacturer's microplate protocol) to calculate protein concentrations.

For SDS-PAGE gel electrophoresis, required volume of 4x Laemmli buffer (with 10% βME) was added to protein samples and incubated at 95°C for 5 mins. 50µg of protein was then loaded into BIORAD Mini-PROTEAN pre-cast gels (12% acrylamide) and run at 60V for 2hrs. Semi-dry transfer onto nitrocellulose or PVDF membrane was performed (Thermo Scientific Pierce G2 fast blotter) for 15 mins at 15V, 1.3A. Membranes were then blocked in 5% non-fat milk for 1hr at RT before adding primary antibodies to desired concentrations and incubating on a roller overnight at 4°C. The following day, membranes were washed 3x with TBS-T before incubating in secondary antibodies in TBS-T for 1hr at RT. Membranes were then washed 2x with TBS-T and 3x with TBS before imaging on LiCor Odyssey CLx and processing on ImageStudio software.

### 2.2.7. BrdU Proliferation Assay

AGS and HFE-145 cells were co-cultivated on glass cover slips in 6 well plates. After 45 hrs co-cultivation, cells were incubated in media containing 10µg/ml BrdU (Abcam, ab142567) for 3 hrs. Cells were then fixed with 4% PFA for 15 mins at RT. After washing 2x with PBS, immunostaining of BrdU was performed using a BrdU immunohistochemistry kit (Abcam, ab125306) according to manufacturer's protocol. Cover slips were then mounted onto slides using Prolong Diamond Antifade mountant and left to dry for 24 hrs. Slides were then imaged on a light microscope with 20x objective and captured using an Olympus EP50 colour camera. BrdU-stained cells (brown) were counted and quantified as a percentage of the total cell population (counterstained blue with haematoxylin).

### 2.2.8. DNA Preparation and Cloning

PCR amplification of DNA for cloning was performed using the following reaction mixture:

10µl 2X CloneAmp, 0.5µl each primer, 2µl vector, 5µl $H_2O$

Restriction enzyme digests of DNA plasmids or purified PCR products for cloning were performed using New England Biolabs (NEB) restriction enzymes in the following reaction mixtures:

Digest of vector: 2µl 10X reaction buffer, 2µl each enzyme, 2µl vector, 14µl $H_2O$

Digest of inserts: 2µl 10X reaction buffer, 2µl each enzyme, 10µl PCR product or insert, 11µl $H_2O$

For ligation reactions of digested products, the following reaction mixtures were incubated at 37$^oC$ for 1hr:

Ligation: 4µl insert, 4µl vector, 1µl T4 DNA ligase, 1µl 10X buffer

DNA plasmids for transfections were generated by transforming 50µl competent *E. coli* cells with 5µl of the desired plasmid or ligation product. Competent cells were defrosted on ice for 20 mins before incubation with DNA for 30 mins. Cells were then

heat shocked at 42ºC for 45s before returning to ice for 2 mins. 600µl of LB media was then added and cells were incubated at 37ºC for 1-2 hrs on a shaker. 100µl of cells were then spread onto antibiotic-containing agar plates and incubated at 37ºC overnight. The next morning, 5-10 colonies were picked and grown in 5ml antibiotic-containing LB media on a shaker at 37ºC. For cloning purposes and subsequent screening, the next day, DNA was extracted and isolated using Qiagen mini prep kit. For isolating existing DNA plasmids for transfection, 50µl of the bacterial broth was used to inoculate 100 ml of antibiotic-containing LB media, which was incubated at 37ºC on a shaker overnight. The next day, DNA was isolated and purified using Qiagen endotoxin-free maxi prep kit according to manufacturer's protocol. DNA was reconstituted in 100µl endo-toxin free TE buffer and concentration measured using a nanodrop.

### 2.2.9. Zebrafish Maintenance and Husbandry

WIK wild-type zebrafish (*Danio rerio*) were maintained at 28°C and on a 14hr light/10hr dark cycle (Brand et al., 2002). Zebrafish care and all experimental procedures were carried out in accordance with the European Communities Council Directive (2010/63/EU) and Animals Scientific Procedures Act (ASPA) 1986. In detail, adult zebrafish for breeding were kept and handled according to the ASPA animal care regulations and all embryo experiments were performed before 120 hrs post fertilization. Zebrafish experimental procedures were carried out under personal and project licenses granted by the UK Home Office under ASPA, and ethically approved by the Animal Welfare and Ethical Review Body at the University of Exeter.

### 2.2.10. Zebrafish microinjections and image analysis

For experiments in zebrafish embryos, indicated DNA plasmids were microinjected at the 1-2 cell stage with 100 ng/µl DNA after dechorination. Embryos were left to develop at 28ºC until 8 hpf (shield stage). For confocal microscopy analysis, live zebrafish embryos were embedded in 0.7% low melting agarose (Sigma-Aldrich) dissolved in 1x Ringer's solution. Images of embryos were obtained with an upright

Leica TCS SP8X microscope equipped with hybrid detectors (HyD) using 63x dip-in objective.

### 2.2.11. In situ hybridisation (ISH)

Pax6a digoxigenin (DIG) antisense RNA probes were generated from linearised plasmids using an RNA labelling and detection kit (Roche) (Scholpp and Brand, 2003). Embryos for ISH were dechorinated and injected with indicated DNA plasmids (100ng/µl) at the 1-2 cell stage. Embryos were left to develop at 28$^{o}$C for 30 hrs before fixation in 4% PFA overnight at 4 $^{o}$C. Embryos were then washed twice in PBST and dehydrated in 100% methanol for 30 mins at RT. Following 2x PBST wash, they were re-fixed in 4% PFA for 30 mins and washed again 2x. Embryos were then incubated in Hyb+ solution at 69$^{o}$C for 4-6 hrs before replacing the solution with probe-Hyb+ mix (1:20 Pax6a probe in Hyb+) and incubated overnight. The next day, embryos underwent several washing steps in 25% Hyb-, Hyb-, 2xSSCT and 0.2xSSCT solutions at 69$^{o}$C, then MABT at RT. Embryos were then incubated in 2% blocking buffer for 4-5 hrs, followed by 1:4000 anti-DIG antibody (Roche) in 2% blocking buffer overnight at 4$^{o}$C. The next day, embryos were washed 5x 15 mins in MABT and then once in NTMT (5 mins). Embryos were transferred to 24 well plates and NTMT-NCP-BCIP mix (1:200 dilution NCP-BCIP:NTMT (Roche)) was added. Embryos were left to develop signal staining for 2 hrs in the dark (RT) prior to NTMT and PBST washes and 30 min re-fix in 4% PFA (RT). Two final washes in PBST were performed before storing embryos in 70% glycerol. Stained embryos were then imaged on a stereo microscope and measurements of the forebrain and midbrain made using Fiji software.

### 2.2.12. Quantifications and Statistical Analyses

All filopodia quantifications were calculated from Z-stack images of cells expressing membrane-mCherry and were done using Fiji software. Filopodia length was measured from the tip of the filopodia to the base, where it contacted the main cell body. In the case of branching protrusions, one branch would be measured. Protrusions that looked like retraction fibres or microspikes were not counted. Cumulative length was calculated by addition of all filopodia lengths per cell to

better summarise the filopodial phenotype, as some cells had few longer filopodia, and some lots of short ones.
Quantifications of Western blot images was performed by measuring the mean gray value of bands (after subtracting background) and normalising to loading controls. Quantification of qPCR data used the Pfaffl equation to calculate fold-change expression from Ct values, after normalising to GAPDH.

Pearson's correlation co-efficient (PCC) quantifications were performed using the "Coloc-2" plugin in Fiji. A region of interest was drawn around the desired cell and PCC was measured with no threshold.

All experiments / conditions were repeated at least in triplicates. For parametric data, significance was tested using student's t-test and for multiple comparisons, Bonferroni correction was used. For non-parametric data, a Mann-Whitney U Test was performed. Error bars on bar charts show standard error of the mean (SEM). For box and whisker plots, the whiskers represent the upper and lower limits of the data and the top and bottom of the box represent the upper and lower quartiles, respectively. The middle line represents the median and the 'x' the mean.

# 3. Chapter 1 – Wnt Cytonemes in Gastric Cancer Cells

## 3.1 Introduction

Adult stem cells represent small populations of undifferentiated cells which undergo long-term self-renewal to provide a source of nascent cells necessary for tissue regeneration or replenishment. In the gastric epithelium, these stem cell populations, found at the base of gastric crypts, are necessary for the replacement of epithelial cells which undergo continual turnover to maintain homeostasis and epithelium integrity (Flanagan *et al.*, 2018). Two sub-populations of stem cells, defined by Axin2 and Lgr5 expression (Axin2+/Lgr5+ and Axin2+/Lgr5-) reside in the gastric crypt. The self-renewal of these stem cells is, in part, regulated by Wnt signalling, which promotes the maintenance of stem cell-like properties (Nusse, 2008). At least 13 of the 19 Wnt ligands expressed in humans have been implicated with the maintenance of stem cell populations in the gastrointestinal tract (Wang *et al.*, 2016b; Flanagan *et al.*, 2018). It is therefore unsurprising that over-expression of Wnt ligands and/or over-activation of the Wnt signalling pathway(s) is a common carcinogenic driver in GC tumourigenesis (Schepers and Clevers, 2012).

Despite decades of research, GC still has one of the highest mortality rates of any cancer type (Sitarz *et al.*, 2018). In recent years, efforts have focused on understanding the molecular underpinnings of GC, at its earlier stages, to identify potential novel therapeutic targets at stages where the disease is more treatable (Xu *et al.,* 2016). Whilst we have discovered the over- or mis-expression of a number of Wnt ligands in GC, the lack of an approved drug targeting Wnt-driven cancers highlights the difficulty in modulating this pathway (Chiurillo, 2015). A more recent avenue of interest involves understanding how Wnt ligands are transported intercellularly, since it is their paracrine signalling function which drives tumourigenesis.

Recently, cytonemes have been discovered as a mechanism for transporting Wnt proteins in GC cells (Mattes *et al.*, 2018). Here, Wnt8a is loaded onto Ror2-expressing cytonemes to promote paracrine activation of Wnt/β-catenin signalling in recipient cells. Extraordinarily, Wnt8a is able to regulate its own dissemination via cytonemes, since its binding to Ror2 and induction of Wnt/PCP signalling in the source cell is what drives cytoneme formation (Mattes *et al.*, 2018).

In this chapter, I expand on these findings to investigate whether GC cells utilise cytonemes to transport Wnt proteins at endogenous levels, including the more physiologically relevant Wnt3, which is frequently overexpressed in GC (Wang *et al.*, 2016). Furthermore, I assess the contribution of cytonemes to paracrine Wnt signalling and subsequent proliferation in GC cells.

## 3.2. Results

### 3.2.1. Gastric cancer cell cytonemes are decorated with Wnt3 ligands

Previous work from our lab suggested that cytoneme-mediated Wnt transport may be an important mechanism in the proliferation of gastric cancer cells (Mattes *et al.*, 2018). Therefore, to begin with I evaluated the gastric adenocarcinoma cell lines AGS, MKN7 and MKN28 and a regular gastric epithelial cell line, HFE-145, for their potential to form actin-based protrusions such as filopodia. Cells were transfected with LifeAct-GFP to visualise actin-based structures (Fig. 9a). For filopodia quantification, cells were transfected with membrane-mCherry (Figure 9b). I found that all GC cells form many dynamic actin-based filopodia, and AGS cells display the longest filopodia, with an average length of 5.2 µm, as well as the greatest number of filopodia per cell (Fig. 9b). These filopodia frequently contact neighbouring cells and were observed at lengths of up to 42 µm (Fig. 9c). Due to their more pronounced filopodial phenotype, AGS cells were used as the primary cell line for later analyses.

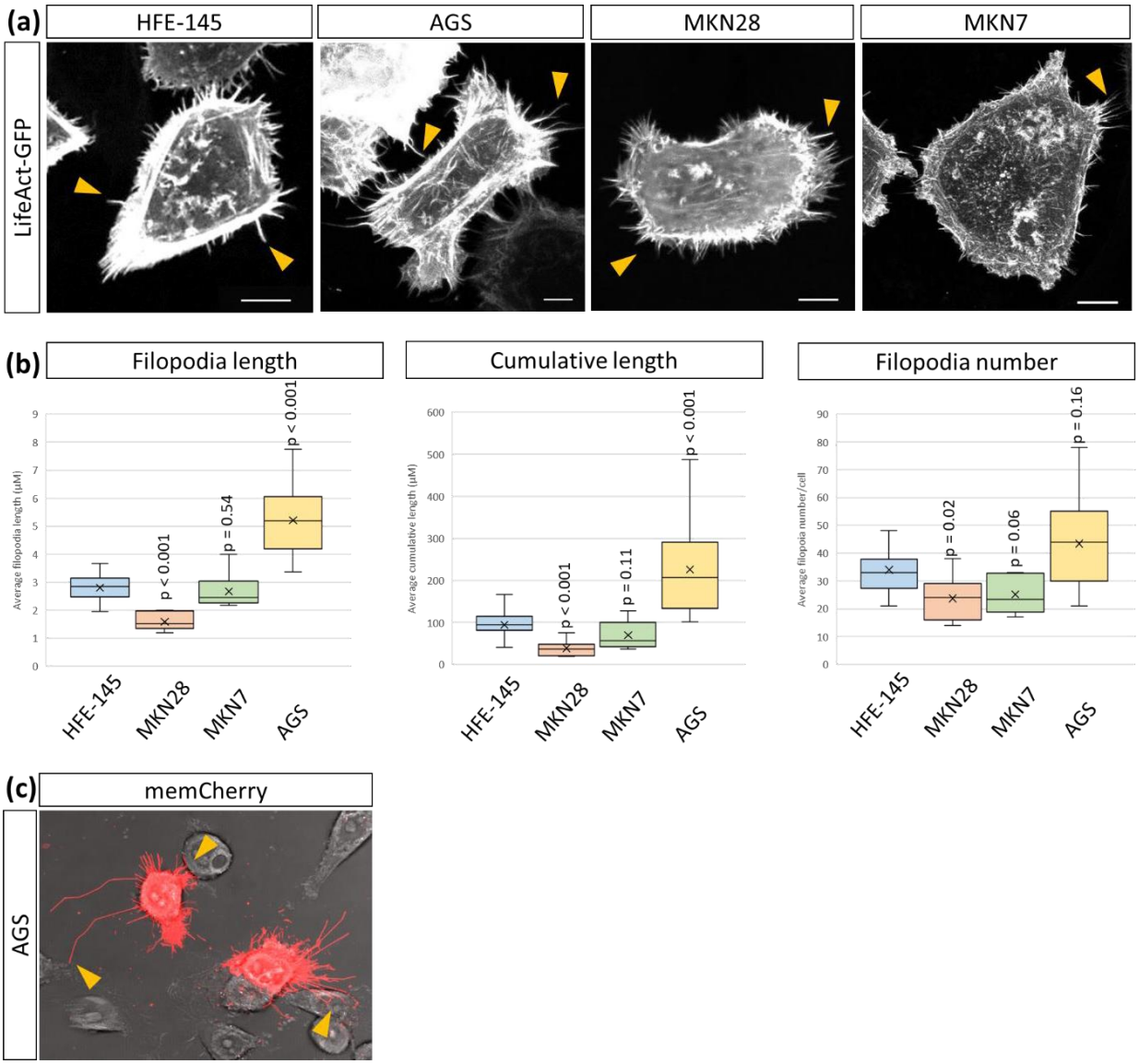


**Figure 9 – Gastric cancer cells display dynamic filopodia**

**(a)** Confocal images of gastric cancer cell lines (AGS, MKN28 and MKN7) and normal gastric epithelial cell line (HFE-145) expressing LifeAct-GFP to visualise actin-based structures. Examples of filopodia are indicated by yellow arrows. Scale bars 10µm.
**(b)** Quantifications of filopodia as shown in (a). Statistical significance calculated by student' T-test. (n = 22, 7, 8, 25; n = number of cells).
**(c)** AGS cells expressing membrane-mCherry with brightfield view. Yellow arrows highlight filopodia contacting neighbouring cells. Scale bar 10um

Next, I wanted to assess whether these filopodia could indeed be Wnt-transporting cytonemes. Due to its overexpression and ability to induce proliferation in GC cells, I chose to evaluate the localisation of Wnt3 ligands (Wang *et al.*, 2016). To assess endogenous Wnt3 expression and localisation in GC cells, immunofluorescent stainings of HFE, MKN28, MKN7 and AGS cells were performed using the MEM-Fix

fixation protocol to preserve cytonemes (Fig. 10a) (Rogers and Scholpp, 2021). Here, Wnt3 can notably be seen localising to cytonemes, along their lengths and tips, and at cytoneme contact sites. Identical IF experiments were attempted for Wnt1, however specificity of antibody staining could not be achieved using the MEM-Fix protocol. However, methanol-fixed cells were assessed for Wnt1 expression and Wnt1 could occasionally be observed on cytonemes (Appendix, Fig. A1).

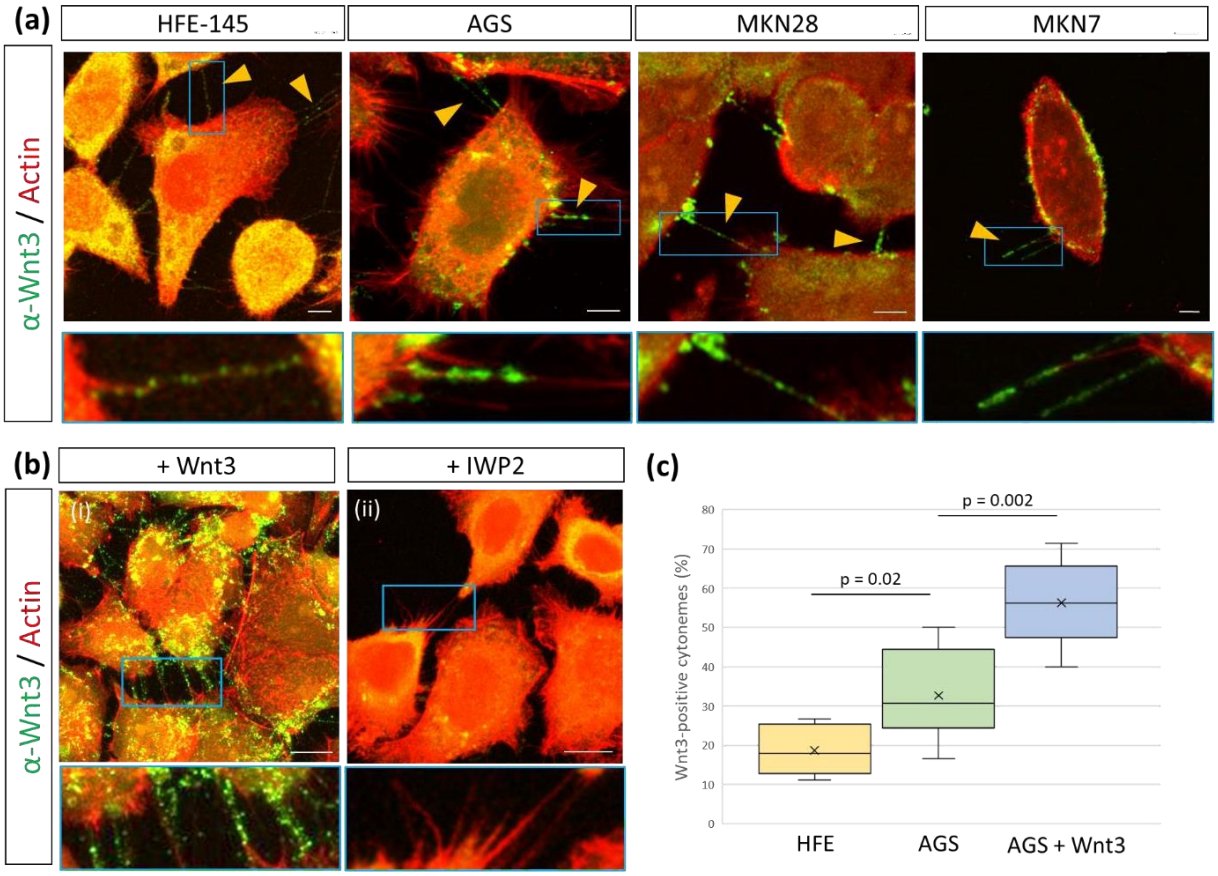


**Figure 10 – Wnt3 localises to cytonemes in gastric epithelial and cancer cells**

**(a)** Confocal images of HFE-145, AGS, MKN28 and MKN7 cells immunofluorescently labelled for endogenous Wnt3 (green) and actin (red) after MEM-Fix fixation. Yellow arrows and blue boxes highlight Wnt3-bearing cytonemes. Scale bars 10µm.
**(b)** Confocal images of AGS cells immunofluorescently labelled for Wnt3 (green) and actin (red). (i) AGS cells over-expressing Wnt3, showing increased Wnt3 cytoneme localisation (blue box). (ii) AGS cells treated with the Porcupine inhibitor IWP2 for 48 hrs prior to antibody staining, showing reduced Wnt3 staining and cytoneme localisation (blue box). Scale bars 10µm.
**(c)** Quantification of Wnt3-positive cytonemes as a proportion of all filopodial protrusions in HFE-145, AGS and Wnt3-overexpressing AGS cells. (n = 6, 8, 6; n = number of cells).

Over-expression of Wnt3 in AGS cells results in increased staining of Wnt3 on cytonemes and confirms antibody specificity (Fig. 10b). Treatment of AGS cells with IWP2, a porcupine (PORCN) inhibitor, strongly reduces Wnt3 staining and localisation of Wnt3 to cytonemes, suggesting that PORCN-dependent palmitoylation is essential for cytonemal localisation of Wnt3. Interestingly, AGS cells displayed a higher proportion of Wnt3-bearing cytonemes (32.7%) compared to HFE-145 cells (18.7%) (Fig. 10c), and this was further increased upon over-expression of Wnt3 (56.3%). Together, these data show that GC and epithelial cells utilise cytonemes to transport Wnt3, and this is more pronounced in GC cells.

### 3.2.2. Characterisation of Wnt3 cytonemes

To further characterise Wnt cytonemes in GC cells, IF stainings were performed to assess the localisation of other cytoneme or Wnt related proteins. This included the intracellular Wnt transport protein Evi, which can be seen at the tips and along the lengths of cytonemes in AGS cells (Fig. 11a(i)), suggesting that Evi may be involved in the delivery of Wnt proteins to or along cytonemes. Indeed, co-staining of Wnt3 and Evi revealed co-localisation in the perinuclear region (likely the ER) as well on cytonemes (Fig. 11b). I also assessed the localisation of Myosin-X (MyoX), a filopodial motor protein involved in Shh cytoneme function, by IF and observed strong localisation on filopodia tips, suggesting MyoX may also be involved in the formation of filopodia in GC cells (Fig. 11a(ii)) (Bohil *et al.*, 2006; Hall *et al.*, 2021).

To visualise the transport of Wnt3 by cytonemes in real-time, I generated a Wnt3-mCherry construct and transfected AGS cells with Wnt3-mCh and LifeAct-GFP for confocal analysis. As shown in Figure 11b, Wnt3-mCh can localise to the tips and along the lengths of cytonemes. These Wnt3-mCh puncta are highly motile and traverse the length of cytonemes. Analogous observations were made using Wnt1-mCh, suggesting this is not a Wnt-specific effect and may be a general mechanism used by GC cells (Appendix, Fig. A1).

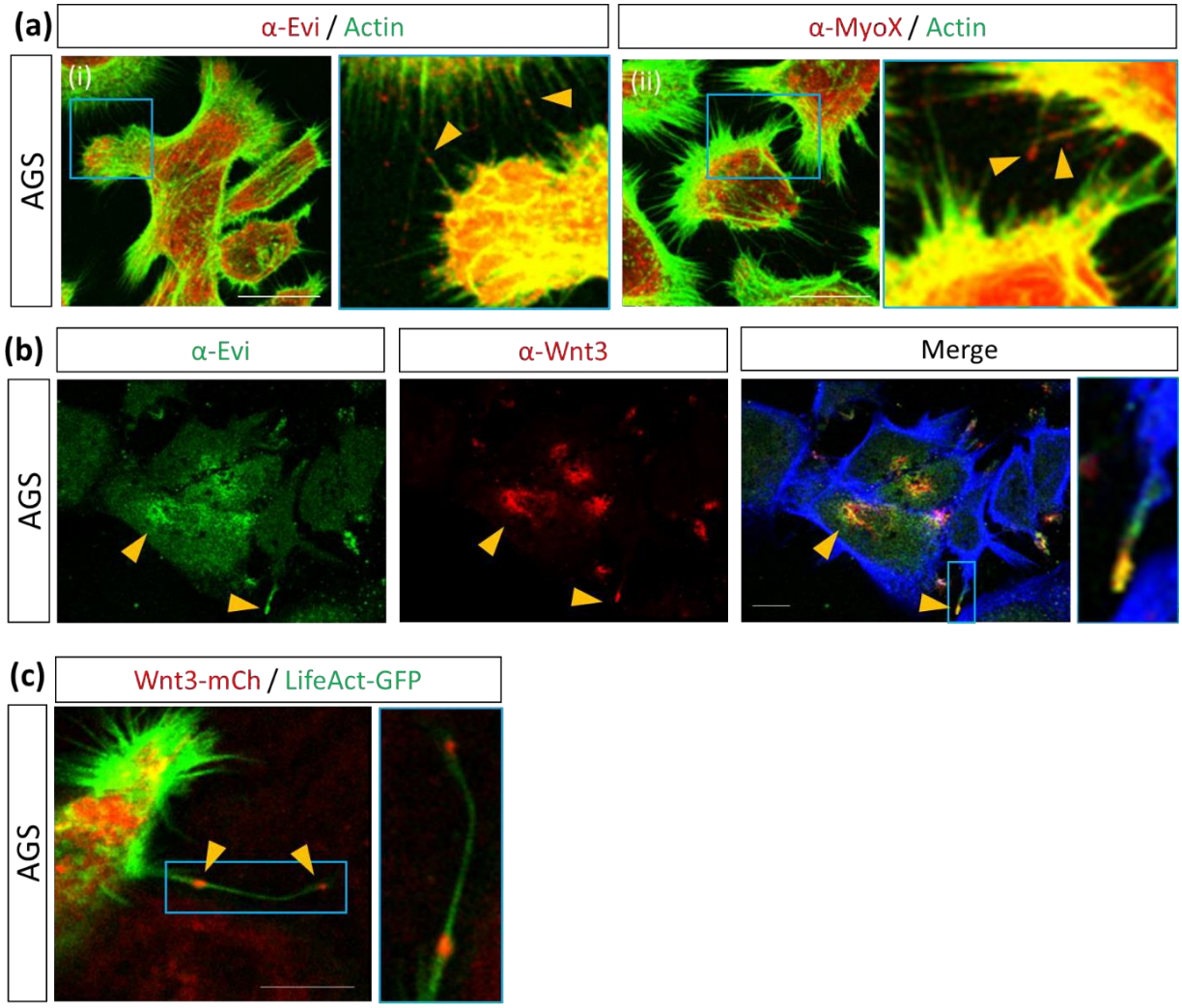


**Figure 11 – Characterising Wnt3 cytonemes in GC cells**
**(a)** Immunofluorescent stainings of AGS cells for (i) Evi (red) or (ii) Myosin-X (MyoX, red) and actin (green). Blue boxes are over-exposed images to show cytonemal stainings. Yellow arrows highlight cytoneme localisations. Scale bars represent 20µm.
**(b)** Immunofluorescent stainings of AGS cells for Evi (green) and Wnt3 (red) after 4% PFA fixation (which differs from MEM-Fix staining in Fig. 10). Actin stained with iFluor405 (blue). Yellow arrows highlight co-localisations of Evi and Wnt3 both intracellularly and on protrusions, the latter of which is highlighted in the blue box. Scale bar represents 10µm.
**(c)** Confocal image of AGS cells expressing Wnt3-mCh and LifeAct-GFP. An example of cytoneme-localised Wnt3-mCh is highlighted (blue box), both along its length and at the tip (yellow arrows). Scale bar 10µm.

To study the effects of cytoneme-mediated Wnt transport on GC cell signalling and proliferation, specific and targeted knockdown of filopodia formation is preferable over the use of chemical inhibitors which target the actin cytoskeleton and may produce off-target effects. To achieve this, I used a dominant-negative mutant of the I-BAR domain protein IRsp53, which normally stabilises filopodia through promotion of actin bundling and Cdc42 activity (Millard *et al.*, 2005). However, the IRSp53$^{4K}$ mutant, which has four lysine residues mutated to glutamic acid in the actin-binding site, prevents Cdc42-mediated filopodia elongation and stabilisation (Disanza *et al.*, 2013; Kast *et al.*, 2014).

To confirm IRSp53$^{4K}$ could block filopodia formation in AGS cells, IRSp53$^{4K}$-GFP and memCherry were co-transfected into AGS cells and their filopodia were quantified (Fig. 12). Here, IRSp53$^{4K}$ significantly reduced filopodia length (by 36%) and number (by 65%) and therefore was used in future experiments for inhibiting cytoneme-mediated signalling.

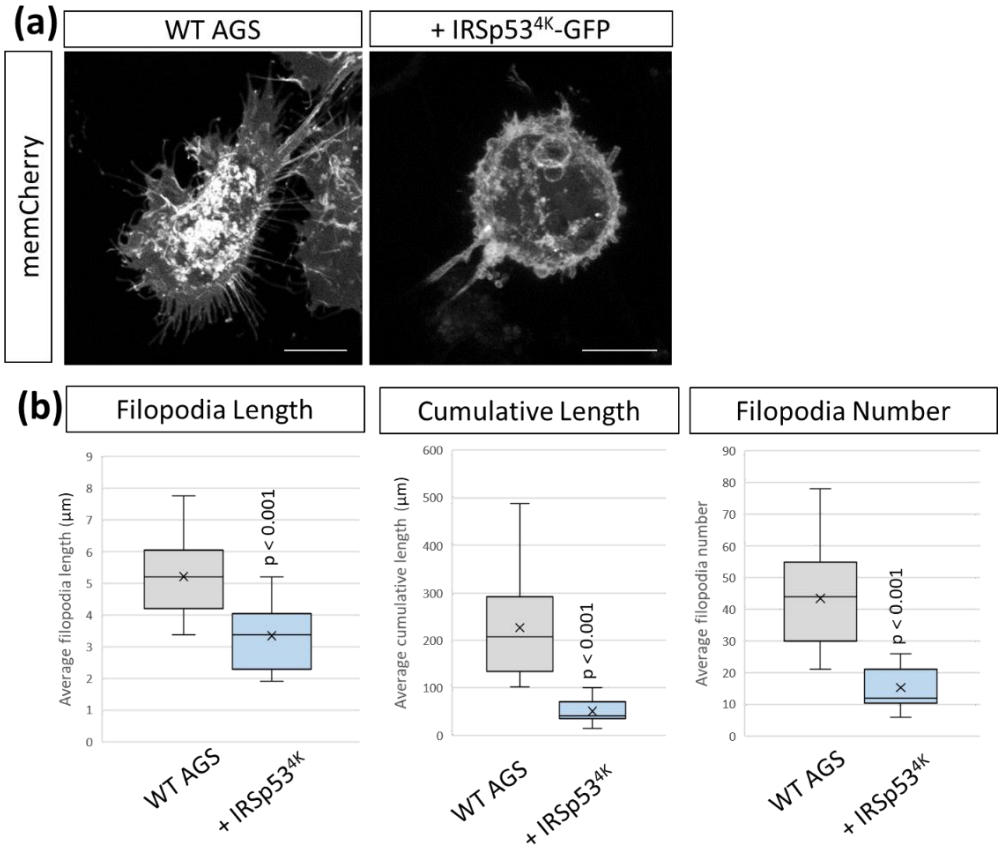


**Figure 12 – IRSp53$^{4K}$ blocks filopodia formation in AGS cells**
**(a)** Representative confocal images of AGS cells expressing memCherry in the presence or absence of IRSp53$^{4K}$-GFP. Scale bar 10µm.
**(b)** Quantifications of filopodia in WT AGS cells and IRSp53$^{4K}$-GFP-expressing cells. (n = 25, 13). Significance calculated by student's t-test.

### 3.2.3. Wnt3 cytonemes regulate paracrine Wnt/β-catenin signalling in gastric cancer cells

Next, I assessed the impact of Wnt3 cytonemes on paracrine Wnt signal activation in GC cells using a Wnt SuperTOPFlash (STF) reporter. This construct contains seven TCF-responsive elements hooked up to a nuclear mCherry (7xTCF-NLS-mCherry) and thus its fluorescence acts as an indicator of Wnt/β-catenin signalling. To measure paracrine activation specifically, a co-cultivation assay was performed (Fig. 13a). HFE-145 cells transfected with the STF reporter (Wnt-receiving cells) were co-

cultivated with AGS cells transfected with the filopodia inhibitor IRSp53$^{4K}$-GFP and/or Wnt3 (Wnt-producing cells). Untransfected AGS cells were used as the control. HFE-145 nuclei fluorescence were then measured as an indicator of Wnt/β-catenin signalling. As shown in Fig. 13b (and quantified in Fig. 13c), over-expression of Wnt3 in AGS cells results in a significant two-fold activation of reporter activity. Conversely, over-expression of IRSp53$^{4K}$-GFP significantly reduced activation by around half. Importantly, co-expression of IRSp53$^{4K}$-GFP with Wnt3 results in attenuation of the increase seen compared to Wnt3 alone. Analogous results were also observed when using Wnt1 (Appendix, Fig. A1). These data suggest cytonemes are important in Wnt transport and concomitant paracrine Wnt signal activation in GC cells.

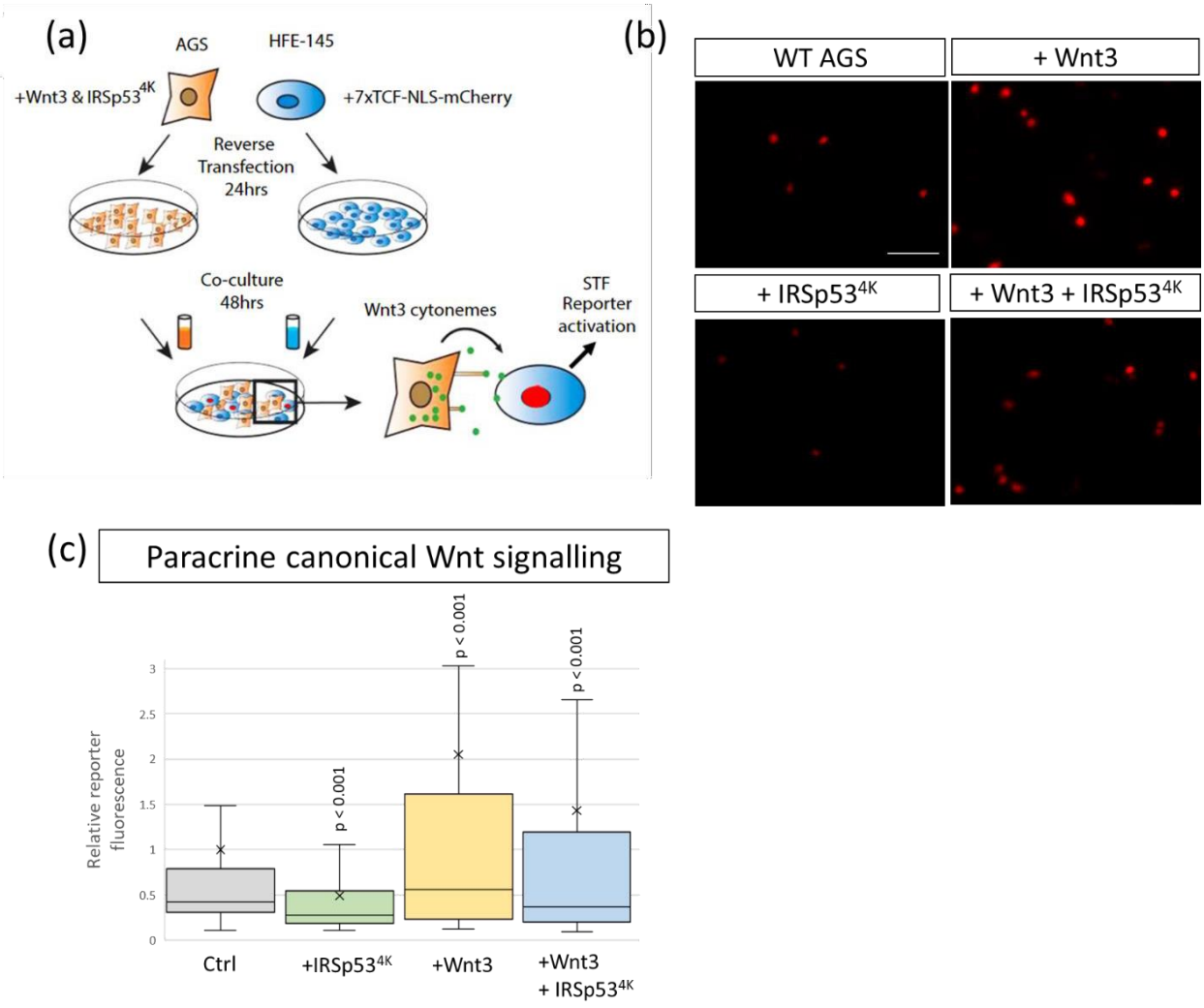


**Figure 13 – Wnt3 cytonemes promote paracrine activation of Wnt/β-catenin signalling in gastric cancer cells**
**(a)** Experimental protocol for measuring paracrine Wnt signalling activation. HFE cells expressing the SuperTOPFlash reporter, 7xTCF-NLS-mCherry, were co-cultivated with AGS cells expressing indicated constructs. Fluorescence of STF mCherry reporter was measured after 48 hrs and compared to untransfected control.
**(b)** Representative images of STF reporter fluorescence for indicated conditions. Scale bar 100μM.
**(c)** Quantification of 7xTCF-NLS-mCherry reporter fluorescence in HFE cells co-cultivated with AGS cells expressing indicated constructs. Quantifications relative to untransfected AGS control. (n per condition = 322, 394, 258, 275)(n = number of cells measured)

### 3.2.4. Wnt3 cytonemes promote proliferation in Wnt-receiving cells

Upregulation of Wnt/β-catenin signalling is often associated with promoting proliferation in GC (Chiurillo, 2015). Therefore, to analyse the functional effects of enhanced paracrine Wnt3 signalling, I counted the number of STF+ cells following each co-cultivation as an indicator of the proliferation of Wnt-receiving HFE-145 cell population (Fig. 14a). Here, the trend follows that of the STF reporter activation; expression of Wnt3 in AGS cells significantly increased the number of HFE-145 cells by 44%, whilst expression of IRSp53$^{4K}$ reduced this by 16%. Concurrently, co-expression of IRSp53$^{4K}$ with Wnt3 in AGS cells reduced the Wnt3-induced increase to only 14%. This suggests cytonemes, through regulation of Wnt3 signalling, can impact the proliferation of cells in a paracrine fashion.

However, cell number cannot strictly be used as an indicator of proliferation, as this could also reflect changes in apoptosis, for example. Therefore, to confirm these effects are proliferative, I performed a BrdU incorporation assay following the same-cocultivations as in Fig. 13a. BrdU stains newly synthesised DNA in actively proliferating cells only and these can be measured as a percentage of the population. Here, in control conditions (untransfected AGS cells), 43% of cells were in a proliferative state (Fig. 14 b,c). Over-expression of Wnt3 increases this to 50%, whilst IRSp53$^{4K}$ reduces this to 37%. Akin to the STF reporter results, IRSp53$^{4K}$ attenuated the effects of Wnt3 and only 47% of cells were undergoing proliferation. These results confirm the formation of cytonemes are important for Wnt3-mediated induction of proliferation in GC cells, and also specifically in Wnt-receiving cells.

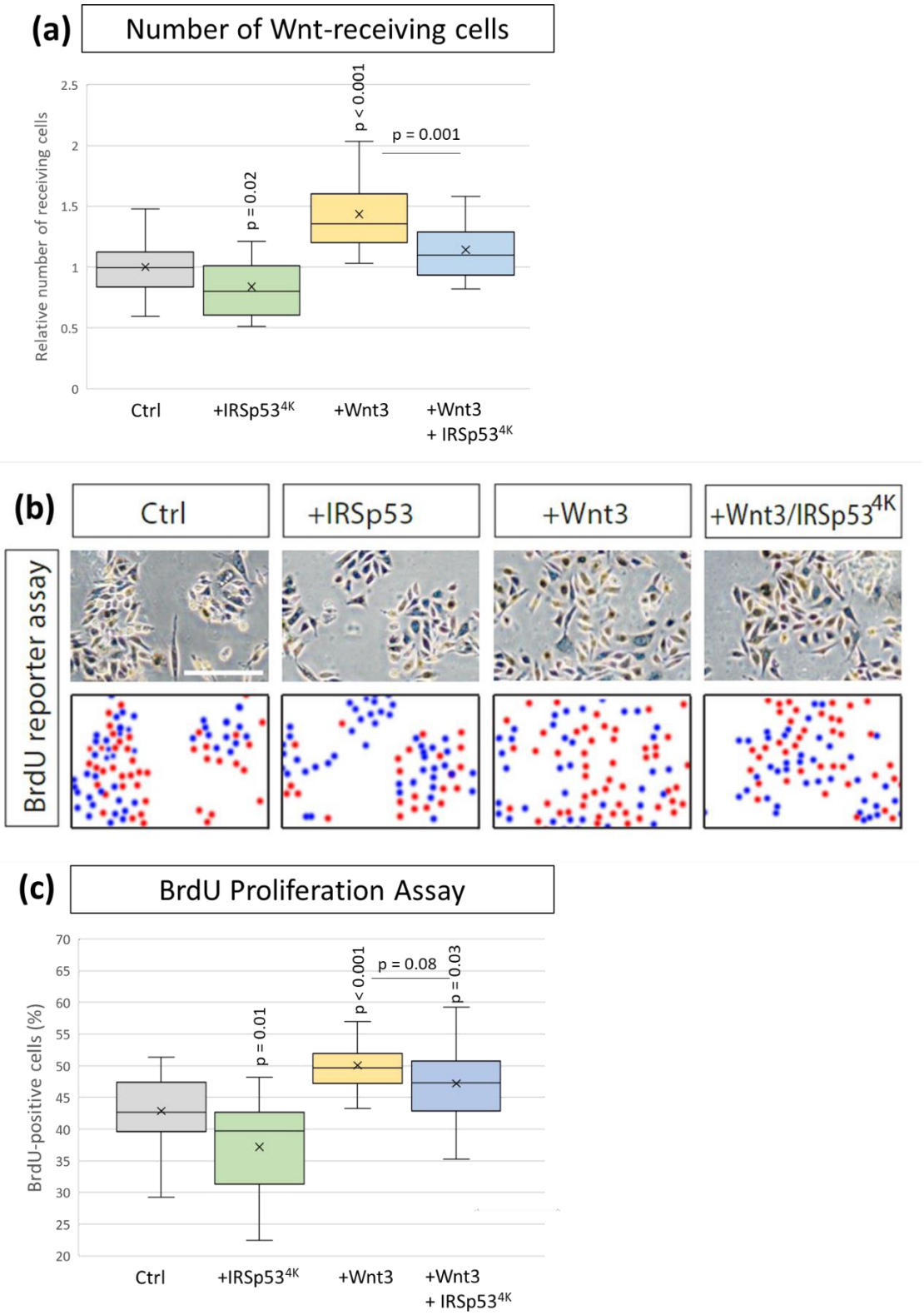


**Figure 14 – Wnt3 cytonemes promote proliferation of GC cells**
**(a)** Relative number of HFE cells per image after co-cultivation with AGS cells expressing indicated constructs. Significance calculated by student's t-test. (n per condition = 28, 26, 27, 17; n = number of images).
**(b)** Representative images of proliferating, BrdU-stained (red); co-cultivated AGS and HFE-145 cells, as described in Fig. 13a. Cells were counterstained with haematoxylin (blue dots). Scale bar 100µm.
**(c)** Quantification of BrdU-stained cells as a percentage of the population. Significance calculated by student's t-test. (n per condition = 20, 20, 20, 20; n = number of images).

## 3.3. Discussion

### 3.3.1. Gastric cancer cells utilise cytonemes to transport Wnt proteins

When deciphering a potential role of Wnt cytonemes in GC cells, I focused on the ligands Wnt1 and Wnt3, which are both commonly implicated with GC. Immunohistochemical analysis of GC patient tissues shows that Wnt1 is frequently over-expressed compared to control matched tissues (Mao *et al.*, 2014). Additionally, Wnt1 expression correlates with CD44 expression; a marker considered to represent 'stem cell-like' populations, which likely represent cancer stem cell (CSC) populations, which are significant contributors to GC tumourigenesis. The authors also show that AGS cell proliferation is enhanced by Wnt1 over-expression, which concurs with my results (Appendix, Fig. A1) (Mao *et al.*, 2014), and that Wnt1 enhances tumorigenicity of GC cells *in vivo* using mouse models. Supporting these findings, it has also been shown that the microRNA miR-140-5p, which targets and degrades Wnt1 mRNA, reduces the proliferation of GC cells. miR-140-5p is often downregulated in GC patients and is associated with poorer prognosis and survival rates (Cha *et al.*, 2018). Furthermore, the downstream activator of Wnt1 signalling, Wnt1-inducible signalling protein-1 (WISP-1), is also over-expressed in GC (Jia *et al.*, 2017). By analysing changes in epithelial-mesenchymal transition (EMT) markers, the authors show that WISP-1 expression induces EMT progression and concomitant invasive and migratory phenotypes. Loss-of-function of WISP-1 reduces GC cell proliferation, migration and invasion (Jia *et al.*, 2017). Together, these findings demonstrate the impact of Wnt1 expression and signalling on GC tumourigenesis and progression.

Similarly, the "canonical" Wnt3 protein is also frequently upregulated in GC. IHC analysis of gastric carcinoma samples shows that Wnt3 is overexpressed compared to non-neoplastic control samples (Wang *et al.*, 2016). siRNA-mediated knockdown of Wnt3 reduces the proliferation of GC cells, induces cell cycle arrest and apoptosis. Using a wound-healing assay, the authors also show that downregulation of Wnt3 reduces migration and invasion of GC cells (Wang *et al.*, 2016). This concurs with my results showing that Wnt3 is highly expressed in GC cells and Wnt3 signalling regulates GC cell proliferation (Fig. 10, 13, 14). Expression of Wnt3 in GC has also been associated with the presence of *Heliobacter pylori*, a bacterium which promotes

GC tumourigenesis through chronic inflammation and induction of oncogenic signalling, including Wnt signals (Song *et al.*, 2015; Zhou *et al.*, 2021). Therefore, Wnt3 is a critical factor in GC pathogenesis and understanding the mechanistic underpinnings of its transport and signalling is important in targeting these processes.

In a similar vein to the debate surrounding the formation of Wnt gradients during embryogenesis, there are questions around how Wnts are intercellularly transported to form gradients within gastric crypts to regulate proliferation and differentiation. Since it was shown that membrane-tethered Wg was functional and did not disturb early *Drosophila* development (Alexandre *et al.*, 2014), this hypothesis of membrane-bound Wnt transport was tested in the context of gastrointestinal crypts. Here, using intestinal organoid “mini-guts”, it was shown that Paneth-cell-derived Wnt3 is transported via direct cell-cell contact with stem cells (Farin *et al.*, 2016). Furthermore, the spreading of Wnt3 away from its source occurs via cell division, not diffusion, where Fzd receptors act as a membrane tether for Wnt3.The authors used chemical inhibitors of EGFR (Gefitinib), MEK (PD-0325901) or CDK4/6 (Palbociclib) to induce cell cycle arrest and observed Wnt3 distribution was restricted to the expressing cells (Farin *et al.*, 2016). They suggested this shows cell proliferation is the primary mechanism by which Wnt3 is spread. However, the picture is not quite so simple because Wnt expression and signalling is itself regulated by the cell cycle (and vice versa). Firstly, Wnt signalling components, including Axin2, GSK3β and β-catenin physically interact with centrosomes and are required for correct distribution of chromosomes during cell division (Bryja *et al.*, 2017). Thus, centrosome formation itself can affect the localisation and function of Wnt signalling components. Additionally, the E3 ubiquitin ligase Jade-1, which localises to centrosomes, is able to ubiquitinate β-catenin and thus target it for proteasomal degradation. Jade-1 activity is negatively regulated by the destruction complex component CK1α (Chitalia *et al.*, 2008; Bryja *et al.*, 2017). Therefore, disruption of centrosome formation, e.g., due to cell cycle arrest, could perturb Jade-1-mediated β-catenin degradation. Secondly, kinases involved in the cell cycle are also involved in the phosphorylation of the Wnt co-receptor Lrp6, including the Cyclin-Y/CDK14 complex and a number of MAP kinases, including p38 and JNK, the latter of which is also important in Wnt/PCP signalling (Červenka *et al.*, 2011). Thus, the cell cycle stage of a cell can modulate its

ability to respond to Wnt signals. Indeed, the ability of Lrp6 to respond to Wnt stimuli is greatest during the G2/M phase, when Cyclin-Y/CDK14 levels and concomitant Lrp6 phosphorylation are highest (Davidson *et al.*, 2009). Therefore, given the interplay between Wnt signal pathways and their ability to regulate their own dissemination, it is conceivable that the secretion or mobilisation of Wnt proteins could be dysregulated in cell cycle arrested cells, and also the ability of neighbouring cells to respond to paracrine stimuli.

An alternative mechanism by which membrane-tethered Wnts could be intercellularly transported throughout the gastric crypt is via cytonemes. Cytonemes have become well-established as an important mediator of Wnt dissemination in a number of tissues (Routledge and Scholpp, 2019). Previous work from our lab first showed the utilisation of cytonemes by GC cells in the mobilisation of Wnt8a ligands (Mattes *et al.*, 2018). These cytonemes are driven by Ror2/PCP signalling and blockage of cytonemes reduced proliferation of GC cells. My research is a continuation of these findings and showed that Wnt1 and Wnt3, which are more physiologically relevant in GC, are also transported via cytonemes and enhance Wnt/β-catenin signalling and consequently promote paracrine proliferation (Fig. 10, 13, 14; Appendix Fig. A1). Importantly, I showed this occurred at endogenous levels and was not an effect of over-expression. Furthermore, blockage of cytonemes with IRSp53$^{4K}$, even in Wnt3- or Wnt1-overexpressing cells, attenuated increases in paracrine Wnt/ β-catenin signalling, suggesting a significant portion of Wnt is transported via these structures. The consistent effect of cytoneme blockage on the transport of Wnt8a, Wnt1 and Wnt3 demonstrate these are not Wnt-specific effects and cytonemes represent a general mechanism of action for transporting Wnts in GC cells.

Other mechanisms of transport, such as exosomes, cannot be ruled out and may also contribute to paracrine Wnt signalling. However, only minimal co-localisation was observed between endogenous Wnt3 and the exosomal markers mCh-CD81 or CD63-GFP (Appendix, Fig. A2), suggesting that this is not the primary mechanism at play. Analysis of the contribution of secreted factors, including exosomes, would need to be conducted to make any such conclusions, however. Whilst exosomal release of proteins and miRNAs has been shown to regulate GC progression, no studies have specifically identified Wnt proteins on these structures (Fu *et al.*, 2019).

### 3.3.2. Characterisation of Wnt3 cytonemes

I also further characterised these cytonemes, showing that the intracellular Wnt chaperone protein Evi is present both at the tips and along the lengths of cytonemes (Fig. 11). In AGS cells, Evi co-localises with Wnt3 intracellularly (likely the ER-Golgi interface) as well as on cytonemes. This raises the question of whether Evi might be transported on cytonemes to facilitate Wnt dissemination. Alternatively, Evi may promote transport of Wnt3 to the tips of cytonemes, where it is released in order to bind to receptors in an autocrine fashion. Indeed, the point at which Evi dissociates from Wnt proteins is unclear, although it is suggested that Evi is not secreted bound to Wnt ligands, since only 10% of Evi and Wnt3a protein secreted on exosomes co-localise extracellularly (Gross *et al.*, 2012). Furthermore, it has been shown that Evi undergoes retromer-dependent recycling from the plasma membrane and is retained in the Wnt-secreting cells (Yang *et al.*, 2008). Thus, in the future, it would be pertinent to assess whether Evi can be detected in Wnt-receiving gastric epithelial cells. If not, it will be useful to understand how and when Wnt3 ligands are released from Evi and allow receptor binding to fulfil their paracrine functions.

As well as Evi, I also showed that the unconventional motor protein MyoX was present on protrusions in GC cells (Fig. 11). MyoX is a filopodial motor protein which has previously been shown to induce filopodia formation (Bohil *et al.*, 2006). More specific to morphogen transport, MyoX is required for Shh cytoneme formation by mobilising vesicular Shh along cytonemes (Hall *et al.*, 2021). Given the observed localisation of MyoX to filopodia in GC cells, it is tempting to speculate that MyoX may be involved in the formation of filopodia or cytonemes here. It would be interesting to see if knockdown of MyoX perturbs transport and/or localisation of Wnt ligands to cytonemes, which would suggest a conserved role of MyoX in transporting morphogens along cytonemes.

Together, the results in this chapter demonstrate that GC cell protrusions do not just represent filopodia, but also dynamic cytonemes, as evidenced by the presence of Wnt3, Wnt1, Evi and MyoX on these structures. Moreover, these Wnt cytonemes significantly contribute to paracrine Wnt signalling and induction of proliferation. Therefore, in the context of gastric crypts, it is conceivable that GC cells may signal to neighbouring normal epithelial cells, via Wnt cytonemes, to promote further

tumourigenesis. Therefore, inhibiting cytonemes to reduce intercellular Wnt transport may offer a novel mechanism for targeting Wnt-driven cancers.

# 4. Chapter 2 – The role of Flotillin-2 in Wnt cytonemes

## 4.1. Introduction

As a membrane scaffolding protein and constituent of lipid rafts, Flot2 is a highly multifunctional protein whose interactome confers its role in a wide variety of signalling pathways and cellular functions (Otto and Nichols, 2011). One of the most observable and notable phenotypes regulated by Flot2, however, is the formation of filopodia. Flot2 was first implicated here when Hazarika *et al* (1999) observed enhanced filopodia formation upon Flot2 overexpression in COS-1 cells, which were described as having a “neuronal appearance”. Since then, numerous studies have corroborated this function, including in a number of epithelial and neuronal cell types (Neumann-Giesen *et al.*, 2004; Langhorst *et al.*, 2008).

As a subset of filopodia, it follows that cytonemes may also be regulated by Flot2 via its ability to promote actin polymerisation (Fig. 8) (Stuermer, 2010). Indeed, Flot2 has been shown to increase the length of Hh cytonemes, and consequently the Hh gradient, in *Drosophila* (Bischoff *et al.*, 2013). The ability of Flot2 to enhance *Drosophila* morphogen gradients can also be seen with Wg, where its overexpression expands the Wg gradient and target gene expression (Katanaev *et al.*, 2008). A potential role for cytonemes in this process, however, was not analysed.

In GC, Flot2 is commonly overexpressed and is associated with poor prognosis (Cao *et al.*, 2013). A common feature of GC is also the overactivation of the Wnt signalling pathway (Flanagan *et al.*, 2018). Given Flot2 has previously been implicated in enhancing Wg signalling, it follows that Flot2 could have a role in promoting Wnt signalling in GC cells, potentially via cytonemes.

In this chapter, I investigate the role of Flot2 in regulating Wnt cytoneme formation in GC cells, specifically its ability to enhance paracrine Wnt3 transport. As well as the effects of Flot2 on Wnt signalling and proliferation, I also investigate how, molecularly, Flot2 achieves this function. Finally, I analyse the ability of Flot2 to perturb Wnt8a signalling *in vivo* during zebrafish development to validate a potentially conserved function of Flot2 in promoting Wnt transport and signalling.

## 4.2. Results

### 4.2.1. Flotillin-2 is over-expressed in GC cells and correlates with filopodial phenotypes

Firstly, I wanted to determine whether the expression levels of Flot2 in different gastric cell lines could explain the difference between gastric epithelial cells and GC cells with regard to filopodia formation and cytoneme-mediated Wnt3 dissemination. To begin with, I assessed the expression levels of Flot2 in three GC cell lines, AGS, MKN28 and MKN7, and compared these to the gastric epithelial HFE-145 cells. As measured by RT-qPCR, AGS cells displayed the highest levels of Flot2 mRNA, over 2-fold greater than HFE-145 (Fig. 15a). MKN7 cells showed a 1.5-fold higher mRNA expression, whilst MKN28 levels were similar to that of HFE-145. Protein expression was then measured by Western blot, which displayed a similar trend; AGS cells have 2.7-fold higher Flot2 protein than HFE-145, followed by MKN7 (1.5-fold) and MKN28, which showed lower Flot2 protein (0.7-fold) than HFE-145 (Fig. 15b). Interestingly, Flot2 expression levels in these GC cell lines correlate with filopodia number and length, where AGS > MKN7 > MKN28 (Fig.9b, 15a,b). Due to their high Flot2 expression and notable filopodial phenotype, AGS cells were chosen as the primary cell type for subsequent experiments.

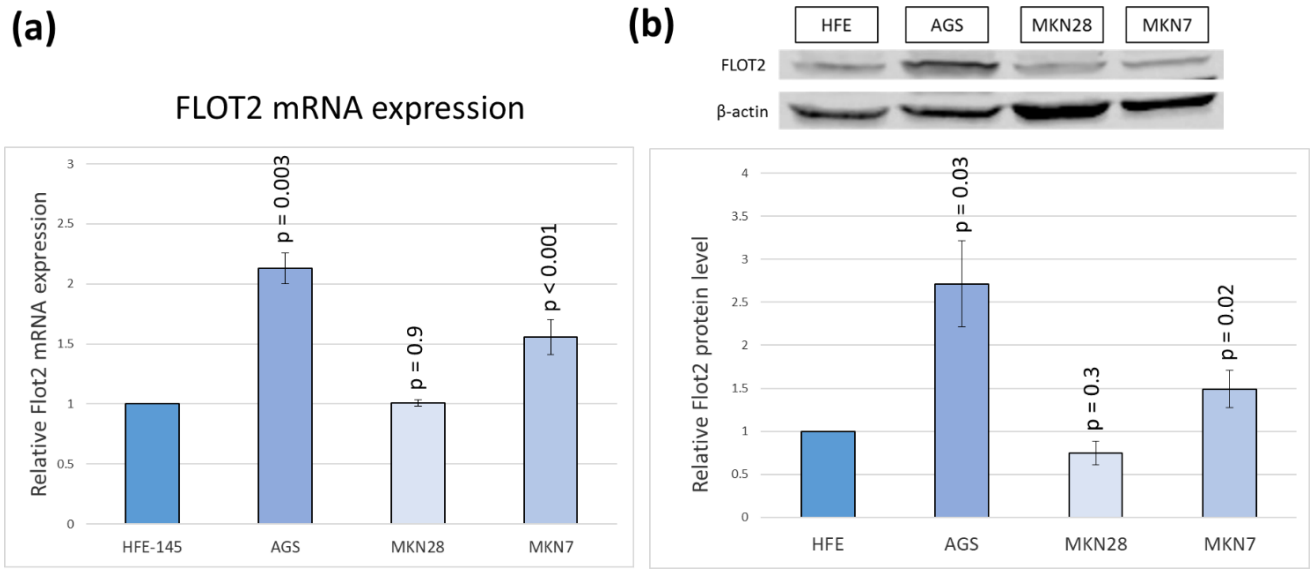


**Figure 15 – Flotillin-2 is over-expressed in GC cells**
**(a)** Relative Flotillin-2 (Flot2) expression as quantified by RT-qPCR after normalising to GAPDH as housekeeping gene. Relative expression levels are compared to HFE-145. Error bars represent standard error of the mean (SEM). Significance calculated by student's t-test. (n = 4, 4, 3, 3, n = number of mRNA samples).
**(b)** Relative Flot2 protein levels as quantified by Western Blot after normalising to beta-actin levels. Relative protein levels are compared to HFE. Error bars represent SEM. Significance calculated by student's t-test. (n = 3).

### 4.2.2. Flot2 localises to filopodia and promotes their formation in AGS and HFE-145 cells

Next, antibody stainings were performed against Flot2 to assess the localisation of endogenous Flot2 in AGS and HFE-145 cells (Fig. 16a). Flot2 displays punctate staining throughout the cell, with notable localisation at the membrane. Here, Flot2 localises at the base and along the lengths of filopodia, more notably in AGS cells, suggesting a physical relationship with these actin-based protrusions. These observations were also made when expressing Flot2-GFP in AGS cells. When co-expressed with memCherry, Flot2-GFP can clearly be seen strongly localising to the membrane (Fig. 16b). Not only did Flot2-GFP also localise to filopodia (Fig. 16b(i)), but its expression domains also appear to correlate with the presence or absence of filopodia; portions of the membrane lacking Flot2-GFP display a notable lack of filopodia (Fig. 16b(ii), blue arrows) and areas with high Flot2-GFP are enriched in filopodia (Fig. 16b(ii), yellow arrows). This strengthens the concept that Flot2

expression dictates filopodial phenotypes in GC cells. It is also possible Flot2 affects cell polarisation and thus the presence of filopodia towards the leading edge of cells.

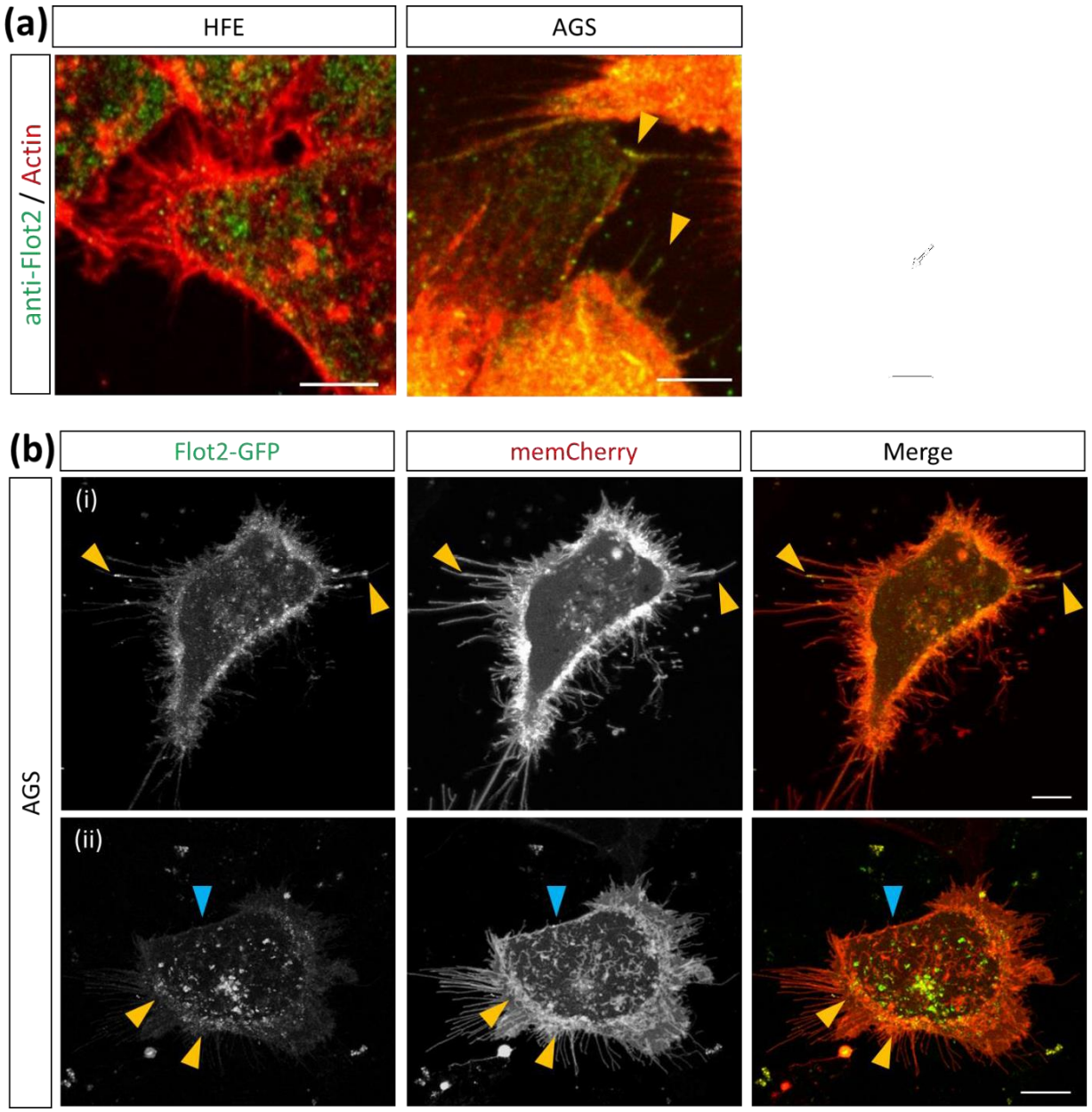


**Figure 16 – Flotillin-2 localises to filopodia-rich portions of the membrane in GC cells**
**(a)** Antibody stainings showing endogenous localisation of Flot2 (green) in HFE and AGS cells. TRITC phalloidin was used to visualise actin. Yellow arrows indicate localisation of Flot2 to filopodia. Scale bars 5μm.
**(b)** Confocal images showing the sub-cellular localisation of Flot2-GFP in AGS cells. (i) Arrows indicate localisation of Flot2-GFP to filopodia. (ii) Yellow arrows indicate presence of Flot2-GFP in filopodia-enriched areas of the membrane. Blue arrows indicate lack of Flot2-GFP expression and filopodia. Scale bar 10μm.

### 4.2.3. Flotillin-2 promotes filopodia formation in GC cells

Flot2 has been shown to promote filopodia formation in a number cell types, including epithelial and neuronal cells (Neumann-Giesen *et al.*, 2004; Munderloh *et al.*, 2009). To decipher whether the same is true for GC cells and to confirm observations made in Fig. 16, Flot2 function was perturbed in HFE-145 and AGS cells and filopodial phenotypes quantified. Cells were transfected with membrane-mCherry to visualise protrusions and filopodia were quantified by number and length (Fig. 17a-d). Here, Flot2-GFP over-expression significantly increases the filopodia number and cumulative length in both HFE-145 and AGS cells. However, the average filopodia length increases in HFE-145 but not AGS cells, which is attributed to a change in distribution of filopodia (discussed in detail later).

To inhibit Flot2 function, two methods were utilised. Firstly, a dominant-negative mutant of Flot2, ΔN-Flot2-GFP, was expressed in cells. This mutant lacks the N-terminus of Flot2, which is necessary for membrane localisation, and is therefore largely soluble and displays a sparse cytosolic localisation. To confirm ΔN-Flot2-GFP disrupts endogenous Flot2 function, an antibody staining was performed against Flot2 in ΔN-Flot2-GFP-expressing AGS cells (Appendix Fig. A3), which does not recognise the mutant Flot2 construct. Here, punctate staining of Flot2 throughout the cell is lost and it consistently aggregates intracellularly, confirming disruption of endogenous Flot2 localisation.

Expression of ΔN-Flot2-GFP strongly reduced the number and length of filopodia in both AGS and HFE-145 cells (Fig. 17a-d). Secondly, Flot2 siRNA was used to transiently knock down Flot2 levels. Here, Flot2 KD produced phenotypes comparable to ΔN-Flot2-GFP, with a significant reduction of filopodia number and cumulative length in both cell types. Filopodia number appears relatively unchanged in Flot2-deficient cells, suggesting the dominant-negative Flot2 mutant construct has a more inhibitory effect on filopodia formation. Since siRNA-mediated knockdowns are not 100% efficient, perhaps residual Flot2 levels are sufficient to permit the formation, but not elongation, of filopodia.

The effects of Flot2 on filopodia formation evidently involve perturbing the actin cytoskeleton. As well as altered filopodial phenotypes, Flot2-deficient cells frequently display larger and greater numbers of lamellipodia, which arise from branching of

actin filaments, as opposed to filopodia, in which filaments are largely unbranched (Fig. 17a). Similarly, Flot2-deficient cells often exhibit membrane ruffles, structures which are also rich in branched actin (Fig. 17e).

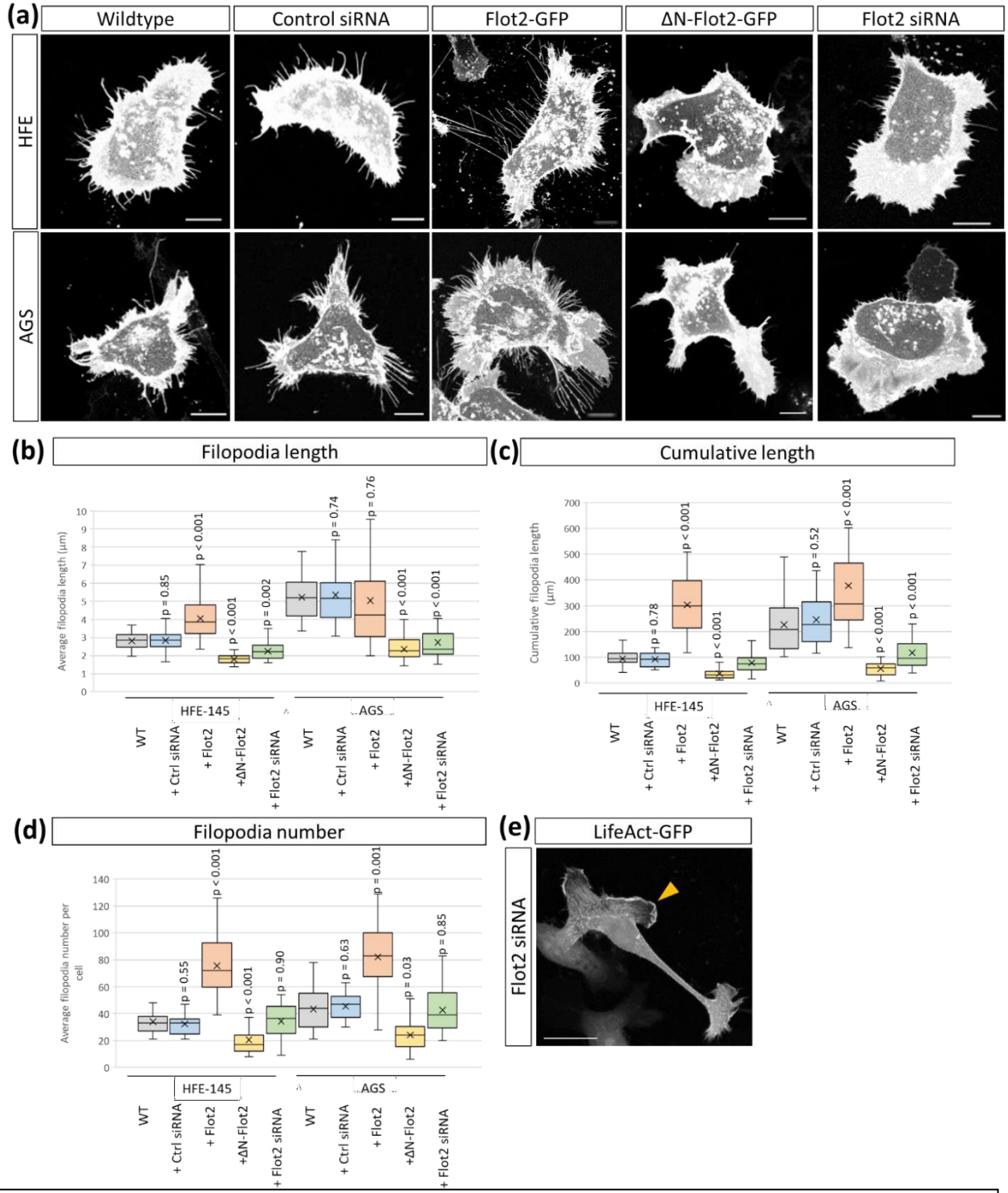


**Figure 17 – Flotillin-2 enhances filopodial phenotypes in HFE-145 and AGS cells**
**(a)** HFE and AGS cells expressing membrane-mCherry and indicated Flotillin-2 (Flot2) constructs or siRNA after 48hrs. Scale bars 10μm.
**(b-d)** Filopodia quantifications of HFE and AGS cells transfected with indicated Flot2 plasmids or siRNA. **(b)** Filopodia length, **(c)** Cumulative length, **(d)** Filopodia number. Significance calculated by Student's t-test with Bonferroni correction for multiple comparisons. (n per condition (HFE) = 22, 19, 25, 23, 24). (n per condition (AGS) = 25, 21, 25, 25, 25; n = number of cells measured).
**(e)** Flot2 siRNA-treated AGS cell expressing LifeAct-GFP to visualise actin-based structures. Yellow arrow highlights membrane ruffles frequently seen in Flot2-deficient cells. Scale bar 10μm.

To assess these changes in filopodia further, I categorised filopodia measurements into different lengths and analysed the proportion of filopodia in each category as a percentage of the total filopodia number (Fig. 18a). This shows that in HFE-145 cells, Flot2-GFP over-expression increased the proportion of longer filopodia (>6um), whilst decreasing the proportion of shorter ones (< 2um). Conversely, Flot2 inhibition (by ΔN-Flot2-GFP or Flot2 siRNA) had opposing effects. This suggests Flot2 promotes the elongation, as well as induction, of filopodia.

As highlighted earlier, however, Flot2-GFP over-expression did not increase the average filopodia length of AGS cells. Even when analysing changes in filopodia distribution (Fig. 18a), no significant changes are observed, particularly for longer filopodia. However, the answer can be found when looking at the raw number of filopodia in each length category. As shown in Fig. 18b, the average number of filopodia per cell < 2um is over 4-fold greater in the presence of Flot2-GFP. At the other extreme, the average number of filopodia per cell >10 um doubles with Flot2-GFP expression, whilst changes for medium-length filopodia are largely unchanged. This suggests Flot2 promotes the elongation and induction of filopodia, as is seen for HFE-145 cells. Overall, the increases in both short and long filopodia appear to cancel each other out and produce a largely similar average length compared to WT AGS cells. Together, these data highlight the significant impact of Flot2 on filopodial phenotypes and thus shows promise as a candidate for regulating cytonemes.

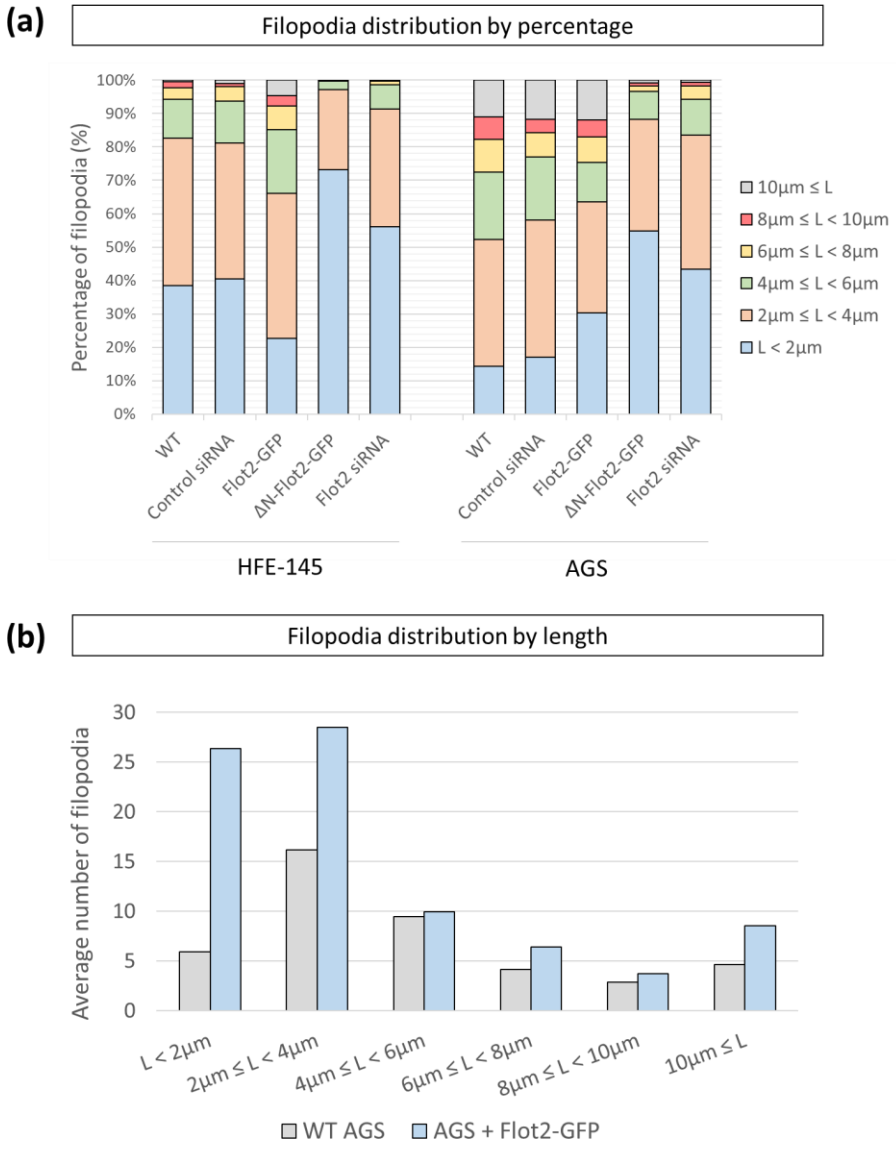


**Figure 18 – Effects of Flot2 on distribution of filopodia lengths**
**(a)** Distribution of categorised filopodia lengths as a percentage of the total filopodia per HFE-145 or AGS cell. (n = 22, 19, 25, 23, 24 (HFE); 25, 21, 25, 25, 25)(n = number of cells)
**(b)** Average number of filopodia, categorised by length, per WT or Flot2-GFP-expressing AGS cell

### 4.2.4. Flotillin-2 co-localises with Wnt3 intracellularly and on cytonemes

Thus far, the hypothesis has been that Flot2 may enhance Wnt cytoneme formation indirectly through its ability to promote actin polymerisation and thus filopodia formation. However, I wanted to investigate if there was a more direct relationship, since Flot2 partially colocalises with Wg in *Drosophila* cultured cells (Katanaev *et al.*, 2008). Thus, I co-expressed Flot2-GFP and Wnt3-mCh in AGS cells and found that they co-localised both intracellularly (in vesicular structures) and on cytonemes (Fig. 19a). Flot2-GFP and Wnt3-mCh clusters can also be seen at cytoneme contact sites (Fig. 19b). This co-localisation persists in the receiving cell, suggesting Flot2 and Wnt3 may be transported together to the receiving cell. To confirm this co-localisation

is not an effect of over-expression, I performed antibody stainings against Flot2 and Wnt3 to assess localisation of endogenous proteins. Since the Flot2 antibody was not functional in MEM-Fix, standard 4% PFA was used for fixation, which results in loss of most fragile structures, such as cytonemes. As shown in Fig. 19c(i), Flot2 and Wnt3 co-localise in a punctate fashion, notably at the membrane, which may represent clustering of Wnt3 at Flot2 microdomains. Where cytonemes were occasionally preserved, Flot2 and Wnt3 can be seen co-localising on these structures (Fig. 19c(ii)). At a cytoneme-cytoneme contact point, Flot2 can be seen on the tips of both protrusions, and Lrp6 (the Wnt co-receptor) can be seen clustering on one side of this contact, suggesting induction of signalling in the recipient cell (Fig. 19d). Together, these results suggest Flot2 has a more direct role in cytonemal Wnt3 transport than first thought.

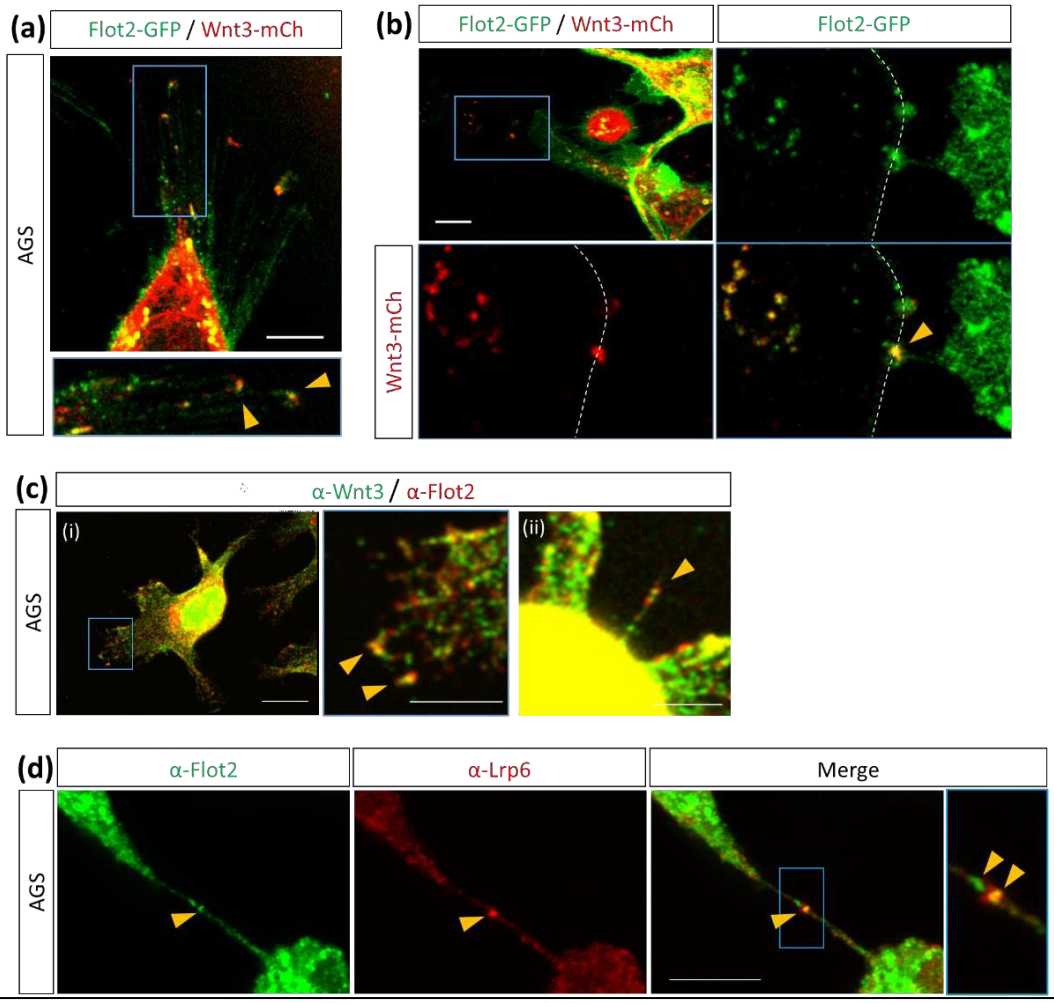


**Figure 19 – Flotillin-2 co-localises with Wnt3 on cytonemes in gastric cancer cells**
**(a)** Confocal image highlighting co-localisation of Flot2-GFP and Wnt3-mCh on cytonemes in AGS cells. Scale bar 10μm.
**(b)** Confocal images highlighting co-localisation and clustering of Flot2-GFP and Wnt3-mCh at a cytoneme contact point. Scale bar 10μm.
**(c)** Antibody stainings of Flot2 and Wnt3, showing co-localisation at the (i) membrane and (ii) on cytonemes. Scale bars 10μm (left) and 5μm (middle, right).
**(d)** Antibody stainings of Flot2 (green) and Lrp6 (red) in AGS cells. Blue box highlights clustering of Lrp6 at a cytoneme-cytoneme contact point. Scale bar 10μm.

Next, I wanted to assess whether inhibiting Flot2 function reduced the number or proportion of Wnt3-positive cytonemes. However, since blocking Flot2 function or expression reduces the number of filopodia, a fair comparison and quantification against WT cells cannot be made. However, I could assess whether Flot2 is involved in the intracellular transport (i.e., secretion) of Wnt3. Therefore, I performed siRNA-mediated knockdown of Flot2 or expressed the dominant-negative ΔN-Flot2-GFP in AGS cells and assessed endogenous Wnt3 sub-cellular localisation by immunofluorescence (Fig. 20). Here, staining of Wnt3 in ΔN-Flot2-GFP-expressing cells shows no significant change compared to surrounding untransfected cells (Fig. 20b). Wnt3 stainings in Flot2-deficient cells also appears largely unchanged (Fig. 20a). Therefore, despite co-localisation of Flot2 and Wnt3, Flot2 is not crucial for Wnt3 intracellular transport.

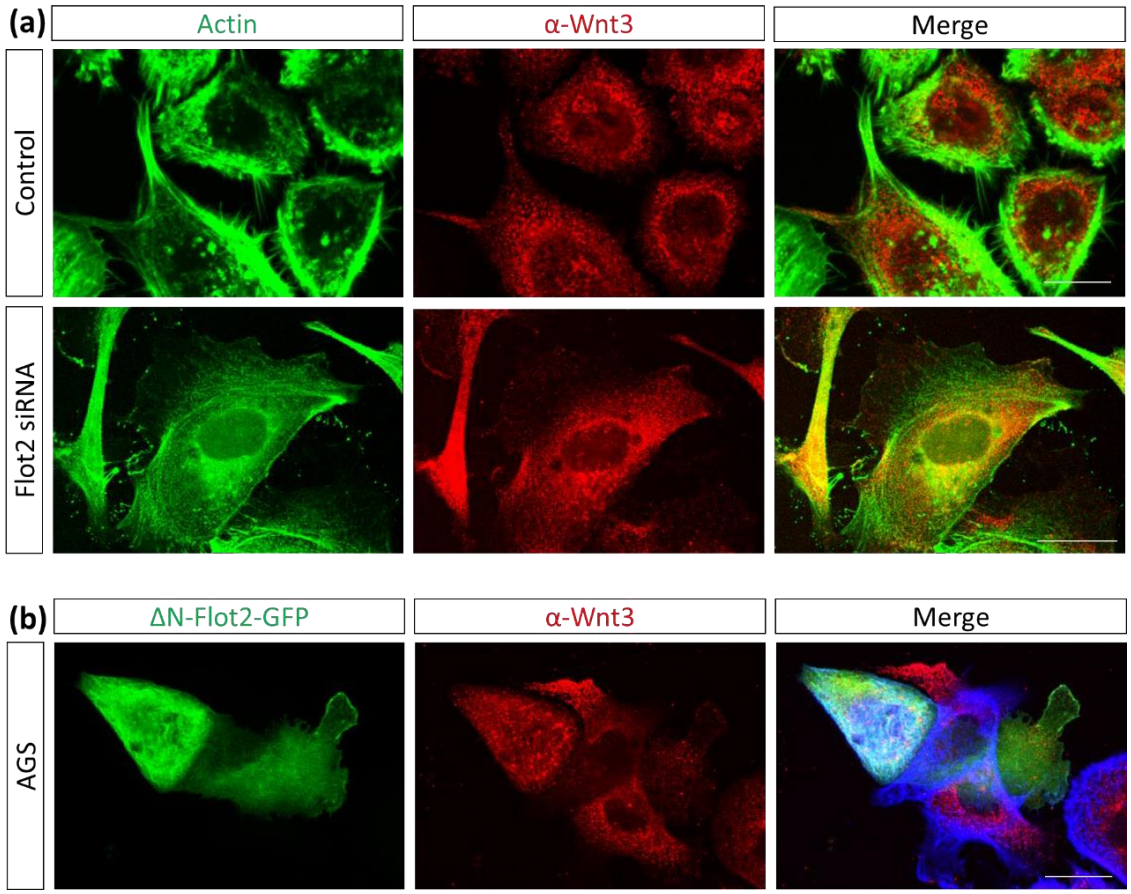


**Figure 20 – Inhibiting Flot2 function does not alter Wnt3 sub-cellular localisation**
**(a)** Antibody staining against Wnt3 (red) in control or Flot2-deficient (siRNA-mediated) AGS cells. Actin stained with phalloidin-iFluor405. Scale bar represents 20μm.
**(b)** Antibody staining against Wnt3 (red) in AGS cells expressing ΔN-Flot2-GFP to assess any changes in sub-cellular localisation of endogenous Wnt3. Actin stained with phalloidin-iFluor405. Scale bar represents 20μm.

### 4.2.5. Flotillin-2 promotes cytoneme-mediated paracrine Wnt3 signalling and proliferation

Next, I wanted to assess the functional impact of Flot2 on Wnt3 cytonemes. To do this, I co-cultivated HFE-145 cells expressing the STF reporter with AGS cells, as described in Fig. 13a. Here, AGS cells were transfected with Flot2-GFP or ΔN-Flot2-GFP with or without over-expressed Wnt3. IRSp53$^{4K}$-GFP was used to inhibit cytoneme-mediated effects and untransfected AGS cells were used as the control. Flot2-GFP alone induces a modest, but insignificant, 39% increase in STF reporter activation, whilst ΔN-Flot2-GFP shows almost no change (Fig. 21a, b). Strikingly, however, when co-expressing Flot2-GFP and Wnt3 in AGS cells, Wnt signal activation in HFE-145 cells is enhanced by 210%, which is significantly greater than Wnt3 alone (100% increase). Conversely, co-expression of ΔN-Flot2-GFP with Wnt3 attenuates this increase to only 41%. The enhanced effect of Flot2 and Wnt3 can also be reduced by co-expression with IRSp53$^{4K}$, which reduces the increase to 100%. Together, these data suggest that Flot2 enhances paracrine Wnt3 signalling, and that this in part is due to significantly altering cytonemal delivery of Wnt3. I cannot rule out other mechanisms by which Flot2 enhances Wnt3 transport, which may account for the remaining signal increase.

The number of STF-mCh positive cells were also counted after co-cultivation as an indicator of cell proliferation. Here, a similar trend to the paracrine Wnt activation is seen, with Flot2-GFP and Wnt3 co-expression producing the greatest increase in cell number, which is significantly reduced by ΔN-Flot2-GFP (Fig. 21c). Consistently, IRSp53$^{4K}$ reduced the effect of Flot2/Wnt3 on cell number, again suggesting a role for cytonemes in eliciting these effects.

To confirm these changes in cell number are due to altered proliferation, a BrdU assay was performed, as in Fig. 14b, after co-cultivation of HFE-145 and AGS cells (Fig. 22). Here, analogous trends are observed for the entire cell population, where Flot2/Wnt3 combined significantly increases the proportion of BrdU-positive cells greater than Wnt3 alone. ΔN-Flot2-GFP and IRSp53$^{4K}$ reliably attenuate Wnt3-mediated changes in proliferation. Together, these data show that Flot2 enhancement of paracrine Wnt3 signalling promotes cellular proliferation and this effect is dependent on Wnt3 cytonemes.

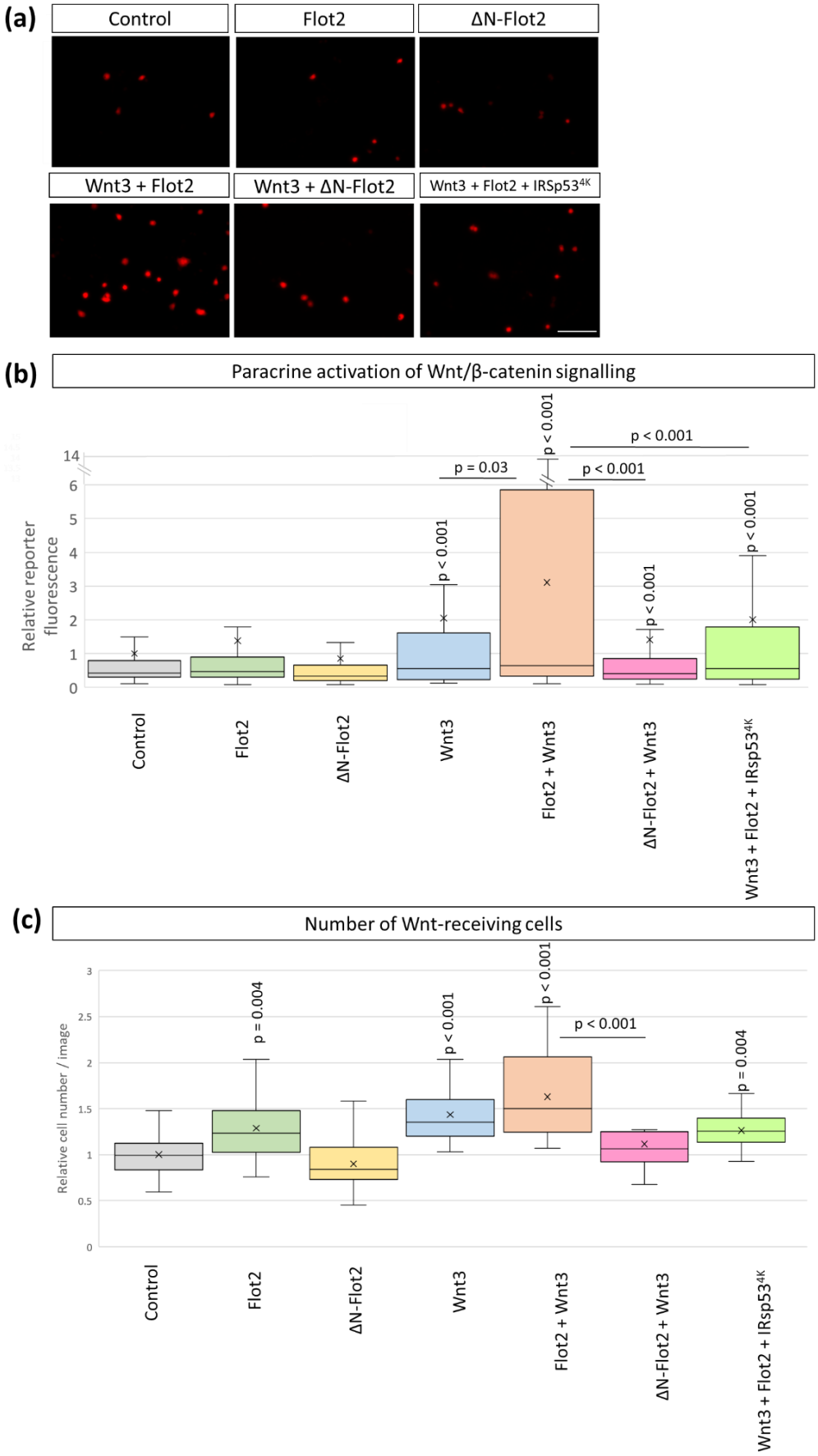


**Figure 21 – Flotillin-2 promotes paracrine Wnt signalling and proliferation via Wnt3 cytonemes**
**(a)** Representative images of HFE-45 cells expressing STF (7xTCF-NLS-mCh) reporter following 48 hr co-cultivation with AGS cells expressing indicated constructs. Scale bar 100μm.
**(b)** Quantification of STF reporter fluorescence. Significance calculated by student's t-test with Bonferroni correction for multiple comparisons. (n = 322, 443, 403, 258, 336, 306, 297; n = number of nuclei measured).
**(c)** Relative number of STF-expressing cells per image following 48 hr co-cultivations. Significance calculated by student's t-test with Bonferroni correction for multiple comparisons. (n = 28, 27, 26, 27, 22, 24, 15; n = number of images).

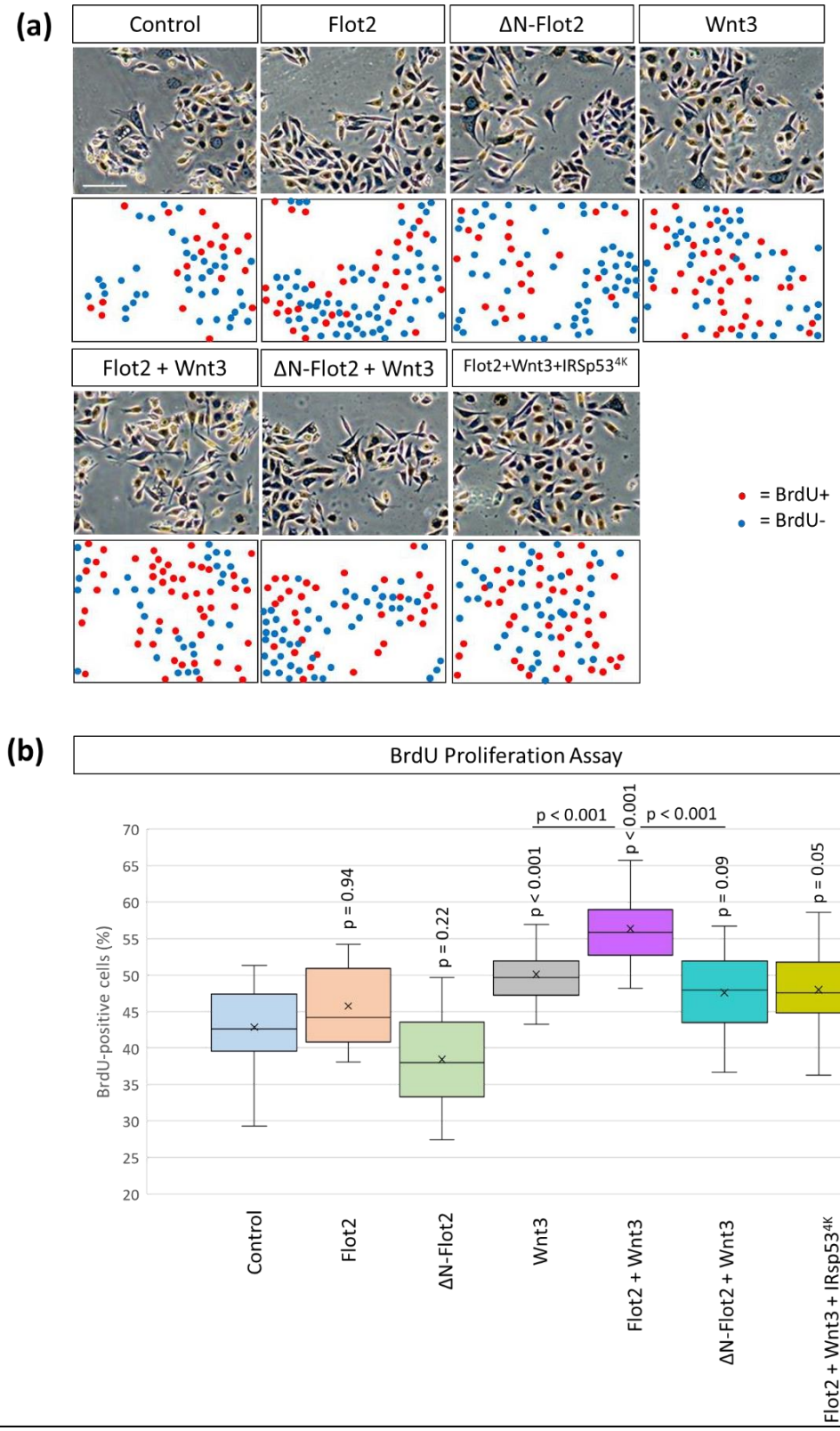


**Figure 22 – Flotillin-2 promotes Wnt3-mediated proliferation in gastric epithelial and cancer cells**
**(a)** Representative images of HFE-145 cells co-cultivated with AGS cells expressing indicated constructs. Cells were stained for BrdU (red) as an indicator of proliferation. Cells were counterstained with haematoxylin (blue). Scale bar 100µm.
**(b)** Quantification of BrdU-positive cells as a percentage of the total population. Significance calculated by student's t-test with Bonferroni correction for multiple comparisons. (n = 20 for all conditions; n = number of images).

### 4.2.6. Flotllin-2 co-localises with Ror2 and is necessary for its membrane localisation

Previous work in *Drosophila* first revealed that over-expression of Flot2 stimulates the secretion and spreading of Wg proteins and concomitant rises in long-range Wg signalling (Katanaev *et al.*, 2008). However, 14 years later and a molecular understanding of this process is yet to be elucidated. Therefore, I aimed to decipher the molecular players involved in Flot2-mediated augmentation of cytonemal Wnt3 signalling.

Previously, the Wnt co-receptor Ror2 has been shown to be critical for Wnt-mediated cytoneme formation and signalling (Mattes *et al.*, 2018; Brunt *et al.*, 2021). Like a number of other RTKs, Ror2 has also been shown to localise to lipid rafts (Sammar *et al.*, 2009). Flot2 has also been shown to regulate the signalling of several RTKs (Moasser, 2007; Amaddii *et al.*, 2012; Tomasovic *et al.*, 2012; Pust *et al.*, 2013; Wang *et al.*, 2017). Therefore, I hypothesised that Flot2 could be interacting with Ror2 in promoting Wnt cytoneme formation.

Firstly, I transfected AGS cells with Flot2-GFP and Ror2-BFP to assess their sub-cellular localisations. Here, Flot2 and Ror2 strongly co-localised, particularly at the PM and along the lengths of cytonemes (Fig. 23a(i)). Unexpectedly, however, when co-expressed with ΔN-Flot2-GFP, Ror2-BFP lost the majority of its membrane localisation and accumulated in the perinuclear region (Fig. 23a(ii)). This effect was mimicked by siRNA-mediated Flot2 KD, which similarly restricted Ror2-BFP localisation (Fig. 23b). These initial findings suggested that Flot2 is necessary for Ror2 membrane localisation.

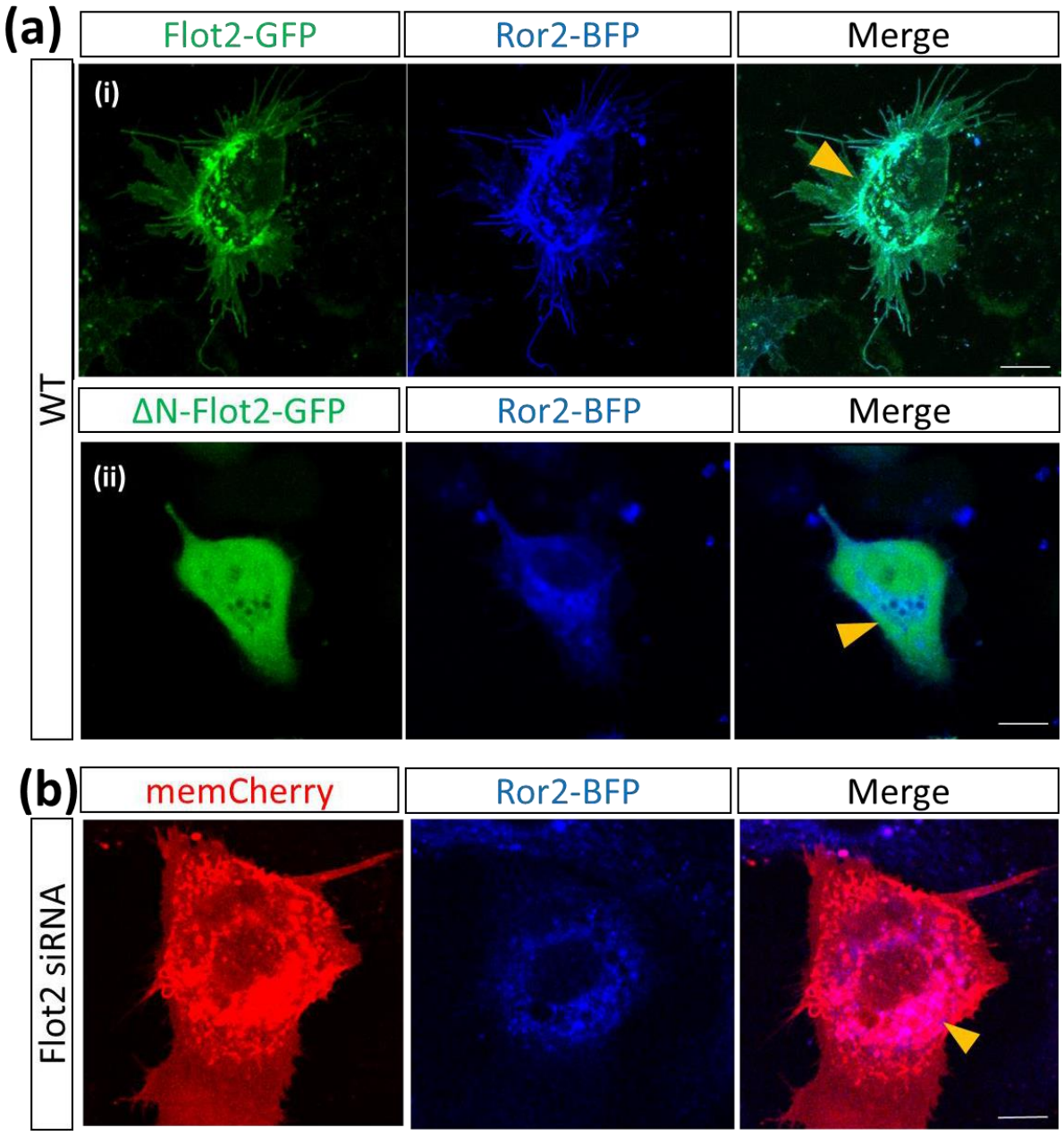


**Figure 23 – Flotillin-2 is necessary for Ror2 membrane localisation**
**(a)** Confocal images of AGS cells expressing Ror2-BFP and (i) Flot2-GFP or (ii) ΔN-Flot2-GFP. Yellow arrows indicate (i) membrane or (ii) intracellular localisation of Ror2. Scale bar 10μm.
**(b)** Confocal images of Flot2-deficient (siRNA) AGS cells expressing membrane-mCherry and Ror2-BFP. Yellow arrow highlights intracellular localisation of Ror2. Scale bar 10μm.

To confirm these findings, I performed antibody stainings against Flot2 and Ror2 to assess endogenous protein localisation (Fig. 24a). Here, Flot2 and Ror2 also co-localise, although more so in the perinuclear region with occasional puncta at the

PM. The difference in these localisations may be due to effects of Flot2 over-expression. To assess this, I transfected AGS cells with Flot2-GFP and performed an antibody stain against endogenous Ror2 (Fig. 24c). Here, membrane localisation of Ror2 is greater than with endogenous Flot2 levels, suggesting Flot2 over-expression promotes the recruitment of Ror2 to the membrane. Similarly to Ror2-BFP, endogenous Ror2 membrane localisation is significantly reduced upon Flot2 KD (Fig. 24b). The accumulation of Ror2 in the perinuclear region suggests that not only is Flot2 necessary for Ror2 membrane localisation, but it is also actively involved in its intracellular transport.

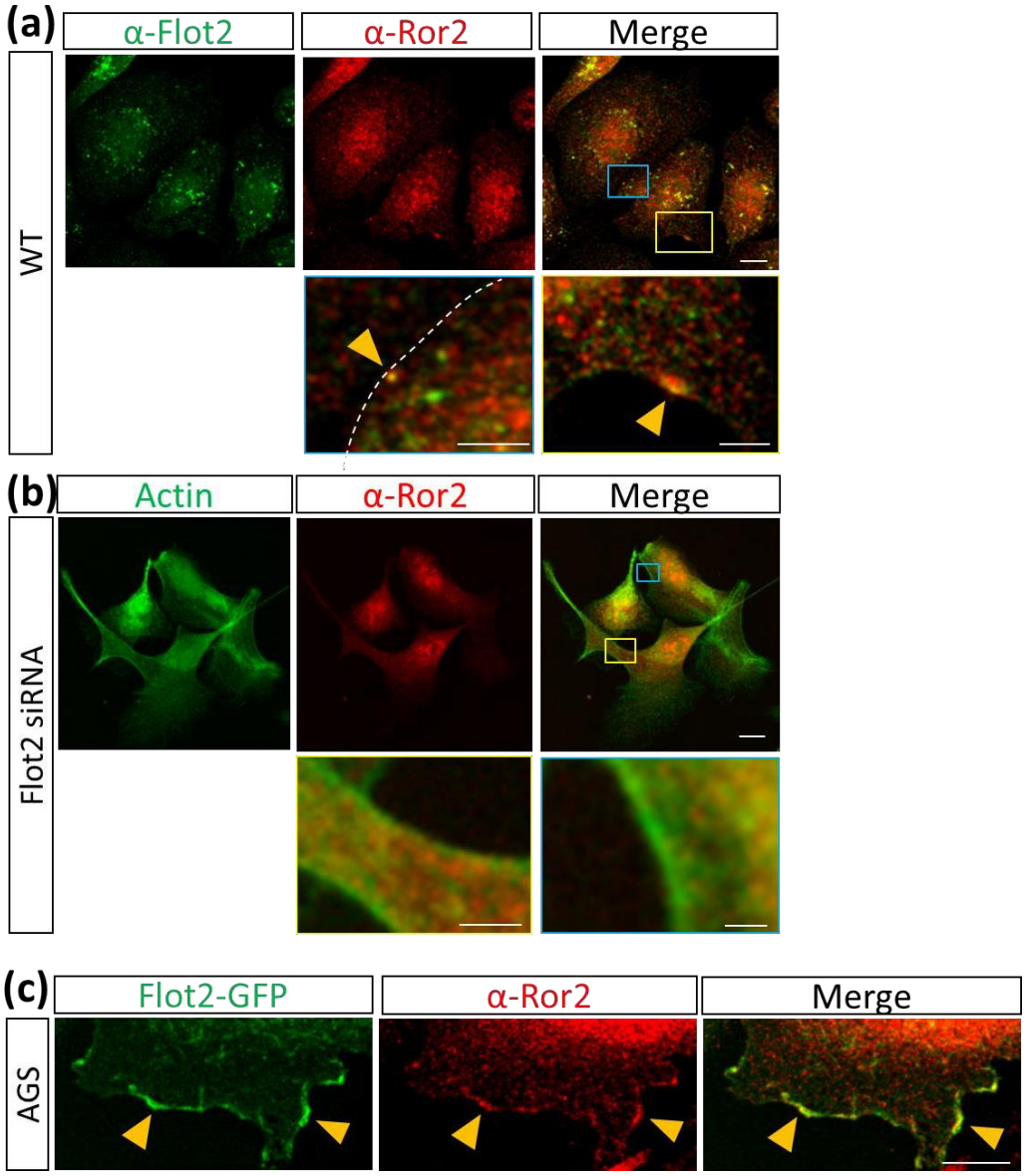


**Figure 24 – Flotillin-2 recruits Ror2 to the plasma membrane**
**(a)** Antibody stainings of AGS cells against Flot2 (green) and Ror2 (red), highlighting co-localising puncta at the membrane (yellow arrows). Dashed line represents the cell boundary. Scale bars 10μm (top) and 5μm (zoomed).
**(b)** Antibody stainings of AGS against Ror2 (red) after siRNA-mediated knockdown of Flot2. Actin is stained with phalloidin (green). Scale bars 10μm (top) and 5μm (zoomed).
**)** AGS cells transfected with Flot2-GFP and antibody stained for Ror2 (red), highlighting co-localisation of Ror2 with Flot2-GFP at the membrane. Scale bar 10μm.

### 4.2.7. Flot2 knockdown causes accumulation of Ror2 in the Golgi apparatus

Next, I sought to identify the compartment(s) where Ror2 was accumulating upon Flot2 KD. AGS cells were transfected with Ror2-mCh and a variety of organelle markers, including Rab5-GFP (early endosomes), Rab7-GFP (late endosomes), mTurq2-LAMP1 (lysosomes) and mTurq2-Golgi (Golgi apparatus). Pearson's correlation coefficient (PCC), which quantifies co-localisation, was then calculated and used to assess changes in co-localisation following Flot2 KD (Fig. 25).

In WT AGS cells, Ror2-mCh and mTurq2-Golgi display some co-localisation, which appears to be in Golgi-derived vesicles, which may represent a portion of Ror2 being transported to the membrane (Fig. 25a, blue arrow). Elsewhere, Ror2-mCh can be seen primarily at the PM and on cytoneme tips (grey and yellow arrows, respectively). Upon Flot2 KD, the majority of PM and cytoneme localisation of Ror2-mCh is lost, but increased co-localisation with mTurq2-Golgi is strikingly visible. This is confirmed by PCC, which shows a significant increase from 0.31 to 0.80 (Fig. 25c). This suggests Flot2 is necessary for exit of Ror2 from the Golgi, and Flot2 KD causes Ror2 accumulation here. Contrastingly, PCC of Ror2-mCh and Rab7-GFP or mTurq2-LAMP1 showed no significant change, whilst Rab5-GFP showed a slightly significant increase in co-localisation (Fig. 25b). Ror2 is known to be internalised via a Rab5-dependent endocytic route (Akbarzadeh *et al.*, 2008), but whether Flot2 is involved in this process or whether this is a knock-on effect of perturbed intracellular transport, is yet to be determined.

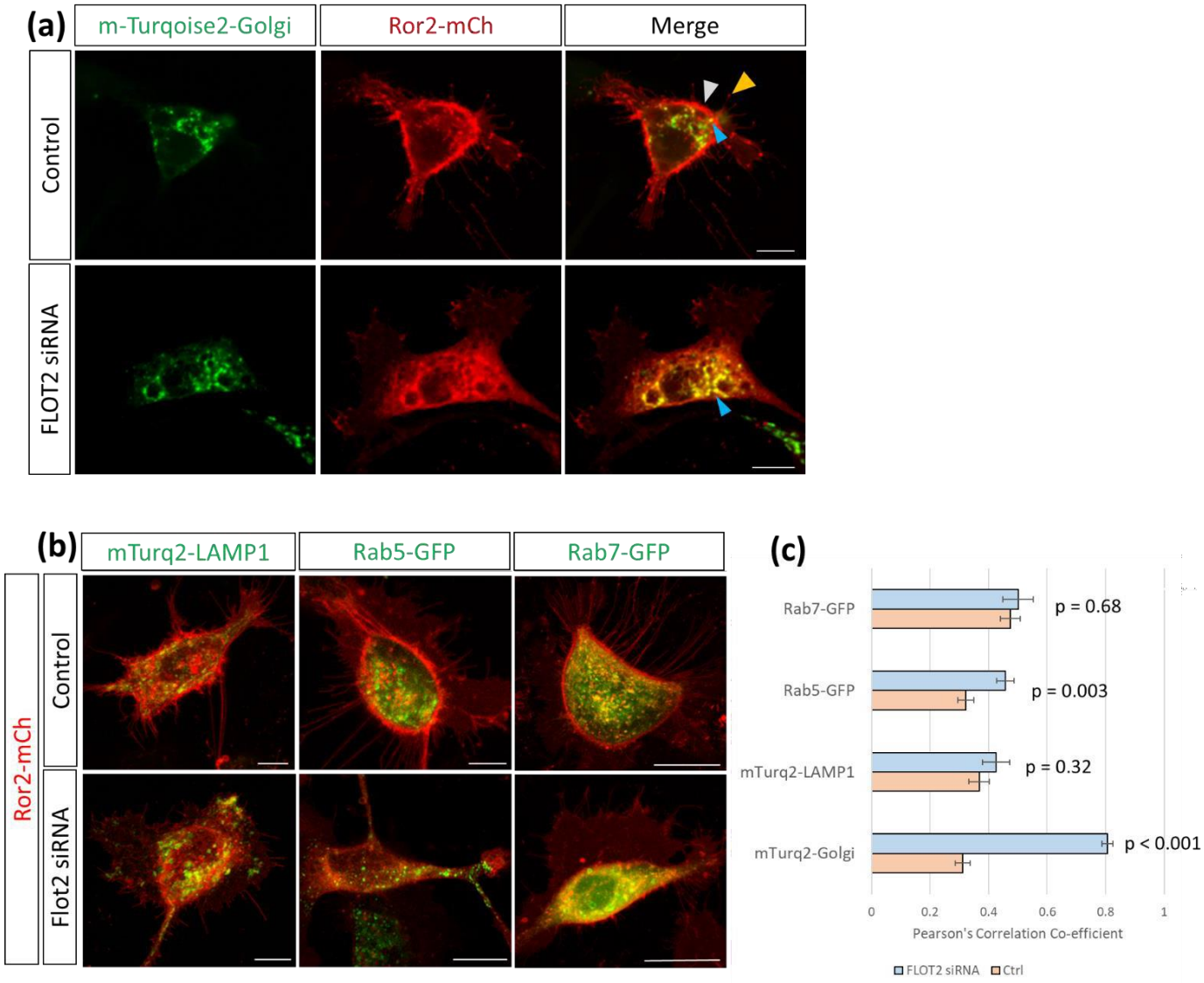


**Figure 25 – Ror2 accumulates in the Golgi apparatus upon Flotillin-2 knockdown**
**(a)** Confocal images of AGS cells expressing Ror2-mCherry and mTurq2-Golgi +/- Flot2 siRNA. Blue arrows highlights co-localisation of Ror2-mCherry and mTurq2-Golgi. Grey and yellow arrows indicate membrane and cytoneme localisation of Ror2-mCh, respectively. Scale bar 10μm.
**(b)** Confocal images of AGS cells expressing Ror2-mCherry and indicated organelle markers +/- Flot2 siRNA. Scale bars 10μm.
**(c)** Quantification of Ror2-mCh co-localisation with indicated organelle markers, as assessed by Pearson's correlation co-efficient (PCC). Error bars represent SEM. Significance calculated by student's t-test. (n = 10 for all conditions)(n = number of cells measured).

### 4.2.8. Ror2/PCP signalling is regulated by Flotillin-2

Activation of Ror2/PCP signalling includes activation of downstream JNK signalling (Fig. 4). Since Flot2 is necessary for Ror2 exit from the Golgi and subsequent membrane localisation, it follows that inhibition of Flot2 function would be expected to inhibit Ror2/PCP and thus JNK signalling, since Ror2 cannot localise to the PM and bind to extracellular Wnt ligands. Therefore, I used an AGS cell line stably expressing a reporter of JNK signalling, the JNK kinase translocation reporter (JNK KTR-mCherry) (Regot *et al.*, 2014; Miura *et al.*, 2018; Brunt *et al.*, 2021). JNK-KTR-mCherry localises to the nucleus (N) in its dephosphorylated state (low JNK activity). Upon activation by phosphorylation, it shuttles to the cytoplasm (C, high JNK activity). The C:N ratio can then be calculated as an indicator of JNK signalling (Fig. 26a).

AGS cells display a low level of basal JNK activity, as indicated by a C:N ratio of 0.49 (Fig. 26b, c). Expression of either Flot2-GFP or Ror2-BFP results in activation of JNK signalling and C:N ratios of 0.76 and 0.71, respectively. Co-expression of Flot2-GFP and Ror2-BFP produces a synergistic effect and significantly increases the C:N ratio to 0.87. Conversely, ∆N-Flot2-GFP alone produces a C:N ratio of 0.53. Additionally, ∆N-Flot2-GFP effectively blocks Ror2-induced JNK activity, producing a C:N ratio of 0.53 even when over-expressing Ror2-BFP (Fig. 26b, c). Together these data (along with localisation studies in Fig. 25) show that Flot2 enhances Ror2/PCP/JNK signalling, likely through promoting membrane localisation and thus Wnt ligand exposure.

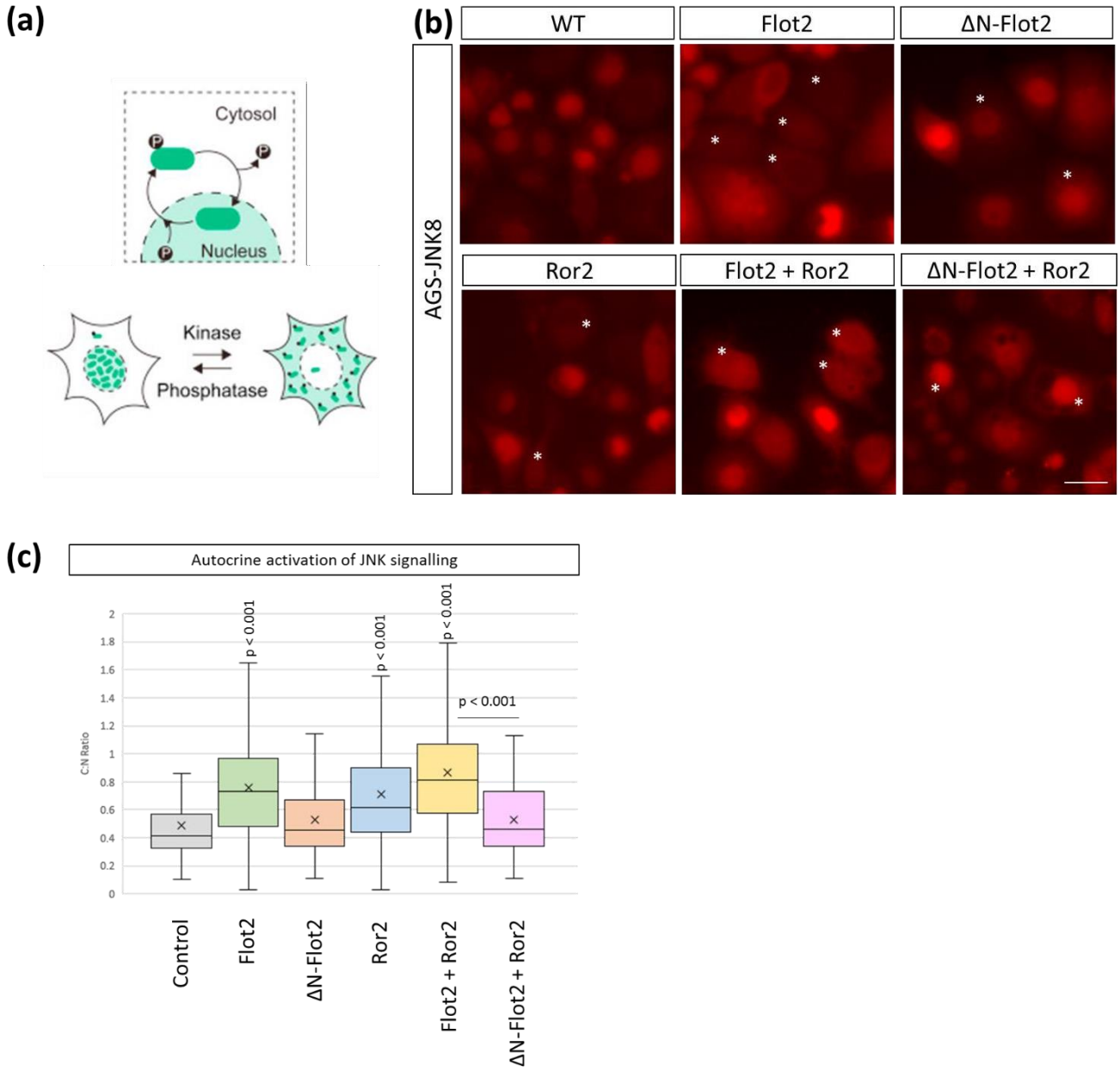


**Figure 26 – Flotillin-2 regulates Ror2/PCP/JNK signalling in gastric cancer cells**
**(a)** Illustration of the JNK-KTR-mCherry reporter system utilised for measuring JNK activity (adapted from Miura *et al.,* 2018).
**(b)** Representative images of AGS cells stably expressing the JNK-KTR-mCherry reporter and indicated constructs after 48 hrs. Asterisks mark transfected cells. Scale bar 20µm.
**(c)** Quantification of the JNK-KTR-mCherry reporter. Nuclear and cytoplasmic fluorescence of cells were measured and the cytoplasmic:nuclear ratio calculated. Significance calculated by one-way ANOVA with Bonferroni correction for multiple comparisons. (n per condition = 136, 109, 74, 109, 82, 79).

### 4.2.9. Flotillin-2 is necessary for Ror2-mediated cytoneme formation

Ror2/PCP signalling is crucial for driving Wnt cytoneme formation (Mattes *et al.*, 2018; Brunt *et al.*, 2021) and I have shown that Flot2 also regulates cytoneme formation in GC cells. I therefore analysed changes in cytonemal phenotypes in AGS cells to assess the functional impact of Flot2 on Ror2-induced cytoneme formation (Fig. 27). AGS cells were transfected with memCherry to visualise and quantify cytonemes.

Expression of Ror2 alone increased the average cytoneme length whilst having no significant effect on cytoneme number (Fig. 27a-d). However, co-expression of Flot2 with Ror2 enhanced this phenotype, significantly increasing cytoneme length in a synergistic fashion. Consistently, blockage of Flot2 function by expression of ΔN-Flot2-GFP considerably decreased the number and length of cytonemes in Ror2-expressing AGS cells. This supports the notion that Flot2 regulates Ror2/PCP signalling and thus cytoneme formation. To test whether the opposite is true, i.e., Flot2 requires Ror2 for cytoneme formation, a mutant construct of Ror2 missing the Wnt-interacting CRD domain (ΔCRD-Ror2) was used, which cannot bind to Wnt ligands and thus transduce a Wnt signal. When expressed in AGS cells, ΔCRD-Ror2-GFP significantly reduced the average length and cumulative length of cytonemes, suggesting Ror2 signalling is important to cytoneme formation in AGS cells. I found that this phenotype can be partially rescued by the expression of Flot2, which moderately restores the average cytoneme length and number, suggesting that Flot2 acts downstream of Ror2-inducing cytonemes. Together with the results in Fig. 26, this shows that Flot2 facilitates Ror2-mediated Wnt/PCP signalling and, consequently, the promotion of Wnt cytonemes.

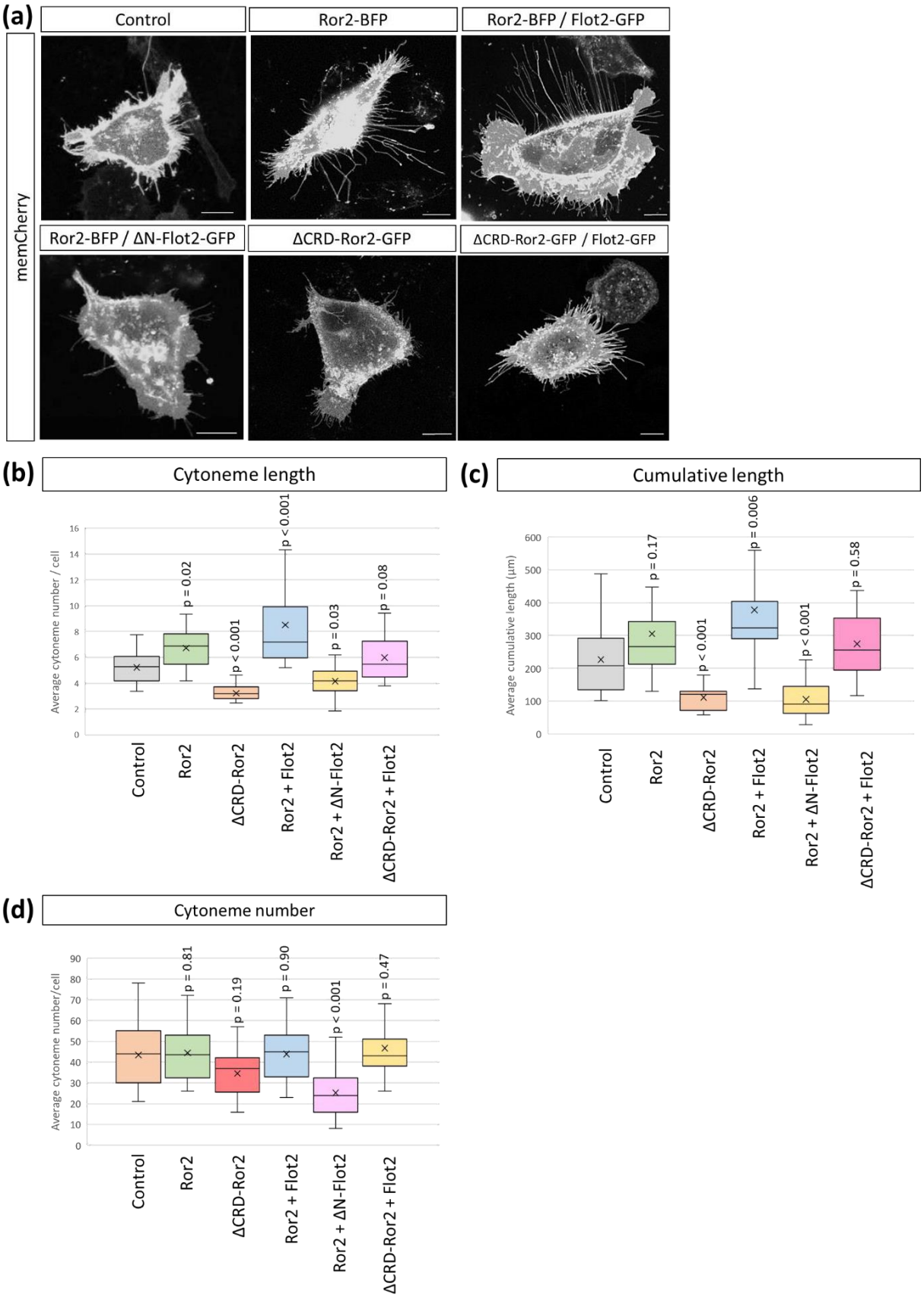


**Figure 27 – Functional Flotillin-2 is necessary for Ror2-mediated cytoneme induction**
**(a)** Representative confocal images of AGS cells expressing memCherry and indicated constructs for 48 hrs. Scale bars 10µm.
**(b-d)** Quantification of cytoneme (a) length, (b) cumulative length and (c) number in AGS cells transfected with constructs indicated. Significance calculated by Student's t-test with Bonferroni correction for multiple comparisons. (n per condition = 25, 22, 21, 23, 25, 21; n = number of cells)

### 4.2.10. Flot2 inhibits autocrine Wnt/β-catenin signalling in GC cells

It is well-documented that the Wnt/β-catenin and Wnt/PCP pathways can act in a mutually repressive manner (Gao and Chen, 2010a; Mattes *et al.*, 2018). Thus, I next investigated whether the autocrine effect of Flot2 on Wnt/PCP signalling perturbs autocrine Wnt/β-catenin signalling. To answer this, I transfected AGS cells with the STF (7xTCF-NLS-mCh) reporter and measured its fluorescence after 48 hrs as a readout of Wnt/β-catenin signalling.

Interestingly, expression of Flot2-GFP had a significant inhibitory effect on Wnt/β-catenin signalling, reducing the fluorescence readout by 39% (Fig. 28a, b). Contrastingly, the ΔN-Flot2-GFP mutant had no inhibitory effect, with a small (but insignificant) 16% increase in reporter activity, suggesting Flot2 membrane localisation is necessary for its ability to inhibit Wnt/β-catenin signalling. To evaluate whether this effect involves Ror2/PCP signalling, I expressed the dominant-negative Ror2-ΔCRD mutant to attenuate Wnt/PCP signalling. Thus, I expected an increase in Wnt/β-catenin signalling by alleviating repression from the Wnt/PCP pathway. Surprisingly, expression of Ror2-ΔCRD displayed a small decrease (10%) in reporter activity. This unexpected result is discussed in detail in section 6.3.3. in the context of results from chapter 4.

However, the presence of Ror2-ΔCRD in Flot2 over-expressing cells effectively abrogates the inhibitory effect of Flot2 on Wnt/β-catenin signalling, producing analogous results to Ror2-ΔCRD alone (10% decrease). This suggests the repression of autocrine Wnt/β-catenin signalling by Flot2 requires functional Ror2 and concomitant activation of the Wnt/PCP pathway.

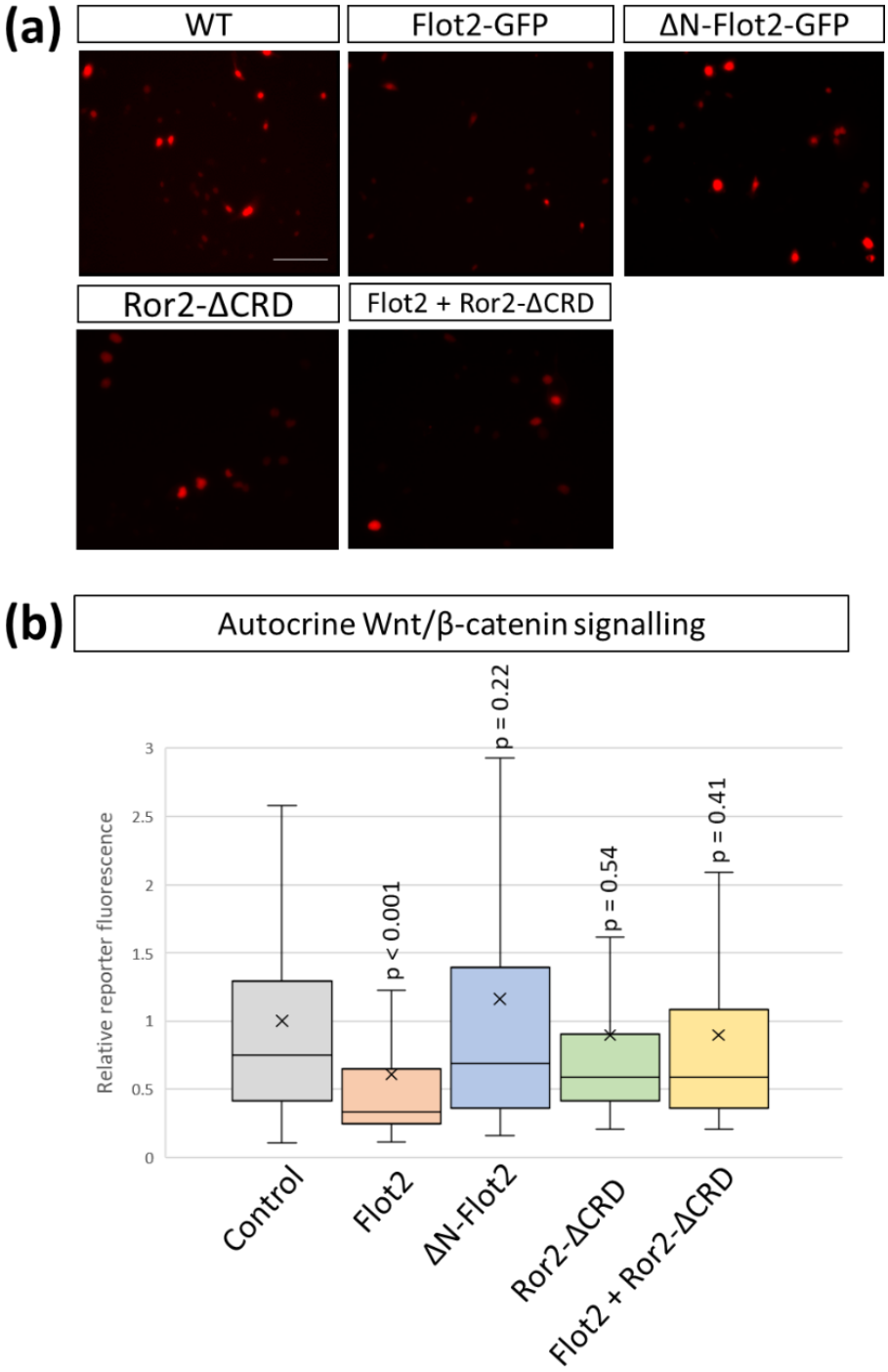


**Figure 28– Flotillin-2 inhibits autocrine Wnt/β-catenin signalling through Ror2/PCP signalling**
**(a)** Representative images of AGS cells expressing STF (7xTCF-NLS-mCh) reporter and indicated constructs after 48 hrs. Scale bar 100μm.
**(b)** Relative quantification of 7xTCF-NLS-mCherry fluorescence compared to untransfected control. Significance calculated by student's t-test. (n per condition = 111, 93, 207, 79, 83; n = number of nuclei measured).

### 4.2.11. Flotillin-2 regulates Wnt8a cytonemes in zebrafish development

To evaluate whether the role of Flot2 in formation of Wnt cytonemes is conserved, I addressed Flot2 function *in vivo* during zebrafish development. Previous work from our lab has shown that cytonemes are essential for the dissemination of Wnt8a in zebrafish embryogenesis (Stanganello *et al.*, 2015) and that these cytonemes can be regulated by the Ror2/PCP pathway (Mattes *et al.*, 2018; Brunt *et al.*, 2021).

First, I microinjected zebrafish embryos with Flot2-GFP DNA and memCherry to map the subcellular localisation of Flot2 in zebrafish epiblast cells (Fig. 29a). As seen in GC cells (Fig.16b), I found that Flot2-GFP displayed strong membrane localisation and was localised to filopodia, including their tips (Fig. 29a, yellow arrows). Flot2-GFP puncta could also be seen in neighbouring recipient cells (Fig. 29a, blue arrow), suggesting intercellular transport. Next, I wanted to assess whether Flot2 also regulates the formation and elongation of filopodia in zebrafish, as was seen in GC cells. Therefore, I altered Flot2 function during zebrafish embryogenesis and observed that the expression of Flot2-GFP significantly increased the average cytoneme length and cumulative length, whilst having no significant effect on cytoneme number (Fig. 29b,c). Consistently, the dominant-negative ΔN-Flot2-GFP attenuated the filopodia number and cumulative length. This data confirms that Flot2 regulates filopodia in the zebrafish embryo, concurrent with our findings in GC cells.

Next, I wanted to assess whether Flot2 is specifically involved in cytoneme-mediated transport of Wnt8a, as well as regulating filopodia in general. I co-injected Flot2-GFP and Wnt8a-mCh into zebrafish embryos and found that Flot2 and Wnt8a co-localise along the lengths and at the tips of cytonemes (Fig. 31), analogous to the co-localisation seen between Flot2 and Wnt3 in GC cells (Fig. 19). These data suggest Flot2 may have a conserved role in the regulation of filopodia and Wnt cytonemes in vertebrates.

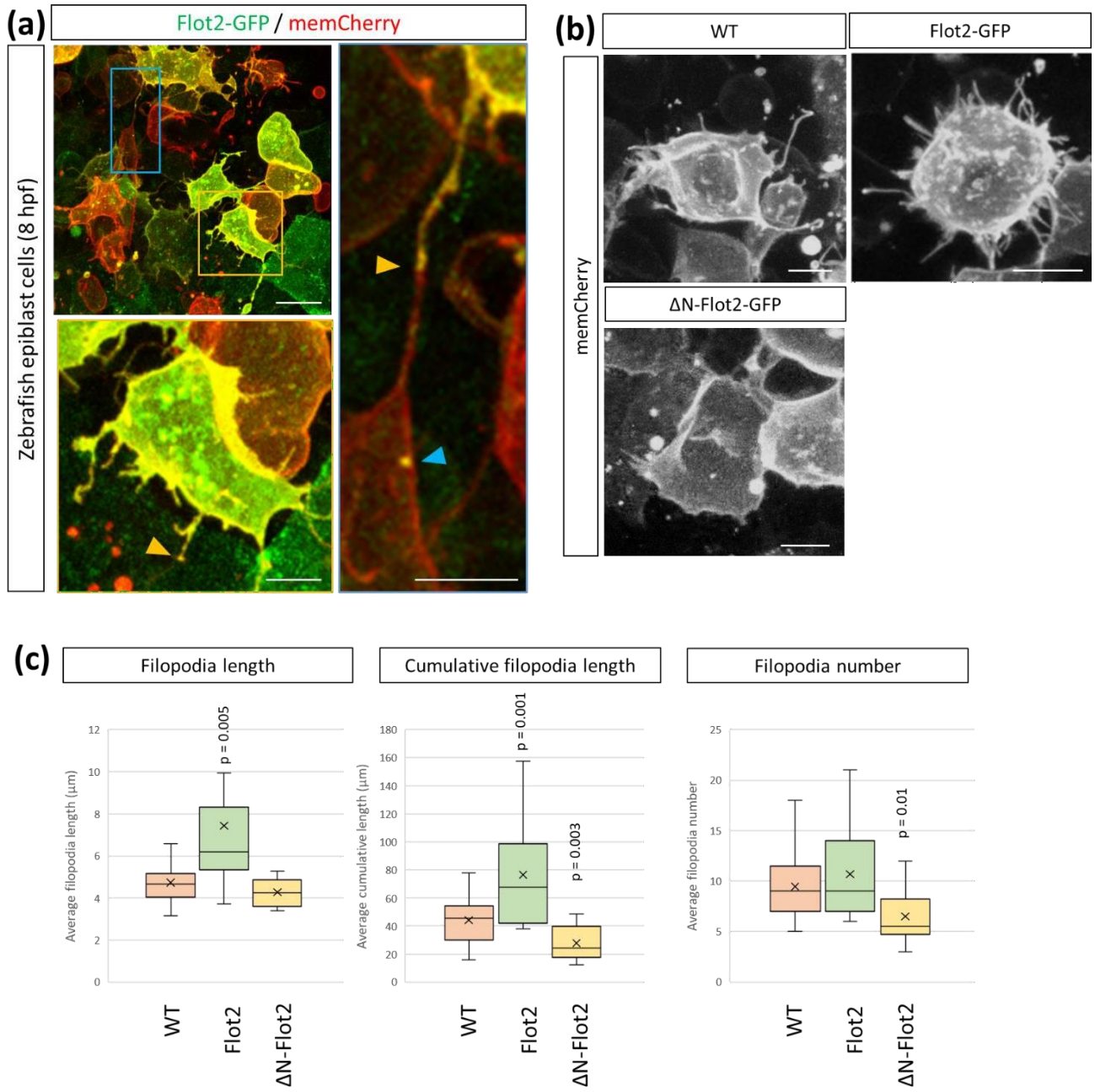


**Figure 29 – Flotillin-2 promotes filopodia formation during zebrafish development**
**(a)** Representative confocal images of zebrafish epiblast cells injected with Flot2-GFP and memCherry (100 ng/µl) and imaged at 8 hpf. Yellow arrows highlight filopodial localisation of Flot2. Blue arrow indicates Flot2 in a neighbouring recipient cell. Scale bar 20µm (top left) and 10µm (zoomed).
**(b)** Representative confocal images of zebrafish epiblast cells (8 hpf) after microinjection with memCherry and Flot2-GFP or ΔN-Flot2-GFP (100 ng/µl). Scale bar 10µm.
**(c)** Quantification of zebrafish epiblast cell filopodia after microinjection with indicated constructs. Significance calculated by student's t-test. (n per condition = 17, 20, 14; n = number of cells).

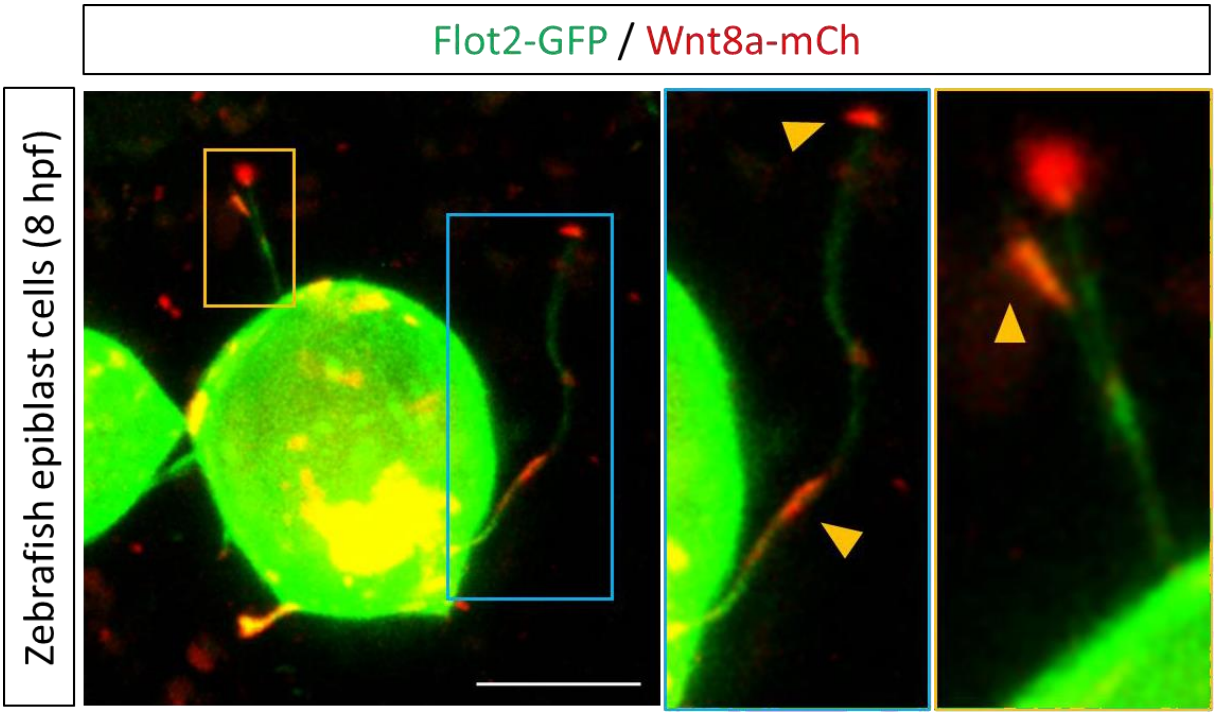


**Figure 30 – Flotillin-2 co-localises with Wnt8a on cytonemes in zebrafish development**
Confocal images of zebrafish epiblast cells (8 hpf) after microinjection of Flot2-GFP and Wnt8a-mCh (100 ng/µl). Localisation of Flot2 and Wnt8a on cytonemes is highlighted by yellow arrows. Scale bar represents 20µm.

### 4.2.12. Flotillin-2 alters brain boundary formation in zebrafish development by promoting Wnt8a signalling

Next, I addressed the consequences of Flot2 function on the formation of the Wnt/β-catenin signalling gradient in zebrafish embryogenesis. Wnt8a is a key Wnt morphogen in determining the positions of the boundaries of the brain anlage, and alteration of Wnt8a cytonemes perturbs brain anlage patterning (Mattes *et al.*, 2018; Brunt *et al.*, 2021). Therefore, I altered Wnt expression levels together with Flot2 levels during neural plate patterning and performed *in situ* hybridisation against *pax6a*, a marker of the forebrain (FB) and hindbrain (HB). Injection of membrane-mCherry was used as the control. Here, I found that the expression of low levels of Wnt8a mRNA results in an anterior shift of the boundaries of the brain anlage and reduces the combined FB and midbrain MB length (Fig. 31a, b). An even more pronounced shift of the position of brain anlage boundaries occurred upon co-injection of Flot2 and Wnt8a, suggesting a synergistic effect of Flot2 and Wnt8a

during neural plate patterning. Consistently, blockage of Flot2 function by expression of the dominant-negative ΔN-Flot2-GFP attenuated the alteration in patterning.

As well as perturbing the length of the primordia of the FB/MB, alteration of Wnt8a signalling can cause defects in the early zebrafish embryo, which can include complete loss of the FB primordium and under-developed or missing eyes (which can be seen in Fig. 31a). I categorised the developmental defects according to severity and found that in embryos expressing Flot2-GFP or Wnt8a-mCh, 31% and 44% display mild defects, respectively (Fig. 31c). Meanwhile around 37% of embryos had severe defects. Co-expressing Flot2-GFP and Wnt8a-mCh significantly increased the number of embryos with severe defects to 67% and only 6% of embryos displayed normal phenotypes. However, co-injection of ΔN-Flot2-GFP with Wnt8a-mCh reduced phenotype severity in all categories to levels comparable with Wnt8a-mCh alone, demonstrating Flot2 membrane localisation is necessary for its enhancement of Wnt8a signalling and thus alterations in phenotypes.

Together, these data suggest Flot2 can enhance the length and number of Wnt8a cytonemes, and thus alter the Wnt8a signalling gradient, leading to a posteriorisation of the zebrafish brain anlage. These findings are concurrent with the results in GC cells showing Flot2 enhances paracrine Wnt signal activation. In both systems, Flot2 also co-localises with the Wnt ligands, Wnt3 and Wnt8a, and promotes cytoneme formation, which suggests a conserved role for Flot2 in promoting cytoneme-mediated Wnt dissemination in vertebrate tissue.

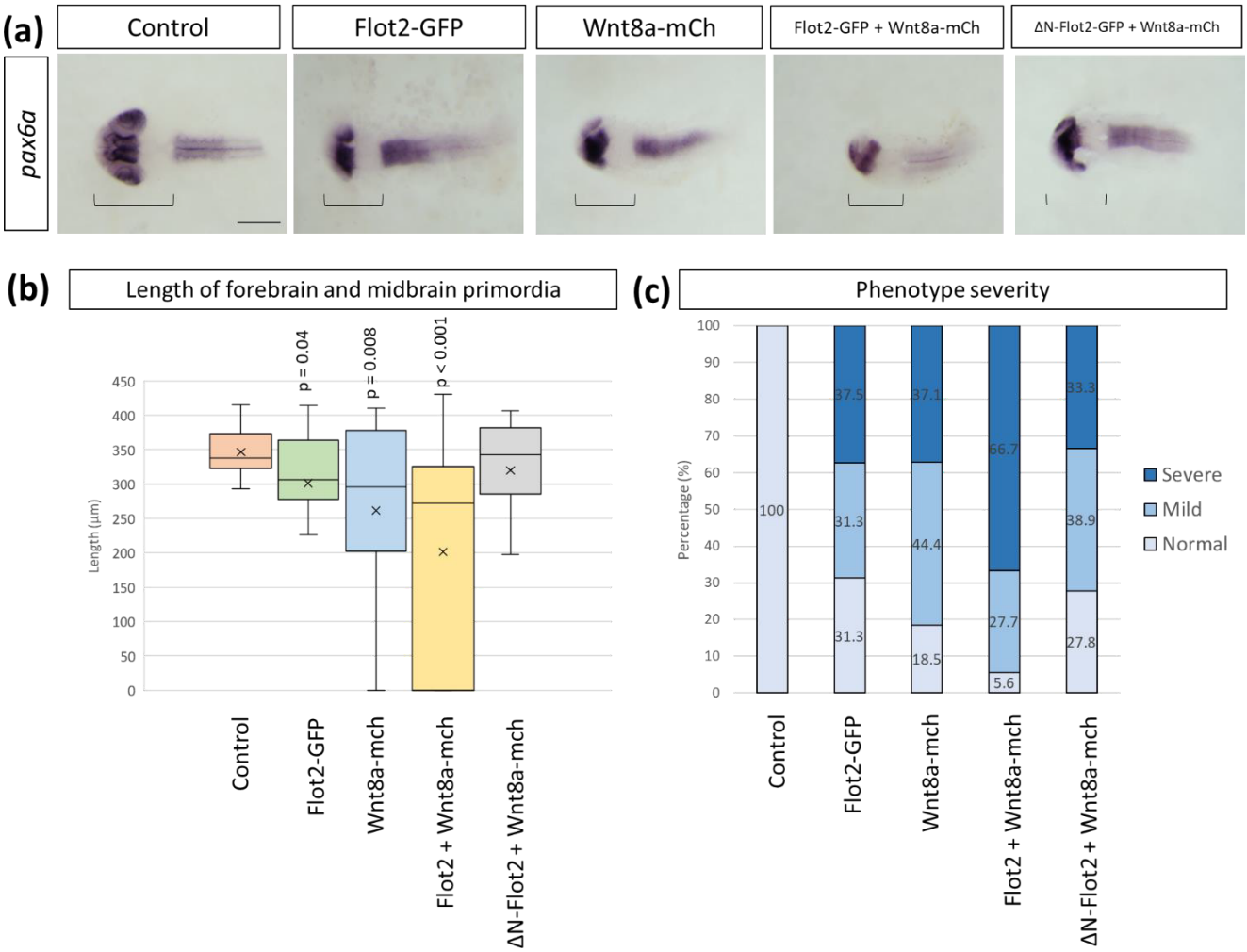


**Figure 31 – Flotillin-2, with Wnt8a, regulates brain boundary formation in zebrafish development**
**(a)** *In situ* hybridisation against pax6a in zebrafish embryos at 30hpf after microinjection of 100ng/µl of indicated DNA constructs. Scale bar represents 100µm.
**(b)** Quantification of forebrain and midbrain primordia length in zebrafish embryos injected as in (a). Significance calculated by Student's t-test. (n per condition = 23, 16, 27, 18, 18; n = number of embryos).
**(c)** Qualitative analysis of phenotype severity in zebrafish embryos injected as indicated in (a) Phenotypes classified into the categories normal, mild and severe. Numbers in bars represent percentages of total embryos.

## 4.3. Discussion

### 4.3.1. Flot2 promotes Wnt cytoneme formation and signalling

As a membrane scaffolding protein and marker of lipid rafts, Flot2 is a functionally diverse protein due to its interactions with a plethora of membrane proteins and receptors. Indeed, Flot2 microdomains are considered key “dating points” for signalling, where protein-protein interactions are encouraged through clustering of ligands and receptors (Gauthier-Rouvière *et al.*, 2020). Thus, by providing platforms for signalling events, Flot2 overexpression often results in a concomitant augmentation of signalling via RTKs, GPCRs and GPI-anchored proteins, among others (Liu *et al.*, 2018). For example, Flot2 overexpression is frequently implicated in GC and is associated with poor prognosis, where it is often used as a prognostic marker (Zhu *et al.*, 2013). Here, Flot2 has been shown to stabilise the RTK ErbB2/HER2 at the PM and thus enhance downstream signals which promote proliferation and tumour cell survival (Moasser, 2007; Zhu *et al.*, 2013). This concurs with other GC studies, which demonstrate that Flot2 expression correlates with increasing cell proliferation, migration and invasion (Cao *et al.*, 2013).

Here, I also showed that Flot2 is overexpressed in GC cell lines (compared to normal gastric epithelial HFE-145 cells), and that Flot2 overexpression or inhibition promotes or attenuates cell proliferation, respectively (Fig. 15, 22). This concurs with previous findings that silencing of Flot2 function, either through siRNA (*in vitro*) or miRNAs (*in vivo*), consistently reduces proliferation and migratory phenotypes, such as invasiveness associated with EMT (Cheng *et al.*, 2014; Li *et al.*, 2015; Wei *et al.*, 2018). Whilst I did not directly assess migration, Flot2 expression levels in HFE-145 and GC cells did correlate with filopodial phenotypes, which concurs with its well-documented ability to induce filopodia in multiple cell types (Fig. 17) (Hazarika *et al.*, 1999; Neumann-Giesen, Falkenbach, Beicht, Claasen, Uers, *et al.*, 2004). Filopodia are important in cell migration and thus may be indicative of increased migratory capacity, although this would need to be assessed (Bischoff *et al.*, 2021).

As well as the aforementioned signalling pathways, Flot2 has also been shown to enhance Wg/Wnt signalling *in vivo*. In *Drosophila*, Flot2 overexpression enhances Wg gradient formation and expression of Wg target genes (Katanaev *et al.*, 2008). Flot2 overexpression also phenocopies that of Wg, perturbing signalling in the wing

imaginal disc and altering adult wing formation (Hoehne *et al.*, 2005). My work therefore supports these findings and presents the first evidence that Flot2 is capable of promoting paracrine Wnt/β-catenin signalling in mammalian cells. Here, I demonstrated that Flot2 overexpression in GC cells can enhance paracrine Wnt3 signalling, as assessed by Wnt reporter activity in receiving gastric epithelial cells (Fig. 21). Consistently, inhibition of Flot2 function (by siRNA or ΔN-Flot2) reduced paracrine Wnt3 signalling, suggesting Flot2 is necessary for efficient Wnt3 dissemination. My data suggest this inhibition is not due to reduced Wnt3 mobilisation within the producing cell, since ΔN-Flot2-GFP or Flot2 KD do not interfere with Wnt3 sub-cellular localisation (Fig. 20).

Whilst *Drosophila* Wg expression regulates developmental processes such as tissue patterning, Wnt signalling in adult epithelial tissues promotes stemness and proliferation (Reya and Clevers, 2005). Indeed, over-expression of Wnt1 or Wnt3 in AGS cells results in increased proliferation in Wnt-receiving HFE cells (Fig. 21, 22). Overexpression of Flot2 significantly enhances both Wnt1- and Wnt3-induced cell proliferation, which confirms a functional role for Flot2 in enhancing Wnt-mediated effects. However, until now, there has been little in the way of a mechanistic explanation for how this is achieved. Suggestions that Flot2 could enhance packaging of Wg into secretory vesicles, such as lipoproteins, have been made (Solis *et al.,* 2013). However, there is no direct evidence to corroborate this hypothesis, other than the observed localisation of the Wnt co-receptor Lrp6 to Flot2 microdomains. This observation was made in the context of LDL signalling and Lrp6 internalisation, however, and not Wnts (Solis *et al.,* 2013; Yamamoto *et al.*, 2017).

Given the ability of Flot2 to enhance filopodial phenotypes and to promote Wnt signalling, it was conceivable that Flot2 may regulate Wnt cytonemes. Flot2 was first implicated in cytoneme-mediated morphogen transport in the context of Hh, where ectopic Flot2 expression enhanced cytoneme formation and the Hh gradient in *Drosophila* (Bischoff *et al.*, 2013). Here, I have provided the first evidence that Flot2 enhances Wnt signalling via its ability to promote Wnt cytoneme formation, since expression of the dominant-negative IRSp53$^{4K}$ attenuated Flot2-mediated enhancement of Wnt signalling (Fig. 21). This function could be conserved, since Flot2 was also able to enhance Wnt8a signalling *in vivo* to perturb brain boundary formation during zebrafish development (Fig. 31). However, the co-localisation of

Flot2 with Wnt3 (and Wnt8a in zebrafish) was a surprising observation. This is because I originally hypothesised Flot2 may enhance cytoneme formation through its ability to regulate the actin cytoskeleton, including actin polymerisation and thus filopodia formation. As a subset of filopodia, I thought cytonemes (and thus Wnt ligand transport) may be passively promoted via this function. This could also explain how both Hh and Wnt cytonemes are promoted, which would not require specificity of morphogen(s) and/or their receptors. However, as well as clustering of Flot2 and Wnt3 at cytoneme contact sites, Flot2-positive cytonemes were also able to induce clustering of the Wnt co-receptor Lrp6 in the recipient cell (Fig. 19), which has previously been observed as an indication of Wnt cytoneme signal transduction (Mattes *et al.*, 2018). Interestingly, Lrp6 also co-localised with Flot2 in the recipient cell at this contact point. Furthermore, co-localisation of Flot2 and Wnt3 persists in Wnt-receiving cells. These observations, which suggest a role for Flot2 in transducing Wnt signals in the receiving cell, are investigated further in chapter 3.

Together, these observations suggest localisation of Wnt proteins, Ror2 and potentially Lrp6 to Flot2 microdomains. It would be interesting to investigate whether Hh and the receptors involved in Hh cytoneme formation, such as Ihog, also localise to Flot2 microdomains, which would suggest a more general function for Flot2 in promoting morphogen-transporting cytonemes (González-Méndez *et al.,* 2017).

### 4.3.2. Flot2 is required for Ror2-mediated cytoneme formation and signalling

Despite the observed co-localisation between Flot2 and Wnt3, I would not expect these two proteins to directly interact or bind, since Flot2 resides on the inner leaflet of the plasma membrane and Wnt3 is presented extracellularly, either bound to its receptor(s) or intracellular chaperone Evi. Therefore, I hypothesised that a more likely scenario involves localisation of Wnt (co-)receptors to Flot2 microdomains, to which Wnt ligands are bound and consequently co-localise with Flot2.

I chose to investigate Ror2 as a candidate receptor in this context, since Ror2 is a known regulator of cytonemes, both in GC cells and zebrafish (Mattes *et al.*, 2018; Brunt *et al.*, 2021). Additionally, Ror2 is an RTK and Flot2 has previously been shown to regulate signalling for a number of RTKs (Banning *et al.*, 2014). Indeed, Ror2-BFP and Flot2-GFP displayed significant co-localisation when co-expressed

and also had a synergistic effect on increasing cytonemal phenotypes in AGS cells (Fig. 27). Flot2 also enhanced Ror2/PCP signalling, as assessed using the JNK-KTR-mCherry reporter, which could explain how Flot2 enhances Ror2-mediated cytoneme formation. The most striking phenotype, however, was upon knockdown of Flot2 by siRNA or inhibition with the dominant-negative ΔN-Flot2-GFP. Here, Ror2 membrane localisation was lost and Ror2 accumulated in the Golgi in the absence of functional Flot2 (Fig. 25). This suggests a defect in Ror2 transport and that Flot2 is necessary for its exit from the Golgi. This is consistent with reports highlighting Flot2 as a regulator of membrane invagination and trafficking between endocytic compartments (Frick *et al.*, 2007). In particular, it has been observed that cargo, including Flot2 itself, accumulate in the Golgi complex of HeLa, Jurkat and PC12 cells when Flot2 function is obstructed (Langhorst, Reuter, *et al.*, 2008). Therefore, whether this effect on Ror2 transport is specific or a consequence of general transport inhibition is debatable. However, there was no significant increase in co-localisation of Ror2 with markers for late endosomes or lysosomes, and only a small increase with the early endosome marker Rab5 (Fig. 25). This could suggest that Flot2 is involved in the internalisation and/or recycling of Ror2. Indeed, Ror2 has been shown to traffic via Rab5-positive endosomes following Wnt5a binding (Akbarzadeh *et al.*, 2008). Concurrently, Flot2 has previously been shown to regulate intracellular trafficking of TCRs via a Rab5-Rab11 axis in T cells and thus it is conceivable that Flot2 could regulate Rab5-mediated Ror2 trafficking (Redpath *et al.*, 2019). Observing alterations in Ror2 localisation (and Rab5 co-localisation) upon expression of the endocytosis mutant Y163F-Flot2 could help decipher this potential function.

Intriguingly, Ror2 internalisation requires phosphorylation by Src kinase, which also localises to and phosphorylates Flot2 to induce its internalisation (Neumann-Glesen *et al.*, 2007; Riento *et al.*, 2009). Therefore, Flot2 could also enhance Ror2 signalling by promoting Ror2-Src interactions and phosphorylation events. It would be interesting to investigate whether the ability of Flot2 to augment Ror2/PCP signalling is dependent on Src activity.

### 4.3.3. Flot2 inhibits autocrine Wnt/β-catenin signalling

It is generally accepted that the Wnt/β-catenin and Wnt/PCP signalling pathways act in a mutually repressive manner, due to competition of receptors and common

intracellular binding proteins, such as Dvl (Gao and Chen, 2010a; Mattes *et al.*, 2018). Here I show that in AGS cells, Flot2 simultaneously enhances Wnt/PCP signalling whilst inhibiting Wnt/β-catenin signalling in the Wnt-producing cells (Fig. 26, 28), supporting this mutual repression model. Here, I hypothesised that Flot2 was achieving this through enhancing Ror2/PCP signalling. Therefore, loss of functional Ror2 signalling, by expression of Ror2-ΔCRD, would relieve inhibition of the Wnt/β-catenin pathway and thus increase STF reporter activity above that of the untransfected control. Co-expression of Flot2 and Ror2-ΔCRD reduced the extent to which Wnt/β-catenin signalling was attenuated compared to Flot2 alone, but still showed a decrease compared to the control. This could suggest that Flot2 can repress Wnt/β-catenin signalling independently from Ror2. Unexpectedly, however, expression of Ror2-ΔCRD alone did not enhance STF reporter activity, but showed attenuation to levels comparable to when Flot2 and Ror2-ΔCRD are expressed together (Fig. 28). This suggests that the inhibition by Flot2 on Wnt/β-catenin signalling does occur via Ror2, but that loss of the Ror2 CRD is also detrimental to Wnt/β-catenin signalling – which is discussed in detail in chapter 4.

Overall, the data presented in this chapter demonstrate that Flot2 is an important regulator of both Wnt signalling pathways and that its effects differ depending on whether it is a Wnt-producing or Wnt-receiving cell. The ability of Flot2 to enhance Wnt/PCP signalling occurs via promoting Ror2 transport, membrane localisation and thus signalling, which in turn enhances Wnt cytoneme formation, paracrine Wnt3 transport and Wnt/β-catenin signalling (summarised in Fig. 32). This work enhances our understanding of the mechanisms by which Flot2 promotes Wnt signalling, which is particularly noteworthy in the context of GC pathogenesis. It would be worthwhile to investigate, by epidemiological studies, whether there is a correlation between Flot2 expression levels and upregulation of Wnt signalling genes and pathways in Wnt-related cancers. Thus, Flot2 could offer a novel therapeutic target for combatting Wnt-related cancers, as well as being a prognostic marker.

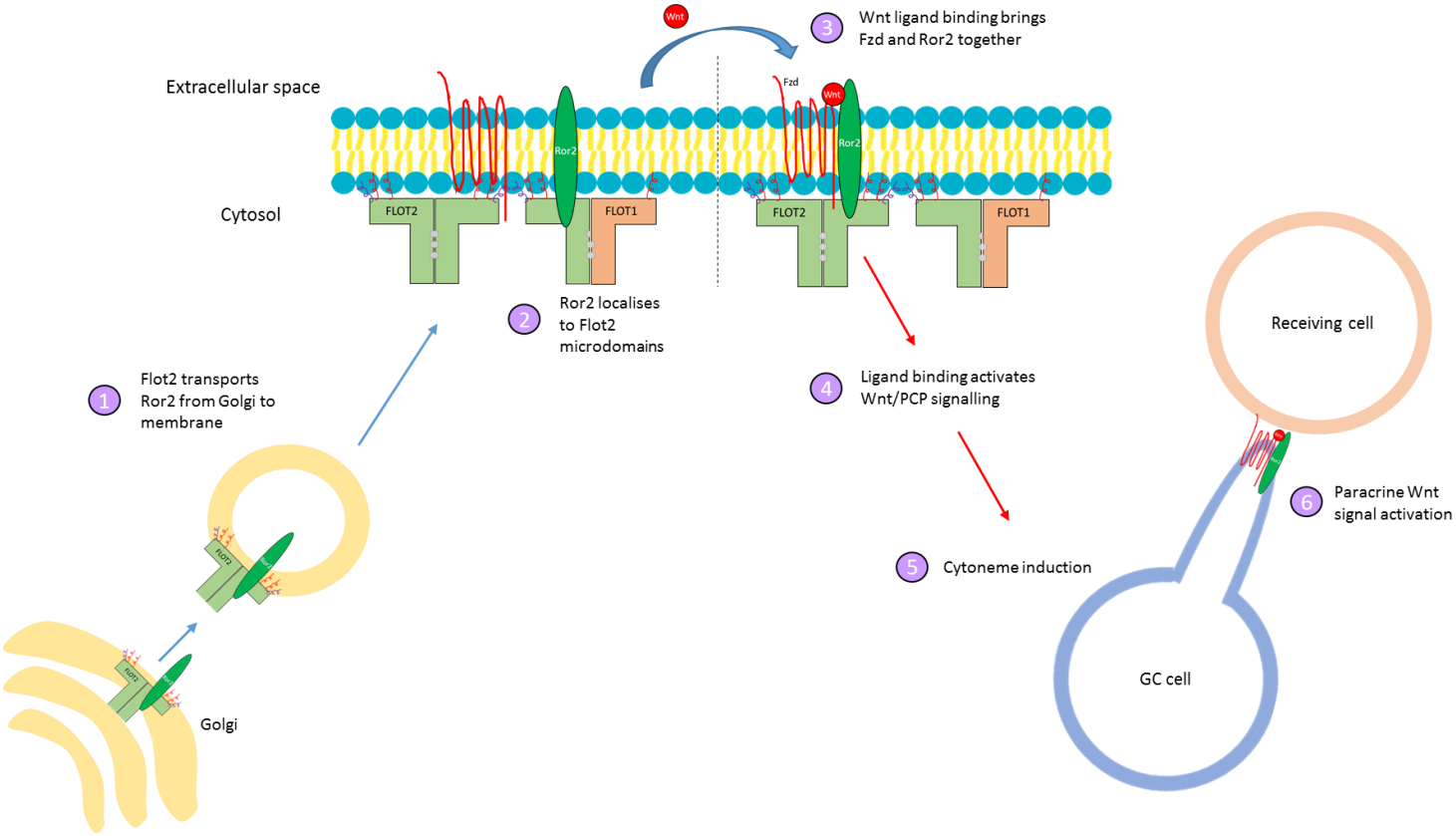


**Figure 32 – Schematic representing the proposed mechanism by which Flot2 enhances Ror2/PCP signalling**
(1) Flot2 interacts with Ror2 in the Golgi apparatus, where it is necessary for its Golgi exit. (2) Flot2 transports Ror2 to the membrane, where Ror2 resides in flotillin microdomains. (3) This promotes surface presentation of Ror2 and binding of Wnt ligands. (4) Wnt/PCP signalling is subsequently transduced and (5) Wnt cytonemes are induced / elongated. (6) This increases paracrine transport of Wnt proteins to neighbouring cells, which are contacted by these cytonemes.

# 5. Chapter 3 – Flotillin-2 and Lrp6 internalisation in GC cells

## 5.1 Introduction

Flotillin-mediated endocytosis is a type of clathrin-independent endocytosis (CIE) thought to mediate the uptake of GPI-anchored proteins and a number of receptors, including the insulin receptor and cholera toxin B receptor GM1 (Baumann *et al.*, 2000; Riento *et al.*, 2009; Saslowsky *et al.*, 2010). More relevant to Wnt signalling, flotillin-mediated endocytosis has previously been implicated with the internalisation of the Wnt co-receptor Lrp6, although this was in the context of LDL signalling in hepatocytes, not Wnt ligands (Yamamoto *et al.*, 2017).

Following my observations that co-localisation of Wnt3 and Flot2 persists in Wnt-receiving cells (Fig. 19b), I began to consider a role of Flot2 in the uptake of Wnt ligands in contacted cells. Given the aforementioned association of Flot2 with Lrp6 uptake, Lrp6 was a good candidate for investigating the role of Flot2 in the uptake of Wnt3, which can bind to and induce Lrp6 endocytosis (Yamamoto *et al.*, 2008). Current models suggest that activation of Wnt/β-catenin signalling in recipient cells occurs via cytonemes inducing clustering of Fzd and Lrp6 receptors and their subsequent internalisation (Stanganello *et al.*, 2015; Mattes *et al.*, 2018), which is necessary for signal transduction (Brunt and Scholpp, 2018).

Thus, I hypothesised that Flot2 may promote Wnt uptake in recipient cells by enhancing Lrp6 internalisation at cytoneme contact sites. This notion is supported by findings from Katanaev *et al.*, (2008), where in *Drosophila* cells, overexpression of Flot2 exclusively in the Wg-producing cells enhances Wg uptake into recipient cells. The authors suggest Flot2 may change the properties of Wg, e.g., enhanced packaging of Wg into vesicles, which are more internalisable by the recipient cells (Katanaev *et al.*, 2008). However, I propose this observation may also be explained by enhanced cytonemal delivery and concomitant uptake of Wnts.

In this chapter, I investigate a potential role for Flot2 in mediating uptake of Wnt3 into recipient cells via endocytosis of Lrp6. Additionally, I assess changes in sub-cellular localisation of Lrp6 in relation to Flot2 function and expression. Finally, I analyse the functions and localisations of dynamin, caveolin and clathrin with respect to Flot2, in order to shed light on the relationship(s) between these endocytic regulators.

## 5.2. Results

### 5.2.1. Internalised Wnt3 localises to Flotillin-2-positive vesicles

Firstly, I wanted to confirm that the co-localisation of Flot2-GFP and Wnt3-mCh in the recipient cells was not an artefact of Flot2 over-expression and thus general membrane marking. Therefore, I transfected AGS cells with Flot2-GFP or mem-GFP and Wnt3-mCh and analysed the surrounding, untransfected cells (Fig. 33a). Here, as observed previously, Flot2 and Wnt3 co-localisation can be seen in recipient cells in a punctate fashion. However, mem-GFP and Wnt3, which co-localise in Wnt-producing cells, form largely discrete puncta in recipient cells with little or no co-localisation, suggesting this is not a general effect of membrane marking. To determine whether Flot2 is actively involved in the uptake of Wnt3, I set up a co-cultivation of Flot2-GFP-expressing HFE-145 cells (Wnt-receiving) and Wnt3-mCh expressing AGS cells (Wnt-producing). Here, Wnt3-mCh can be seen in Flot2-GFP-positive vesicles in the recipient cells (Fig. 33b), suggesting Flot2 is directly involved in Wnt3 internalisation, likely through endocytic events.

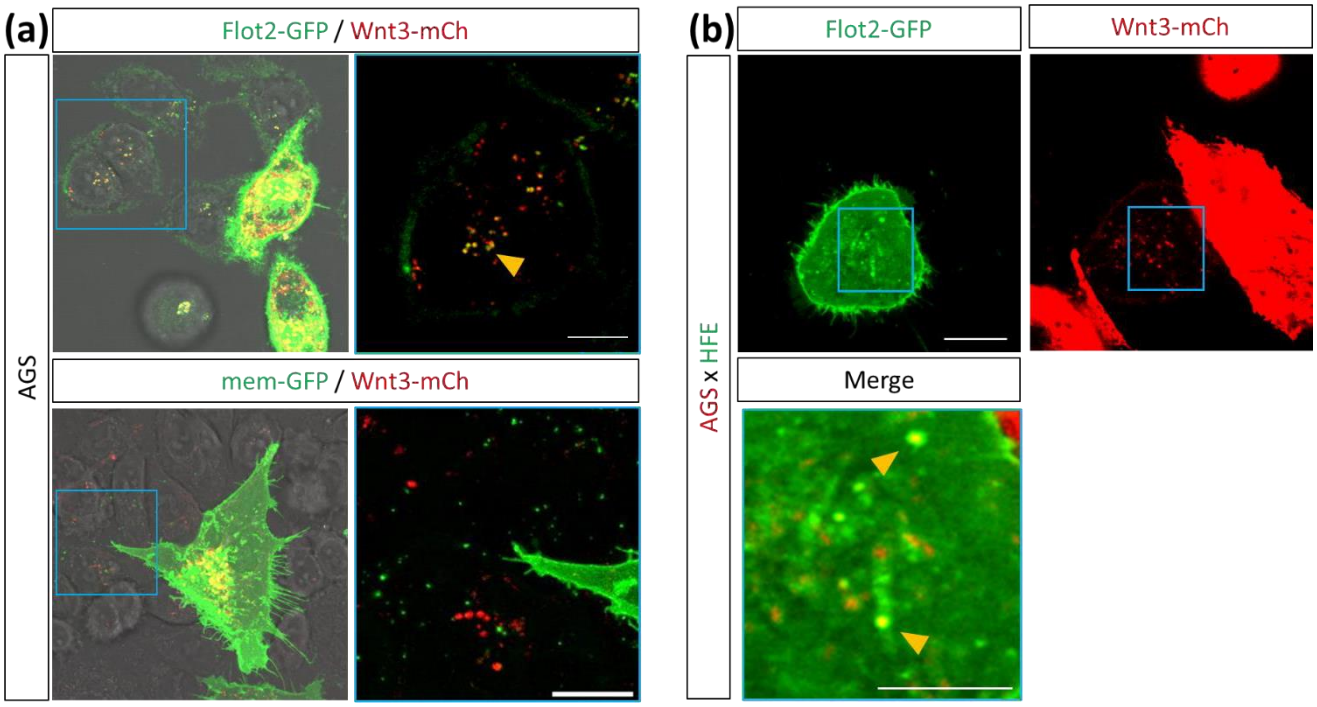


**Figure 33 – Flotillin-2 is involved in the internalisation of Wnt3 in Wnt-receiving cells**
**(a)** Confocal images of AGS cells transfected with Flot2-GFP or mem-GFP and Wnt3-mCh. Yellow arrow indicates co-localisation of Flot2 and Wnt3 in a recipient cell. Scale bars represent 10µm.
**(b)** Confocal images of co-cultivated HFE-145 and AGS cells transfected with Flot2-GFP and Wnt3-mCh, respectively. Yellow arrows highlight presence of Wnt3-mCh in Flot2-positive vesicles. Scale bars represent 10µm (top) and 5µm (zoomed).

### 5.2.2. Flotillin-2 co-localises with the Wnt co-receptor Lrp6 and influences its sub-cellular localisation

Flot2, through its endocytic functions, has previously been shown to mediate the internalisation of Lrp6 in the presence of LDL in HepG2 cells (Yamamoto *et al.*, 2017). Following Wnt ligand binding, Lrp6 is endocytosed as part of the signal transduction process (Brunt and Scholpp, 2018). Thus, I wanted to assess the relationship between Flot2 and Lrp6, starting with their sub-cellular localisations. Therefore, I began by transfecting AGS cells with Flot2-GFP and Lrp6-mCh (Fig. 34a). Lrp6-mCh predominantly resides in the cytosol, where it is localised to vesicular structures. Here, Lrp6-mch co-localises with Flot2-GFP in a number of vesicles. I also performed antibody stainings against Lrp6 and Flot2 to determine whether these proteins co-localise at endogenous levels. As shown in Fig. 34b, Lrp6 displays a greater membrane association, where it notably localises to Flot2-positive regions of the membrane. Next, I performed siRNA-mediated knockdown of Flot2 to assess any

changes in Lrp6 sub-cellular localisation. Strikingly, Flot2 KD resulted in a strong accumulation of Lrp6 in the perinuclear region and significant loss of membrane localisation (Fig. 35c). Together, along with Fig. 33, these findings suggest that Flot2 may be involved in the intracellular transport and/or membrane localisation of Lrp6.

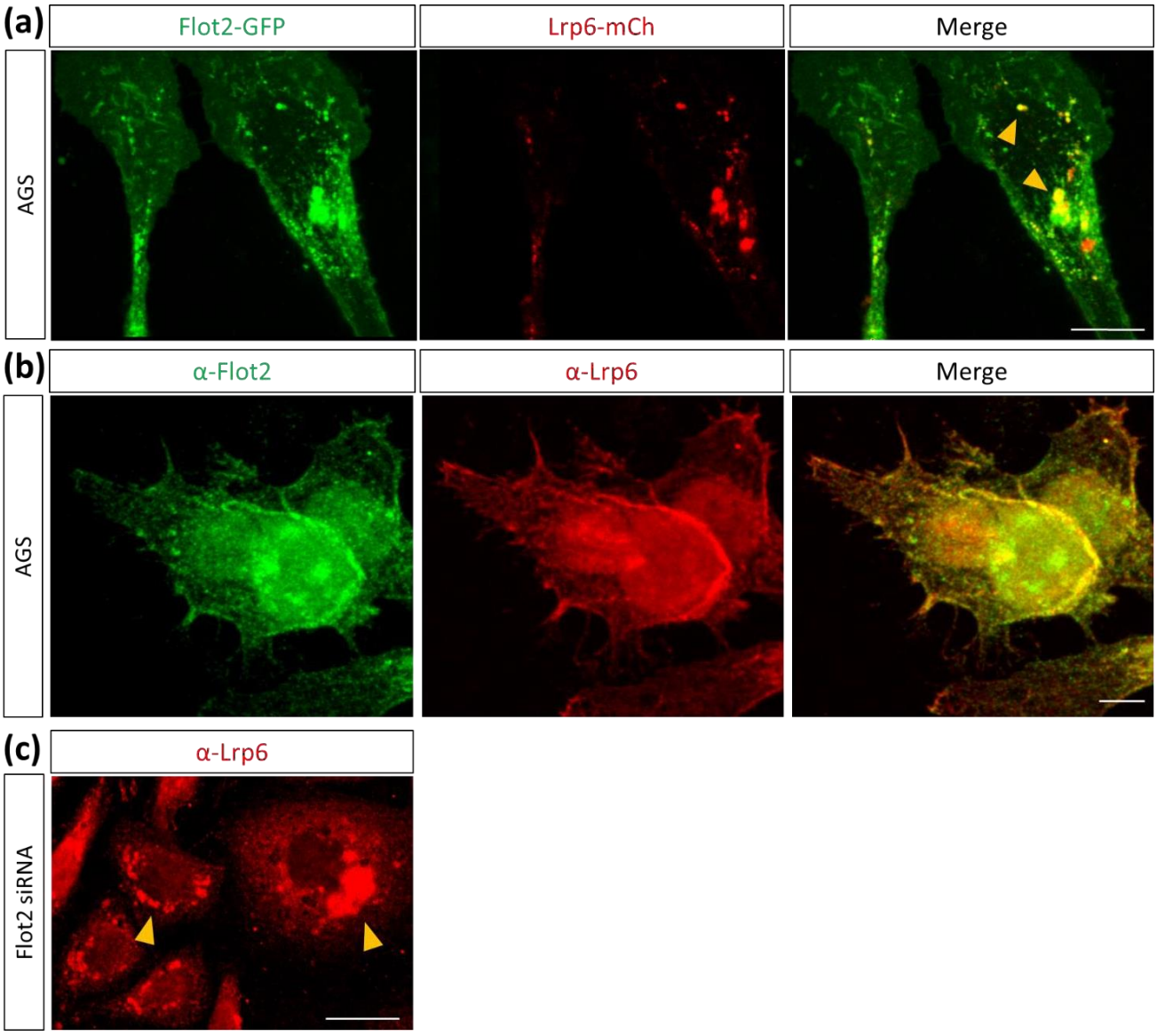


**Figure 34 – Flotillin-2 co-localises with Lrp6, and its knockdown alters Lrp6 localisation**
**(a)** Confocal images of AGS cells transfected with Flot2-GFP and Lrp6-mCh. Yellow arrows indicate co-localisation. Scale bar 10µm.
**(b)** Antibody stainings against Flot2 (green) and Lrp6 (red) in AGS cells. Scale bar 10µm.
**(c)** Antibody staining against Lrp6 after siRNA-mediated knockdown of Flot2 in AGS cells. Yellow arrow highlights accumulation of Lrp6 in perinuclear region. Scale bar 20µm.

### 5.2.3. Wnt3-Lrp6 complexes localise to cytonemes and cluster in Flot2-positive microdomains

Thus far, I have shown that Flot2 co-localises with both Lrp6 and Wnt3 in GC cells. Therefore, I wanted to confirm whether Lrp6 and Wnt3 localise together in Flot2 microdomains. To analyse this, I first performed antibody stainings against Lrp6 and Wnt3 in AGS cells, where significant co-localisation can be visualised (Fig. 35a). Perhaps unsurprisingly, due to the nature of Wnt receptors to cluster, Wnt3 and Lrp6 can be seen in clusters of up to around 800nm (Fig. 35a, blue box). These clusters appear to occur in Flot2 microdomains, as transfection of AGS cells with Flot2-GFP prior to fixation and staining revealed these Wnt3/Lrp6 clusters localise to Flot2-GFP-positive regions (Fig. 35b). Unexpectedly, however, Wnt3 and Lrp6 can be seen co-localising on cytonemes, which appeared to be a common feature (Fig. 34a, yellow box). This finding is discussed in section 7 in the context of data from chapter 4. However, these images show that Flot2 is involved in the localisation and/or clustering of Wnt3/Lrp6 complexes.

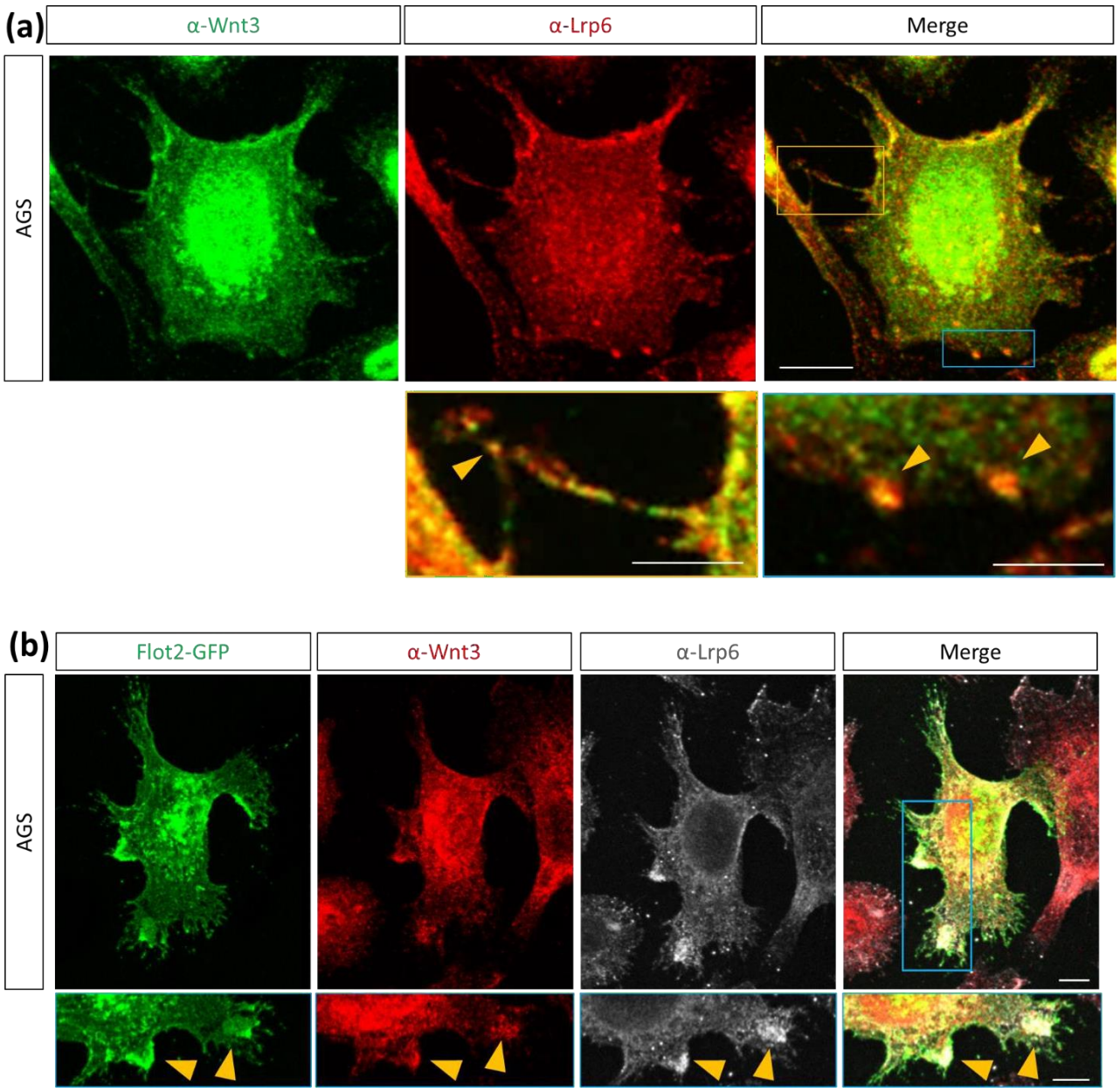


**Figure 35 – Wnt3-Lrp6 complexes localise to cytonemes and cluster in Flot2 microdomains**
**(a)** Antibody stainings against Wnt3 (green) and Lrp6 (red) in AGS cells. Yellow box highlights cytonemal localisation and blue box highlights Wnt3-Lrp6 clusters. Scale bars 10μm (top) and 5μm (zoomed).
**(b)** Antibody stainings against Wnt3 (red) and Lrp6 (grey) in AGS cells after transfection with Flot2-GFP. Blue box and yellow arrows highlight Flot2-Wnt3-Lrp6 clusters. Scale bars 10μm (top) and 5μm (zoomed).

### 5.2.4. Wnt/β-catenin signalling and Lrp6 internalisation are inhibited by a Flotillin-2 endocytosis mutant

Next, I wanted to functionally assess the impact of Flot2 on the endocytosis of the Wnt co-receptor Lrp6. To do this, I used a dominant-negative Flot2 endocytosis mutant, which has a key tyrosine phosphorylation residue, necessary for its endocytosis, mutated to phenylalanine (Y163F-Flot2) (Neumann-Glesen *et al.*, 2007; Babuke *et al.*, 2009). This mutant can still localise to and cluster at the membrane, but is incapable of internalising and translocating to endosomes and thus is a useful tool for studying the role of Flot2 in Lrp6 endocytosis.

Firstly, I transfected AGS cells with Flot2-GFP and then performed an antibody stain against Lrp6 (Fig. 36a). Here, co-localisation between Flot2 and Lrp6 can be seen both intracellularly in the perinuclear region (Fig. 36a, yellow box) and at the membrane (Fig. 36a, blue box). However, expression of Y163F-Flot2-GFP causes a striking phenotype, with significant accumulation of Lrp6 at the membrane (Fig. 36b). Here, large Y163F-Flot2-positive vesicles containing Lrp6 can be seen building up at the membrane (Fig. 36b, blue box). It appears as though these vesicles are attempting, but failing, to undergo successful endocytosis and thus the cargo is also accumulating. This suggests that Lrp6 internalisation occurs in a Flot2-dependent manner. Interestingly, the intracellular co-localisation of Lrp6 and Flot2 in the perinuclear region is lost upon Y163F-Flot2 expression and there is a notable decrease in intracellular Lrp6 staining (Fig. 36b, yellow box), suggesting the intracellular relationship of Flot2 and Lrp6 is an endocytic one, as one would expect co-localisation to persist if Flot2 was involved in the anterograde transport of Lrp6 to the membrane.

To assess the impact of Flot2 endocytosis on Wnt/β-catenin signalling, I transfected AGS cells with the STF (7xTCF-NLS-mCh) reporter and Y163F-Flot2-GFP (Fig. 36c). Untransfected AGS cells were used as the control. As reported in section 4.3.10., wildtype Flot2-GFP causes a decrease in autocrine Wnt/β-catenin signalling, likely through its role in Ror2/PCP signalling. However, Y163F-Flot2-GFP more potently inhibited STF reporter activity, causing an 86% decrease compared to the control. This demonstrates that Flot2 endocytosis is important in Wnt/β-catenin signal transduction, potentially through the endocytosis of Lrp6.

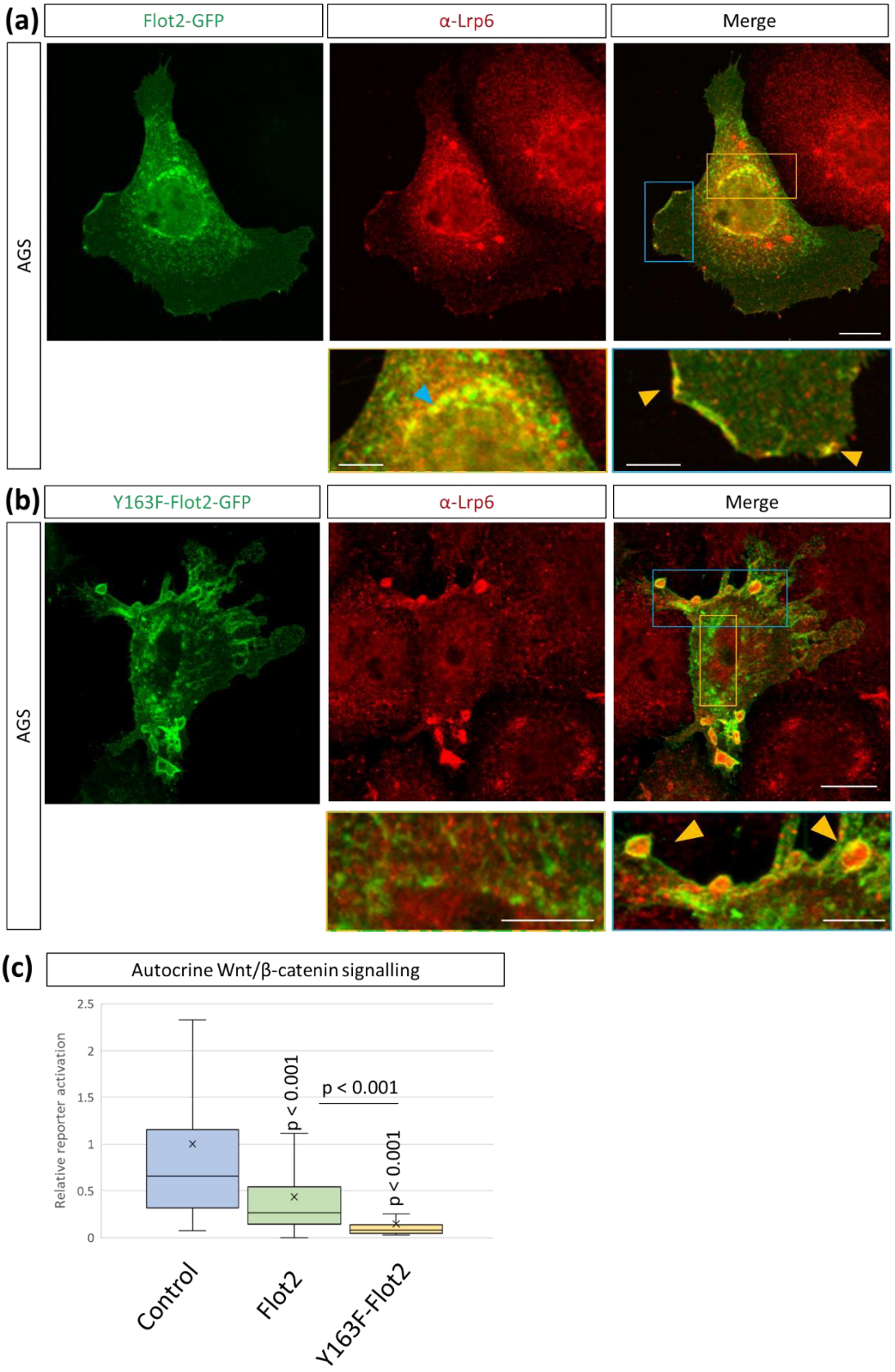


**Figure 36 – Blocking Flot2 endocytosis inhibits Lrp6 internalisation and Wnt/β-catenin signalling**
**(a)** Antibody staining against Lrp6 (red) after transfection of AGS cells with Flot2-GFP. Yellow box highlights intracellular co-localisation and blue box highlights membrane co-localisation. Scale bars 10μm (top) and 5μm (zoomed).
**(b)** Antibody staining against Lrp6 (red) after transfection of AGS cells with endocytosis mutant Y163F-Flot2-GFP. Yellow box highlights loss of intracellular co-localisation and blue box highlights accumulation of Lrp6 in Flot2-positive vesicles. Scale bars 10μm (top) and 5μm (zoomed).
**(c)** Quantification of STF (7xTCF-NLS-mCh) Wnt reporter in AGS cells following transfection with Flot2-GFP or Y163F-Flot2-GFP. Significance calculated by student's t-test. (n = 111, 93, 157; n = number of nuclei measured).

### 5.2.5. Flot2 and Lrp6 co-localise intracellularly in response to Wnt3a-, but not Dkk1-induced, Lrp6 endocytosis

Lrp6 internalisation has previously been reported to occur via two different routes, a clathrin-mediated degradation route and a caveolin-mediated recycling route (Liu *et al.*, 2014). These routes are dictated by the ligands which bind to Lrp6, with Wnt3a promoting recycling, and the Lrp6 inhibitor, Dkk1, promoting degradation (Yamamoto *et al.*, 2008). Thus, I decided to investigate which internalisation route, degradative or recycling, Flot2-mediated endocytosis might be regulating. To achieve this, I treated serum-starved AGS cells with recombinant Wnt3a or Dkk1 protein and analysed changes in Flot2 and Lrp6 localisations by immunofluorescence. DMSO-treated cells were used as the control.

In serum-starved, DMSO-treated AGS cells, Flot2 and Lrp6 co-localise predominantly at the membrane, as previously observed (Fig. 37). Following treatment with recombinant Wnt3a (30 mins, 250ng/µl), the localisation of both Flot2 and Lrp6 is notably more intracellular, where their co-localisation can be observed in the perinuclear region. Similarly, following Dkk1 treatment (30 mins, 250ngl/µl), the majority of Flot2 and Lrp6 staining appears to be intracellular. However, co-localisation between Flot2 and Lrp6 is lost and they appear to be residing in distinct compartments of the cell, perhaps due to clathrin-mediated endocytosis being the primary mechanism for Dkk1-induced Lrp6 endocytosis. Levels of Lrp6 staining also appear reduced, suggestive of Dkk1-induced degradation. These results suggest that Flot2 is involved in Wnt3a-induced, but not Dkk1-induced, Lrp6 endocytosis.

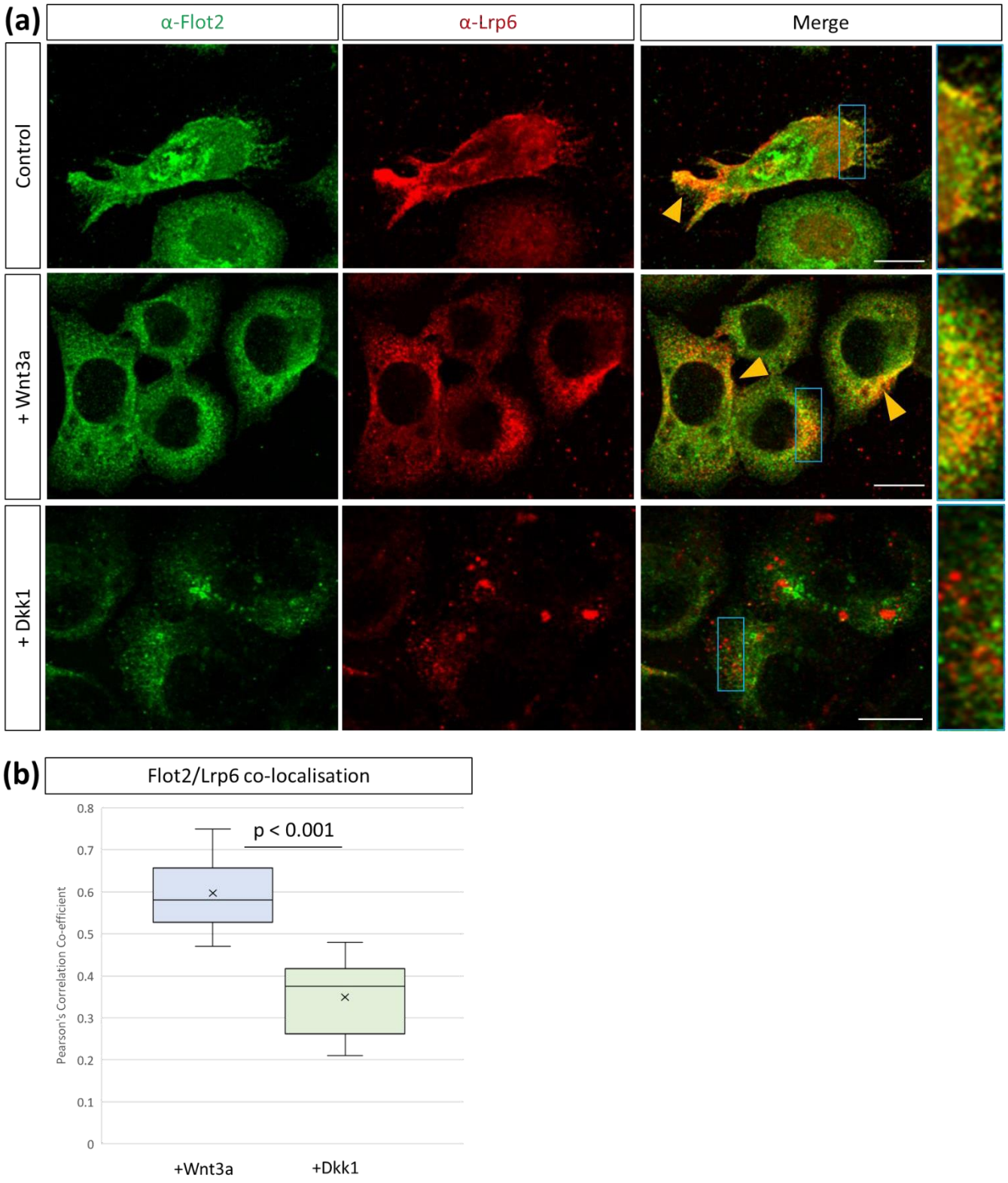


**Figure 37 – Flot2 co-localises with Lrp6 in Wnt3a-, but not Dkk1-induced, Lrp6 internalisation**
**(a)** Antibody stainings against Lrp6 (red) and Flot2 (green) following recombinant Wnt3a or Dkk1 treatment (30 mins, 250ng/µl). AGS cells were serum-starved for 24 hrs prior to treatment. DMSO was used as the control. Scale bars 10µm.
**(b)** Quantification of Flot2 and Lrp6 co-localisation, as assessed by Pearson's correlation co-efficient (PCC), following addition of rWnt3a or rDkk1. (n = 10, 10; n = number of cells).

### 5.2.6. Flotillin-2 knockdown reduces localisation of Lrp6 to Rab11+ endosomes

To further investigate the endocytic route by which Flot2 regulates Lrp6 internalisation and attempt to identify the compartment Lrp6 accumulates in upon Flot2 KD (Fig. 34c), I performed antibody stainings against Lrp6 in the presence of a number of organelle / compartment markers. Then, I repeated these stainings following siRNA KD of Flot2 to assess changes in localisation. The following markers were used; anti-calnexin (ER), mTurq2-Golgi (Golgi), anti-EEA1 (early endosomes), Rab7-GFP (late endosomes), Rab4-mCh (fast recycling endosomes) and LAMP1- mTurq2 (lysosomes). For all of these markers, there was no notable increase or decrease in co-localisation with Lrp6 between control and Flot2 KD AGS cells (Fig.
38). Despite the perinuclear accumulation of Lrp6 being evident in all Flot2 KD cells, I could not identify the compartment or organelle in which this was occurring.

There was, however, a loss of co-localisation between Lrp6 and Rab11-mCh, which marks slow recycling endosomes, following Flot2 KD (Fig. 39). In control cells, Lrp6 and Rab11-mCh co-localise, at the membrane and intracellularly, suggesting Lrp6 is internalised and shuttled through a Rab11-dependent axis. The loss of co-localisation in Flot2-deficient cells suggests Flot2 is required for the loading of Lrp6 into Rab11+ slow recycling vesicles or that Flot2 regulates Rab11 function and/or localisation.

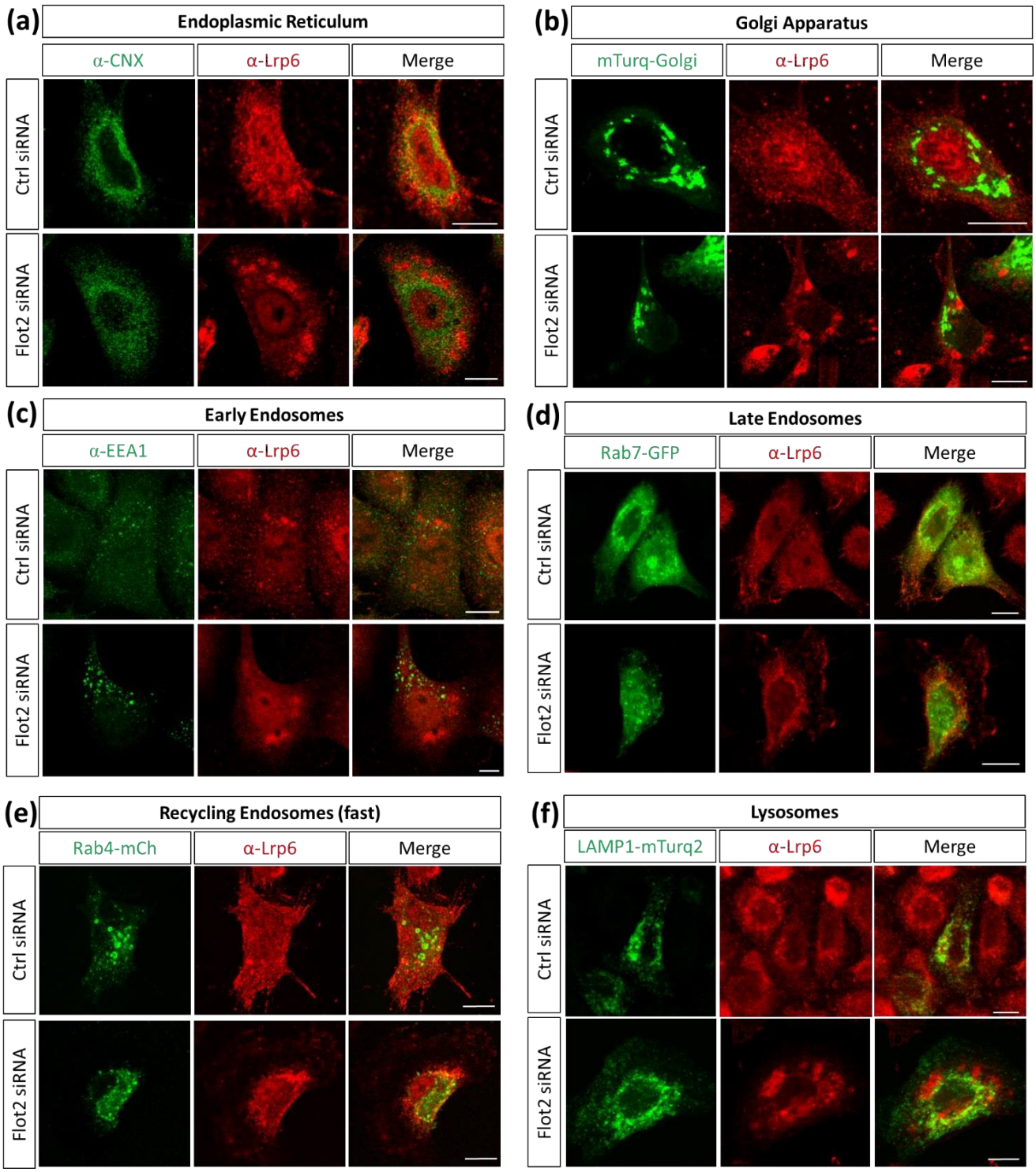


**Figure 38 – Accumulation of Lrp6 in Flot2-deficient cells does not occur in a number of compartments**
Antibody stainings against Lrp6 (red) in AGS cells (+/- Flot2 siRNA) to track localisations with the following intracellular markers (either transfected or antibody stained); **(a)** Calnexin, **(b)** mTurq2-Golgi, **(c)** EEA1, **(d)** - Rab7-GFP, **(e)** mCh-Rab4, **(f)** LAMP1-mTurq2. Scale bars 10µm.

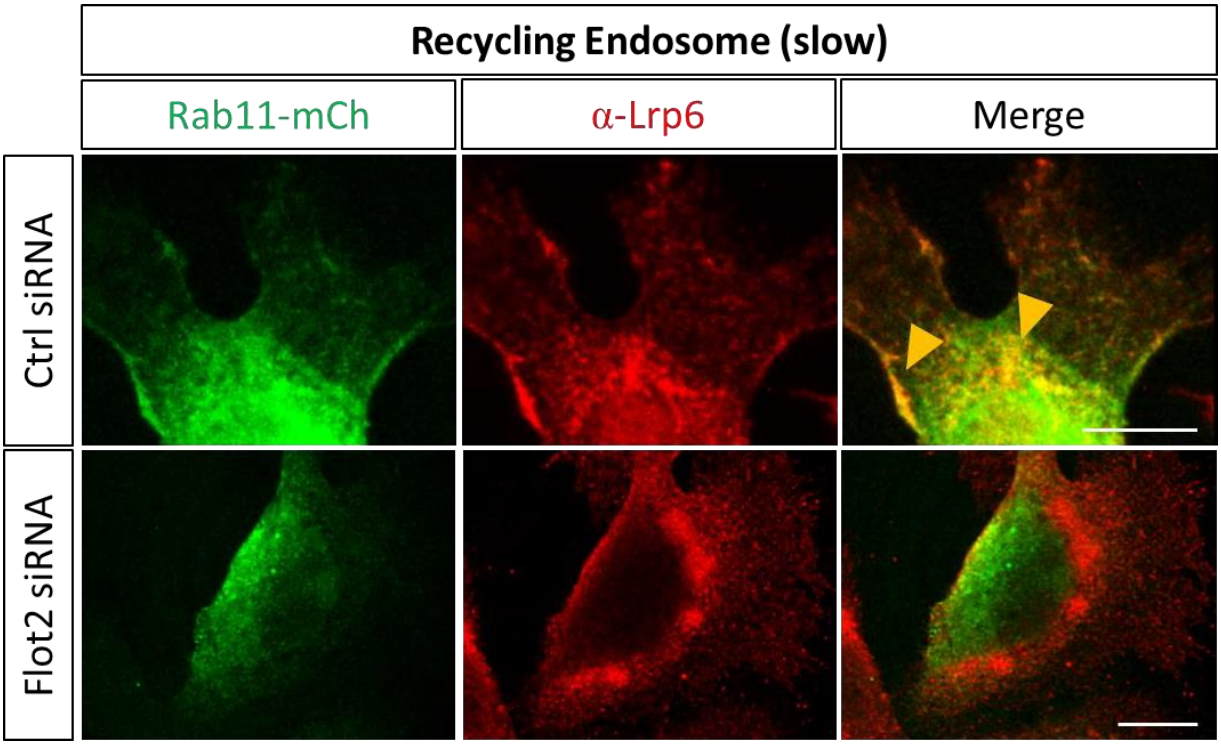


**Figure 39 – Flotillin-2 knockdown results in loss of localisation of Lrp6 to Rab11+ endosomes**
Antibody stainings against Lrp6 (red) in AGS cells transfected with mCh-Rab11. Yellow arrows highlight co-localisation of Lrp6 and Rab11-mCh. Scale bars 10μm.

### 5.2.7. Flotillin-2-mediated internalisation of Lrp6 is dynamin-dependent

To further characterise the mechanism by which Flot2 is assisting Lrp6 internalisation and trafficking, I next investigated the necessity for dynamin in these processes. Dynamin is a membrane scission protein and has been shown to be necessary for both clathrin- and caveolin-mediated endocytosis and is also implicated in the intracellular trafficking of Flot2 itself (Meister *et al.*, 2014).

Firstly, I used a chemical inhibitor of dynamin, dynasore, to inhibit dynamin-mediated cleavage of endocytic vesicles. Serum-starved AGS cells were treated with dynasore for 1hr and rWnt3a was added for the final 30 mins to induce internalisation of Lrp6. Cells were then fixed and stained for Flot2 and Lrp6. DMSO was used as the negative control, and cells treated with only Wnt3a (no dynasore) as a positive control. As seen in Fig. 40, treatment with Wnt3a causes internalisation of Lrp6, where it co-localises with Flot2. However, when AGS cells were pre-treated with dynasore, this co-localisation is lost. Unexpectedly, Lrp6 still predominantly localises in the perinuclear region. Since dynamin is required for cleavage of endocytic

vesicles at the PM, one would expect accumulation of endocytosis cargo here following addition of dynasore. Whether Lrp6 has still been internalised via a different endocytic route, or whether its anterograde transport is impaired, is not clear.

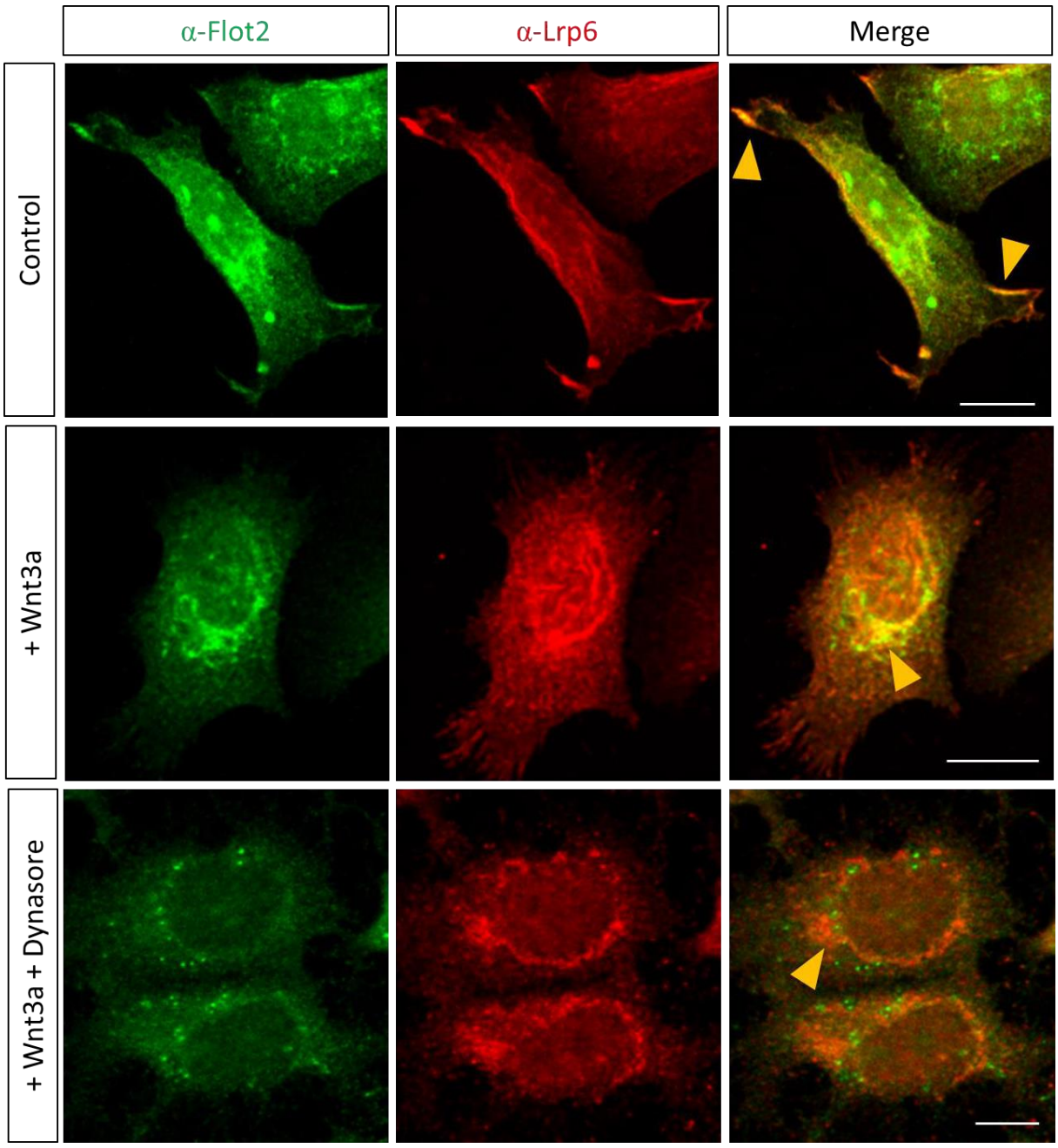


**Figure 40 – Flotillin-2-mediated internalisation of Lrp6 is perturbed by the dynamin inhibitor dynasore**
Antibody stainings against Flot2 (green) and Lrp6 (red) in AGS cells after treatment with DMSO (control), Wnt3a (30 mins, 250ng/µl) or dynasore pre-treatment (1hr, 100µM) and Wnt3a. Yellow arrows highlight co-localisation of Flot2 and Lrp6 (top and middle), or loss of co-localisation (bottom). Scale bars 10µm.

Recently, the specificity of dynasore for dynamin has been questioned, and it is reported to also perturb the actin cytoskeleton and disrupt lipid rafts (Preta *et al.*, 2015). Therefore, I next inhibited dynamin via a more specific method, by expressing a dominant-negative dynamin mutant, Dyn$^{K44A}$, which is deficient in GTP binding and hydrolysis (Damke *et al.*, 2001).

AGS cells were transfected with RFP-Dyn$^{K44A}$ and then antibody stained against Flot2 and Lrp6 to assess its impact on their localisation. As shown in Fig. 41, RFP-Dyn$^{K44A}$ displays significant co-localisation with both Flot2 and Lrp6 at the PM, which may be due to impaired endocytosis (Fig. 41, yellow arrows). There is also notable accumulation of Flot2, Lrp6 and Dyn$^{K44A}$ intracellularly, suggesting impairment of intracellular transport (Fig. 41, blue arrow). These phenotypes differ greatly from those seen with dynasore, perhaps due to the differences in their mechanisms of inhibition or off-target effects of dynasore treatment. Despite the disparities in their phenotypes, it is clear that dynamin plays a significant role in the relationship between Flot2 and Lrp6. Whether this involves endocytosis, endocytic trafficking or both, remains to be deciphered.

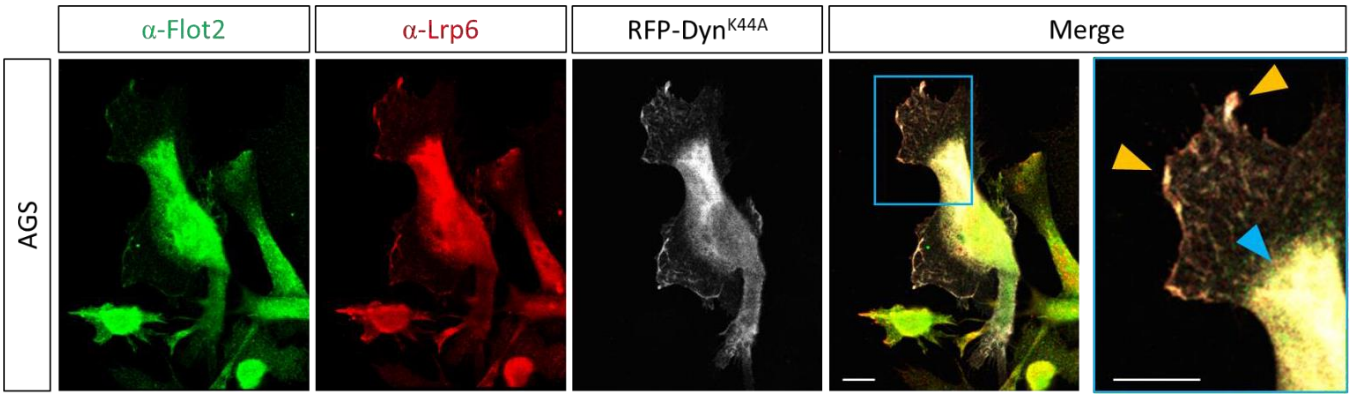


**Figure 41 – Dynamin$^{K44A}$ mutant impairs Flotillin-2 and Lrp6 intracellular transport**
Antibody stainings against Flot2 (green) and Lrp6 (red) in AGS cells after transfection with RFP-Dyn$^{K44A}$. Yellow arrows highlight co-localisation at the membrane. Blue arrow represents intracellular accumulation and co-localisation. Scale bars 10μm.

### 5.2.8. Flotillin-2 and caveolin partially co-localise and Lrp6 localises to both Flot2- and caveolin-positive microdomains

To date, it is still debated whether Flot2 and caveolin are functionally and spatially distinct from one another, due to conflicting evidence over their co-operation (Volonté *et al.*, 1999; Frick *et al.*, 2007; Hansen and Nichols, 2009). There is also significant literature demonstrating that Lrp6 is internalised via a caveolin-mediated route following Wnt3a stimulation (Yamamoto *et al.,* 2006). Therefore, I analysed the relationship between Flot2 and caveolin in GC cells to assess whether these proteins form discrete structures or whether they may co-operate in the internalisation of Lrp6.

To begin with, I performed IF stainings for Flot2 and caveolin in AGS cells to assess their localisations at endogenous levels. At first glance, Flot2 and caveolin appear to co-localise, as they accumulate in the same regions of the cell (Fig. 42a, yellow arrow). However, upon closer inspection, they appear to occupy distinct regions of the membrane and their puncta show minimal overlap (Fig. 42a, blue box). Next, I wanted to determine whether Flot2 has any functional impact on the localisation of caveolin. Flot2 KD (by siRNA) had no significant impact on the localisation of caveolin compared to the control (Fig. 42b), suggesting caveolin does not require Flot2 for its localisation.

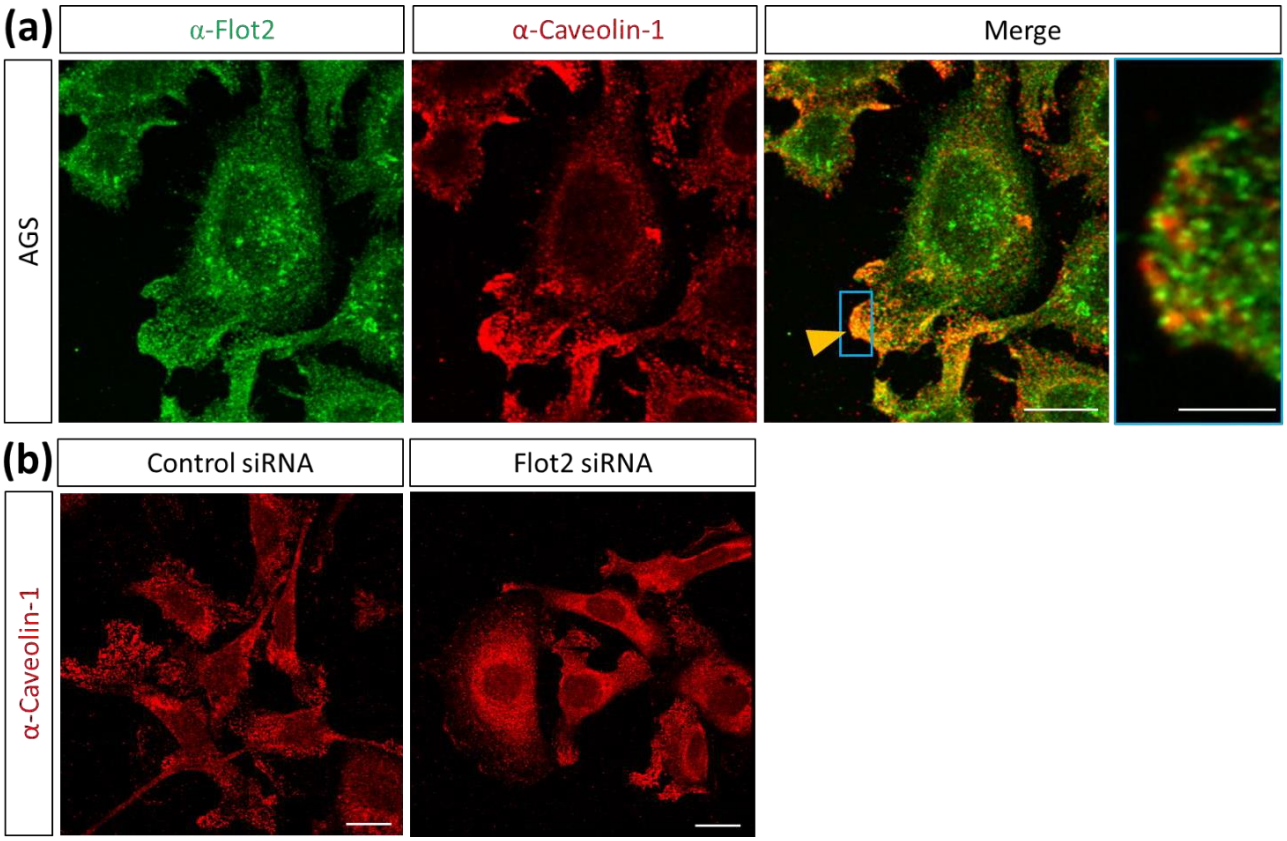


**Figure 42 – Flotillin-2 and caveolin localise adjacently and Flotillin-2 knockdown does not alter caveolin localisation**

**(a)** Antibody staining against Flot2 (green) and caveolin-1 (red) in AGS cells. Yellow arrow indicates Flot2- and caveolin-positive membrane region. Blue box highlights the largely distinct nature of Flot2 and caveolin puncta. Scale bar 10μm (blue box 2.5μm).

**(b)** Antibody staining against caveolin-1 (red) after treatment with control or Flot2 siRNA in AGS cells. Scale bars 10μm.

Lrp6 has previously been reported to localise with caveolin, yet I have observed strong co-localisation of Lrp6 with Flot2. Hence, I wanted to determine whether Lrp6 displays a greater affinity for one or the other, or whether it is capable of localising with both. Therefore, I performed a triple antibody stain against Flot2, Lrp6 and caveolin-1 (Fig. 43a). Here, all three proteins localise to similar regions of the cell membrane, perhaps DRM domains. Interestingly, whilst mostly discrete puncta can be seen for both Flot2 and caveolin, there are occasions where both Flot2 and caveolin co-localise with Lrp6 (Fig. 42a, yellow arrows). This partial co-localisation of Flot2 and caveolin is infrequent, but consistently observable. Larger clusters of Flot2, Lrp6 and caveolin can also occasionally be observed (Fig. 42b).

In general, it appears that Flot2 and caveolin can and do function discretely. However, their partial co-localisation and consistent adjacent localisation in particular membrane regions, and co-localisation with Lrp6, reasons that a co-operative function in Lrp6 internalisation cannot be ruled out.

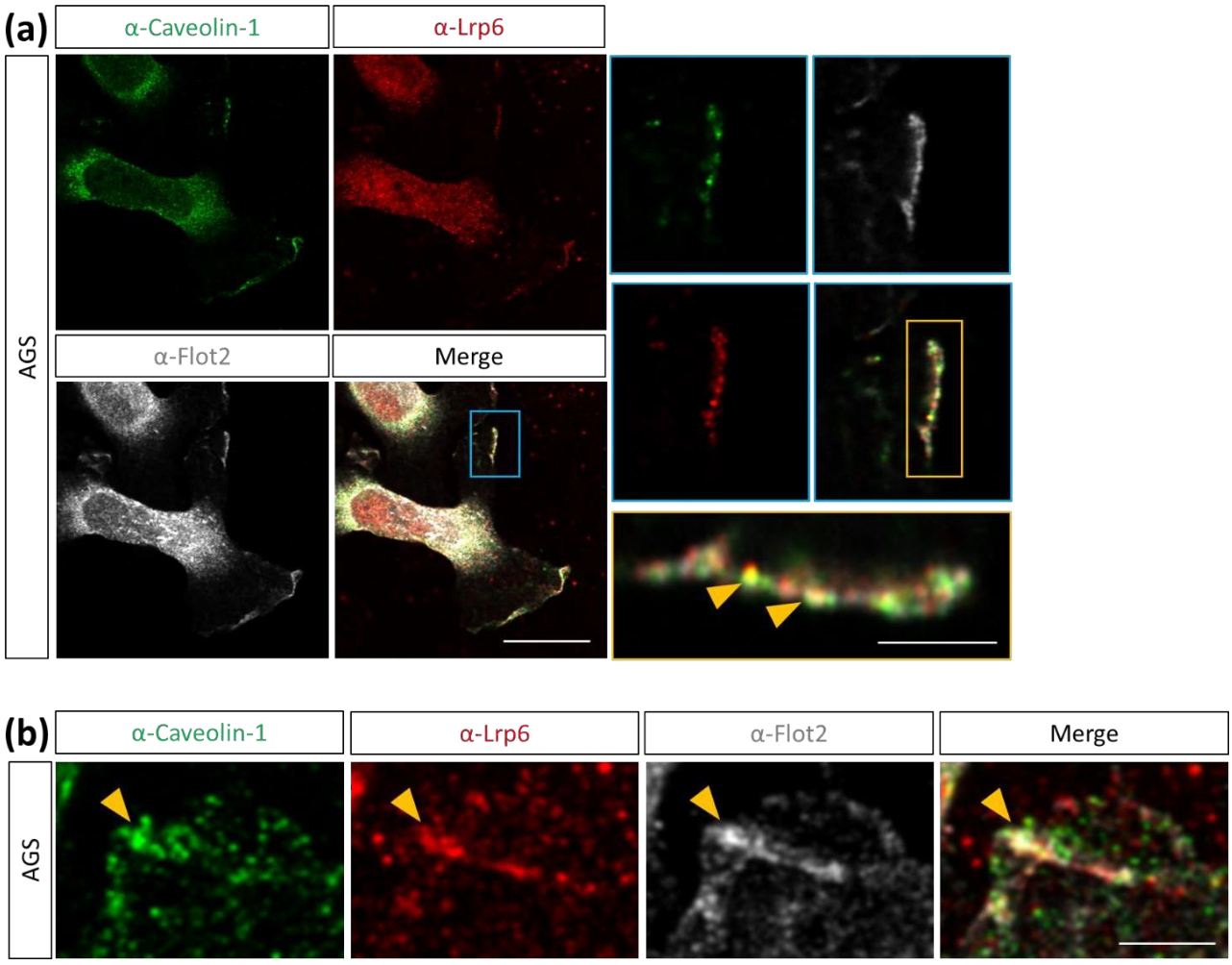


**Figure 43 – Lrp6 co-localises with both Flotillin-2 and caveolin-1, which also partially co-localise**
**(a)** Antibody stainings of caveolin-1 (green), Lrp6 (red) and Flot2 (grey) in AGS cells. Blue and yellow boxes highlight membrane regions rich in all three proteins. Yellow arrows highlight puncta where caveolin-1, Flot2 and Lrp6 all co-localise. Scale bars 10μm (zoomed out) and 2.5μm (yellow box).
**(b)** Antibody stainings as described in (a). Yellow arrow highlights a larger cluster of caveolin-1, Lrp6 and Flot2 co-localising. Scale bar 5μm.

### 5.2.9. Flotillin-2 does not affect clathrin localisation

Flot2 has been shown to occupy distinct regions of the membrane to clathrin and flotillin-mediated endocytosis is widely accepted to be a CIE mechanism (Glebov *et al.*, 2006). To confirm that perturbation of Flot2 function is not also affecting clathrin, I co-expressed Flot2-GFP and mRFP-Clathrin in AGS cells to assess their localisations. As expected, there was no co-localisation between these two proteins (Fig. 44). Expression of the dominant-negative ΔN-Flot2-GFP also had no effect on mRFP-clathrin localisation. Therefore, I concluded that Flot2 has no significant role in the localisation or function of clathrin.

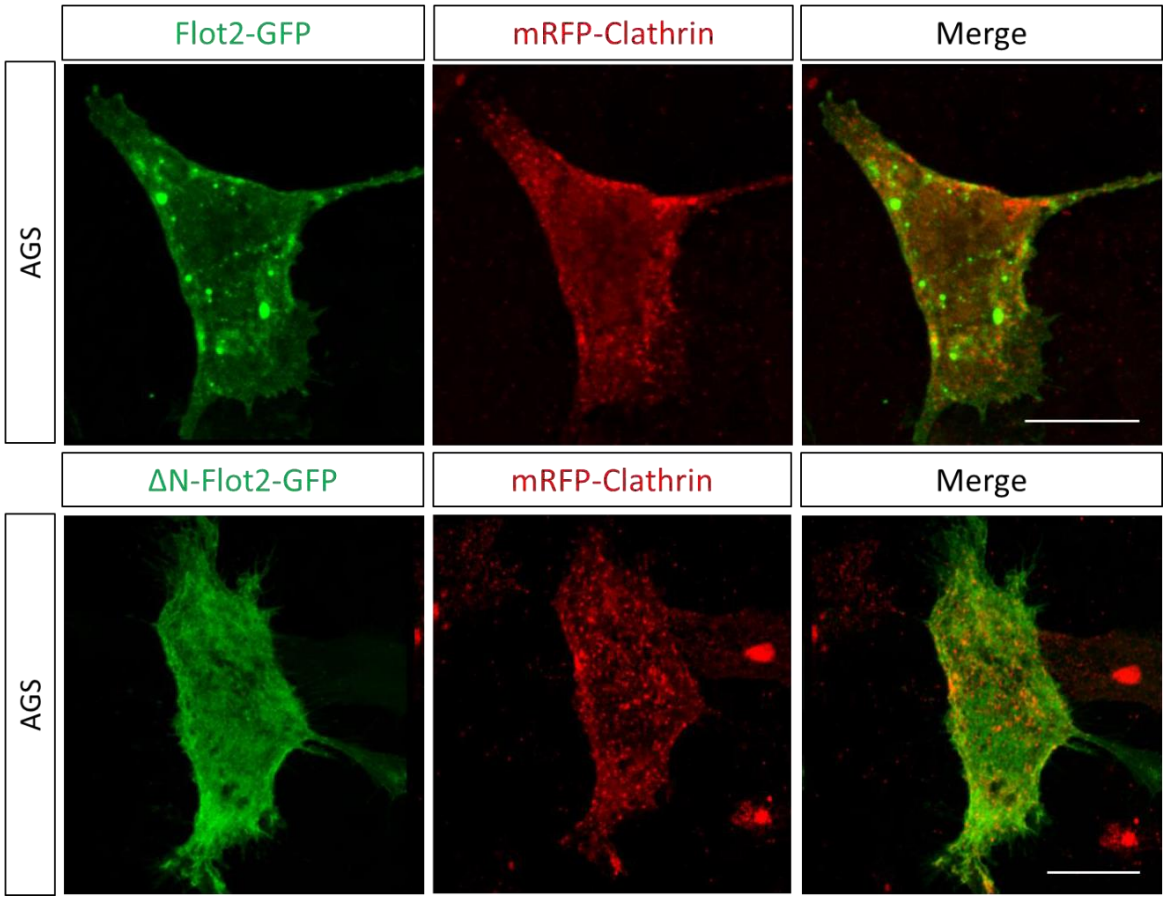


**Figure 44 – Flotillin-2 does not affect clathrin localisation**

**(a)** Confocal images of AGS cells transfected with Flot2-GFP or ΔN-Flot2-GFP and mRFP-Clathrin. Scale bars 10μm.

## 5.3. Discussion

### 5.3.1. Flot2 is involved in Wnt3 uptake in recipient cells

In recent years, there has been increasing evidence for the role of cytonemes in transporting Wnt proteins intercellularly, and this is now a generally accepted mechanism of dissemination (Routledge and Scholpp, 2019). However, little is known about how exactly the Wnt proteins are transported from the Wnt-producing to Wnt-receiving cell at the contact site; this is known as the “handover problem”. Previously, it has been hypothesised that a difference in binding affinity of Wnt ligands for receptors on the producing cell (e.g. Fzd-Ror2) and receiving cell (e.g. Fzd-Lrp6) allow release of Wnts and transfer to the higher affinity receptor(s) (Routledge and Scholpp, 2019). The Wnt-Fzd-Lrp6 complexes are then internalised by endocytosis and the signal is transduced. However, when analysing the role of Flot2 in Wnt3 cytonemes, I observed that the co-localisation of Flot2-GFP and Wnt3-mCh persisted in the Wnt-receiving cell, suggesting these proteins are handed over together (Fig. 33a). This is concurrent with very recent findings from our lab which demonstrate that Wnt-receptor complexes, namely Wnt5a-Ror2 (in GC cells) and Wnt5b-Ror2 (in zebrafish), are handed over together into the Wnt-receiving cell (Rogers *et al.*, 2022; Zhang *et al.*, 2022). Here, Wnt5a-Ror2 complexes display persistent binding affinities and are capable of inducing Wnt/PCP signalling in recipient cells. Live cell imaging also appears to show that in some cases, the entire tip of cytonemes is broken off and internalised into the recipient cell, and so a portion of the Wnt-producing membrane, which contains Wnt-receptor complexes, is transferred (Rogers *et al.*, 2022). However, whilst Flot2-GFP/Wnt3-mCh co-localisation persisted, when I co-expressed Wnt3-mCh with memGFP, to generally mark the membrane, I saw minimal co-localisation in the recipient cells (Fig. 33a). Therefore, I propose that Wnt-Ror2 complexes specifically localise to Flot2 microdomains at the tips of cytonemes, and these components are transferred to and endocytosed in the recipient cell. This model could explain why in *Drosophila*, over-expression of Flot2 in the Wg-producing cells, but not receiving population, enhances Wg uptake in the recipient cells (Katanaev *et al.*, 2008). As well as promoting dissemination via cytonemes, Flot2 may then aid the internalisation of Wg following handover.

Confirming a role for Flot2 in the uptake of Wnt3, when Wnt3-mCh-expressing AGS cells were co-cultivated with Flot2-GFP-expressing HFE-145 cells, Wnt3-mCh could be seen in Flot2-GFP-positive vesicles in the Wnt-receiving cells (Fig. 33b). This suggests Flot2 is involved in the endocytosis of Wnt3. Alongside the observed co-localisation of the Wnt co-receptor Lrp6 with Flot2, I hypothesised that Flot2 may be mediating Wnt3 uptake via endocytosis of Lrp6 following Wnt3 binding (Fig. 34). Indeed, antibody stainings show that Wnt3 and Lrp6 co-localise at endogenous levels at the PM, intracellularly and on cytonemes (Fig. 34, 35). Both Wnt3 and Lrp6 also appear to cluster in Flot2-rich portions of the membrane (Fig. 35b), further supporting my hypothesis that Flot2 could regulate Lrp6 internalisation during Wnt signalling.

### 5.3.2. Flot2 regulates the localisation and internalisation of Lrp6

Endocytosis of Wnt-Fzd-Lrp6 complexes from the membrane has been shown to be necessary for signal transduction, where subsequent endosomal acidification promotes Lrp6 phosphorylation and signal maintenance (Brunt and Scholpp, 2018). Two endocytosis routes have primarily been associated with Lrp6 internalisation; clathrin-mediated endocytosis (CME) and caveolin-mediated (or clathrin-independent endocytosis, CIE). Which route Lrp6 is internalised by is dependent on ligand binding, where Wnt3a induces CIE (promoting Wnt signalling) and the Lrp6 inhibitor Dkk1 induces CME (inhibiting Wnt signalling) (Yamamoto *et al.*, 2008). A third route of endocytosis I believe has been overlooked is flotillin-mediated endocytosis. Only one group has previously linked Lrp6 internalisation to flotillins, which showed that Lrp6 is endocytosed via CME in the presence of LDLs and this switches to a flotillin-mediated route in the absence of LDLs (Yamamoto *et al.*, 2017). My observations that internalised Lrp6 co-localises with Flot2 following Wnt3a, but not Dkk1, treatment supports the notion that Lrp6 internalisation routes differ depending on ligand binding, however I identify Flot2 as an important player in Lrp6 endocytosis (Fig. 37).

In previous studies, it has been shown that knockdown or inhibition of caveolin proteins leads to inhibition of Lrp6 internalisation following Wnt3a treatment and Lrp6 appears stuck on the cell surface (Yamamoto *et al.,* 2006). Therefore, when I performed a similar experiment with Flot2 siRNA, I expected to observe a similar phenotype. Surprisingly, Flot2 KD resulted in drastically altered Lrp6 localisation,

where it strongly accumulated intracellularly in the perinuclear region (Fig. 34). I was unable to identify the compartment in which Lrp6 was accumulating, since it did not co-localise with markers for the ER, Golgi, early endosomes, late endosomes, lysosomes, or fast recycling endosomes upon Flot2 KD (Fig. 38). At endogenous Flot2 levels, Lrp6 co-localises with the slow recycling endosome marker Rab11 (Fig. 39). This co-localisation is reduced upon Flot2 KD, suggesting Flot2 may play a role in transporting Lrp6 from the PM via a Rab11-mediated route. Indeed, Flot2 has previously been implicated with trafficking cargo, such as integrins, in coordination with Rab11 (Hülsbusch *et al.*, 2015; Redpath *et al.*, 2019). In the future, it would be pertinent to assess whether inhibition of Rab11 function or expression has any effect on Flot2 and Lrp6 co-localisation, which would provide further evidence for a role of Flot2 in Lrp6 recycling following endocytosis.

How or why Lrp6 accumulates in the perinuclear region, however, is unclear. It cannot be ruled out that disruption of lipid raft / flotillin microdomains upon Flot2 KD could be the cause of major redistribution of Lrp6. However, Lrp6 is capable of localising to both caveolin- and clathrin-rich regions of the membrane, which are distinct from flotillins, and so I would expect if this were the case, Lrp6 would still be capable of localising to the PM.

Confirming the relationship between Flot2 and Lrp6 is an endocytic one, antibody stainings against Lrp6 in Flot2-GFP expressing AGS cells displayed co-localisation both at the PM and perinuclear region. However, upon expression of Y163F-Flot2-GFP, as well as a significant accumulation of Lrp6 at the cell surface, there is loss of co-localisation in the perinuclear region (Fig. 36a,b). Furthermore, expression of Y163F-Flot2-GFP also significantly inhibits Wnt/β-catenin signalling, as assessed by Wnt reporter activity (Fig. 36c). These data suggest Flot2 is not involved in the anterograde transport of Lrp6 to the cell surface, but the internalisation and trafficking of Lrp6 which is necessary for signal transduction.

I did plan to quantify the effects of Flot2 function and expression on Lrp6 endocytosis using an antibody-feeding assay, as described here (Rizzolio and Tamagnone, 2017). This experiment allows the tracking of surface receptors and quantification of their endocytic uptake. However, difficulties in obtaining an antibody targeting the

extracellular domain of Lrp6, which was necessary for this experiment, prevented me from performing this experiment.

### 5.3.3. Flotillin-mediated trafficking of Lrp6 is dynamin-dependent

Dynamin is a GTPase which mediates membrane fission during both CME and CIE (Henley *et al.*, 1998; Sundborger and Hinshaw, 2014). Therefore, dynamin is crucial to the internalisation of surface receptors, and knockdown or functional inhibition of dynamin results in accumulation of Lrp6 on the cell surface due to defective endocytosis (Yamamoto *et al.*, 2008). Blockage of dynamin function using a Dyn2$^{K44E}$ mutant, which is defective in GTP hydrolysis, also attenuates Wnt3a-induced stabilisation of β-catenin, indicating its necessity for Wnt/β-catenin signal transduction (Blitzer and Nusse, 2006).

Consistently, dynamin is also necessary for flotillin-mediated endocytosis, since the chemical inhibitor dynasore attenuates EGF-induced Flot1 and Flot2 internalisation and accumulation at the PM is observed (Meister *et al.*, 2014). My data also indicates a role for dynamin in Flot2-mediated trafficking, however there are some discrepancies. Firstly, upon addition of Wnt3a, Flot2 and Lrp6 both internalise and co-localise intracellularly in AGS cells. However, upon treatment with dynasore, unlike the findings from Meister *et al.,* (2014), I do not see accumulation of Flot2 at the cell surface. Instead, both Lrp6 and Flot2 are seen intracellularly, but they no longer co-localise and appear to reside in distinct regions (Fig. 40). A possible explanation for this is that in the presence of dynasore, Lrp6 is internalised via a dynamin-independent route which does not involve flotillins.

To investigate this further, I chose to use the Dyn$^{K44A}$ mutant, since dynasore has been reported to have potential off-target effects on lipid rafts and the actin cytoskeleton (Preta *et al.*, 2015). Upon expression of RFP-Dyn$^{K44A}$, Flot2 and Lrp6 both co-localise at the PM. However, more prominently, there is a strong intracellular accumulation of Flot2, Lrp6 and Dyn$^{K44A}$ (Fig. 41). These findings concur with previous reports that Dyn$^{K44A}$ expression causes sub-cellular redistribution of flotillins, where they accumulate in late endosomes (Meister *et al.,* 2014). Consistently, dynamin is implicated with mediating membrane scission events during endosomal trafficking and Dyn$^{K44A}$ has previously been reported to cause aberrant accumulation

of several cargo in different intracellular compartments (Kreitzer *et al.*, 2000; Nicoziani *et al.*, 2000). Therefore, I believe dynamin is important in the intracellular trafficking of flotillins through endosomal compartments, and consequently, $Dyn^{K44A}$ impairs Lrp6 trafficking.

### 5.3.4. Flot2 and caveolin partially co-localise and may co-ordinate in Lrp6 endocytosis

Despite the evidence presented here that Flot2 is important in the localisation, internalisation and trafficking of Lrp6, there is a mountain of evidence which highlights caveolin as the key mediator of Wnt3a-induced Lrp6 internalisation (Yamamoto *et al.*, 2006; Liu *et al.*, 2014). Therefore, the data presented here could be contradictory to previous findings. Additionally, it is still unclear to what extent caveolins and flotillins function discretely or co-operatively in endocytosis. Here, in AGS cells, I observed strong accumulation of caveolin-1 and Flot2 at the PM in the same regions of the cell (Fig. 42a). Upon inspection at higher resolution, caveolin-1 and Flot2 appear to form largely distinct puncta. However, partial co-localisation of caveolin-1 and Flot2 was observable, and Lrp6 also co-localises at these sites (Fig. 43).

It is tempting to speculate that whilst Flot2 and caveolin-1 mostly reside in distinct regions of the PM, they may both be involved in the endocytosis of Lrp6. One possible explanation could be the previously described model of flotillin-assisted endocytosis, in which flotillins are necessary for pre-endocytic clustering of receptors, which then undergo endocytosis in a flotillin-independent manner (Meister and Tikkanen, 2014). Additionally, this may explain why Flot2 and caveolin-1 cluster / accumulate in the same regions of the membrane with minimal co-localisation; they do not directly interact, but they are both required for the co-ordinated internalisation of Lrp6. Supporting this notion, siRNA-mediated KD of Flot2 did not alter the sub-cellular distribution of caveolin-1 and thus it is unlikely to reside with flotillin microdomains (Fig. 42). This contradicts a previous report that Flot1 regulates the stability of caveolin-1 in intestinal epithelial cells (Vassilieva *et al.*, 2009). However, this could represent a distinct function of Flot1 from Flot2. Similarly, Flot2 KD neither

affected the distribution of clathrin, confirming these two function in discrete membrane regions and do not regulate one another's localisations (Fig. 44).

The idea of flotillin-mediated pre-endocytic clustering is conducive with known functions of Flot2 in the clustering of receptors, such as RTKs, for signalling events (Amaddii *et al.*, 2012). One observation which is not so clearly explained by this hypothesis is that Lrp6 accumulates at the cell surface in Y163F-Flot2-expressing cells, suggesting successful endocytosis of Flot2 is necessary for Lrp6 internalisation. Perhaps phosphorylation of Flot2 at Y163 regulates other Flot2 functions or interactions with intracellular machinery necessary for membrane curvature or budding.

Additional questions need answering to decipher this relationship. For example, does perturbing caveolin expression or function alter Flot2-Lrp6 interactions? Was the Wnt3a-induced intracellular co-localisation of Flot2 and Lrp6 due to Flot2-mediated endocytosis, or does Flot2 first interact with Lrp6 in a different compartment? For example, in Rab11 recycling endosomes, where it then mediates the recycling of Lrp6 back to the cell surface? This could explain why Lrp6 accumulates in the perinuclear region, rather than at the PM, in Flot2-deficient cells. Despite these uncertainties, I have demonstrated that Flot2 is an important regulator in the intracellular trafficking of Lrp6, and a potential co-operation between Flot2 and caveolin should not be ruled out.

# 6. Chapter 4 – The Wnt Receptor Supercomplex

## 6.1 Introduction

In schematics representing the Wnt signalling network and its pathways, the Wnt/β-catenin and Wnt/PCP pathways are often depicted as completely separate entities, where Fzd/Lrp6 or Fzd/Ror2 complexes share little commonality other than that they both bind to Wnt ligands (Ackers and Malgor, 2018). Here, traditionally “canonical” Wnts (e.g. Wnt3a) or “non-canonical” Wnts (e.g. Wnt5a) are depicted binding to Fzd/Lrp6 or Fzd/Ror2, respectively. The mutually repressive nature of these pathways is often associated with competition over the shared downstream effector protein Dvl (Gao and Chen, 2010).

However, there is increasing evidence that this model of the Wnt signalling pathways is becoming obsolete, as more and more examples of unconventional activation of these pathways are observed. For example, numerous studies have implicated Ror2 with the ability to bind to “canonical” Wnts and/or regulate Wnt/β-catenin signalling in a manner not dependent on Dvl (Billiard *et al.*, 2005; Rasmussen *et al.*, 2013). Conversely, Lrp6 has been shown to regulate Fzd8-Wnt5a interactions by physically interacting with Fzd8 away from its CRD (Ren *et al.*, 2015). Additionally, “non-canonical” phenotypes can be observed in Lrp6-/- mice, where Wnt5a signalling is impaired (Gray *et al.*, 2013).

These findings, along with my own observations that Ror2 and Lrp6 both co-localise with Flot2 at the membrane and on cytonemes, led me to question whether there may be a more direct relationship between these two Wnt co-receptors. In this chapter, I investigate the sub-cellular and membrane localisations of Wnt3, Ror2, Lrp6 and Fzd receptors with one another, and Flot2, to examine the possibility that these receptors associate with one another, and that these interactions occur in flotillin microdomains.

## 6.2. Results

### 6.2.1. Wnt co-receptors Ror2 and Lrp6 co-localise in Flot2 microdomains

In chapters 2 and 3, I showed that Flot2 is capable of regulating both the Wnt/PCP and Wnt/β-catenin signalling pathways. Furthermore, the two Wnt co-receptors Ror2 and Lrp6 can both localise to Flot2 microdomains. To address the spatial relationship between Ror2 and Lrp6, I began by performing an antibody stain against these two co-receptors (Fig. 45). To my surprise, there was significant and strong co-localisation of Ror2 and Lrp6 on both cytonemes (Fig. 45a(i)) and at the PM (Fig. 45a(ii)). Next, I asked whether this co-localisation occurred in Flot2 microdomains. Therefore, I transfected AGS cells with Flot2-GFP prior to staining (Fig. 45b). Here, Ror2 and Lrp6 strongly co-localised with each other and Flot2-GFP, suggesting both Ror2 and Lrp6 localise to lipid rafts and that Flot2 may act as a platform for both these Wnt co-receptors.

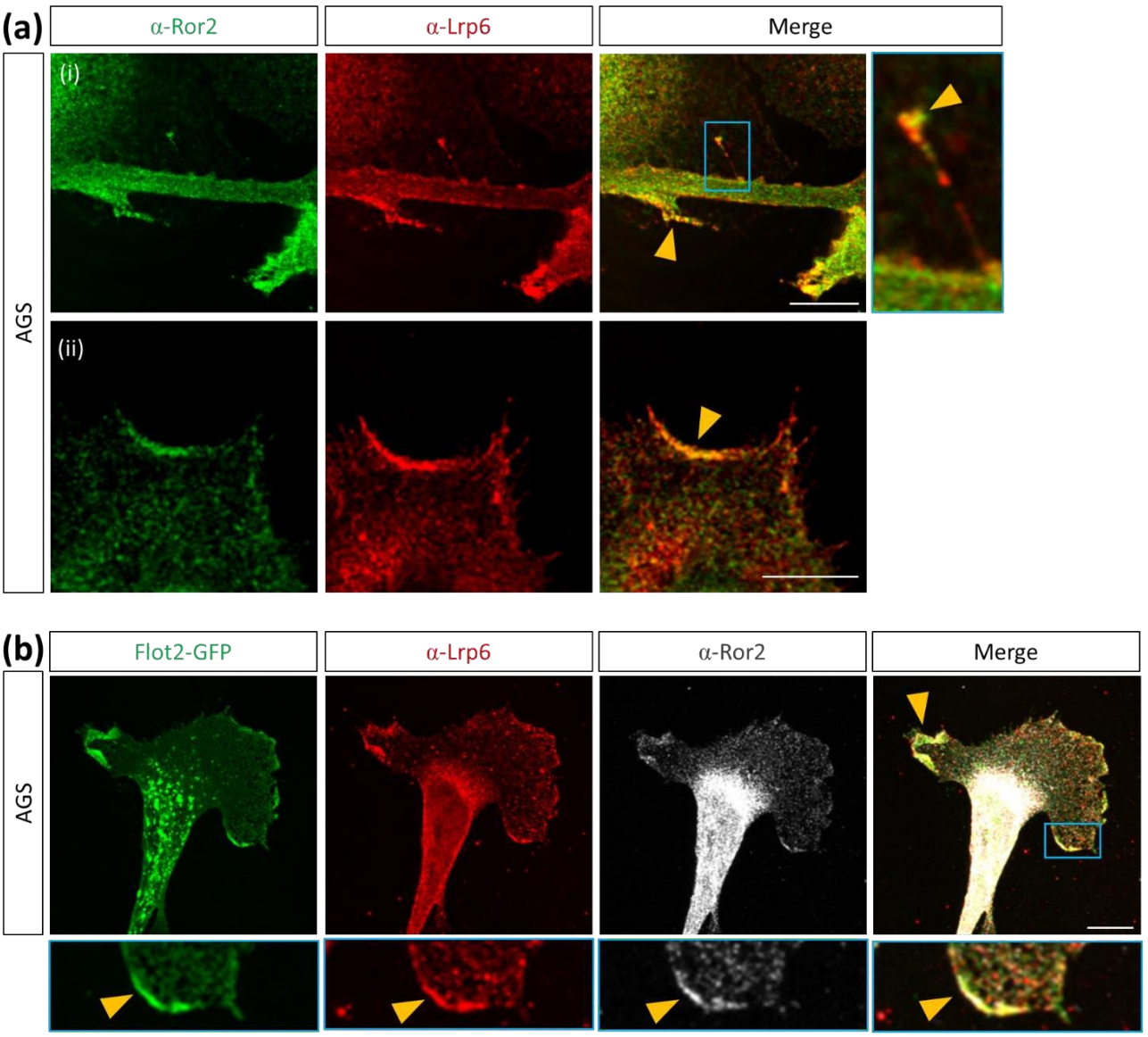


**Figure 45 – Ror2 and Lrp6 co-localise in Flotillin-2 microdomains**

**(a)** Antibody stainings against Ror2 (green) and Lrp6 (red) in AGS cells.(i) Cytonemal localisations of Ror2 and Lrp6 (highlighted in the blue box). (ii) Membrane localisations of Ror2 and Lrp6. Scale bar 10μm.

**(b)** Antibody stainings against Ror2 (grey) and Lrp6 (red) in AGS cells after transfection with Flot2-GFP. Blue boxes and yellow arrows highlight co-localisation of Lrp6 and Ror2 in Flot2-GFP-positive microdomains. Scale bar 10μm.

### 6.2.2. Fzd7 localises to Flot2 microdomains

The observation that both Ror2 and Lrp6 can (co-)localise to Flot2 microdomains raised the question of whether Flot2 may act as a platform for Wnt (co-)receptors in general. Furthermore, rather than Ror2 and Lrp6 directly interacting with one another, it is conceivable they may be associated via a further component, namely the Fzd receptors, which can interact with both Ror2 and Lrp6 (Nishita *et al.*, 2010; Nile *et al.*, 2018). Therefore, I next investigated the relationship between Flot2 and Fzd7, which is highly expressed in GC (Flanagan *et al.*, 2019). Initially, I attempted to assess endogenous Fzd7 localisation with antibody stainings, however specificity of antibodies towards Fzd7 using IF could not be achieved. Therefore, AGS cells were transfected with Fzd7-GFP and antibody stained for Flot2 (Fig. 46a). Here, Flot2 and Fzd7-GFP strongly co-localise intracellularly, at the membrane and on cytonemes, suggesting Flot2 could also be involved in the transport and/or localisation of Fzd7. To assess this, I performed siRNA-mediated knockdown of Flot2 in AGS cells and transfected Fzd7-GFP and memCh. Consistent with phenotypes seen with Ror2 and Lrp6, Fzd7-GFP also strongly accumulated in the perinuclear region in Flot2-deficient cells (Fig. 46b). This suggests Flot2 could also required for the intracellular transport and membrane localisation of Fzd7. Whether these defects in transport of Fzd7, Ror2 and Lrp6 are specific for these Wnt (co-)receptors or whether this is due to a general inhibition of intracellular transport is unclear. However, the strong co-localisation of all three (co-)receptors with Flot2 is indicative of a more specific and direct effect. Furthermore, Ror2 accumulates in the Golgi in Flot2-deficient cells, and Lrp6 accumulates in a different (yet unidentified) compartment (Fig. 25, 38). Additionally, Wnt3, which is secreted via the Golgi-ER axis, shows no notable change in sub-cellular localisation in Flot2-deficient cells either (Fig. 20). Together, these observations suggest it is unlikely Flot2 KD is having a general inhibitory effect on intracellular trafficking.

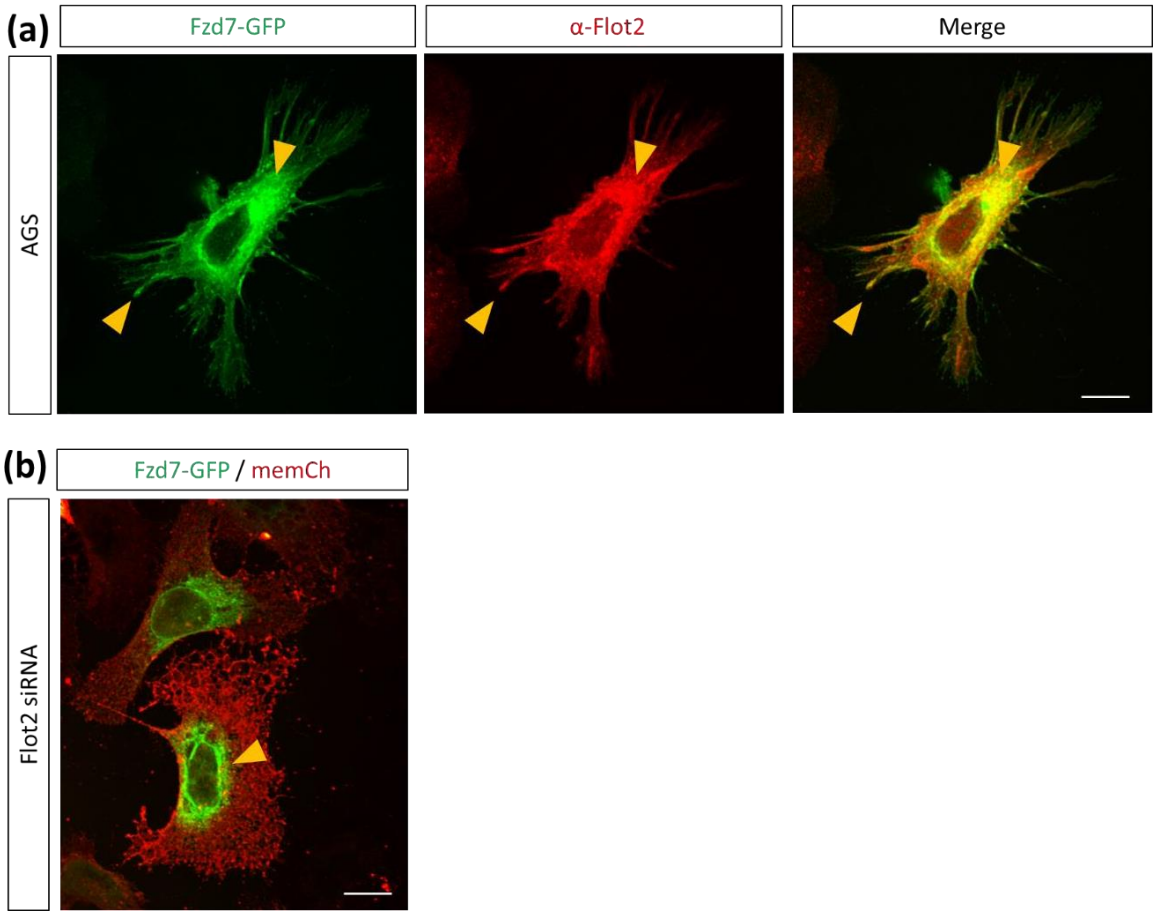


**Figure 46 – Fzd7 co-localises with and requires Flotillin-2 for its membrane localisation**
**(a)** Antibody stainings against Flot2 (red) in AGS cells after transfection with Frizzled7-GFP. Yellow arrows highlight co-localisation events. Scale bar 10µm.
**(b)** Confocal image of AGS cells transfected with Fzd7-GFP and memCh after siRNA-mediated knockdown of Flot2. Yellow arrow highlight perinuclear accumulation of Fzd7-GFP. Scale bar 10µm.

### 6.2.3. Ror2 and Lrp6 both co-localise with Fzd7

Fzd7 is an example of a Fzd receptor which is capable of interacting with both Ror2 and Lrp6 to mediate activation of the Wnt/PCP and Wnt/β-catenin pathways, respectively (Nish*ita et al*., 2010; Nile *et al*., 2018). Whilst mostly depicted as separate complexes, there is increasing evidence that Ror2 and Lrp6 could exist in one complex and regulate one another's signalling capabilities. Therefore, I wanted to investigate whether the co-localisation of Ror2 and Lrp6 might be mediated by both their interactions with Fzd7. I transfected AGS cells with Fzd7-GFP and performed antibody stainings against Lrp6 and Ror2. Here, all three (co-)receptors can be seen co-localising intracellularly, at the PM and on cytonemes (Fig. 47a). The

cytonemal and membrane localisations are highlighted in greater detail in Fig. 47b. Since Fzd7, Ror2 and Lrp6 have been shown to co-localise with one another and Flot2, it is possible that Flot2 microdomains may act as a platform for promoting interactions of these Wnt (co-)receptors.

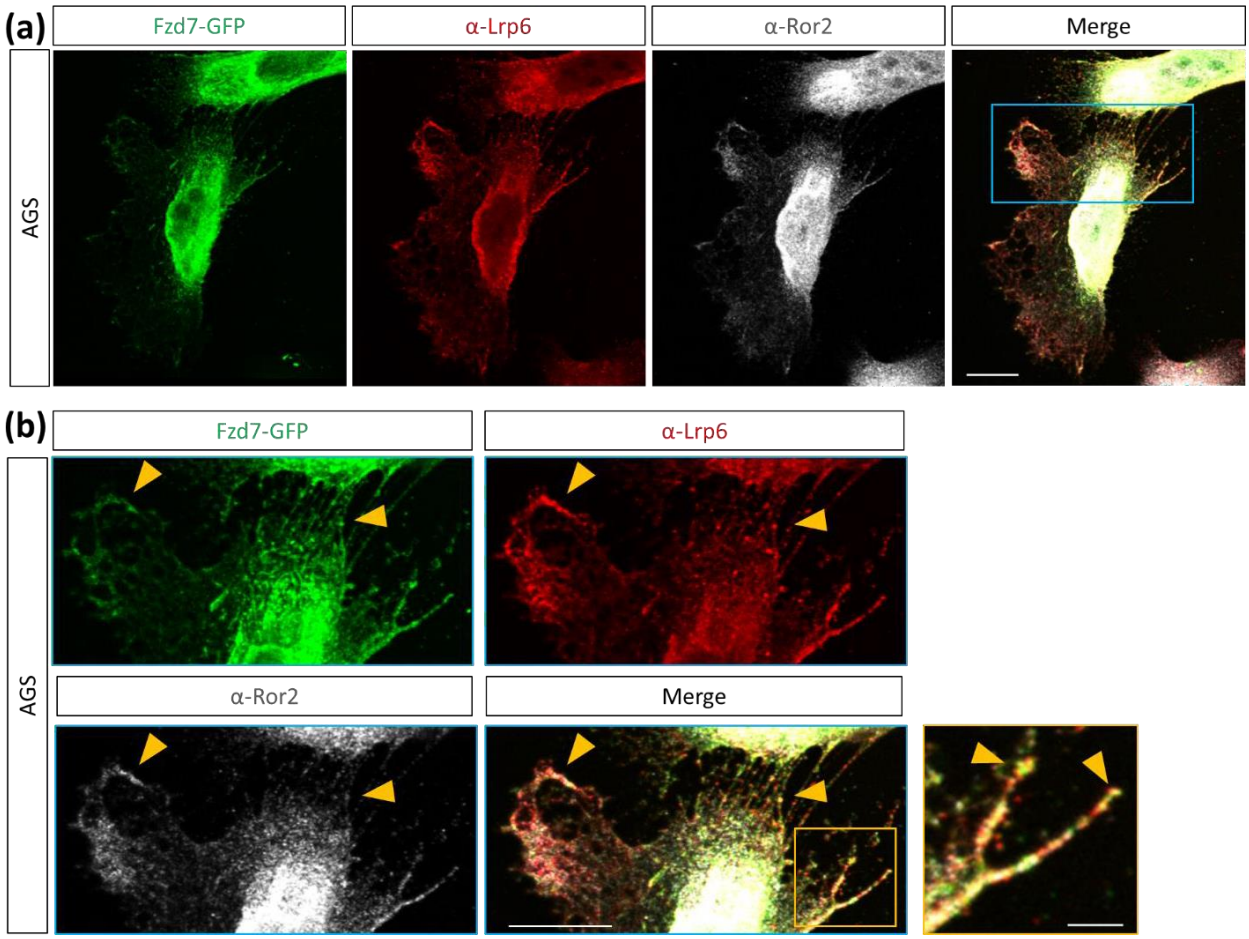


**Figure 47 – Fzd7, Ror2 and Lrp6 co-localise on cytonemes in GC cells**

**(a)** Antibody stainings against Ror2 (grey) and Lrp6 (red) in AGS cells transfected with Fzd7-GFP. Scale bar 10µm.

**(b)** Antibody staining as in (a), highlighting cytonemal and membrane co-localisation of Fzd7-GFP, Lrp6 and Ror2 (yellow box, yellow arrows). Scale bar 10µm (blue box), 2.5µm (yellow box).

### 6.2.4. Ror2 and Wnt3 form clusters, but do not co-localise, in GC cells

I have shown that there is co-localisation between Wnt3/Lrp6 (Fig. 35) and Ror2/Lrp6 in GC cells (Fig. 45). However, it has previously been reported that Wnt3 can interact with Ror2 (Billiard *et al.*, 2005). Therefore, I asked whether Wnt3 and Ror2 would also co-localise, or whether Wnt3 might associate with Ror2 via binding to Lrp6. I therefore performed antibody stains against Wnt3 and Ror2 to assess their localisations (Fig. 48a). Interestingly, Wnt3 and Ror2 consistently localise in the same regions of the cell, however there is little co-localisation observed. Rather, they appear to cluster together (Fig. 48a(i)). This clustering can be seen at both the PM and in the perinuclear region (Fig. 48a(ii)).

To assess this further, I quantified the fluorescent intensities of Wnt3 and Ror2 along a line and compared these with intensities of Wnt3 and Lrp6 (Fig. 49a). Here, broad increases in fluorescent intensities of Wnt3 and Ror2 occur simultaneously, however the peaks of intensity of individual puncta are consistently contiguous. Contrastingly, the intensity peaks of Wnt3 and Lrp6 puncta are largely synchronous and coincide with one another (Fig. 49b). This suggests that perhaps Wnt3 is binding to Lrp6 and thus has strong co-localisation, and that Ror2 is close by but not directly interacting with Wnt3. Indeed, when measuring the fluorescence intensity peaks of Ror2 and Lrp6, they are also consistently aligned (Fig. 49c). This is conducive of the idea that Ror2 and Lrp6 could be interacting or in complex with one another. Thus, Ror2 appears to cluster with Wnt3 due to being in close proximity with Lrp6, potentially via Fzd7.

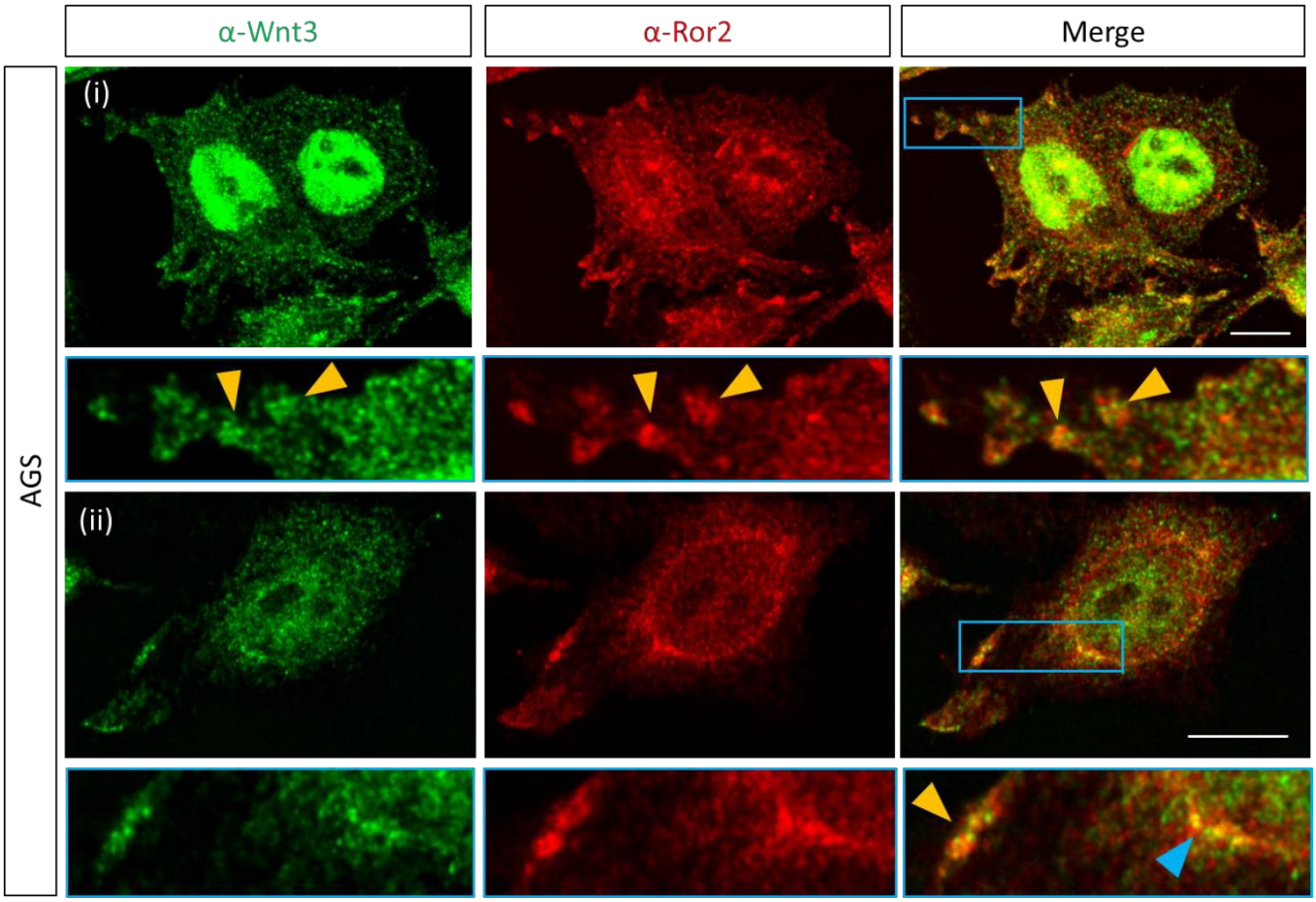


**Figure 48 – Wnt3 and Ror2 form clusters in GC cells**

Antibody stainings against Wnt3 (green) and Ror2 (red) in AGS cells. (i) Yellow arrows highlight clusters of Wnt3 and Ror2. (ii) Yellow and blue arrows highlight membrane and perinuclear clustering, respectively. Scale bars 10μm.

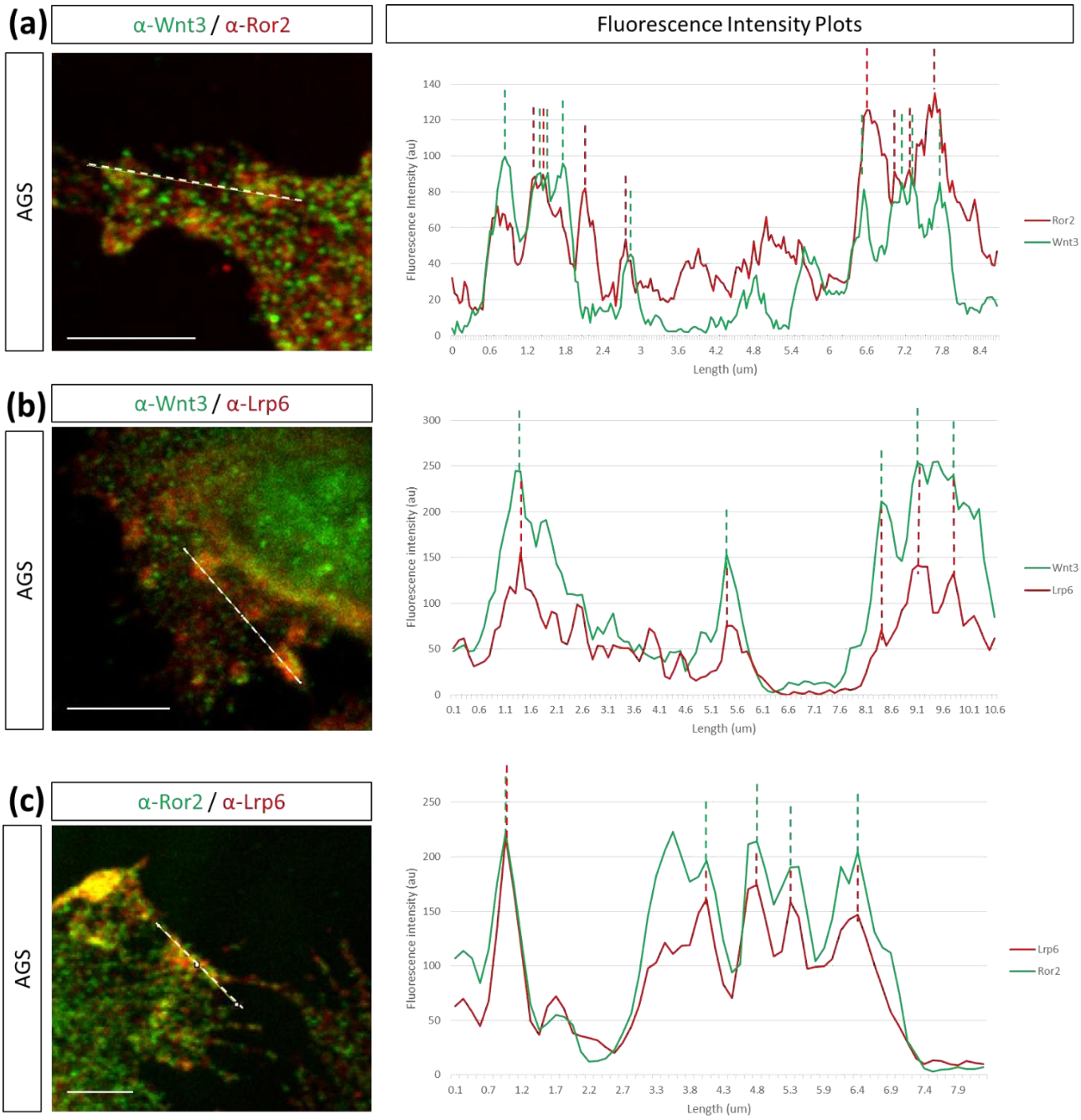


**Figure 49 – Ror2 associates with but does not co-localise with Wnt3**

**(a-c)** Antibody stainings for **(a)** Wnt3 and Ror2, **(b)** Wnt3 and Lrp6, **(c)** Ror2 and Lrp6, and associated fluorescence intensity plots along the dashed white lines. Red and green dashed lines highlight alignments of intensity peaks for each channel. Scale bars 5μm.

### 6.2.5. Ror2 and Lrp6 co-localisation is dependent on the Ror2-CRD

Ror2 and Lrp6 are both known to interact with Fzd receptors in their Wnt binding and concomitant signalling. Physical interactions between Ror2 and Fzd receptors occur via the extracellular cysteine rich domain (CRD) (Griffiths *et al.*, 2022). Therefore, I used a mutated Ror2 construct which lacks its CRD to assess any changes in its co-localisation with Lrp6 and thus decipher whether the CRD is required for these observed co-localisations.

AGS cells were transfected with Ror2-GFP or Ror2-ΔCRD-GFP and antibody stained for Lrp6. Here, strong co-localisation of Ror2-GFP and Lrp6 can be observed throughout the cell, including cytonemes (Fig. 50a). This was quantified and produced a PCC value of 0.67 (Fig. 50c). However, expression of Ror2-ΔCRD-GFP resulted in significantly less co-localisation with Lrp6 (Fig. 50b), producing a PCC of only 0.30 (Fig. 50c). This suggests that the CRD of Ror2 is necessary for its co-localisation with Lrp6. Whether this is due to loss of Ror2 interactions with Fzd7 is not known and this would require further investigation.

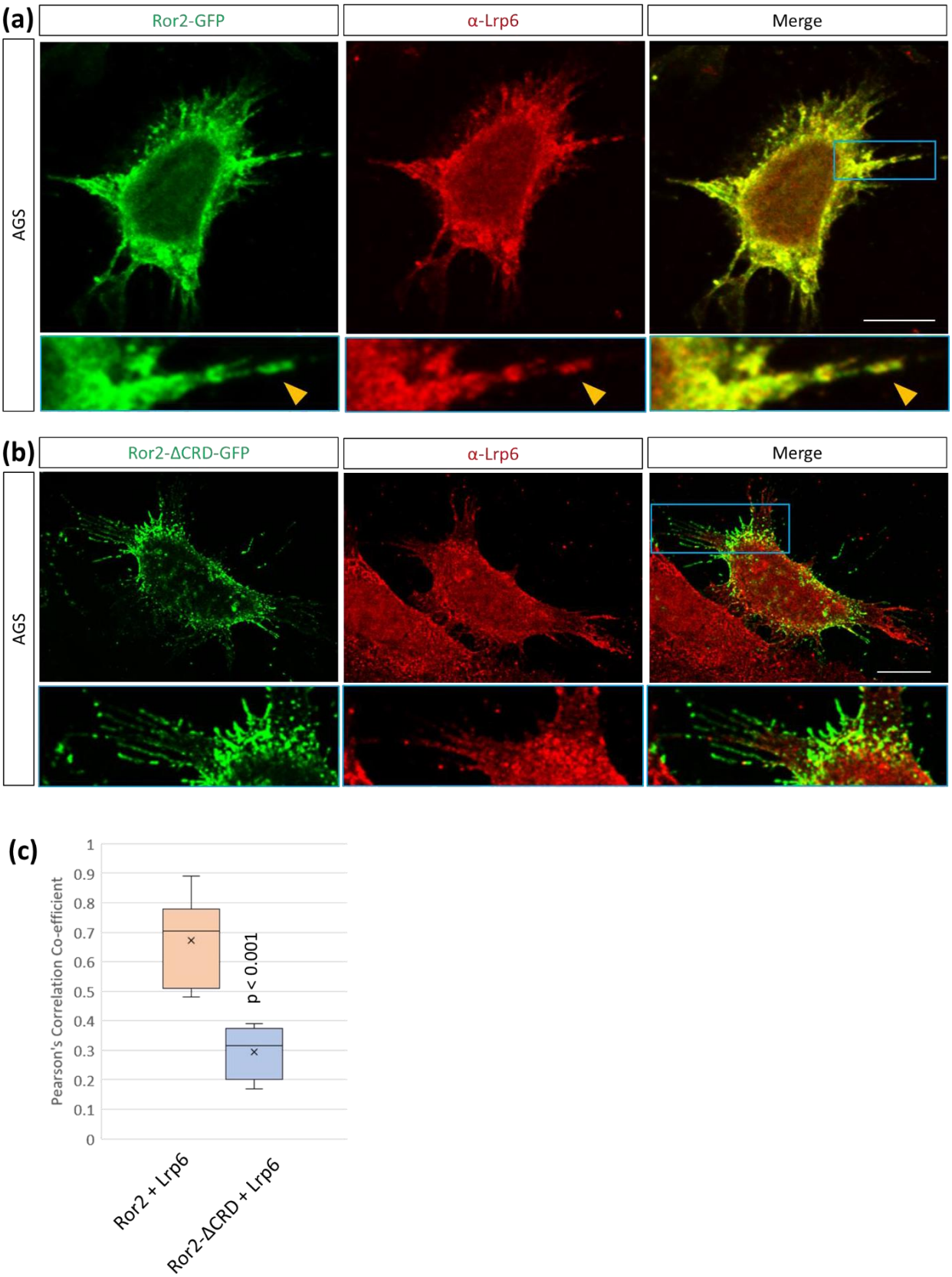


**Figure 50 – Ror2 CRD domain is needed for co-localisation with Lrp6**

**(a)** Antibody stainings against Lrp6 (red) in AGS cells after transfection with Ror2-GFP. Yellow arrows highlight cytonemal co-localisation of Ror2 and Lrp6. Scale bar 10µm.

**(b)** Antibody staining as in (a) with Ror2-ΔCRD-GFP mutant showing less co-localisation with Lrp6. Scale bar 10µm.

**(c)** Pearson's correlation co-efficient (PCC) quantification of Ror2-GFP or Ror2-ΔCRD-GFP co-localisation with Lrp6. Statistical significance calculated by student's t-test. (n = 6, 6; n = number of cells measured).

## 6.3. Discussion

### 6.3.1. Ror2 and canonical Wnt signalling

In chapter 2, I showed that Flot2 enhanced cytoneme-mediated delivery of Wnt3 in AGS cells, and that Flot2 promotes Wnt/PCP signalling in the producing cell via Ror2. However, at this point, it was unclear whether Wnt3 was acting as a passenger on cytonemes or whether it was directly binding with Ror2. Indeed, there is evidence that Wnt3, despite being a “canonical” Wnt, can bind to Ror2 (Billiard *et al.*, 2005). However, antibody stainings in AGS cells showed that whilst Ror2 and Wnt3 frequently clustered in the same regions of the cell membrane, there was only partial co-localisation (Fig. 48, 49). This suggested that Wnt3 may be binding to or interacting with another component which associates with Ror2.

Here, I believe these observations may be explained by Lrp6 binding with Wnt3 but interacting with Ror2, based on the following observations: (1) Lrp6 co-localises with both Wnt3 and Ror2, including on cytonemes and at the PM: (2) Whilst WT Ror2 inhibited Wnt/β-catenin signalling in AGS cells, expression of the Ror2-ΔCRD mutant did not have the reverse effect and still showed an inhibitory effect on reporter activity (Fig. 28): (3) Expression of the Ror2-ΔCRD mutant showed loss of co-localisation with Lrp6 compared to WT Ror2, suggesting the CRD domain regulates its ability to associate with Lrp6. Together, these data suggest that Ror2 may interact with Lrp6 (either directly or indirectly) to regulate its signalling capabilities, and that this requires the Ror2 CRD.

In support of a role for Ror2 in regulating “canonical” Wnt signalling, Ror2 has been shown to modulate canonical Wnt signals via interactions with Wnt1 and Wnt3 in osteoblastic cells (Billiard et al., 2005). Here, Ror2 antagonised Wnt1- and Wnt3-mediated stabilisation of β-catenin but regulated more distal signalling mechanisms, namely Wnt target gene transcription. These actions require the Ror2 CRD, which concurs with my findings. This also required tyrosine kinase activity, but neither Wnt1 nor Wnt3 induced Ror2 autophosphorylation. Interestingly, Ror2 had opposing effects on Wnt1 and Wnt3, potentiating and attenuating Wnt-responsive gene reporter activity, respectively. The authors speculate that Wnt1 and Wnt3 binding to Ror2 activates currently unidentified signalling cascades, which require RTK activity and regulate Wnt-responsive promoter activity independently from β-catenin (Billiard et

al., 2005). However, I believe these findings could be explained by a more direct relationship between Ror2 and Lrp6, in which Ror2 regulates Lrp6-Wnt binding and signalling.

Ror2 has also been shown to potentiate Wnt3a-mediated signalling in renal cancer cells (Rasmussen et al., 2013). Overexpression of Ror2 correlated with Axin2 expression, acting as part of a Wnt feedback loop. Conversely to Billiard et al., however, Ror2 is found to increase the pool of free β-catenin in the cell, producing a partially activated or “poised” state in which Wnt signal activation is primed and thus stimulation of Wnt/β-catenin signalling is enhanced by Ror2. The authors suggest that Ror2 enhances the pool of β-catenin by activation of Dvl proteins, since Ror2 over-expression increases the levels of phosphorylated Dvl2 and Dvl3. Interestingly, whilst inhibition of Lrp6 (by siRNA or Dkk1) attenuated the Wnt3a-mediated response, it did not interfere with the increased β-catenin levels associated with Ror2, suggesting an Lrp6-independent mechanism by which Ror2 regulates Dvl proteins and thus β-catenin (Rasmussen et al., 2013).

In agreement with these findings, Ror2 is also able to potentiate Wnt3a signalling in the lung carcinoma cell line H441 (Li et al., 2008). Here, Ror2 is shown to interact with Fzd2, but not Fzd7, to potentiate the Wnt3a signal. This contradicts my own findings that Ror2 co-localises with Fzd7 in AGS cells, along with Lrp6. However, I cannot rule out this could be due to effects of over-expression or cell-specific receptor and ligand availabilities. I attempted to look at endogenous Fzd2 and Fzd7 localisations, however, to date no antibodies with specificity for these receptors have been successfully generated for IF. If time had permitted, I would have liked to assess the impact of perturbing Fzd7 function or expression on Ror2-Lrp6 interactions and localisations, to more confidently determined whether Fzd7 could be the central mediator of these interactions.

In agreement with my data, the authors find that regulation of “canonical” signalling by Ror2 requires its CRD, but not the intracellular PRD domain (Li *et al.*, 2008). In contrast to Rasmussen et al., however, the authors find that this function of Ror2 in canonical Wnt signalling is dependent on Lrp6 function. Inhibition of Lrp6, by siRNA or addition of Dkk1 and its co-receptor Kremen1 (Krm1), attenuated the ability of Ror2 to augment the Wnt3a signal. These discrepancies may be due to cell-specific

effects, such as receptor expression or basal levels of Wnt signalling. Additionally, the authors' measure of Wnt signal activation differ; Rasmussen *et al.,* (2013) use Axin2 expression, whereas Li *et al.,* (2008) use a luciferase reporter system to assess changes in Wnt signalling. Despite the disagreement over the mechanistic underpinnings, these papers provide evidence that Ror2 is not exclusively a "non-canonical" receptor and can act to regulate the "canonical" Wnt signalling pathway.

### 6.3.2. Lrp6 and non-canonical Wnt signalling

Promoting the notion that the Wnt signalling pathways and receptors are not so discrete, there is also evidence that the traditionally "canonical" Wnt co-receptor Lrp6 can directly modulate "non-canonical" Wnt signals. Firstly, Lrp6 has been shown to interact with Fzd8; a Fzd receptor known for its ability to activate both the Wnt/β-catenin and Wnt/PCP pathways (Ren et al., 2015; Yang et al., 2017). This interaction involves all three Lrp6 ectodomains; E1-2, E3-4 and LDLR. Whilst E1-2 and E3-4 are known to bind Wnt proteins, LDLR is not, and the authors speculate that this ectodomain is responsible for regulating Fzd8-Lrp6 interactions. Using a Bioluminescence Resonance Energy Transfer (BRET) technique with mutants lacking particular ectodomains of Fzd8 and Lrp6, the authors demonstrate that Fzd8-Lrp6 interactions occur in a Wnt-independent manner and that the Fzd8-CRD domain is dispensable. Interestingly, Lrp6 binding inhibits activation of Wnt/PCP signals stimulated by Wnt5a-Fzd8 binding in a manner dependent on its LDLR domain. Addition of recombinant Lrp6 ectodomain (Lrp6N) to HepG2 cells decreased the ability of Wnt5a to induce cell migration *in vitro* and over-expression of Lrp6N in a mouse model decreases metastasis of breast cancer cells *in vivo*. Together, these findings demonstrate that Lrp6 can inhibit Wnt5a-mediated Wnt/PCP signalling and the authors suggest a novel interaction of Lrp6 with Fzd8 in modulating this response. Here, the ectodomain of Lrp6 interacts with Fzd8 (away from the CRD) and the presence of "canonical" or "non-canonical" Wnts determines the conformational changes which occur to permit the relevant Wnt signals (Ren et al., 2015).

These findings concur with research showing that Wnt5a-mediated activation of RhoA activity is impaired in Lrp6(-/-) mouse embryonic fibroblasts (MEFs) (Gray et

al., 2013). Lrp6 loss-of-function also resulted in defective neurulation of the mouse neural tube, where cellular defects commonly associated with Wnt/PCP signalling, such as cell shape and polarity, were observed. Furthermore, Lrp6 co-immunoprecipitated with Dishevelled-associated activator of morphogenesis 1 (DAAM1), an intracellular protein commonly associated with the Wnt/PCP pathway. These data suggest that Lrp6 can regulate “non-canonical” signalling and has a significant impact on subsequent cellular processes, such as actin cytoskeletal regulation (Gray et al., 2013). In the future, it would be interesting to see whether perturbing Lrp6 function and expression impairs Ror2-mediated activation of Wnt/PCP signalling, which would suggest a bidirectional regulation.

### 6.3.3. Integrating Wnt Signals: A Wnt receptor supercomplex?

In a similar vain to the model proposed by Ren *et al.,* (2015), whereby Fzd8-Wnt5a binding was regulated by Lrp6 interactions, I believe that Fzd7-Wnt3a binding may be regulated by Ror2 interactions. Firstly, I have shown that Ror2 and Lrp6 can both co-localise with Fzd7, a key Fzd receptor in GC (Fig. 47) (Flanagan *et al.*, 2019). Secondly, it has previously been shown that the Ror2 CRD is key to its interactions with Fzd (Griffiths *et al.*, 2022). Thus, loss of the CRD domain and loss of interactions with Fzd could explain why there is a decrease in co-localisation between Ror2 and Lrp6 (Fig. 50). Finally, expression of Ror2-ΔCRD, whilst decreasing Wnt/PCP signalling, does not disinhibit Wnt/β-catenin signalling. I hypothesise this is because Fzd-Ror2 interactions somehow regulate Fzd-Lrp6 interactions and/or Fzd-Wnt-Lrp6 bindings.

Therefore, I propose a model whereby Fzd receptors, in this case Fzd7, can interact with both Ror2 and Lrp6 even in the absence of Wnt ligands. These Ror2-Fzd7 interactions regulate Fzd7-Wnt3a-Lrp6 bindings, and Lrp6-Fzd7 interactions, away from the Fzd CRD, regulate Ror2-Wnt5a-Fzd7 bindings. Depending on which Wnt ligands bind, determines the consequential conformational changes, which are permissive of binding to Dvl in a manner which promotes Wnt/PCP signalling (via its DEP domain) or Wnt/β-catenin signalling (via its DIX domain). Finally, I propose that this signalling complex, which I have named the Wnt Receptor Supercomplex (WRS), resides in flotillin microdomains, which promote the clustering and formation of these

receptors (Fig. 51). This is based on the observation that all three of these receptors (and Wnt3) co-localise with Flot2 and that upon Flot2 KD, their localisations are drastically altered. This model could also explain why Ror2 co-localises with Flot2, Fzd7 and Lrp6, but always clusters adjacent to Wnt3; because Wnt3 is in close proximity, bound to Fzd-Lrp6.

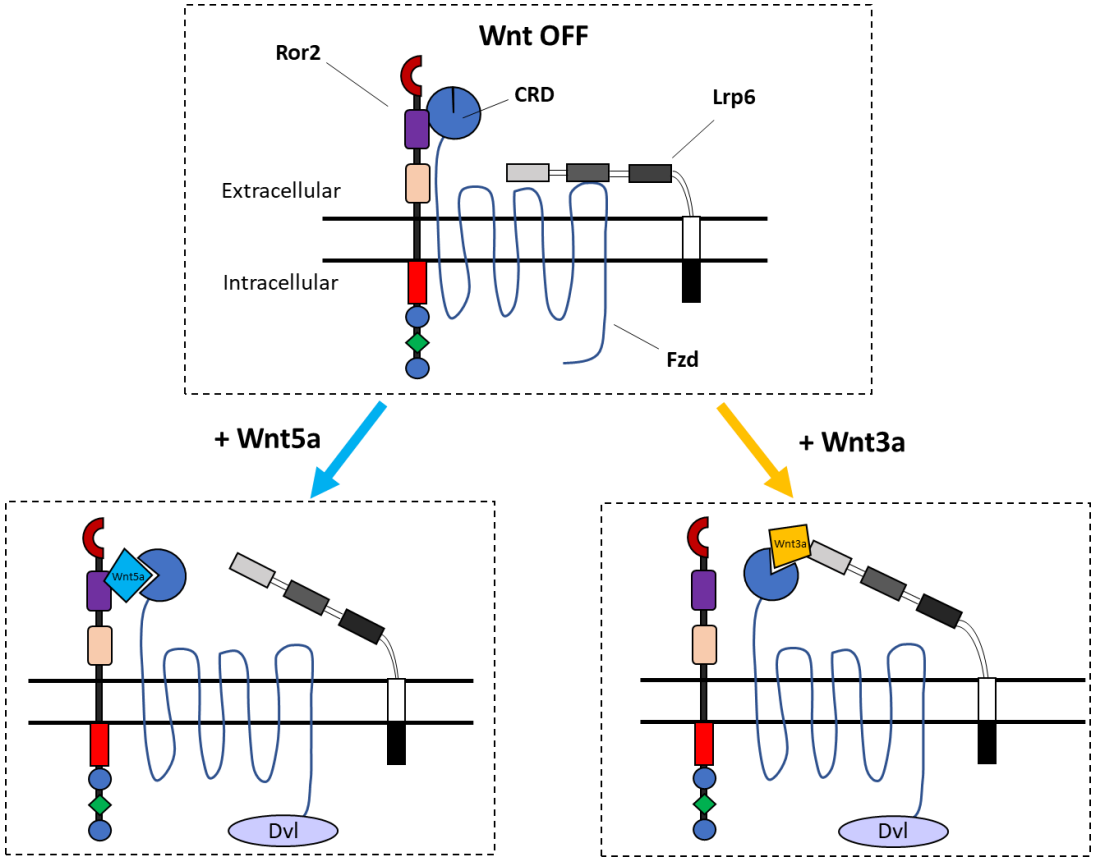


**Figure 51 – Schematic representing the proposed Wnt Receptor Supercomplex (WRS) model** The WRS model suggests that Ror2 and Lrp6 both interact with the same Frizzled receptor(s) to modulate one another's binding to either "canonical" or "non-canonical" Wnt ligands. Wnt/β-catenin and Wnt/PCP signalling can still be transduced via Dishevelled (Dvl), but interactions within the WRS determine how that signal is transduced (e.g., by altering domain interactions of Dvl). The schematic depicts Ror2 or Lrp6 dissociating from the complex upon Wnt binding, however this is purely conceptual and if these interactions do occur, they may well persist following ligand binding.

The data I have presented here to produce this hypothesis are largely preliminary and further experiments are necessary to assess these possibilities. One limitation of these experiments and the interpretation is that these observations were made only in AGS cells. Thus, I cannot infer that this could be a general mechanism and further investigation using other cell lines or model organisms needs to be conducted to validate these findings. For example, validation experiments would need to be conducted to confirm physical interactions between these receptors, such as using

immunoprecipitation and/or elaborating on the BRET system used by Ren *et al.,* (2015). Additionally, super-resolution microscopy would be useful for analysing the arrangements of these receptors at the cell surface and to gauge if there are any differences in the presence / absence of Wnt ligands. For example, in Fig. 51, I depict Ror2 or Lrp6 dissociating from the WRS after binding of Wnt3a or Wnt5a, respectively. However, it is possible that these receptors continue to interact even upon binding of Wnt ligands. Whether this is the case and if so, how this is functionally achieved, remains unclear. Additionally, it remains to be understood whether these interactions are positively or negatively regulating Wnt bindings.

## 7. General Discussion and Concluding Remarks

In this work, I have shown that Flot2 is an important player in the localisation, trafficking and signalling of Wnt (co-)receptors in GC cells. Firstly, that Flot2 is required for Ror2 membrane localisation and promotes Wnt/PCP signalling necessary for cytoneme formation. Secondly, that Flot2 is important in the paracrine transport of Wnt3 and subsequent internalisation of Lrp6 during endocytosis. Finally, I have shown that Fzd7 localises, along with Ror2 and Lrp6, to flotillin microdomains, potentially as a WRS. So how does this all fit together?

It remains possible that the function(s) of Flot2 with respect to Ror2 and Lrp6 may be distinguished by their roles in Wnt-producing and Wnt-receiving cells. However, the observation that Lrp6 can also localise to cytonemes (with Ror2 and Fzd7) presents the possibility that the entire WRS may be present on cytonemes and that the handover events we see include Fzd and Lrp6 receptors. Hence, the clustering of Lrp6 receptors seen at cytoneme contact sights could represent Lrp6 which has been handed over. Whether subsequent endocytosis of Fzd and Lrp6 in the recipient cell also includes Ror2, or whether this somehow dissociates during this process, would be an interesting line of investigation. However, since Ror2 accumulates in the Golgi upon Flot2 KD, but Lrp6 does not, it is clear the relationship between Flot2 and these co-receptors differs, and so it may still be possible to distinguish their functions based on where interactions are first made and which Wnt ligands are bound.

In the context of deciphering and diverging functions of Ror2 and Lrp6, the WRS model may seem counter-intuitive or even contradictory to previous models. But it is

important to highlight that this is still conducive of the idea of Wnt/β-catenin and Wnt/PCP pathways. Rather, instead of depicting Ror2-Fzd and Lrp6-Fzd as separate entities, they may function as one complex. Similarly, this model still works around the idea that Dvl is the central mediator of signal transduction, but Fzd-Dvl interactions, which determine which pathway is activated, are regulated by the availability of not only the Wnt ligands and their cognate receptors, but also Wnt co-receptors the ligands may not directly bind to. This could explain the aforementioned examples of unconventional activation of these pathways as well as some of the discrepancies in the mechanistic understandings of Wnt signalling events.

Finally, in the context of Wnt-driven cancers, this could have important implications in understanding tumourigenesis and phenotypic characteristics of cancer cells. For example, the Wnt/β-catenin pathway is most commonly associated with promoting cell proliferation and thus tumour growth (Zhan *et al.*, 2017). Conversely, the Wnt/PCP pathway is associated with increasing cell migration and invasiveness (Chen *et al.*, 2021). In melanomas, hypoxia induces a phenotypic “switch” from a more proliferative state to a more migratory and metastatic phenotype, which is associated with upregulation of Ror2 and Wnt/PCP signalling (O’Connell *et al.*, 2013; Webster *et al.*, 2015). Cells which have undergone this “switch” show a 10-fold decrease in responsiveness to BRAF inhibitors, which are used to treat melanoma cells. In contrast, in Triple-negative breast cancer (TNBC) cells, which are naturally high in Ror2, downregulation of Ror2 increases cell migration and invasion by increasing expression of ECM components such as integrins and fibronectin, which aid cell migration (Henry *et al.*, 2015). Here, loss of Ror2 in TNBC tumours shows an increase in *Axin2* expression, suggesting upregulation of Wnt/β-catenin signalling through loss of repression. However, only some of the expression changes of ECM and EMT components, seen following loss of Ror2, can be rescued by inhibition of the Wnt/β-catenin pathway. This indicates some interplay between the pathways, but a level of Ror2-mediated regulation of the ECM which is not part of a simple “ON/OFF” Wnt signal switch, as is often depicted in diagrams. Here, the existence of a WRS may be involved in regulating these pathways in a more complex manner which has not previously been considered. Thus, it may affect the efficacy and downstream effects of therapeutic inhibitors which target Wnt receptors, such as those targeted against Fzd7 in gastrointestinal cancers (Flanagan *et al.,* 2017).

## 8. Appendix

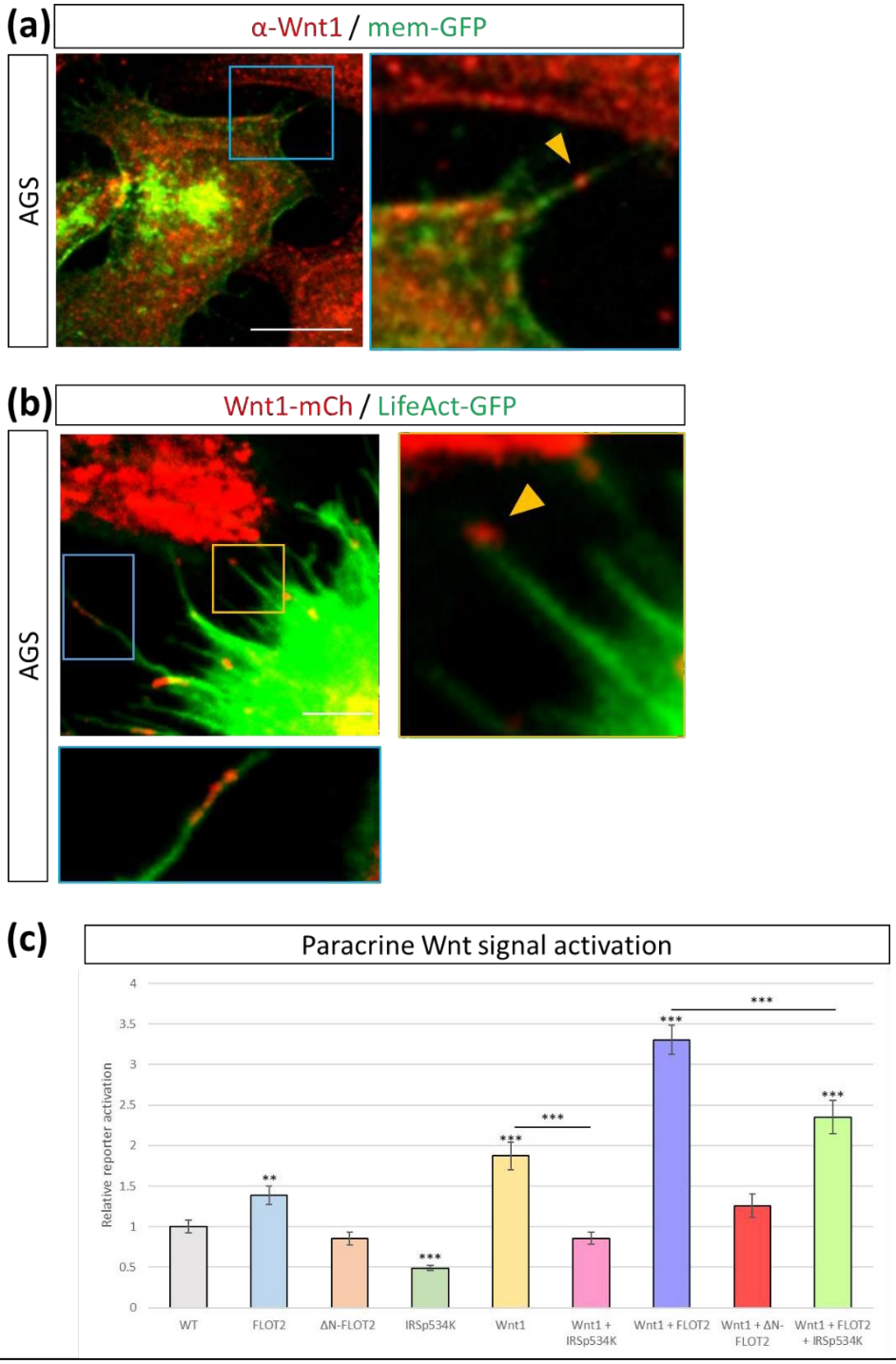


**Figure A1 – Cytoneme-mediated paracrine Wnt1 signalling**

**(a)** Antibody staining of mem-GFP-expressing AGS cells stained against Wnt1 (red) after methanol fixation. Scale bar represents 10μm. Blue box and yellow arrow highlight cytoneme-localised Wnt1. Scale bar represents 10μm.

**(b)** Confocal image of AGS cells expressing Wnt1-mCh and LifeAct-GFP. Boxes and yellow arrows highlight cytoneme-localised Wnt1-mCh. Scale bar represents 5μm.

**(c)** Quantification of STF reporter fluorescence following 48 hr co-cultivation of STF-mCh-expressing HFE-145 cells with AGS cells expressing indicated constructs. Significance calculated by student's t-test with Bonferroni correction for multiple comparisons. (n = 322, 443, 403, 258, 167, 388, 469, 362, 221; n = number of nuclei measured).

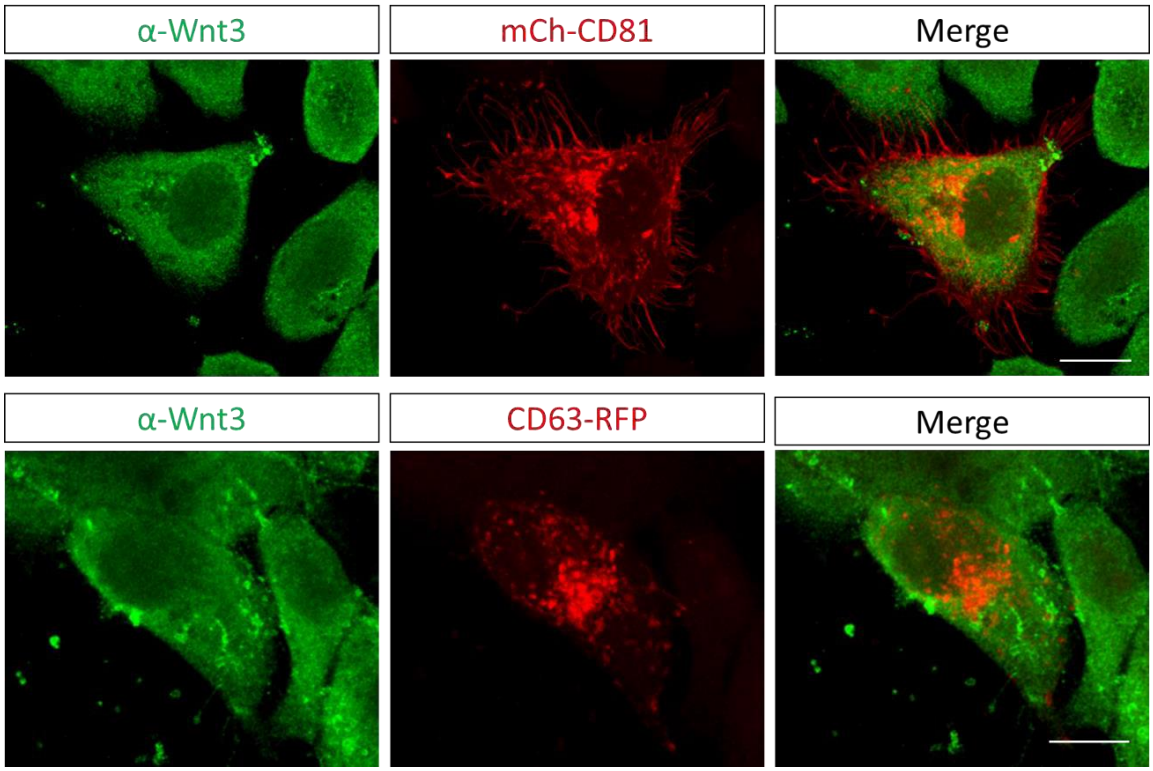


**Figure A2 – Wnt3 shows minimal co-localisation with exosomal markers in GC cells**
Antibody stainings of AGS cells expressing mCh-CD81 or CD63-RFP to mark exosomes, stained for endogenous Wnt3 (green). Scale bars represent 10μm.

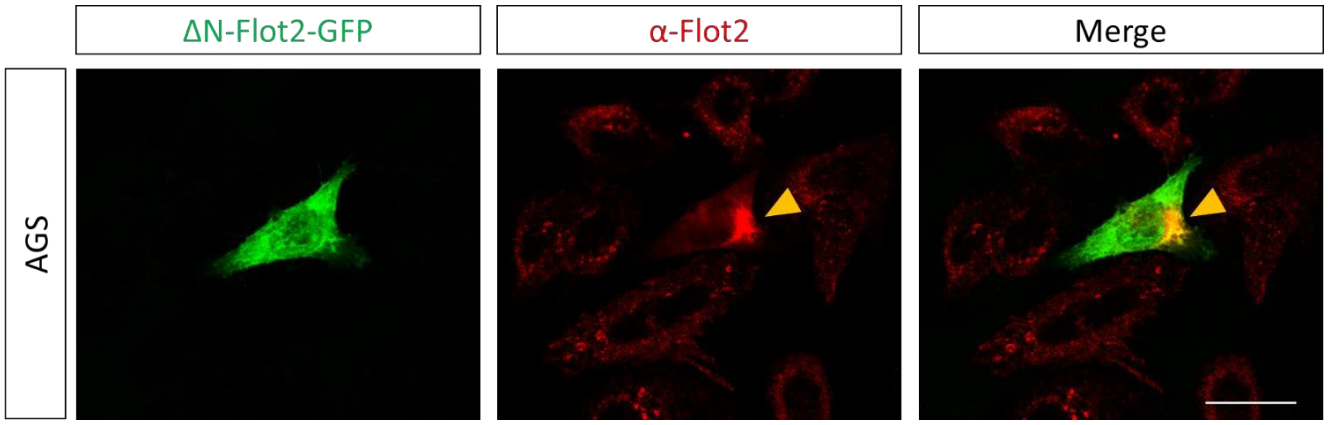


**Figure A3 – Dominant-negative ΔN-Flot2-GFP disrupts endogenous Flot2 localisation**
Antibody staining of endogenous Flot2 (red) in AGS cells expressing the dominant-negative ΔN-Flot2-GFP mutant. Yellow arrow highlights the altered localisation of endogenous Flot2, where punctate staining throughout the cell is lost compared to surrounding untransfected cells. Scale bar represents 20μm.

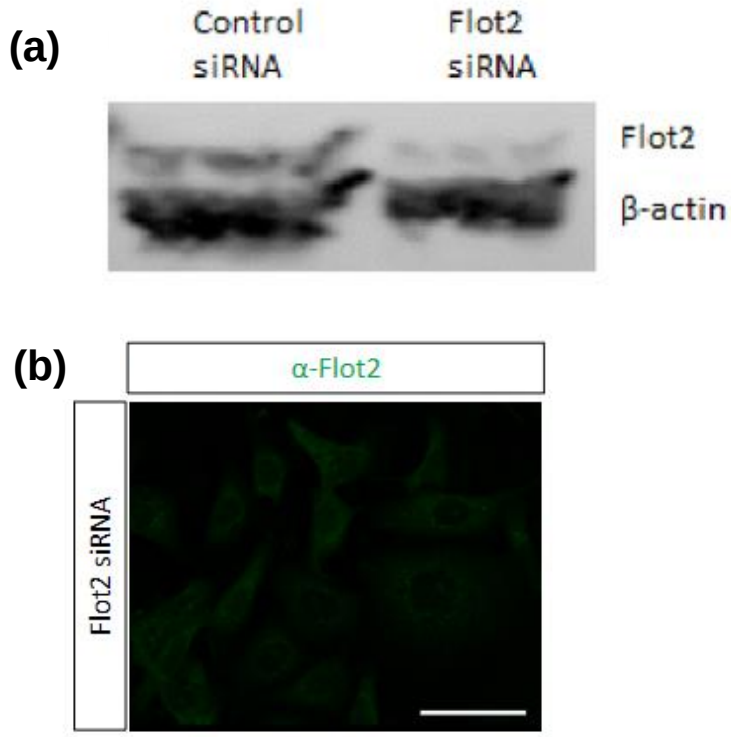


**Figure A4 – Flot2 siRNA successfully knocks down Flot2**

(a) Western blot showing successful knockdown of Flot2 in AGS cells after 24 hr treatment with Flot2 siRNA (30 pmol final concentration). β-actin was used as the loading control.

(b) Confocal image of Flot2 siRNA treated AGS cells stained for Flot2 (green). Minimal staining is visible, indicating successful knockdown. Scale bar 50μm.

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