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To my family and friends

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ABSTRACT

The movement of cells and the formation of tissues is dependent on the individual cell's ability to attach to its surroundings. The attachment of cells to the surrounding extracellular matrix is principally mediated by integrin adhesion receptors. Integrins mediate bidirectional signalling across the plasma membrane, and by binding to components in the extracellular matrix, transform cues from their surroundings to changes in cell behaviour, such as dynamic restructuring of the cytoskeleton. The endosomal network is responsible for transporting molecules to their correct cellular locations. As cells migrate, integrins undergo continuous cycles of internalisation and endosomal recycling back to the cell surface to facilitate the dynamic assembly and disassembly of adhesions that enables sustained cell motility.

Cancer cells regulate endosomal receptor traffic to promote their malignancy. This includes promotion of integrin trafficking to facilitate cancer cell migration and invasion, and the regulation of growth factor receptor trafficking to promote oncogenic signalling. However, large gaps remain in our understanding of the mechanistic details behind these processes.

This thesis presents new insights into the regulation of integrin and human epidermal growth factor receptor HER2 traffic in breast cancer. In the first project, we aimed to determine the impact of the endosomal sorting receptor SORLA on the trafficking and oncogenic signalling of HER2, and found that SORLA directs endocytosed HER2 back to the cell surface to drive growth and survival signalling. In the second project, we investigated how Swiprosin-1 and Rab21 co-facilitate integrin endocytosis, and revealed that they direct integrins through the clathrin- and dynamin-independent CLIC/GEEC endocytic pathway, thus promoting integrin-mediated cell migration and invasion. In the third project, we studied the role of the actin-binding protein EPLIN and its isoforms in the context of integrin trafficking, and discovered that EPLINα localises to endosomes carrying integrin cargo, where it facilitates integrin recycling back to the cell surface to promote cell migration. Taken together, the results presented here increase our understanding of how breast cancer cells control receptor trafficking to promote their growth and motility, highlighting the significant impact that receptor trafficking has on cancer cell malignancy.

KEYWORDS: Integrin, HER2, endosome, endocytosis, recycling, SORLA, Swiprosin-1, EPLIN, LIMA1, actin, cytoskeleton, cancer, cell migration

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TIIVISTELMÄ

Solujen liike ja kudosten muodostuminen riippuu yksittäisen solun kyvystä kiinnittyä ympäristöönsä. Solujen kiinnittyminen ympäröivään soluväliaineeseen välittyy pääasiassa integriiniadheesioreseptorien kautta. Integriinit välittävät kaksisuuntaista signalointia solukalvon läpi, ja sitoutumalla soluväliaineen komponentteihin, säätelevät solujen käyttäytymistä muuntamalla ympäristöstään tulevia signaaleja solunsisäisiksi muutoksiksi, kuten solun tukirangan uudelleenjärjestelyksi. Solunsisäinen endosomaalinen verkosto vastaa molekyylien kuljettamisesta solun eri osiin. Kun solu liikkuu, se ylläpitää jatkuvaa integriinien kierrätystä, mikä mahdollistaa soluadheesioiden dynaamisen säätelyn ja solujen jatkuvan liikkuvuuden.

Syöpäsolut säätelevät reseptorien liikennettä edistääkseen pahanlaatuisuuttaan. Syöpäsolut käyttävät hyväkseen integriinien sisäistämiseen ja kierrätykseen liittyviä mekanismeja edistääkseen niiden liikkuvuutta, ja säätelevät kasvutekijäreseptoreiden solunsisäistä liikennettä onkogeenisen signaloinnin edistämiseksi. Monet näiden prosessien taustalla olevista mekanismeista tunnetaan kuitenkin edelleen suhteellisen huonosti.

Tämä väitöskirja tuo esiin uutta tietoa integriinien ja HER2-kasvutekijäreseptorin liikennöinnin säätelystä rintasyövässä. Ensimmäisessä osatyössä tutkimme SORLA-proteiinin vaikutusta HER2:n liikennöintiin, ja havaitsimme, että se toimii HER2:n liikenteen säätelijänä ja ohjaa HER2-reseptorin takaisin solun pinnalle tehostaakseen kasvua edistävää signalointia. Toisessa osatyössä halusimme selvittää, miten Swiprosin-1 ja Rab21 yhdessä säätelevät integriinien endosytoosia, ja löysimme että ne ohjaavat integriinien endosytoosia klatriinista ja dynamiinista riippumattoman CLIC/GEEC-reitin kautta, mikä stimuloi integriinivälitteistä solujen migraatiota ja invaasiota. Kolmannessa osatyössä halusimme selvittää, miten EPLIN vaikuttaa integriinien liikennöintiin, ja havaitsimme, että sen isoformi EPLINα lokalisoituu integriinejä kuljettaviin endosomeihin, ja edistää rintasyöpäsolujen migraatiota kierrättämällä integriinejä takaisin solun pinnalle. Nämä tulokset tarjoavat tärkeää lisätietoa siitä, miten rintasyöpäsolut säätelevät solupinnan reseptorien liikennöintiä edistääkseen kasvua ja migraatiota, ja korostaa solunsisäisen liikennöinnin keskeistä roolia syövässä.

ASIASANAT: Integriini, HER2, endosomi, endosytoosi, kierrätys, SORLA, Swiprosin-1, EPLIN, LIMA1, aktiini, solun tukiranka, syöpä, solumigraatio

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Abbreviations

ADP	Adenosine diphosphate
AP2	Adaptor protein 2
APPL1	Adaptor protein containing a pleckstrin-homology domain, phosphotyrosine binding domain and leucine zipper motif 1
Arf1	ADP-ribosylation factor 1
Arhgap21	Rho GTPase activating protein 21
Arp2/3	Actin-related protein 2/3 complex
ATP	Adenosine triphosphate
BAR	Bin/amphiphysin/rvs
BiCAP	Bimolecular complementation affinity purification
BiFC	Bimolecular fluorescence complementation
BSA	Bovine serum albumin
CAD	Cationin amphiphilic drug
Cdc42	Cell division control protein 42
CG	CLIC/GEEC
CLIC	Clathrin-independent carrier
CLIC3	Chloride intracellular channel protein 3
CME	Clathrin-mediated endocytosis
CNN	Calponin
CORO1C	Coronin 1C
CTTN	Cortactin
Dab2	Disabled-2
DAPI	4',6-diamidino-2-phenylindole
DN	Dominant negative
DOK1	Docking protein 1
ECM	Extracellular matrix
EEA1	Early endosome antigen 1
EGFR1	Epidermal growth factor receptor 1
EHD1	Eps15 homology domain protein 1
EHD2	Eps15 homology domain protein 2
ELM	Eukaryotic linear motif

EMCCD	Electron-multiplying charge-coupled device
EPLIN	Epithelial protein lost in neoplasm
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ESCRT	Endosomal sorting complex required for transport
FA	Focal adhesion
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FCHSD2	FCH and double SH3 domains protein 2
FLN	Filamin
FMNL2	Formin-like protein 2
G-actin	Globular actin
GAP	GTPase-activating protein
GBF1	Golgi-specific brefeldin A resistance factor 1
GEEC	GPI-AP-enriched early endosomal compartment
GEF	Guanine-nucleotide exchange factor
GEF	Guanine-nucleotide exchange factor
GFP	Green fluorescent protein
GFR	Growth factor receptor
GGA	Golgi-localised gamma-ear containing Arf-binding protein
GLUT1	Glucose transporter type 1
GPI-APs	Glycosylphosphatidylinositol-anchored proteins
GRAF1	GTPase regulator associated with focal adhesion kinase-1
GTPase	Guanosine triphosphate hydrolase
HAX-1	HS1-associated protein 1
HeLa	Henrietta Lacks
HER2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor
Hip1R	Huntingtin-interacting protein 1-related
Hsc70	Heat-shock cognate protein 70
IAC	Integrin adhesion complex
I-BAR	Inverse BAR
ICAP1	Integrin cytoplasmic domain-associated protein 1
ILK	Integrin-linked kinase
ILV	Intraluminal vesicle
IRSp53	Insulin-responsive protein of mass 53 kDa
KIF15	Kinesin family member 15
LAMP	Lysosome-associated membrane protein
LIM	Lin-11, Isl-1, Mec-3
LRP1	Low-density lipoprotein receptor-related protein 1

LRP12	Low-density lipoprotein receptor-related protein 12
MAPK	Mitogen-activated protein kinase
MDGI	Mammary-derived growth inhibitor
MHCI	Major histocompability complex I
MICAL-L1	Molecule interacting with CasL-like 1
MMP-13	Matrix metalloproteinase-13
mRNA	Messenger RNA
NA	Numerical aperture
NME2	NME/NM23 nucleoside diphosphate kinase 2
NPF	Nucleation promoting factor
N-WASP	Neuronal Wiscott-Aldrich syndrome protein
PARP1	Poly ADP ribose polymerase 1
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
PICK1	Arp2/3 inhibitor and BAR domain-containing protein interacting with C kinase 1
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKCα	Protein kinase C alpha
PKD1	Protein kinase D1
PLA	Proximity ligation assay
PtdIns(3)P	Phosphatidylinositol 3-phosphate
RCP	Rab-coupling protein
RGD	Arginine-glycine-aspartic acid
SCAR	Suppressor of cyclic AMP receptor
sCMOS	Scientific complementary metal-oxide semiconductor
SDS	Sodium dodecyl sulfate
SHARPIN	SHANK-associated RH domain interactor
SIM	Structured illumination microscopy
siRNA	Small interfering RNA
SNX	Sorting nexin
SORLA	Sortilin-related receptor with A-type repeats
SVIL	Supervillin
TGN	Trans-Golgi network
TIRF	Total internal reflection fluorescence
TMA	Tissue microarray
TNBC	Triple-negative breast cancer
VASP	Vasodilator-stimulated phosphoprotein
VCA	Verprolin homology central acidic
VPS	Vacuolar protein sorting
WASH	Wiscott-Aldrich syndrome protein and SCAR homologue

WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP family verprolin-homologous
WT	Wild-type

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Pietilä M., Sahgal P., Peuhu E., Jäntti N.Z., Paatero I., Närvä E., Al-Akhrass H., Lilja J., Georgiadou M., Andersen O.M., Padzik A., Sihto H., Joensuu H., Blomqvist M., Saarinen I., Boström P.J., Taimen P. and Ivaska J. SORLA regulates endosomal trafficking and oncogenic fitness of HER2. *Nature Communications*, 2019; 10(1): 2340.

II Moreno-Layseca P., Jäntti N.Z., Godbole R., Sommer C., Jacquemet G., Al-Akhrass H., Conway J.R.W., Kronqvist P., Kallionpää R.E., Oliveira-Ferrer L., Cervero P., Linder S., Aepfelbacher M., Zauber H., Rae J., Parton R.G., Disanza A., Scita G., Mayor S., Selbach M., Veltel S. and Ivaska J. Cargo-specific recruitment in clathrin- and dynamin-independent endocytosis. *Nature Cell Biology*, 2021; 23(10): 1073-1084.

III Jäntti N.Z., Moreno-Layseca P., Chastney M.R., Dibus M., Conway J.R.W., Leppänen V-M., Hamidi H., Eylmann K., Oliveira-Ferrer L., Veltel S. and Ivaska J. EPLINα controls integrin recycling from Rab21 endosomes to drive breast cancer cell migration. *Developmental Cell*, 2025; 60(22): 3018-3033.

1 Introduction

The eukaryotic cell is the basic unit of multicellular life, and is composed of a cell membrane, the cytoplasm, and an array of membrane-bound organelles, including the nucleus, the endoplasmic reticulum, the Golgi apparatus, and endosomes and lysosomes. In order to function, the cell orchestrates the concerted functions of all its parts, while supplying each part with their respective constituents. This crucial task is performed by the endo-lysosomal network, a system of specialised membranous organelles which coordinate the transport and sorting of both internalised and newly synthesised molecules between the different membrane-bound compartments of the cell. Endosomal cargo is sorted through maturing and increasingly acidic endosomal compartments, finishing in lysosomes, where the cargo is degraded. Cargo is actively diverted from this route by the action of endosomal retrieval machinery that mediate their recycling. The accurate sorting of cargoes to their correct cellular locations ensures the proper function of the cell, and enables intra- and extracellular communication, cell adhesion and maintenance of homeostasis. The internalisation and recycling of receptors from and to the plasma membrane represent vital mechanisms of endosomal traffic, allowing the cell to communicate with its surroundings through interactions at the cell surface.

Integrins are transmembrane receptors that mediate the connection between cells and their surrounding extracellular environment. By coupling the outer extracellular matrix to the intracellular cytoskeleton, integrins enable cells to sense and adapt to their extracellular environment, mediating signals that facilitate processes such as cell migration, growth and survival. To accomplish dynamic assembly and disassembly of integrin-mediated cell adhesions, integrins are constantly internalised from the cell surface, and recycled back for another round of attachment. During cell migration, the precise regulation of these processes ensures that integrins are trafficked to the correct location in the cell, promoting sustained movement. Dynamic changes in the actin cytoskeleton mediates changes in cell shape and enables cell motility. In addition, local regulation of actin on endosomes is imperative for the endosomal sorting of cargoes. The regulation of actin network dynamics on endosomes allows the reshaping of endomembranes, and facilitates cargo retrieval and vesicle transport.

Given the importance of endosomal traffic, it is perhaps not surprising that the trafficking of receptors plays an important role in a wide range of diseases, such as cancer. Cell migration is dysregulated in cancer, favouring invasion and metastasis, and the endocytosis and recycling of integrins promotes the migration and invasion of cancer cells. The human epidermal growth factor receptor HER2, an established oncogene, is amplified in 15-20% of breast cancers. Residing on the cell surface, it forms homo- and heterodimers with other members of its receptor family upon growth factor or ligand-independent binding, resulting in growth- and survival signalling. However, much is still unknown regarding the trafficking of both HER2 and integrins in cancer. The purpose of this thesis is to present new insights into integrin and HER2 receptor trafficking, and to identify new players that regulate the presence of these receptors on the cell surface of breast cancer cells.

2 Review of the Literature

2.1 The endo-lysosomal network

The endo-lysosomal network consists of an array of specialised membranous organelles which coordinate the transport and sorting of both internalised and newly synthesised molecules between the different membrane-bound compartments of the cell. This tightly regulated transport system enables vital functions such as intracellular and extracellular communication, cell migration and maintenance of cellular homeostasis (Miaczynska, 2013; Wang et al., 2018; Moreno-Layseca et al., 2019). Following receptor internalisation via one of several endocytic mechanisms, the endocytosed cargoes are transported to early endosomes, where their initial fate is assigned (Cullen and Steinberg, 2018; Naslavsky and Caplan, 2018). From here, the cargo can be directed back to the cell surface or to the trans-Golgi network (TGN) to be re-used, or sorted for degradation through increasingly mature and acidic endosomal compartments, culminating in the lysosome (Huotari and Helenius, 2011; Cullen and Steinberg, 2018). The fate of endosome-bound cargo is tightly regulated, with each endosome presenting a unique and dynamic composition of proteins, phosphatidylinositol phosphates and lipids that give them identity, determining their trafficking route (Wandinger-Ness and Zerial, 2014; Elkin, Lakoduk and Schmid, 2016).

2.1.1 Mechanisms of endocytosis

2.1.1.1 Clathrin-mediated endocytosis

Cells employ several different complementing methods of cargo endocytosis (Figure 1). The most thoroughly studied of these routes of internalisation is the clathrin-mediated endocytic (CME) pathway. Notable examples of receptors that are internalised via this route include the transferrin receptor, the low-density lipoprotein receptor, epidermal growth factor receptors, G protein-coupled receptors and integrins (Wolfe and Trejo, 2007; Sigismund et al., 2008; Ezratty et al., 2009; Mayle, Le and Kamei, 2012; Islam, Hlushchenko and Pfisterer, 2022).

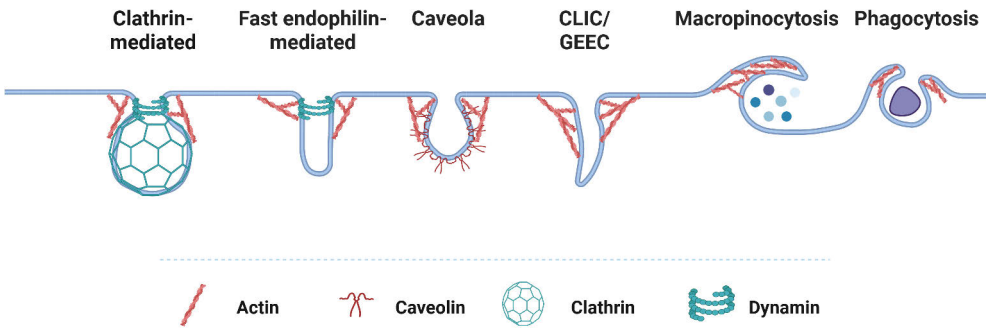

Figure 1. Simplified illustration of the main pathways of endocytosis, which include clathrin-mediated, fast endophilin-mediated, caveolae-mediated and CLIC/GEEC endocytosis, as well as macropinocytosis and phagocytosis. CLIC = Clathrin-independent carrier, GEEC = GPI-anchored protein-enriched early endosomal compartment.

The cargo is initially selected for internalisation based on signal sequences on the cytoplasmic domains of the cargo, recognised by adaptor protein 2 (AP2) complexes that are recruited from the cytosol to plasma membrane regions enriched in phosphatidylinositol 4,5-bisphosphate (PIP_2). It is important to note, that many other adaptor proteins exist in addition to AP2, allowing for more versatility in cargo selection through the pathway. Indeed, multiple different receptors can be selectively internalised simultaneously through one endocytic event (Traub and Bonifacino, 2013). Actin has also been reported to be present already early on during clathrin-coated pit formation, forming a branched network surrounding the site of endocytosis. The formation of the invagination is supported by local actin polymerisation, which increases along with increased membrane tension in the forming pit (see section 2.3.2 for a more detailed description of actin in CME).

The AP2 complexes act as adaptors between the plasma membrane lipids and the lattice of clathrin molecules that give the endocytic pathway its name. AP2 also binds to specific internalisation motifs in the cytoplasmic tails of protein cargoes, selecting them for endocytosis. When AP2 binds to PIP_2 on the plasma membrane, AP2 undergoes a conformational change from a locked to an open state. This facilitates interaction with clathrin, and starts a nucleation event of more and more clathrin and AP2 complexes, resulting in a clathrin lattice made of clathrin trimers (triskelions), AP2 and other adaptors and accessory proteins (Kirchhausen, Owen and Harrison, 2014; Mettlen et al., 2018). These accessory proteins are important for the stability of the growing clathrin lattice, which increases in curvature as it grows. The release of the clathrin-coated vesicle is facilitated by the GTPase dynamin, which constricts and reorganises the neck that connects the budding pit to the plasma membrane, allowing vesicle pinching and release. When membrane tension reaches a certain threshold, for example due to a cell's cytoskeletal attachments, dynamin-

mediated vesicle scission is further supported by local actin polymerisation (Kirchhausen, Owen and Harrison, 2014; Antonny et al., 2016).

Following the internalisation of the clathrin-coated vesicle, it is quickly uncoated. This is facilitated by the protein auxilin, which recruits the heat-shock cognate protein Hsc70 to the clathrin lattice. Powered by cycles of ATP hydrolysis, a clamp-like domain of Hsc70 grabs onto a clathrin triskelion in the lattice. This process repeats itself until the strain produced by the cumulative clamping events of several Hsc70 proteins leads to disassembly of the clathrin lattice, freeing the now uncoated primary vesicle for fusion with an early endosomal compartment *(*Xing et al., 2010; Kirchhausen, Owen and Harrison, 2014). Finally, Hsc70 holds on to the released clathrin molecules, preventing them from aggregating in the cytoplasm (Jiang et al., 2000).

2.1.1.2 Caveolae-mediated endocytosis

Caveolae are distinct, cup-shaped plasma membrane invaginations that, similarly to CME, concentrate cargo destined for endocytosis. Unlike clathrin-coated pits, which are generally found in similar numbers in different cell types, the prevalence of caveolae can vary significantly from one cell type to another. In skeletal muscle tissue, over half of the plasma membrane surface can be occupied by caveolae, whereas caveolae are almost completely absent in the cells of the liver or in proximal tubule cells of the kidney (Parton, 2018). Moreover, unlike clathrin-coated pits, caveolae can form higher-order clusters of several interlocked caveolae (Yeow et al., 2017).

The endocytic capacity of caveolae is considered to be significantly lower compared to clathrin-mediated endocytosis, and only a relatively small fraction of endosomal cargoes originate from caveolar endocytosis. Current evidence suggests that caveolae can internalise glycosylphosphatidylinositol-anchored proteins (GPI-APs) and ordered plasma membrane domains, whereas transmembrane proteins are excluded from caveolae (Parton, 2018). Certain bacteria and viruses have also been shown to induce caveolae-mediated endocytosis as a means to enter the cell (Marjomäki et al., 2002; Xing et al., 2020; Barman and Drolia, 2025). The phenomenon of caveolar mechanosensing and flattening in reaction to increased membrane tension is another emerging function of caveolae, where cells can change their shape and protect themselves from physical stress by using caveolae as buffers against plasma membrane strain (Echarri and Del Pozo, 2015). Moreover, caveolae have been reported to support cancer invasion by promoting invadosome formation (Sotodosos-Alonso and del Pozo, 2024).

The core proteins that are essential for the caveolar structure are the caveolins (caveolin-1 and caveolin-2), cavin1, Eps15 homology domain (EHD) proteins

(primarily EHD2), and the pacsins (pacsin2 and pacsin3). Caveolin-1 and pacsin2 are enriched in non-muscle cells, whereas caveolin-2 and pacsin3 are found in muscle cells (Parton, 2018). Caveolins are small oligomeric cholesterol-binding proteins, and caveolae form at plasma membrane domains enriched in cholesterol, PIP_2 and phosphatidylserines. Caveolin oligomeric complexes are first formed in the endoplasmic reticulum before they are sent to the Golgi apparatus, where they are assembled into larger complexes and stored, awaiting release. The release of caveolin carriers from the Golgi to the plasma membrane is dependent on cholesterol sensing. When cholesterol levels are sufficient, carriers of caveolin oligomers reach the plasma membrane, where they interact with plasma membrane cholesterol and induce membrane bending (Hayer et al., 2010). Cavin1 oligomers bind to both caveolins and PIP_2 and phosphatidylserines at the site of caveolae formation, and give stability to the forming invagination (Hill et al., 2008; Kovtun et al., 2014). While cavin1 gives stability to the bulbous part of caveolae, EHD proteins stabilise the neck region, and a loss of EHD proteins leads to a dysfunctional neck morphology and reduced caveolar clustering (Yeow et al., 2017). Finally, pacsins act as another crucial component, enabling caveolar maturation by providing increased structural stability to the invagination (Hansen, Howard and Nichols, 2011; Senju et al., 2011).

Caveolae-mediated endocytosis is regulated by cell adhesion to the extracellular matrix, as loss of adhesion results in caveolin-1 relocation from the plasma membrane to the perinuclear area (Echarri and Del Pozo, 2015). The actin cytoskeleton, microtubules and intermediate filaments are all associated with caveolae, providing them with further stability and regulating shape changes (Parton, 2018). Finally, the GTPase dynamin, which facilitates fission at the site of clathrin-mediated endocytosis by constricting the neck of the invagination, has been considered to play a similar role during caveolar endocytosis, thus being an important player during the fission process. However, recent studies in HeLa cells and mouse embryonic fibroblasts have challenged this model (Matthaeus et al., 2022; Larsson et al., 2023; Parton, Taraska and Lundmark, 2024). These findings report that, compared to clathrin-coated pits, only a subset of caveolae contain dynamin, where it localises to the bulbous part of the invagination instead of the neck region. Loss of dynamin did not lead to changes in caveolae, and was even found to increase caveolae budding, suggesting that dynamin could have an inhibitory role in caveolae-mediated endocytosis. As HeLa cells have a history of some contradictory results within the field of clathrin-independent endocytosis (Thottacherry et al., 2019), further studies are needed to get the complete picture and to finetune the model of endocytosis through caveolae.

2.1.1.3 The CLIC/GEEC endocytic pathway

In addition to clathrin- or caveolae-mediated endocytosis, cells can internalise cargo through plasma membrane invaginations that lack distinct membrane coats. These alternative, less-characterised routes of endocytosis can be broadly divided into dynamin-dependent and dynamin-independent pathways. Fast Endophilin-mediated endocytosis depends on the scission function of dynamin, and is thus classified as dynamin-dependent. Dynamin also participates in phagocytosis, but here, dynamin is not considered to be required for the scission event. Dynamin-independent pathways include CLIC/GEEC (CG) endocytosis, macropinocytosis, and other, even less understood pathways. While these pathways have not been as extensively studied as CME or caveolae-mediated endocytosis, both of which depend on local actin polymerisation, the clathrin- and dynamin-independent endocytic pathways are also considered to share a reliance on actin dynamics to facilitate cargo uptake (Thottacherry et al., 2019).

The CG pathway is important for several vital cellular processes. It has a high endocytic capacity, and is responsible for large plasma membrane rearrangements (Thottacherry et al., 2019). The CG pathway can remove excess plasma membrane, and can regulate membrane tension. Moreover, the pathway is important for membrane turnover during cell shape changes that occur during cell movement (Sigismund et al., 2021). The CG endocytic pathway is considered to be the primary route of internalisation for the extracellular fluid phase and several GPI-APs (Shafaq-Zadah, Dransart and Johannes, 2020). Other confirmed cargos include dextran, CD44, Thy-1, viral particles and toxins (Howes et al., 2010; Ferreira and Boucrot, 2018; Thottacherry et al., 2019; Shafaq-Zadah, Dransart and Johannes, 2020). The pathway is characterised by GPI-AP-containing tubular invaginations, called clathrin-independent carriers (CLICs), which often form at the leading edge of migrating cells (Howes et al., 2010). These CLICs fuse together and mature into GPI-AP-enriched early endosomal compartments (GEECs).

In the current, still incomplete model for CG endocytosis (Sathe et al., 2018), recruitment of the CG machinery begins with accumulation of the small GTPase ADP-ribosylation factor 1 (Arf1) and its guanine-nucleotide exchange factor (GEF) Golgi-specific brefeldin A resistance factor 1 (GBF1). This is accompanied by recruitment of the actin-related protein 2/3 (Arp2/3) actin nucleation complex, which is kept inactive by the Arp2/3 inhibitor and BAR domain-containing protein interacting with C kinase 1 (PICK1). Another bin/amphiphysin/rvs (BAR) domain-containing protein, insulin-responsive protein of mass 53 kDa (IRSp53), is also recruited to the site. IRSp53 binds to negatively curved membranes with its inverse BAR domain, and its inherent curved shape can induce membrane tension and invagination (Prévost et al., 2015).

In the second phase of the model, Arf1 is activated, and acts as an inhibitor of PICK1, preventing PICK1 from inhibiting the Arp2/3 complex. At the same time, IRSp53 is activated by cell division control protein 42 (CDC42), enabling IRSp53 to recruit activators of the Arp2/3 complex. The net result is activation of Arp2/3-mediated actin polymerisation (Sathe et al., 2018). The regulation of Arp2/3-mediated actin polymerisation presented in this model could accomplish two things: Initially, repression of Arp2/3 activity could enable the early stages of membrane invagination, whereas Arp2/3 activation and the subsequent actin polymerisation could act to constrict the neck of the invagination, leading to scission and release of the vesicle (Thottacherry et al., 2019). Importantly, the above-mentioned model does not describe all known players in CG endocytosis. For example, CG endocytosis has been reported to require the activity of the small GTPase GRAF1 and the Rho-GTPase activating protein ARHGAP21, a deactivator of CDC42 (Kumari and Mayor, 2008; Lundmark et al., 2008). Moreover, CG endocytosis is dependent on the presence of cholesterol-rich plasma membrane domains (Chadda et al., 2007).

Following scission, CLICs fuse together to form GEECs. These early endosomal compartments are more acidic than the classical sorting endosome, and have the ability to rapidly recycle their cargo back to the plasma membrane before fusion with the classical sorting endosome. Presumably, this higher acidity could facilitate the release of internalised receptor-bound ligands. Finally, GEECs can associate with the small GTPase Rab5, and both Rab5 and its effector phosphoinositide 3-kinase (PI3K) mediate the fusion of GEECs with the classical sorting endosome (Kalia et al., 2006).

2.1.2 Cargo recycling

There are an estimated 5500 integral membrane proteins encoded by the human genome (Uhlén et al., 2015). Together, these proteins enable crucial functions such as cell signalling, cell adhesion, nutrient sensing, cell polarity and migration, through interactions at the cell surface (Cullen and Steinberg, 2018). In order for a cell to function normally, it needs to maintain an optimal amount of membrane proteins on its surface. It achieves this by upholding a balance between the secretory pathway and the uptake and sorting of cell surface proteins (Figure 2). For this sorting to work as intended, cells depend on an array of sorting machinery that decide the fate of endosomal cargo. Disturbances in this balance are linked to many diseases, such as neurodegeneration and cancer (O'Sullivan and Lindsay, 2020). Historically, cargo recycling back to the plasma membrane has been regarded as a default, constitutive route for internalised cargo, while the degradation of cargo has been seen as an active and selective fate decision. This view has since then changed, and we now know that cells can recycle cargo in a selective manner.

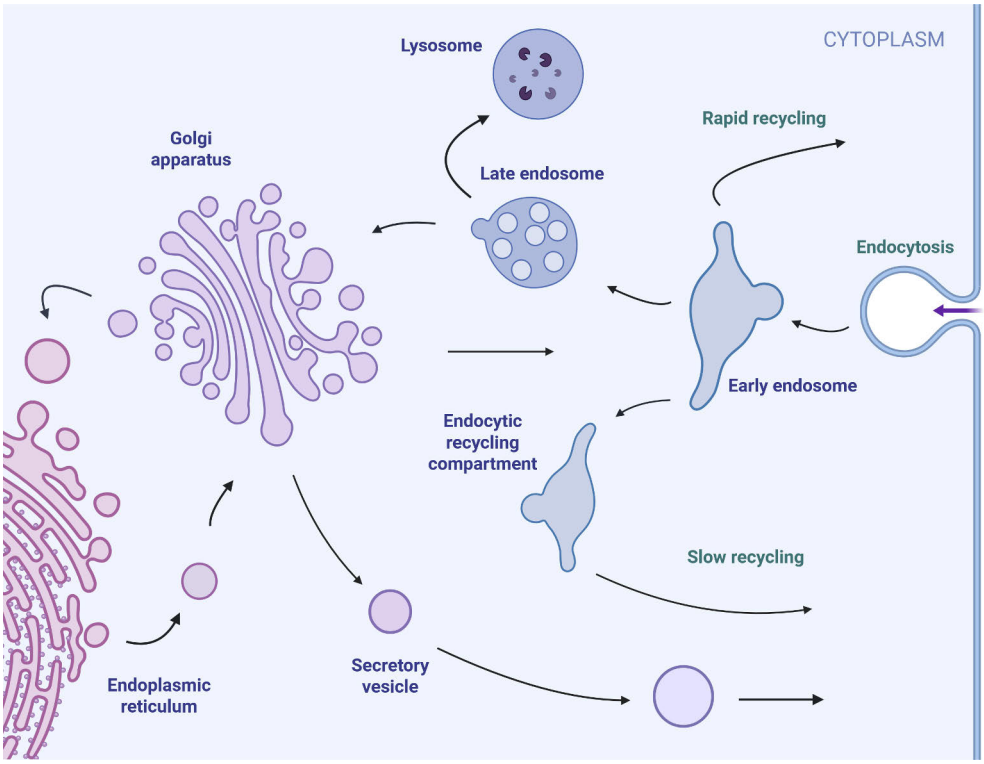

Figure 2. Simplified overview of the endo-lysosomal sorting routes. Following endocytosis at the plasma membrane, the internalised cargo can be actively retrieved for recycling back to the plasma membrane, or directed to the Golgi (trans-Golgi network), from where it can be dispatched to different parts of the cell. Alternatively, cargo can be targeted for degradation in the lysosome.

Proteins destined for degradation are marked by ubiquitylation, a reversible protein modification (Clague, Liu and Urbé, 2012). This mark is recognised by members of the endosomal sorting complexes required for transport (ESCRT) family, which collaborate to cluster ubiquitylated cargo into subdomains on the early endosome. Indeed, the partitioning of cargo destined for degradation and cargo destined for recycling into separate domains on the early endosome is key for successful sorting. The ESCRT machinery orchestrates the formation of intraluminal vesicles (ILVs) that contain the cargo destined for degradation. This ESCRT-mediated mechanism of ILV formation is not the only way that cargo destined for degradation can be incorporated into ILVs, but it is the most thoroughly characterised (Cullen and Steinberg, 2018). Several rounds of ILV formation occur during the maturation of early endosomes into late endosomes, and the abundance of ILVs in late endosomes has inspired the often-used name multivesicular bodies.

While cargo destined for degradation is concentrated into ILVs, cargo destined for recycling is sequestered into elongated tubular structures on the early endosome. This retrieval of cargo for recycling is made possible by an array of retrieval machinery that recognise specific sorting motifs on the cytosolic regions of the protein cargo and facilitate changes in endosomal membrane shape. The main complexes responsible for the retrieval of cargo are the retromer complex, the retriever complex, the COMMD–CCDC22–CCDC93 (CCC) complex and the Wiscott-Aldrich syndrome protein and SCAR homologue (WASH) complex (Gomez and Billadeau, 2009; Burd and Cullen, 2014; McNally et al., 2017; Boesch et al., 2024). Together with the sorting nexins SNX27 and SNX17, these components form two main pathways for cargo retrieval: The SNX27-retromer-WASH pathway and the SNX17-retriever-CCC-WASH pathway (Cullen and Steinberg, 2018).

Retromer is a heterotrimer consisting of the vacuolar protein sorting family proteins VPS35, VPS29 and VPS26 (VPS26A or VPS26B) (Seaman, 2021). In addition to this core trimer, a second, heterodimeric unit of SNX-proteins (SNX1 or SNX2 with SNX5 or SNX6) has been historically regarded as part of retromer. However, because the VPS trimer and the SNX dimer have been shown to be able to act separately, the name retromer has evolved to refer to just the core VPS trimer (Harterink et al., 2011; Kvainickas et al., 2017; Simonetti et al., 2017, 2019; Seaman, 2021; Solinger, Rashid and Spang, 2022). Retromer is recruited to early endosomes via SNX3-mediated binding of phosphatidylinositol 3-phosphate (PtdIns(3)P) in the endosomal membrane (Leneva et al., 2021). On late endosomes, binding of retromer is mediated by Rab7 activity (Rojas et al., 2008). Another SNX protein, SNX27, acts as an adaptor protein and cooperates with retromer to select endosomal cargo for retrieval (Temkin et al., 2011; Steinberg et al., 2013). Because of its ability to associate with both early and late endosomes, retromer can retrieve cargo from a degradative fate even at later stages of endosome maturation. Cargo retrieved by retromer has been shown to recycle either directly to the plasma membrane, or to the TGN. It is evident that retromer plays an important role in cargo retrieval, as loss of retromer tends to change the fate of retromer-associated cargo from recycling to lysosomal degradation (Cullen and Steinberg, 2018). Examples of cargo retrieved by retromer include the glucose transporter GLUT1, the transferrin receptor and the sorting receptor SORLA (Fjorback et al., 2012; Chen et al., 2013; Steinberg et al., 2013).

The retriever complex is a heterotrimer, which shares one component, VPS29, with the retromer complex. The other two components are VPS26C and VPS35L. Cargo retrieval by the retriever complex is mediated by SNX17, which associates with the early endosome through interactions with cargo and PtdIns(3)P (McNally et al., 2017; Cullen and Steinberg, 2018). To perform its retrieval function, retriever collaborates with the CCC complex, forming a larger regulatory complex called commander (Laulumaa et al., 2024). Examples of cargoes that are recycled by this

machinery include integrins, amyloid precursor protein (APP), and the low-density lipoprotein receptor-related protein 1 (LRP1) (van Kerkhof et al., 2005; Lee et al., 2008; McNally et al., 2017).

The WASH complex is a unifying factor in both above-mentioned retrieval pathways. It is a heteropentameric complex that promotes Arp2/3-mediated polymerisation of branched actin filaments (Gomez and Billadeau, 2009). In cancer, WASH-mediated regulation of endosomal trafficking is known to facilitate cancer cell migration and invasion (Zech et al., 2011; MacDonald et al., 2018). The formation of branched actin networks reshapes the endosomal membrane and enables the partitioning of cargo into subdomains together with the retromer and retriever complexes. WASH associates with phospholipids in the endosomal membrane, as well as with both the retromer and commander complexes, and is required for their proper localisation and function (Phillips-Krawczak et al., 2015; McNally et al., 2017; Cullen and Steinberg, 2018). During retriever-mediated cargo retrieval, the CCC complex controls WASH recruitment by regulating PtdIns(3)P levels through the action of the lipid phosphatase myotubularin-related protein-2 (MTMR2) (Singla et al., 2019). Interestingly, WASH can bind to the ESCRT-0 member of the ESCRT family on endosomes. Through this binding, WASH competes with ESCRT-0, promoting cargo retrieval instead of degradation. This mechanism seems to be dependent on the actin-binding function of the cargo (MacDonald et al., 2018).

WASH-driven branched actin polymerisation promotes the formation of membrane tubules, which are shaped and stabilised further by BAR domain-containing proteins. Once the tubule containing retrieved cargo is formed, it can be cut off from the sorting endosome through membrane scission, catalysed by EHD1 or dynamin (Cullen and Steinberg, 2018; Naslavsky and Caplan, 2023). From here, the cargo can be recycled directly back to the plasma membrane, or it can take a slower route and fuse with the endosomal recycling compartment. Alternatively, it can be directed to the TGN, which dispatches the cargo to various cellular destinations (O'Sullivan and Lindsay, 2020). Each route is governed by Rab GTPases (covered in section 2.2.4) and their effectors, which give endosomes their diverse identities, recruit specialised sorting machinery, and ensure the correct sorting of cargo (Wandinger-Ness and Zerial, 2014).

2.2 Integrin adhesion complexes

The attachment of cells to their surroundings is a prerequisite for multicellular life, and is principally mediated by a group of heterodimeric transmembrane adhesion receptors called integrins. Together with an array of associated proteins, integrins form integrin adhesion complexes (IACs), which act as links between the outer extracellular matrix and the cytoskeleton. This allows the cell to sense and adapt to

its surroundings by activation of signalling pathways that regulate processes such as cell migration, growth and survival (Chastney, Conway and Ivaska, 2021). In diseases such as cancer, dysregulation of these integrin-mediated processes can influence the properties of cancer cells, causing them to become more aggressive and treatment-resistant (Chastney et al., 2025). The composition of IACs can vary depending on the specific pairing of integrin subunits that make up the receptor and on both the bound extracellular matrix ligand (such as fibronectins, collagens or laminins) and intracellular signalling events (Humphries et al., 2019). Integrin activation and inactivation is tightly regulated to ensure proper signalling, and the endocytosis and recycling of integrins plays an important role in regulating adhesion assembly and disassembly. Migrating cells rely on the dynamic assembly and disassembly of IACs, and the intracellular trafficking of integrins is tightly regulated to ensure that integrins are dispatched to the correct cellular locations to facilitate further movement (Moreno-Layseca et al., 2019).

2.2.1 Integrin structure and ligand specificity

There are 24 different known heterodimeric integrin receptors in humans, created from a combination of 18 larger α-subunits and 8 smaller β-subunits (Hynes, 2002). The many combinations of integrin αβ-pairs enable integrins to recognise a broad range of different ECM ligands. Integrins can be divided into four subfamilies based on their ligand specificity – collagen-binding integrins, laminin-binding integrins, leukocyte-specific integrins and RGD (arginine-glycine-aspartic acid) sequence-binding integrins. The most common integrin subunit is β1-integrin, which forms heterodimers with 12 different integrin α-subunits. The αβ1 heterodimers represent the main ECM-binding integrins, and it is the specific α-subunit in the αβ1 pair that determines ligand specificity. While some overlap in ligand binding exists between the different integrin subfamilies, most integrins are considered to serve important individual roles (Chastney, Conway and Ivaska, 2021).

Each integrin subunit consists of a large multi-domain extracellular part, a single transmembrane helix, and a cytoplasmic tail. The large extracellular domain engages the ECM, while the cytoplasmic tail contains both shared and subunit-specific sequences that recruit proteins that form the IAC and enable integrin heterodimer-specific signalling. The initial pairing of integrin heterodimers occurs in the endoplasmic reticulum (ER), from where integrins are transported to the Golgi apparatus for post-translational modification prior to being dispatched (Tiwari et al., 2011). Alternatively, some integrins can be trafficked to the cell surface directly from the ER, circumventing the Golgi (Lerche et al., 2025). Integrins travel to the plasma membrane in an inactive, calcium (Ca^{2+})-bound state, and upon arrival to the cell surface, can swap their Ca^{2+} to Mg^{2+} or Mn^{2+} to allow integrin activation (Tiwari et al., 2011).

2.2.2 Formation and regulation of IACs

IAC formation is regulated on several different levels. This includes ligand availability, receptor clustering, the regulation of integrin activity by integrin activators and inactivators, growth factor signalling, regulation of the cytoskeleton and mechanical forces, as well as transcriptional control of gene expression (Chastney, Conway and Ivaska, 2021). Integrin-mediated cell adhesion begins with integrin receptor activation, which is closely tied to the conformation of the receptor. Inactive integrins exist in a bent, closed conformation, with the head domain folded back against the integrin structure, which prevents ECM ligands from interacting with the integrin head domain. While in this conformation, the cytoplasmic tails of the integrin subunits are in close proximity to each other. Activation of the integrin receptor by ligand binding or by binding of intracellular adaptors induces a conformational change to an intermediate, extended closed state, where the integrin head unbends and swings out. The sequence of conformational changes results in an active state with high ligand-affinity, where the cytoplasmic tails of the subunits have moved farther apart (Figure 3). A defining feature of integrins is their ability to transmit signals bidirectionally, both from the outside to the inside of the cell ("outside-in signalling") and from the inside of the cell to the outside ("inside-out signalling") (Campbell and Humphries, 2011; Kolasangiani, Bidone and Schwartz, 2022).

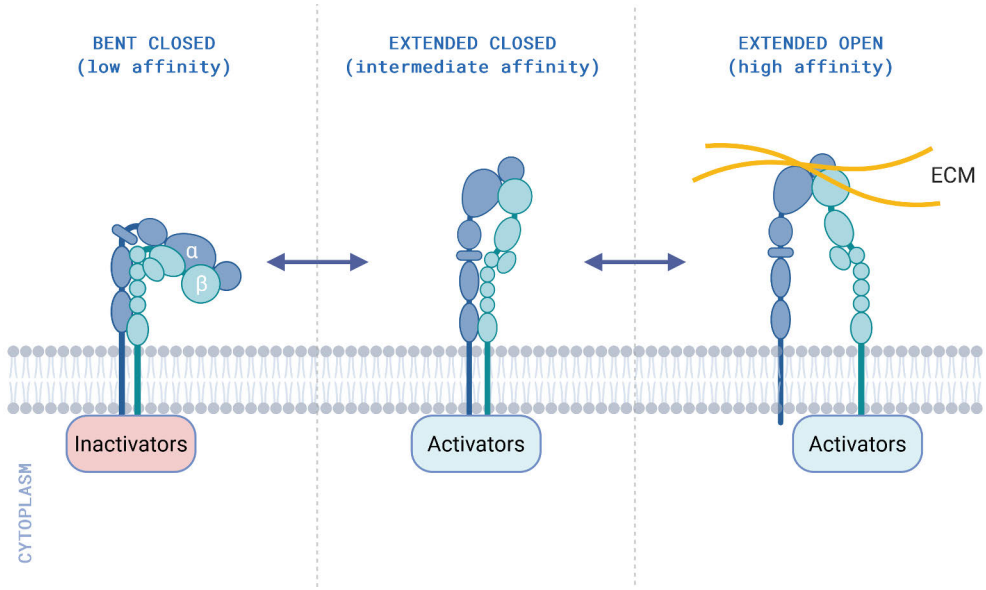

Figure 3. The sequence of conformational changes during integrin activation. Integrins are inactive in their bent closed state, and are kept inactive by intracellular integrin inactivators. Binding of integrin activators promotes the bent headpiece to swing out (extended closed), and the subsequent separation of the integrin legs and cytoplasmic tails (extended open), promotes ligand binding.

Integrins are kept in their inactive state by inactivating proteins that bind to the integrin tails and hinder the binding of intracellular integrin activators. Inactivators that bind to the α-tails include SHARPIN, LRP12 and MDGI (Nevo et al., 2010; Rantala et al., 2011; Huang et al., 2023), and inactivators that bind to the β-tails include filamin A, integrin cytoplasmic domain-associated protein 1 (ICAP1) and docking protein 1 (DOK1) (Bouvard et al., 2003; Kiema et al., 2006; Wegener et al., 2007). Intracellular "inside-out" integrin activation is driven by the scaffold protein talin, which binds to integrin β-tails, facilitating the separation of the tails of the αβ heterodimer, and by doing so, promoting the open extended integrin conformation with high ligand affinity. Talin also binds to actin filaments, acting as a link between integrins and the actin cytoskeleton (Wegener et al., 2007). The resulting link that extends from the ECM ligand all the way to the intracellular cytoskeleton enables the cell to exert forces on the ECM. A complex consisting of a talin dimer and the adaptor proteins paxillin and kindlin acts to reinforce the link between the ECM and the cytoskeleton, facilitating the clustering of integrin receptors and IAC maturation (Lu et al., 2022). These so-called nascent adhesions are the first IACs that a cell forms at its lamellipodia during cell migration, and contain an estimated 50 integrin receptors (Changede et al., 2015). Integrin activation is also accompanied by the activation of intracellular signalling pathways, such as signalling by the kinases FAK and Src, which associate with IACs, phosphorylating other IAC components and promoting cell migration, survival and proliferation via downstream signalling pathways. Indeed, the phosphorylation of IAC components is key for regulating adhesion lifetime and function (Chastney, Conway and Ivaska, 2021; Conway et al., 2025).

While the lifetime of an average nascent adhesion is relatively short, the clustering of integrins and the reinforcement of the link between the ECM and the actomyosin machinery can lead to nascent adhesion maturation into a focal adhesion (FA). This maturation is dependent on tension, and occurs through changes in protein composition and an increase in IAC size. Examples of proteins that reinforce the ECM-actin cytoskeleton link during adhesion maturation include the actin-binding proteins vinculin, VASP and α-actinin (Chastney, Conway and Ivaska, 2021). FAs are crucial signalling hubs that enable the cell to sense and react to changes in its environment, and to create traction forces that allow movement. The compositional complexity of IACs and their ability to undergo dynamic changes is reflected in the vast number of components that have been linked to the integrin adhesome, encompassing over 2400 proteins. These have been narrowed down to define the consensus adhesome, comprising 60 proteins (Horton et al., 2015).

While different classes of IACs, including FAs, are highly dynamic with a diverse set of associated proteins, the core nanoscale architecture of FAs has been elucidated using modern three-dimensional imaging methods, complementing the proteomics studies and revealing the vertical distribution of multiple, partially

overlapping layers with specific functions (Kanchanawong et al., 2010). These layers, spanning the space between plasma membrane integrins and actin stress fibres, are the integrin signalling layer, the force-transduction layer and the actin regulatory layer. The integrin signalling layer occupies the space closest to the integrin cytoplasmic tails, and is characterised by the presence of FAK, paxillin and integrin-linked kinase (ILK). This is followed by the force-transduction layer, which contains talin and vinculin. The third and uppermost layer closest to the actin stress fibres is the actin regulatory layer, and is characterised by the presence of actin regulators such as VASP, α-actinin and zyxin. While talin is categorised as a component of the force-transduction layer, it is in fact oriented vertically in FAs, and is relatively long (roughly 100 nm), oriented from N-terminal head to C-terminal tail and spanning all the three layers of FAs from the signalling layer to the FA/stress fibre interface. Talin has several binding sites for both actin, other adaptor proteins and self-dimerisation, and is generally considered to be the backbone in FAs. These binding sites become increasingly more available as tension increases, providing a regulatory mechanism for FA maturation (Liu et al., 2015).

2.2.3 IAC function

The principal function of IACs is to mediate the cell's attachment to its surroundings, be it the ECM or a neighbouring cell. Tight regulation of cell adhesion is critical for homeostasis, and cell detachment from its ECM leads to a type of programmed cell death called anoikis that is responsible for eliminating detached and misplaced cells (Gilmore, 2005). The ability to circumvent anoikis and grow independent of an anchor, is a hallmark of cancer (Shaw et al., 2025). Proper cell-ECM and cell-cell attachment gives structure to tissues and enables multicellular life. In addition, cells use IACs to probe and regulate the ECM through deposition, removal or reorganisation of the matrix (Chastney, Conway and Ivaska, 2021). A second, major function of IACs is their ability to facilitate movement. This is a vital attribute in healthy tissues, as it enables tissue repair, immune responses and embryonic development, but is also a defining characteristic of metastatic cancer cells (Chastney et al., 2025).

Migrating cells probe the ECM using both sheet-like and finger-like actin-rich protrusions called lamellipodia and filopodia, respectively (Jacquemet, Hamidi and Ivaska, 2015; Paul, Jacquemet and Caswell, 2015). Integrins at the leading edge of these structures bind to the ECM and form adhesions, which mature into stable anchorage points for the cell. The cell uses actomyosin contractility to create forward movement, while simultaneously disassembling adhesions at the back of the cell. Indeed, the cell's ability to polarise, forming a front and a rear, is key for mesenchymal cell migration (SenGupta, Parent and Bear, 2021). The regulation of

integrin trafficking is an important mechanism that cells use to maintain dynamic actin protrusions, and integrin endocytosis and recycling is crucial for adhesion turnover during cell migration (covered further in 2.2.5). Focal adhesions mediate stable connections between the ECM and the cell, and enable mechanosensing by transforming extracellular physical cues into intracellular biochemical signals. This enables directed cell migration, as cells use mechanosensing to recognise gradients in the cell's surroundings, such as ECM ligand density and substrate rigidity (SenGupta, Parent and Bear, 2021). By sensing outside cues, integrins can also regulate cell survival and proliferation, by activating downstream pathways such as MAPK/ERK and PI3K/AKT signalling (Yee, Weaver and Hammer, 2008; Zhang et al., 2022). In cancer, this signalling can be hijacked to promote cancer malignancy.

2.2.4 Integrin trafficking

The composition and abundance of integrins at the cell surface governs the cell's capacity to react to cues in the ECM, and the transport of integrins to and from the plasma membrane is crucial for controlling integrin availability. The trafficking of integrins regulates cell behaviour by maintaining a continuous cycle of integrin endocytosis and recycling, enabling cells to quickly adapt to changes in their environment. By increasing or decreasing the transport of specific integrins to the plasma membranes, cells can promote or reduce their affinity for different ECM ligands. During cell migration, cells finetune the balance between integrin uptake and recycling to regulate FA turnover, enabling forward movement (Moreno-Layseca et al., 2019).

Integrins are internalised through several mechanisms. CME of integrins is mediated by adaptors at the clathrin-coated pit. The core clathrin adaptor AP2 recognises a Yxxϕ (where ϕ is a bulky hydrophobic residue L/I/M/V/F) amino acid sequence found in the cytoplasmic tails of integrin α-subunits, enabling the selective uptake of these receptors (De Franceschi et al., 2016). The clathrin adaptors Numb and Dab2, on the other hand, recruit integrins for CME by recognising the NxxY tail motif in integrin β-subunits (Nishimura and Kaibuchi, 2007; Ezratty et al., 2009; Eskova et al., 2014). During directional cell migration, Numb facilitates integrin endocytosis at the leading edge in a phosphorylation-dependent manner by binding to integrin β-subunits (Nishimura and Kaibuchi, 2007). The integrin inactivator ICAP1 drives integrin endocytosis by controlling the association of NME/NM23 Nucleoside Diphosphate Kinase 2 (NME2) to clathrin-coated pits (Kyumurkov et al., 2022). Moreover, microtubules, which act as tracks for cargo trafficking, have been shown to regulate CME of integrins, where the microtubule motor protein KIF15 recruits the clathrin adaptor Dab2 to promote FA disassembly during cell migration (Ezratty et al., 2009; Eskova et al., 2014).

Integrin uptake through CLICs has been reported to require Galectin-3 for CLIC biogenesis (Lakshminarayan et al., 2014). Galectin-3 binds to glycans attached to the extracellular side of integrins, and has been shown to lock α5β1 integrins in their inactive conformation, clustering them for endocytosis (Furtak, Hatcher and Ochieng, 2001; Yang et al., 2017; Shafaq-Zadah et al., 2025). Moreover, the rho GTPase-activating protein GTPase regulator associated with focal adhesion kinase-1 (GRAF1) has been implicated in CLIC formation, promoting membrane remodelling at the leading edge of migrating cells (Lundmark et al., 2008; Doherty et al., 2011). The small GTPase Rab21 was the first Rab protein identified to directly bind integrins, regulating β1-integrin uptake through a mechanism that does not affect transferrin internalisation, suggesting a clathrin-independent route (Pellinen et al., 2006). Multiple studies have also linked integrins with caveolar endocytosis, which promotes fibronectin turnover and wound healing (Shi and Sottile, 2008; Bass et al., 2011). Another endocytic mechanism, macropinocytosis, has been implicated in integrin internalisation during growth factor-induced cell migration (Gu et al., 2011).

The recycling of integrin is a selective process, incorporating different recycling machineries and endosomal trafficking routes. The Rab family of small GTPases and their effectors regulate all steps of endosomal transport, and the different routes of integrin traffic can be characterised by the identity of the specific Rab protein involved. Rabs perform their functions by acting as molecular switches that can be turned on and off. This is achieved by Rab GEFs (guanine-nucleotide exchange factors) and GAPs (GTPase-activating proteins), which catalyse the cycling between the Rab proteins active (GTP-bound) and inactive (GDP-bound) forms, respectively. The activation of Rabs enables them to recruit assemblies of adaptor proteins to the endosomes, specific to each individual Rab, thus directing cargo flow through the endosomal pathways (Wandinger-Ness and Zerial, 2014).

Integrins are relatively long-lived, and are primarily recycled back to the plasma membrane for reuse, instead of degradation (Paul, Jacquemet and Caswell, 2015). The general consensus seems to be that the recycling of integrins throughout endosomal maturation is an active, selective process, as loss-of-function experiments have shown that integrins are diverted towards lysosomal degradation when retrieval is blocked (Böttcher et al., 2012; Steinberg et al., 2012; McNally et al., 2017). An important driving machinery behind integrin retrieval is the cargo adaptor SNX17 (which binds to the NPxY/NxxY motif present in β-integrin cytoplasmic tails), the retriever complex, and WASH complex-powered actin polymerisation (covered in section 2.1.2). Integrins are collected in Rab5-positive early endosomes after endocytosis. From here, integrins can be retrieved for recycling either via a Rab4-mediated rapid recycling route (short loop) to the plasma membrane, or a slower Rab11-mediated recycling route (long loop) through the perinuclear endosomal recycling compartment, before being dispatched back to the cell surface (Moreno-Layseca et al., 2019).

Integrins are selectively shuttled to the perinuclear endosomal recycling compartment through the action of VPS3 and VPS8, members of the CORVET endosomal tethering complex (Jonker et al., 2018). Alternatively, a Rab13-mediated, GGA2-dependent pathway can direct active β1-integrins back to the cell surface, an example of integrin activity-dependent traffic (Sahgal et al., 2019). Integrins can be retrieved and recycled from both early and late endosomes. Integrins are retrieved from late endosomal/lysosomal compartments and recycled directly back to the plasma membrane in a Rab25- and CLIC3-dependent manner (Dozynkiewicz et al., 2012). In addition, integrins can be directed to the TGN via retrograde transport, from where they are dispatched to the plasma membrane (Shafaq-Zadah et al., 2016). Integrins which are not retrieved, are transported with the maturing endosome, finally ending up in lysosomes for degradation (Lobert et al., 2010; Böttcher et al., 2012; Steinberg et al., 2012; Shafaq-Zadah et al., 2016; McNally et al., 2017).

Whether an integrin receptor is internalised in its active or its inactive state affects its intracellular transport. Inactive integrins are maintained on the plasma membrane and recycled more quickly compared to endocytosed active integrins, which generally take a longer recycling route, as the increased acidity in late endosomes promotes ligand dissociation (De Franceschi et al., 2015). Internalised ligand-unbound integrins can be kept in their active state by association with talin and FAK on endosomes, enabling directed recycling to assemble FA in a polarised manner for continued migration (Nader, Ezratty and Gundersen, 2016). FAK also mediates endosomal signalling from ligand-bound active integrin-containing endosomes, promoting cell survival through anoikis-resistance (Alanko et al., 2015). The binding of ECM ligands is another factor that influences integrin traffic, and active integrins regulate ECM turnover by co-trafficking with matrix metalloprotease-cleaved ECM-ligand fragments (Lobert et al., 2010; Shi and Sottile, 2011; Dozynkiewicz et al., 2012). In chondrocytes, endocytosed fibronectin-bound active integrins signal from endosomes to stimulate the production of MMP-13 for increased ECM remodelling (Miao et al., 2023).

2.2.5 Integrin trafficking during cancer cell migration

Mesenchymal cell migration can be considered the classical mode of cell motility, and is the main mode of cell migration on 2D substrates, but is also used by many cell types to migrate in a 3D matrix environment. This includes stromal fibroblasts and several types of cancer cells, such as breast adenocarcinoma cells (Caballero et al., 2017; Wang et al., 2019; Doyle et al., 2021; Duggan and Petrie, 2025). Cells that move using this mode of migration are characterised by an elongated, polarised shape, with a front (“leading edge”) and a rear. Cells grab onto ECM components at the leading edge, mediate cytoskeletal tension via their adhesions, and contract their

rear to facilitate forward movement (Figure 4). Cells form actin-rich structures at the leading edge, including lamellipodia and filopodia, which they use to probe their environment for migratory cues. These structures contain integrins, and the integrin-mediated sensing of ECM signals is a key mechanism for giving directionality to cell motility, through the formation of cell polarity parallel to the axis of movement (Jacquemet, Hamidi and Ivaska, 2015; Paul, Jacquemet and Caswell, 2015). To enable continuous forward movement, cells need to finetune their traction to their substrate by carefully regulating both their adhesion attachment and detachment. Membrane trafficking of integrins plays an important role in these processes, with continuous cycles of targeted integrin endocytosis and recycling controlling cell motility (Moreno-Layseca et al., 2019).

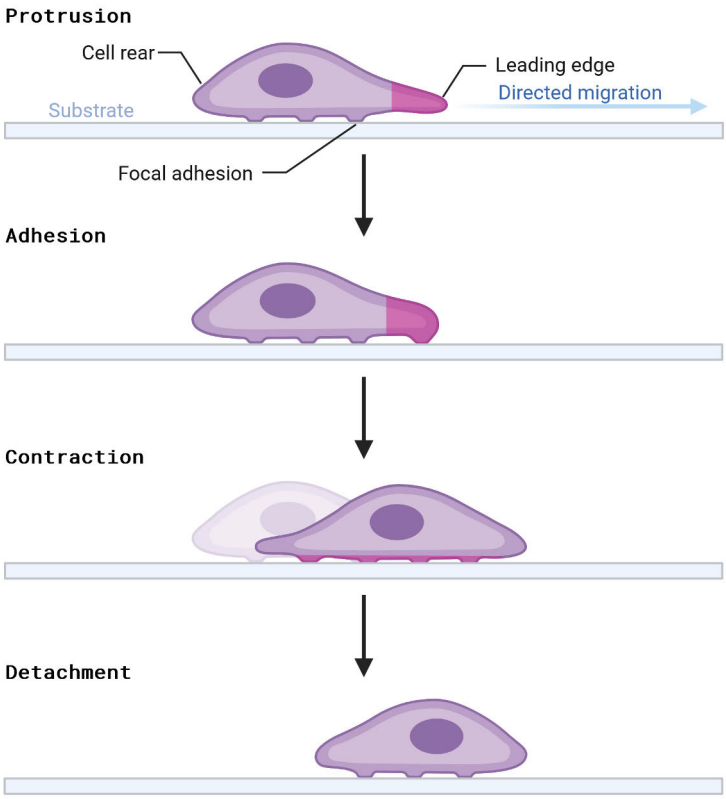

Figure 4. The steps of directed mesenchymal cell migration. Actin-rich protrusions at the leading edge probe the extracellular environment, and integrins at the leading edge bind to the substrate (extracellular matrix, ECM), forming nascent adhesions, which can mature into focal adhesions that couple the ECM to the actin cytoskeleton. This allows the actomyosin-dependent generation of traction forces, which pull the cell forward, while actomyosin-based contraction at the back of the cell retracts the rear upon focal adhesion detachment.

Several proteins have been implicated in the regulation of integrin endocytosis and focal adhesion disassembly during cell migration. The clathrin adaptors Numb and Dab2 interact with the NxxY tail motif on integrin β-subunits, and localise to FAs together with clathrin and dynamin to regulate clathrin-mediated integrin endocytosis and FA turnover (Nishimura and Kaibuchi, 2007; Ezratty et al., 2009; Eskova et al., 2014). In this context, FAK regulates integrin endocytosis by recruiting dynamin to FAs. Numb facilitates integrin endocytosis at the leading edge in a phosphorylation-dependent manner by binding to integrin β-subunits (Nishimura and Kaibuchi, 2007). Dab2 promotes internalisation of inactive β1-integrins at the dorsal side of migrating cells, enabling their translocation to the ventral side of the cell. The microtubule motor protein KIF15 recruits Dab2 to large FAs located in the middle regions of the cell, promoting endocytosis of active β1-integrins and FA disassembly during cell migration (Teckchandani et al., 2009). In the context of FA disassembly, the recruitment of Dab2 for integrin endocytosis can be dependent on traction force, as loss of tension between actomyosin, integrins and their ECM ligand stimulates endocytosis of β3-integrins (Yu et al., 2015). This illustrates how cells can signal to internalise integrins upon adhesion disassembly, facilitating movement. FMNL2 is another identified regulator of β1-integrin uptake and cancer cell invasion, mediating integrin endocytosis upon phosphorylation by PKCα (Wang et al., 2015). In oral squamous cell carcinoma cells, the HS1-associated protein X-1 (HAX-1) binds to the tail of β6 integrins and facilitates migration and invasion through clathrin-mediated uptake of αvβ6 integrins (Ramsay et al., 2007). The activity of both Arf and Rab family of small GTPases and their effectors adds another layer of control, regulating the endocytosis of integrins and FA turnover during cancer cell migration. For example, both Arf6 and Arf5, through regulation by their effectors, regulate β1-integrin internalisation (Dunphy et al., 2006; Moravec et al., 2012), and the early endosomal marker Rab5 associates with vinculin and paxillin at FAs, promoting FAK dephosphorylation and FA disassembly (Mendoza et al., 2013). Rab5-mediated integrin endocytosis is inhibited by APPL1 (the adaptor protein containing a pleckstrin-homology (PH) domain, phosphotyrosine binding (PTB) domain and leucine zipper motif 1), resulting in downregulation of Rac activity and decreased motility (Diggins et al., 2018). Moreover, tensins, components of fibronectin-remodelling fibrillar adhesion, regulate active α5β1 integrin endocytosis via an Arf4-dependent pathway, targeting the integrins and their ECM ligands to late endosomes/lysosomes (Rainero et al., 2015).

As mentioned earlier, integrins can be recycled via different routes after internalisation (Figure 5), the main ones being the Rab4-mediated “short loop” and the Rab11-mediated “long loop”. SNX17 is a key player in retrieving integrins for recycling by binding to the NxxY motif present in β-integrin cytoplasmic tails, coupling with the retriever, CCC and WASH complexes to direct integrins for

recycling (McNally et al., 2017). During migration, cells can utilise the Rab4- and Rab11-loops to finetune integrin activity at the leading edge, and the activity of these two recycling pathways can be regulated by specific signalling events. The short Rab4-loop is stimulated by growth factors and serum (Paul, Jacquemet and Caswell, 2015), and entry into this route is governed by the phosphorylation status of the Rab5 effector Rabaptin-5 in a PKD1-dependent manner, with Rabaptin-5 acting as a link between Rab5 and Rab4 (Christoforides et al., 2012). Entry into the long Rab11-loop, on the other hand, can be controlled by Rab-coupling protein (RCP), which has been shown to shuttle α5β1 integrins together with the epidermal growth factor receptor 1 (EGFR1) (Caswell et al., 2008). As an example of differential usage of the two recycling routes, cells can regulate their motility by differential recycling of the RGD-binding receptors integrin αvβ3 and α5β1. Integrin αvβ3 recycling via the short Rab4-loop stimulates Rac-activity and slow, directional migration, whereas recycling of α5β1 via the long Rab11-loop favours RhoA-activity and rapid, random migration (Danen et al., 2005). The same integrin receptor can also be differentially

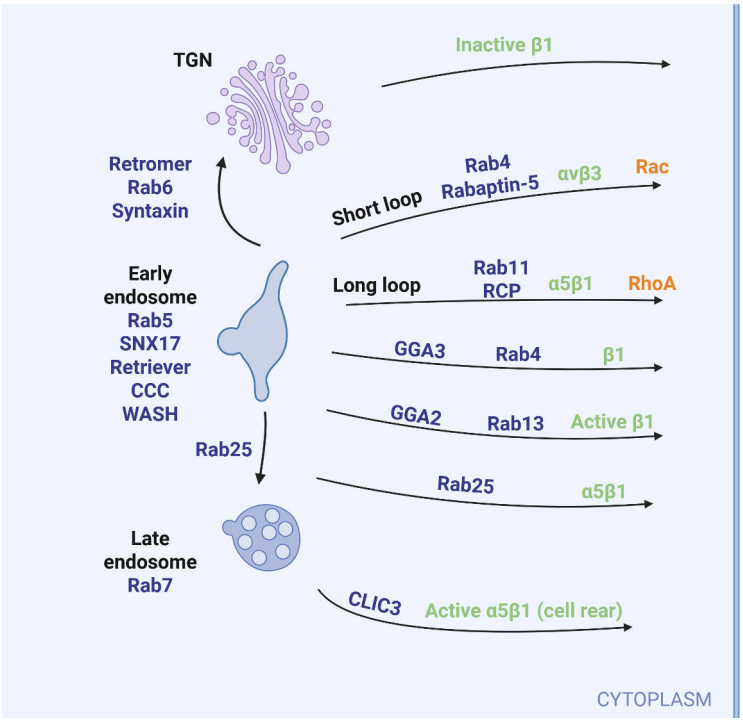

Figure 5. Simplified schematic summary of the integrin recycling pathways back to the cell surface (described in section 2.2.5), which cells use to promote their motility. Written next to the arrows are identified mediators of the respective recycling routes, along with identified integrin cargo (green) and in the case of the long and short loops, the resulting promotion of Rho GTPase activity (red), which regulates migratory behaviour.

recycled depending on its activation state. For example, in 3D matrices, Rab25 facilitates invasion by binding to the β1-integrin tail and directing internalised inactive α5β1 to the leading edge of the cell, while delivering internalised active α5β1 from the leading edge to the cell rear (Caswell et al., 2007; Dozynkiewicz et al., 2012). Recently, the Rab25-mediated delivery of β1-integrins to the plasma membrane was shown to enable the formation of filopodia-like actin protrusions in a 3D matrix, promoting invasive cancer cell migration (Gemperle et al., 2025).

In addition to the Rab4- and Rab11-mediated loops, integrins have been shown to recycle via other, alternative pathways during cell migration. In HeLa cells, inactive β1-integrins can be retrieved by the retromer complex and undergo retrograde trafficking to the TGN, where they are collected by Rab6 and syntaxin-16 before being dispatched in a polarised manner to the leading edge of the cell, promoting cell migration (Shafaq-Zadah et al., 2016). Members of the Golgi-localised gamma-ear containing Arf-binding protein (GGA) family have also been implicated in integrin recycling during cancer cell migration. GGA3 promotes recycling of the collagen receptor α2β1 via the Rab4-mediated route, preventing degradation of the collagen receptor. This was dependent on an intact Arf binding site in GGA3 (Ratcliffe et al., 2016). GGA2, on the other hand, promotes the recycling of active β1-integrins through an alternative, Rab13-mediated pathway (Sahgal et al., 2019).

It is important to note that integrins are not trafficked in isolation. Growth factor signalling is an important regulator of cell migration and integrin activity, and growth factor receptors (GFRs) and integrins can be co-trafficked to promote cell migration, as alluded to earlier. Integrins can associate with GFRs and induce GFR signalling, as well as internalisation. Likewise, GFRs can influence integrin activity and promote both integrin recycling and expression. Mutant p53 has been shown to promote RCP-mediated co-trafficking of integrins and the GFRs EGFR and hepatocyte growth factor receptor MET (Muller et al., 2009, 2013). In this way, these GFRs tag along with the integrins as they are recycled back to the cell surface together. This facilitates increased growth factor binding and signalling that supports cell motility, proliferation and survival. Stimulation with hepatocyte growth factor (HGF) induces co-internalisation of MET and β1-integrins. Once inside the cell, the two receptors are co-trafficked to autophagy-related endomembranes, where β1-integrin acts as a scaffold, bringing MET together with the adaptor protein Shc, which activates survival signalling through ERK. This mechanism enables the anchorage-independent survival and invasion of cancer cells (Barrow-McGee et al., 2016). Indeed, the ability of integrins to continue signalling on endosomes after internalisation, has become increasingly recognised as an important pro-invasive tool of cancer cells. FAK signalling on integrin-containing Rab5- and Rab11-endosomes has been shown to keep integrins in their active state after FA

disassembly by stimulating endosomal PIP_2 production, which keeps talin associated with integrins on endosomes (Nader, Ezratty and Gundersen, 2016). Through this signalling, FAK enables the recycling of integrins to the leading edge of migrating cells, forming FAs in a polarised, directed manner. Furthermore, FAK signalling from integrin-containing early endosomal compartments has been shown to promote anchorage-independent growth and breast cancer cell metastasis (Alanko et al., 2015). Altogether, it is clear that the endocytosis and directed recycling of integrins is crucial for cancer cell migration. However, it is a highly complex landscape with many variables, and cancer cells may employ different integrin trafficking pathways depending on the cell type and context.

2.3 Actin and endosomal traffic

The actin cytoskeleton is one of the four main components of the cytoskeleton, along with microtubules, intermediate filaments and septins. The actin cytoskeleton serves a multitude of roles in the cell – its dynamic nature allows it to change the shape of the cell, form protrusions, establish cell polarity, transmit forces and enable cell migration. Moreover, it plays an important role in the transport of cargoes, regulating both endocytic events and intracellular endosomal sorting. Actin is a monomeric protein (G-actin) that can bind ATP and ADP, and can form actin filaments (F-actin) through polymerisation. The formation of actin filaments is powered by ATP hydrolysis, and begins with the formation of actin dimers and trimers, a process called nucleation. These structures are unstable, and though actin nucleation and polymerisation can occur on its own (slowly and inefficiently), the nucleation events are reinforced through the activity of actin-nucleating proteins, making the process significantly more efficient. Actin is polymerised in a polarised manner, with a fast-growing barbed (+) end and a slower-growing pointed (-) end. In a process called actin treadmilling, ATP-bound monomers are added to the growing barbed end, where they undergo ATP-hydrolysis, eventually dissociating at the pointed (-) end, due to the weaker affinity of the ADP-bound form (Pollard, 2016). This process, which allows the constant turnover of G-actin and enables dynamic reorganisation of the actin cytoskeleton, is regulated by several actin-binding proteins.

2.3.1 Actin-binding proteins regulate actin filament assembly and disassembly

The Arp2/3 complex is an actin-nucleating protein complex, consisting of seven subunits, that promotes actin polymerisation. Its two catalytic subunits, Arp2 and Arp3, mimic an actin dimer, while the remaining five (Arpc1, Arpc2, Arpc3, Arpc4 and Arpc5) act as scaffolds. By binding to the side of existing actin filaments, it

forms branches of F-actin (jutting out in a 70° angle from the side of the existing filament), orchestrating the creation of branched actin networks (Gautreau et al., 2022). The Arp2/3 complex is not inherently efficient in promoting actin polymerisation. Instead, it is aided in its task by nucleation promoting factors (NPFs), which bring actin filaments, actin monomers and the Arp2/3 complex together. Well-established NPFs include the Wiskott-Aldrich Syndrome protein (WASP), the neuronal-enriched homologue of WASP (N-WASP), the WASP family verprolin-homologous (WAVE) complex, also known as suppressor of cyclic AMP receptor (SCAR), and the WASP and scar homologue (WASH) complex (Gautreau et al., 2022). While the Arp2/3 complex nucleates actin to create branched filaments, another family of actin nucleators, the formins, promote the polymerisation of linear actin filaments. Formins homodimerise to form a functional unit, and bind monomeric actin with their formin homology 2 (FH2) domain, encircling the barbed end of the actin filament (Oosterheert et al., 2024). With their proline-rich formin homology 1 (FH1) domain, formins recruit profilin, another actin-binding protein that binds G-actin. Profilin prevents spontaneous actin nucleation, and instead, directs ATP-bound G-actin for nucleation and filament elongation by binding to proline-rich domains in actin polymerisation machinery such as formins, Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) and WASP family of proteins (Pollard, 2016).

The elongation of actin filaments is regulated by actin-binding proteins that facilitate filament disassembly or make the barbed end unavailable for continued growth. Actin filament disassembly can be induced by the action of actin severing proteins such as cofilins. Cofilins bind preferentially to ADP-bound actin, and promote dissociation of ADP-actin monomers from the pointed end of the filament. Capping proteins, on the other hand, halt filament elongation by binding to the barbed end of actin filaments, preventing the addition of further actin monomers (Pollard, 2016). The formation of actin networks is regulated by several actin-binding proteins that can mediate the bundling and crosslinking of filaments to form higher-order actin structures. Filamins act as scaffolds and crosslink actin filaments at angles, forming actin networks, and can link membrane-bound receptors, such as integrins, with the actin cytoskeleton (Kiema et al., 2006; Nakamura, Stossel and Hartwig, 2011). The Lin-11, Isl-1 and Mec-3 (LIM) domain-containing protein EPLIN can crosslink actin filaments using its two actin binding sites, and has been shown to inhibit Arp2/3-mediated actin polymerisation (Maul et al., 2003). Another actin-binding protein, Swiprosin-1, inhibits cofilin-mediated filament disassembly, regulating actin dynamics in lamellipodia (Huh et al., 2013).

2.3.2 Actin and endocytosis

The regulation of actin filament dynamics plays an important role in facilitating endocytosis. The polymerisation of actin filaments can be used to exert pushing forces on the membrane, and as tracks for myosin motor-based force production (Chakrabarti, Lee and Higgs, 2021). Moreover, actin filament anchoring proteins at the endocytic site enable the conversion of pushing forces to pulling and squeezing forces (Serwas et al., 2022). Actin nucleators enable the dynamic assembly of actin filaments, with the Arp2/3 complex and its regulators playing a central role for actin polymerisation during endocytosis, enabling the formation of branched actin networks (Chakrabarti, Lee and Higgs, 2021).

During clathrin-mediated endocytosis (CME), the Arp2/3 complex is recruited following the initiation of clathrin coat assembly, with an estimated 200 Arp2/3 complexes being recruited during the whole endocytic process (Akamatsu et al., 2020). The activity of Arp2/3 during CME is facilitated by N-WASP, which is recruited to clathrin-coated pits by the FCH and double SH3 domains protein 2 (FCHSD2), a member of the BAR protein superfamily (Almeida-Souza et al., 2018). Actin has been reported to be present already early on during clathrin-coated pit formation, forming a branched network surrounding the site of endocytosis. Actin polymerisation at the clathrin-coated pit is facilitated by tension, and actin polymerisation, directed towards the base of the pit, increases when an increase in tension at the invagination dictates it, resulting in increased actin network density (Akamatsu et al., 2020; Kaplan et al., 2022). Indeed, disruption of actin polymerisation has been shown to results in stalled, U-shaped pits, highlighting the role of actin during the later stages of CME (Abouelezz and Almeida-Souza, 2022). The actin-binding linker protein Huntingtin-interacting protein 1-related (Hip1R) can bind to both actin filaments and the plasma membrane at CME sites, and is thought to facilitate pulling forces between actin filaments and the membrane at the neck of the pit, supporting neck constriction (Serwas et al., 2022). The rate of actin polymerisation is at its highest during the scission event, when the budding invagination is cut off from the plasma membrane. This is also the stage with the largest number of actin regulatory proteins present, which cooperate to orchestrate the late-stage events. This includes proteins such as cortactin, which enhances branched actin polymerisation and stabilises actin branches, as well as the motor proteins myosin IE and myosin VI, which are recruited together with dynamin (Abouelezz and Almeida-Souza, 2022). Myosin IE is thought to promote the completion of late-stage CME by potentially recruiting NPFs to increase Arp2/3 activity, and/or by generating force (Krendel, Osterweil and Mooseker, 2007; Cheng, Grassart and Drubin, 2012). Myosin VI moves towards the pointed end of actin filaments, and is thought to pull the pit inwards (Spudich et al., 2007). Non-muscle myosin II is also recruited, and has been suggested to promote CME by creating local

tension in the actin cortex (Chandrasekar et al., 2014). Finally, cofilin is also recruited to the pit, and its association with the endocytic site peaks just after dynamin, indicating that it plays a role in actin filament disassembly after the scission event (Taylor, Perrais and Merrifield, 2011; Abouelezz and Almeida-Souza, 2022).

While it is known that actin plays an important role in clathrin-independent endocytosis, such as the CG pathway, these pathways have not been studied to the same extent as CME. As a consequence, in-depth knowledge of the details of actin behaviour in these pathways is still lacking. Disruption or stabilisation of actin polymerisation, as well as inhibition of the Arp2/3 complex blocks CG endocytosis, underlining the importance of branched actin dynamics during CG endocytosis (Chakrabarti, Lee and Higgs, 2021). Recently, a TIRF microscopy-based study determined the sequence of arrival and disappearance of CG machinery components, giving rise to a stepwise model of CG endocytosis (Sathe et al., 2018). Many of the implicated proteins regulate actin dynamics, illustrating the importance of actin during the endocytic process. During initiation of CG endocytosis, the Arp2/3 inhibitor PICK1 is recruited to the endocytic site via its BAR domain, and keeps Arp2/3 in an inactive state. IRSp53 is recruited to the site, potentially by binding to the plasma membrane using its I-BAR domain, and is activated by Cdc42. Once activated, IRSp53 promotes Arp2/3-mediated actin polymerisation. Interestingly, while IRSp53 has been shown to interact directly with N-WASP (Lim et al., 2008), N-WASP was neither recruited nor required for CG endocytosis, contrary to its role in CME. Thus, it seems that IRSp53 promotes Arp2/3 activity via another, yet unidentified NPF. Another contributing factor for the increase in Arp2/3 activity is the loss of PICK1-mediated Arp2/3 inhibition, which occurs through the activity of GTP-Arf1. Actin polymerisation at the endocytic site occurs throughout the maturation process of the invagination, presumably to provide the forces needed for the shaping of the invagination, and F-actin buildup continues even after scission. Myosin II-mediated actomyosin contractility was recently shown to regulate the uptake of CG cargoes, suggesting a mechanism where myosin II regulates plasma membrane tension at the endocytic site by acting on the cortical actin cytoskeleton (Wayt et al., 2021). Finally, it is unclear if the scission event itself during CG endocytosis is performed by actin, but Arp2/3 complexes localise to the site of scission and dissociate after scission completion, indicating that actin polymerisation plays a role here as well (Sathe et al., 2018).

2.3.3 Actin and endosomal sorting of cargo

Actin plays a vital role in the sorting of endosomal cargo. Endosomal actin polymerisation is driven principally by the WASH complex, which facilitates

Arp2/3-mediated branched actin network assembly to support the tubulation of endosomes, the partitioning and retrieval of endosomal cargoes, as well as endosomal transport. Consequently, disruption of actin dynamics and loss of WASH function results in major defects in the endosomal network (Simonetti and Cullen, 2019).

The pentameric WASH complex associates with both retromer and retriever/commander complexes, and binds to Arp2/3 via its verprolin homology central acidic (VCA) domain, promoting actin polymerisation on endosomes. Ubiquitylation and deubiquitylation, as well as phosphorylation and dephosphorylation of WASH regulates its activity, finetuning actin nucleation at the endosome (MacDonald, Savage and Zech, 2020). The phospholipid composition of endosomes acts as another mechanism for regulating actin network turnover, as it determines the recruitment of actin polymerisation machinery (Hong, Qi and Weaver, 2015; Simonetti et al., 2019; Singla et al., 2019). The endosomal formation of branched actin networks serves multiple purposes. Together with cortactin, WASH localises to the base of forming tubules, where they are thought to facilitate tubule formation by shaping the membrane through Arp2/3-mediated branched actin network assembly (Derivery et al., 2009; Duleh and Welch, 2010; Chakrabarti, Lee and Higgs, 2021). The actin network stabilises tubules, making them sufficiently long-lived for collection of cargoes destined for recycling (Puthenveedu et al., 2010; Chakrabarti, Lee and Higgs, 2021). The segregation of cargoes into endosomal subdomains is another suggested function of WASH-mediated actin polymerisation, where cargoes are selected for retrieval based on specific sequence motifs in their cytoplasmic tails, such as the NPxY/NxxY motif recognised by SNX17 and SNX27 (Simonetti and Cullen, 2019). In addition, actin itself is thought to be able to herd cargo into tubular subdomains for retrieval, through interactions with actin-binding proteins (Puthenveedu et al., 2010; Healy and Collins, 2023). WASH binds to and competes with ESCRT-0 on endosomes, and rescues cargo from a degradative fate by segregating the cargo into retrieval domains through actin polymerisation (MacDonald et al., 2018). Altogether, WASH facilitates cargo recycling by bringing retrieval machinery (such as commander and retromer) and partitioned cargo together on tubular endosomal subdomains.

WASH is also implicated in the scission of endosomes together with dynamins and EHD proteins. Loss of WASH results in accumulation of tubular structures on endosomes, suggesting that the complex is required for scission to occur (Derivery et al., 2009; Gomez and Billadeau, 2009; Duleh and Welch, 2010). It has been suggested that WASH supports scission by local force production through actin polymerisation (Rottner, Hänisch and Campellone, 2010). Recently, the actin-binding protein coronin 2A was reported to facilitate cargo recycling by cooperating with endosomal EHD1 to promote endosome scission (Dhawan, Naslavsky and

Caplan, 2022). In this model, coronin 2A binds to F-actin on the tubule, allowing EHD1 to come in and cut off the vesicle. The endosomal scaffold protein MICAL-L1 is also implicated in this process, as it recruits FCHSD2 to promote Arp2/3-mediated actin polymerisation early on in the scission process, followed by EHD1 recruitment to finalise scission (Frisby et al., 2024). Overall, the scission process is a multi-step process which requires multiple actin regulators combined with EHD1-mediated membrane constriction.

Actin filaments have also been shown to play a role in the transport of endosomal vesicles by providing tracks for myosin motor protein-based movement. The unconventional myosin VI has been shown to position APPL1- and Rab5-positive endosomes close to the plasma membrane, associating with cortical actin (Masters et al., 2017). It also moves cargo, such as the transferrin receptor, along actin filaments from early endosomes to the endosomal recycling compartment (Chibalina et al., 2007). Myosin V has been reported to cooperate with the actin nucleator Spir at Rab11-positive endosomes, coupling myosin motor movement with actin filament track assembly and promoting Rab11-positive vesicle movement (Pylypenko et al., 2016). Furthermore, in addition to serving as tracks, actin has been reported to induce movement through N-WASP-stimulated Arp2/3-mediated actin polymerisation, forming a so-called actin comet tail as local actin polymerisation propels the vesicle forward (Taunton et al., 2000). A similar actin comet tail propulsion mechanism has been proposed to facilitate the movement of autophagosomes at the ER membrane interface (Kast et al., 2015).

3 Aims

The cell balances receptor endocytosis and recycling as a means to regulate the amount of available receptors on the plasma membrane, which, in turn, influences the cell's capacity to relay signals from the cell surface. The influence of trafficking on the signalling activity of receptor tyrosine kinases is well-established, but despite being an established driver of oncogenicity, the trafficking of HER2 in breast cancer cells has remained poorly understood, and its contribution to HER2 signalling activity has been questioned. Integrins mediate cell adhesion, and the trafficking of integrins facilitates cancer cell migration and invasion. However, our understanding of the mechanisms of integrin endocytosis and recycling is far from complete. We have identified the sorting receptor SORLA as a regulator of HER2 recycling, and the actin-binding proteins Swiprosin-1 and EPLINα as interactors of the established integrin trafficking protein Rab21. In the projects of this thesis, I have investigated the role of SORLA as a regulator of HER2 recycling, and its impact on oncogenic signalling. I have also studied the role of Swiprosin-1 in Rab21-mediated integrin endocytosis, and characterised the role of EPLINα in integrin recycling, in the context of breast cancer cell migration. In a broader sense, the goal of this thesis work has been to increase our understanding of how breast cancer cells utilise different trafficking mechanisms to promote their malignancy.

The specific aims of the thesis were:

I. To determine the impact of SORLA on the recycling and oncogenic signalling of HER2 in HER2-amplified breast cancer.
II. To investigate how Swiprosin-1 and Rab21 co-facilitate integrin endocytosis in triple-negative breast cancer.
III. To study the role of EPLIN and its isoforms in the context of integrin recycling and breast cancer cell migration.

4 Materials and Methods

This section describes the materials and methods used to produce the data in this thesis, with an emphasis on the methods where I have taken part in the planning and conducting of the experiments. More detailed information can be found in the original publications (I, II, III).

4.1 Cell culture (I, II, III)

Table 1 lists cell lines and culture media used in the projects of this thesis. The cells were cultured in +37 °C, 5% CO_2 in a humidified incubator, and were routinely tested for mycoplasma contamination.

Table 1. Cell lines and culture media used in the experiments. TNBC = Triple-negative breast cancer, RPMI = Roswell Park Memorial Institute, FBS = Fetal bovine serum, EMEM = Eagle's minimum essential medium, HER2 = Human epidermal growth factor receptor 2, ER = Estrogen receptor, PR = Progestrone receptor, DMEM = Dulbecco's modified Eagle's medium, NEAA = Non-essential amino acid.

Cell line	Culture medium	Cancer type	Original publication
5637	RPMI-1640, 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin	Bladder cancer	I
BT-20	EMEM, 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin	TNBC	II
BT-474	RPMI-1640, 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin	HER2+, ER+, PR+ breast cancer	I
BT-549	RPMI-1640, 10% FBS, 2 mM L-glutamine, 0.023 U/ml human insulin, 1% penicillin-streptomycin	TNBC	III
HCC1937	RPMI-1640, 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin	TNBC	III
JIMT-1	DMEM, 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin	HER2+ breast cancer	I
MCF7	DMEM, 10% FBS, 2 mM L-glutamine, 0.01 mg/ml human insulin, 1% penicillin-streptomycin	ER+, PR+ breast cancer	I, III
MDA-MB-231	DMEM, 10% FBS, 2 mM L-glutamine, 1% NEAAs, 1% penicillin-streptomycin	TNBC	I, II, III

Cell line	Culture medium	Cancer type	Original publication
MDA-MB-361	DMEM, 20% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin	HER2+, ER+, PR+ breast cancer	I
MDA-MB-468	DMEM, 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin	TNBC	II
MFM-223	DMEM, 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin	TNBC	I
T-47D	RPMI-1640, 10% FBS, 2 mM L-glutamine, 0.2 U/ml human insulin, 1% penicillin-streptomycin	ER+, PR+ breast cancer	III

4.2 Transfections, siRNA and plasmids (I, II, III)

The expression of proteins of interest in cells was transiently suppressed by transfecting 20-40 nM siRNA oligonucleotides (Table 2) using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Cells were used in experiments 72 hours after siRNA-transfection. Plasmid DNA was transiently transfected using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Table 3 lists the plasmid DNA constructs used in the experiments.

Table 2. siRNA oligonucleotides used in the experiments.

Target	Source	Identifier	Original publication
Allstars negative control siRNA	Qiagen	1027281	I, II, III
Arf1 siRNA1	Qiagen	SI02757734	II
Arf1 siRNA2	Qiagen	SI02654470	II
CORO1C siRNA1	Horizon Discovery	ON-TARGETplus J017331-05-0002	III
CORO1C siRNA2	Horizon Discovery	ON-TARGETplus J017331-06-0002	III
EPLIN total siRNA1	Horizon Discovery	Accell A-010663-14	III
EPLIN total siRNA2	Horizon Discovery	ON-TARGETplus J010633-09-0002	III
EPLINβ siRNA1	Horizon Discovery	Dharmacon_A010663-13	III
EPLINβ siRNA2	Invitrogen	N/A, custom-made	III
IRSp53 siRNA1	Qiagen	SI02637271	II
IRSp53 siRNA2	Qiagen	SI00087675	II
siHER2 #2	Horizon Discovery	ON-TARGETplus J-003126-17	I
siHER2 #4	Horizon Discovery	ON-TARGETplus J-003126-20	I
siSORLA #1	Horizon Discovery	ON-TARGETplus J-004722-08	I
siSORLA #2	Horizon Discovery	ON-TARGETplus J-004722-06	I
siSORLA #3	Horizon Discovery	ON-TARGETplus J-004722-07	I
siSORLA #4	Horizon Discovery	ON-TARGETplus J-004722-05	I
siSORLA 3'UTR	Qiagen	SI05039888	I
Swip1 siRNA1	Sigma-Aldrich	SASI_Hs01_00186848	II
Swip1 siRNA2	Sigma-Aldrich	SASI_Hs01_00186847	II

Table 3. Plasmid DNA constructs used in the experiments.

DNA construct	Source	Original publication
Arf1-GFP	Addgene, 49578	II
CORO1C-mEmerald	J.E. Bear, University of North Carolina, USA	III
GFP-EPLINα	Addgene, 40947	III
GFP-EPLINα-ΔΔ	In-house	III
GFP-EPLINβ	Addgene, 40948	III
GFP-EPLINβ-ΔΔ	In-house	III
GFP-Swip1	P. Cervero, UKE, Hamburg, Germany	II
GFP-Swip1 ΔEF1	In-house	II
GFP-Swip1 ΔEF2	In-house	II
HA-Arf1	Addgene, 79409	II
IRSp53-GFP	Genome biology unit, Univ. of Helsinki	II
IRSp53-mCherry	Genome biology unit, Univ. of Helsinki	II
mScarlet- EPLINα	In-house	III
mScarlet- EPLINβ	In-house	III
mScarlet-EPLINα-ΔΔ	In-house	III
mScarlet-EPLINβ-ΔΔ	In-house	III
mScarlet-I-Swip1	In.house	II
mScarlet-I-Swip1 ΔEF1	In-house	II
mVenus	Addgene, 154899	II, III
PB-TagBFP-T2A-myc-BirA*	In-house	III
PB-TagBFP-T2A-myc-BirA*- EPLINα	In-house	III
PB-TagBFP-T2A-myc-BirA* EPLINβ	In-house	III
Rab21-GFP CA (Q76L)	In-house	II
Rab21-GFP DN (T33N)	In-house	II
Rab21-GFP WT	Addgene, 83421	II, III
Rab21-V1	In-house	II, III
SORLA-GFP ECD + TM	In-house	I
SORLA-GFP full length	In-house	I
SORLA-GFP TM + CD	In-house	I
StrepII-EPLINα	In-house	III
StrepII-EPLINα-ΔΔ	In-house	III
Swip1-V2	In-house	II
V2-EPLINα	In-house	III

4.3 Western blot (I, II, III)

Cells were washed once with cold phosphate-buffered saline (PBS) and scraped in lysis buffer made of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton-X, 0.5% glycerol, 1% SDS, complete protease inhibitor (Sigma-Aldrich) and phos-stop tablet (Sigma-Aldrich). The lysates were homogenised by sonication, and relative protein concentrations were determined using a DC protein assay kit (Bio-Rad), before normalising sample protein concentrations by addition of lysis buffer. After adding SDS sample buffer, lysates were heated for 10 minutes at +90 °C on a heating block, loaded into a precast 4–20% gradient polyacrylamide gel (Bio-Rad) and separated by gel electrophoresis. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad) using the Trans-Blot Turbo system (Bio-Rad). The membrane was blocked using StartingBlock blocking buffer (Thermo Fsher Scientific) for one hour at room temperature, after which primary antibody solutions against proteins of interest were added to the membrane and incubated overnight at +4 °C. After washing away the excess primary antibody solution with Tris-buffered saline containing 0.1% Tween 20 (TBST), a fluorophore-conjugated secondary antibody solution was added to the membrane and incubated for one hour at room temperature. The membrane was then washed again with TBST. All antibodies used for western blot were diluted in StartingBlock blocking buffer, and are listed in Table 4. Finally, the membrane was scanned using an Odyssey infrared imaging system (LI-COR Biosciences), and analysed with Fiji (ImageJ, National Institutes of Health).

Table 4. Primary antibodies used in the experiments. For further details, see the original publications (I, II, III).

Antibody	Host	Source	Identifier	Application	Original publication
4E-BP1	Rabbit	Cell Signaling Technology	9452	WB	I
AKT	Rabbit	Cell Signaling Technology	9272	WB	I
Alexa Fluor 488(quenching antibody)	Rabbit	Thermo Fisher Scientific	710369	IF	III
Arf1	Rabbit	Genetex	GTX113385	WB	II
Arp2/3 subunit 1B	Rabbit	Abcam	ab99314	WB	III
Calnexin	Rabbit	Enzo Life Sciences	ADI-SPA-865-F	WB	II
CD63 (LAMP-3)	Mouse	Developmental Studies Hybridoma Bank	H5C6	IF	I
Cleaved PARP1	Rabbit	Abcam	ab32064	WB	I

Antibody	Host	Source	Identifier	Application	Original publication
CORO1C	Mouse	J.E. Bear, University of North Carolina, USA	N/A	WB, IF, PLA	III
CORO1C	Rabbit	Proteintech	14749-1-AP	WB	III
Cyclin D1	Mouse	Santa Cruz Biotechnology	sc-450	WB	I
E-cadherin	Rabbit	Cell Signaling Technology	3195	WB	III
EEA1	Goat	Santa Cruz Biotechnology	sc-6415	IF	I
EEA1	Rabbit	Abcam	ab2900	IF	II
EEA1	Rabbit	Cell Signaling Technology	3288	IF	III
EPLIN (total)	Rabbit	Novus Biologicals	NB100-2305	WB, IF, PLA	III
EPLIN β	Mouse	Thermo Fisher Scientific	MA5-27016	IF	III
ERK1/2	Rabbit	Cell Signaling Technology	9102	WB	I
GAPDH	Mouse	HyTest	5G4cc	WB	I, II, III
GFP	Rabbit	Thermo Fisher Scientific	A11122	WB	II
GFP	Mouse	Abcam	ab1218	IF	II
Goat IgG	Goat	Sigma-Aldrich	I5256	PLA	II
HER2	Mouse	Thermo Fisher Scientific	MA5-14057	WB, IP	I
Integrin β1 (total)	Mouse	Developmental Studies Hybridoma Bank	P5D2	WB, IF	II
Integrin β1 (total)	Mouse	BD Biosciences	610468	WB	II
Integrin β1 12G10	Mouse	K.M. Yamada, NIH, USA	N/A	IF, PLA	II, III
Integrin β1 12G10, Alexa Fluor 488-conjugated	Mouse	Abcam	ab202641	IF	III
Integrin β1 9EG7	Rat	BD Biosciences	553715	IF	II
Integrin β1 mAb13	Rat	BD Biosciences	552828	IF	II
IRSp53	Rabbit	Atlas Antibodies	HPA023310	WB, PLA	II
IRSp53	Mouse	G. Scita, IFOM, Italy	N/A	IP	II
LAMP-1	Mouse	Santa Cruz Biotechnology	sc-20011	IF	I, II
MHCI (HLA class I)	Mouse	Sigma-Aldrich	SAB4700637	IF	II
Mouse IgG	Mouse	Diagenode	C15400001	IP	II
Mouse IgG	Mouse	Thermo FIsher Scientific	02-6502	PLA	III
Myc-tag	Rabbit	Cell Signaling Technology	2276	IF	III

Antibody	Host	Source	Identifier	Application	Original publication
PARP1	Rabbit	Santa Cruz Biotechnology	sc-7150	WB	I
Phospho-4E-BP1 (Thr37/46)	Rabbit	Cell Signaling Technology	2855	WB	I
Phospho-AKT (S473)	Rabbit	Cell Signaling Technology	9271	WB	I
Phospho-ERK1/2 (T202/Y204)	Rabbit	Cell Signaling Technology	4370	WB	I
Rab11	Rabbit	Cell Signaling Technology	5589	IF	I, II
Rab21	Rabbit	In-house	N/A	WB, IF, PLA	II
Rab5	Rabbit	Cell Signaling Technology	3547	IF	I, II, III
Rab7	Rabbit	Cell Signaling Technology	9367	IF	II
SORLA	Rabbit	C.M. Petersen, Aarhus Univ., Denmark	N/A	IF	I
SORLA (LR11)	Mouse	BD Biosciences	612633	WB	I
Strep II-tag	Mouse	Novus Biologicals	NBP2-43735	WB	III
Swiprosin-1	Goat	Everest Biotech	EB06552	WB, IF, PLA	II
Swiprosin-1	Rabbit	Atlas Antibodies	HPA048961	WB	II
Trastuzumab (HER2), Alexa Fluor 568-conjugated	Humanised	Roche	CAS 180288-69-1	IF	I
Vimentin	Mouse	Santa Cruz Biotechnology	sc-6260	WB	III
Vinculin	Mouse	Sigma-Aldrich	V9131	IF	II
VPS35	Goat	Abcam	ab10099	IF	I, II
α-tubulin	Mouse	Developmental Studies Hybridoma Bank	12G10	WB	I
β-actin	Mouse	Sigma-Aldrich	A1978	WB	II, III

4.4 Trafficking assays (I, II, III)

4.4.1 HER2 internalisation and recycling (I)

Cells plated on microscopy dishes (ibidi) were incubated with anti-HER2 Trastuzumab-568 (Tz-568) in cold Hank's Balanced Salt Solution on ice for 1 hour for cell surface labelling. Internalisation was triggered by adding warm serum-free medium and incubating the cells for 0, 30 and 60 minutes at +37 °C, before fixation with 4% formaldehyde. To assess the role of recycling, cells were treated the same as above, but internalisation and receptor trafficking was allowed to occur for 45

minutes before removing any Tz-568 on the cell surface by performing an acid wash (0.2 M acetic acid, 0.5 M NaCl, pH 2.5). Recycling of remaining intracellular Tz-568-bound HER2 was then allowed to occur for 30 minutes at +37 °C before fixation. Samples were imaged with an LSM780 laser scanning confocal microscope (Zeiss), and the intracellular signal was quantified using Fiji (ImageJ, National Institutes of Health).

4.4.2 Integrin internalisation (II, III)

Cells plated on microscopy dishes (Cellvis) were incubated with anti-integrin β1 (12G10) antibody in cold full growth medium on ice for 1 hour for cell surface labelling. Samples were washed with cold full growth medium, and internalisation was triggered by adding warm full growth medium and incubating cells at +37 °C for 0 and 15 minutes. Receptor trafficking was stopped by changing to cold medium. Cell surface-bound antibodies were then removed with an acid wash (0.2 M acetic acid, 0.5 M NaCl, pH 2.5), and samples were fixed with 2% formaldehyde. Cells were permeabilised and incubated with fluorescent secondary antibodies in order to detect the internalised integrins. Samples were imaged with a Marianas spinning disk confocal microscope (Intelligent Imaging Innovations), and the fluorescent signal from the whole cell was quantified using the 'spots detection' function in IMARIS (Oxford Instruments).

4.4.3 Integrin recycling (III)

Cells plated on microscopy dishes (Cellvis) were starved for 45 minutes in warm serum-free medium before cooling them down and incubating with Alexa Fluor 488-conjugated anti-integrin β1 12G10 antibody (12G10-488) in cold serum-free medium for 1 hour on ice, in order to label active cell surface β1-integrins. Cells were washed with cold serum-free medium, and integrin endocytosis was triggered by adding warm serum-free medium and incubating cells at +37 °C for 0 and 30 minutes. Endocytosis was stopped by adding cold serum-free medium and placing samples on ice. To remove cell-surface signal, samples were incubated with an anti-Alexa 488 quenching antibody diluted in cold serum-free medium for 1 hour on ice. To start integrin recycling, warm full growth medium containing the quenching antibody was added to the cells, and cells were incubated at +37 °C for 30 minutes. Lastly, samples were fixed with 2% formaldehyde, and stained with Phalloidin-Atto 647N (Sigma-Aldrich) to visualise cell boundaries. Samples were imaged with a Marianas spinning disk confocal microscope (Intelligent Imaging Innovations) and the intracellular intensity sum of 21 slices from the middle of the cell was quantified for each cell, normalised to cell area, using Fiji (ImageJ, National Institutes of Health).

4.5 Proximity ligation assay (PLA) (II, III)

Cells plated on microscopy dishes (Cellvis) were fixed with formaldehyde, washed with PBS and permeabilised with 0.3% Triton X-100 in PBS for 15 min at room temperature. Samples were incubated with primary antibodies against two proteins of interest (one from a mouse host and the other from a rabbit host), diluted in PBS containing 5% horse serum for 1 hour at room temperature. A PLA kit (Sigma-Aldrich) was used to perform the proximity ligation, according to the manufacturer's instructions. The samples were imaged using a Marianas spinning disk confocal microscope (Intelligent Imaging Innovations) and the number of PLA spots per 1000 µm3 per cell was quantified with IMARIS (Oxford Instruments). To distinguish early endosomal PLA spots from PLA spots further away, EEA1 staining was used as a marker for early endosomes, and the 'spots close to surface' Xtension in IMARIS was used, with 1 µm as the threshold between surface object (EEA1) and spots object (PLA spot).

4.6 DQ BSA assay (I)

The DQ Red BSA assay kit (Thermo Fisher Scientific) was used to assess the proteolytic activity of lysosomes. Cells plated on microscopy dishes (ibidi) were incubated for 48 hours in full growth medium containing 25 µg/ml DQ Red BSA, a fluorogenic substrate that is quenched until it is broken down into single peptides, which relieves the quenching effect, producing a fluorescent signal. The stronger the signal, the higher the proteolytic activity. The samples were fixed with 4% formaldehyde and imaged with an LSM780 laser scanning confocal microscope (Zeiss).

For flow cytometry, cells plated on 6-well plates were incubated with DQ Red BSA for 24 hours. When treating cells with DQ Red BSA together with bafilomycin (Calbiochem), the incubation time was shortened to 4 hours. Cells were detached using HyQtase (HyClone) and washed, before fixation with 4% formaldehyde. The DQ Red BSA signal was then measured using an LSRFortessa (BD Biosciences) flow cytometer, and analysed with Flowing software (Cell imaging and cytometry core, Turku Bioscience Centre).

4.7 Microscopy (I, II, III)

4.7.1 Immunofluorescence sample preparation (I, II, III)

Cells were fixed with either 2% or 4% formaldehyde for 20 or 15 minutes, respectively (depending on the experiment), at room temperature, before permeabilisation with 0.3% Triton X-100 in 10% horse serum (Gibco) for 15

minutes. Samples were further blocked in 10% horse serum for 20 minutes and incubated with primary antibodies (Table 4) diluted in 10% horse serum overnight at +4 °C. Samples were washed with PBS and incubated with secondary antibodies diluted in 10% horse serum for 1-2 hours at room temperature, followed by PBS washes. An additional post-fixation step (4% formaldehyde for 10 minutes) was performed when preparing samples for structured illumination microscopy (SIM). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and the actin cytoskeleton was stained with Phalloidin-Atto 647N (Sigma-Aldrich), Alexa Fluor 488 Phalloidin (Thermo Fisher Scientific) or SiR-actin (Spirochrome), depending on the experiment. These stains were added together with the secondary antibody solution.

4.7.2 Light microscopy (I, II, III)

A Marianas spinning disk confocal microscope controlled by SlideBook 6 software (Intelligent Imaging Innovations), equipped with a CSU-W1 scanning unit (Yokogawa), an ORCA-Flash4.0 v2 scientific complementary metal-oxide semiconductor (sCMOS) camera (Hamamatsu Photonics) and a back-illuminated 10 MHz EMCDD camera (Photometrics Evolve) served as the "workhorse" microscope for imaging fluorescent samples. The following objectives were used: 63x/1.4 NA oil Plan-Apochromat (Zeiss) and 100x/1.4 NA oil Plan-Apochromat (Zeiss). Live imaging was performed inside a microscope cage incubator at +37 °C and 5% CO_2.

Airyscan super-resolution imaging was performed with an LSM880 laser scanning confocal microscope controlled by Zen Black 2.3 software (Zeiss), equipped with an Airyscan detector and 63x/1.4 NA oil C Plan-Apochromat objective. The imaging was performed in standard super-resolution mode.

SIM super-resolution imaging was performed with a Deltavision OMX v4 (GE Healthcare Life Sciences) controlled by softWoRx for Linux, equipped with a front-illuminated pco.edge sCMOS camera (pixel size 6.5 mm, readout speed 95 MHz; PCO AG) and a Plan-Apochromat 60x/1.42 NA oil objective (immersion oil RI of 1.516). The imaging was performed in SIM illumination mode (five phases x three rotations).

For live imaging of cell migration, an Eclipse Ti-E epifluorescence microscope was used, controlled by NIS-Elements AR 4.60 or AR 5.11 software (Nikon). The microscope was equipped with a sCMOS Orca Flash4.0 camera (Hamamatsu Photonics) and a 10x/0.3 NA CFI Plan-Fluor objective (Nikon). Imaging was performed inside a microscope cage incubator at +37 °C and 5% CO_2.

4.7.3 Bimolecular fluorescence complementation (II, III)

Bimolecular fluorescence complementation (BiFC) microscopy was performed by co-transfecting cells with plasmid constructs of proteins of interest tagged with complementary halves of the Venus fluorescent protein. The following morning, cells were seeded to microscopy dishes, allowed to attach for 5 hours, and fixed with 4% formaldehyde for 15 minutes. After following the above-mentioned sample preparation and staining steps, samples were imaged with a Marianas spinning disk confocal microscope (Intelligent Imaging Innovations).

4.8 Migration assays (II, III)

4.8.1 Wound-healing assay (II)

Cells were seeded into silicone culture inserts (ibidi) made of two wells separated by a wall, and grown until confluency. The inserts were then carefully removed using forceps, and cells were washed twice with warm PBS to remove floating cells, leaving only the two patches of confluent cells separated by a gap. Warm full medium was then added, and live imaging of the cells closing the gap was performed with an Eclipse Ti-E epifluorescence microscope (Nikon), using a 20-minute imaging interval over 24 hours. The speed of gap closure was analysed using Fiji (ImageJ, National Institutes of Health), by measuring the gap area at 0, 6, 8, 12, 16 and 18 hour timepoints.

4.8.2 Random migration assay (III)

Cells plated on 6-well plates, seeded sparsely enough to allow single-cell migration, were imaged with an Eclipse Ti-E epifluorescence microscope (Nikon), using a 10- or 20-minute imaging interval over 6 hours. Cells were tracked using the MTrackJ plug-in for Fiji, and migration tracks were plotted using RStudio (Posit).

4.9 Focal adhesion analysis (II)

Cells plated on microscopy dishes coated with collagen I, fibronectin or laminin (Sigma-Aldrich) were allowed to attach and spread, before fixation with 4% formaldehyde for 15 minutes. Previously mentioned sample preparation steps were followed, and cells were stained for the focal adhesion protein vinculin. Samples were imaged with an LSM880 laser scanning confocal microscope (Zeiss). Cell masks of focal adhesions were created from the vinculin signal after background

subtraction using Fiji, and the number and area of focal adhesions per cell were quantified.

4.10 BioID sample preparation (III)

Cells (MDA-MB-231 and HCC1937) stably expressing the promiscuous biotin ligase BirA* alone or coupled to either EPLINα or EPLINβ were grown on 10 cm dishes (two per condition) and incubated in the presence of biotin (50 µM) for 24 hours at +37 °C and 5% CO_2 to allow biotinylation of proximal proteins. Cells were washed three times with PBS, before scraping them in lysis buffer made of 50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% SDS, 0.5 mM dithiothreitol (DTT) and complete protease inhibitor (Roche). 800 µl of lysate per condition was added to tubes containing 80 µl 20% Triton X-100, and kept on ice. Samples were homogenised by passing them four times through a 19G needle, before adding 720 µl 50 mM Tris-HCl (pH 7.4) and homogenising four more times through a 27G needle. Samples were then centrifuged for 10 minutes at maximum speed, and the supernatant was incubated with 30 µl MagReSyn magnetic streptavidin beads (ReSyn Biosciences) overnight at +4 °C. The next day, samples were washed with three stringent buffers (buffers 1-3): twice with buffer 1 (10% SDS), once with buffer 2 (500 mM NaCl, 50 mM HEPES, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholic acid) and once with buffer 3 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% NP-40, 0.5% deoxycholic acid). The proteins were then eluted from the magnetic streptavidin beads by incubating the samples in 2x SDS sample buffer and 10 µM biotin at 70°C for 10 minutes before performing western blot to verify the presence of biotinylated proteins in the samples.

In-gel trypsin digestion was performed to prepare samples for mass spectrometry. Samples were loaded into wells of a precast 4–20% gradient polyacrylamide gel (Bio-Rad), and subjected to gel electrophoresis until the samples had entered the gel. After staining with Coomassie blue, the gel was washed with double-distilled H_2O: first four times for 10 minutes, and then overnight at +4 °C. Bands of protein were cut out from the gel, washed twice with 0.04 M NH_4HCO_3/50% acetonitrile (ACN) for 15 minutes, and incubated in 100% ACN for 10 minutes. Samples were incubated in DTT reducing agent (Sigma-Aldrich) for 30 minutes at 56 °C, before adding 100% ACN and incubating for 10 minutes. Proteins were then alkylated with 55 mM iodoacetamide (Sigma-Aldrich) in 100 mM NH_4HCO_3 for 20 minutes at room temperature, protected from light. Next, samples were washed twice with 100 mM NH_4HCO_3, before adding 100% ACN, and centrifuging in a vacuum centrifuge. Proteins were digested with 0.005 µg/µl trypsin (Promega) in 40 mM NH_4HCO_3/10% ACN, first for 20 minutes at +4 °C, and then for 16 hours at +37 °C. Peptide extraction was done by incubating samples in 100%

ACN for 15 minutes at +37 °C, followed by incubation with 50% ACN/5% formic acid (Thermo Fisher Scientific) for 15 minutes at +37 °C. After each extraction step, the supernatant was collected and dried in a vacuum centrifuge. Finally, the extracted peptides were dissolved in 2% formic acid before mass spectrometry.

4.11 Proliferation assay (I)

An equal number of cells were seeded into wells on 96-well plates, with one plate per timepoint measurement. At each timepoint, cell growth was measured by adding WST-8 reagent (Sigma-Aldrich) directly to the cells. As the WST-8 is bioreduced by cellular dehydrogenases, an orange formazan dye is produced. This colour shift, which is proportional to the amount of viable cells, was measured using an absorbance plate reader (Thermo Fisher Scientific). The relative proliferation of cells was analysed by normalising the subsequent timepoint measurements to the first.

4.12 Colocalisation analysis (I, II, III)

Colocalisation between proteins of interest in microscopy images was analysed using two different plug-ins for Fiji. The ComDet plug-in was used to detect spot-to-spot colocalisation in regions of interest, where a user-defined distance threshold determines whether or not two spots colocalise. Spots are first detected in each image channel independently based on particle size thresholds, before their location is compared to each other. The analysis tells you the fraction of spots from one channel that colocalise with the spots in another channel, and vice versa, expressed as a percentage. The Coloc2 plug-in was used to determine colocalisation based on the pixel intensities of two image channels in regions of interest. The resulting Pearson's coefficient indicates how well the pixel intensities in the two channels correlate with each other.

4.13 Figures and illustrations (I, II, III)

Figures and illustrations for the original publications were created using Adobe Illustrator. The schematic illustrations in this thesis were created using the BioRender software https://BioRender.com under an active academic license.

5 Results

5.1 SORLA promotes HER2 recycling and sustained oncogenic signalling (I)

5.1.1 SORLA co-traffics with HER2 and facilitates HER2 signalling from the plasma membrane

Even though HER2 is a well-established oncogenic receptor tyrosine kinase, there have been relatively few studies that investigate its trafficking. We noticed that the sorting protein SORLA was highly expressed in breast cancer cell lines with a HER2 amplification compared to non-HER2-amplified breast cancer cell lines (I, Fig. 1a). Among the HER2-amplified cell lines, HER2 protein expression levels also correlated with levels of SORLA on the cell surface (I, Fig. 1b). This prompted us to look further into the potential link between SORLA and HER2.

Using microscopy, we examined the subcellular localisation of HER2 in HER2-amplified breast cancer cell lines with high (BT-474), intermediate (MDA-MB-361) and low (JIMT-1) expression of SORLA, and found that HER2 was localised to the plasma membrane in BT-474 cells, but was increasingly more intracellular in the SORLA-intermediate and SORLA-low cell lines (I, Fig. 1c). Co-staining of endosomal markers together with HER2 and/or SORLA in the SORLA-intermediate and SORLA-low cell lines revealed that the proteins localise to early endosomes (EEA1 and Rab5), retrograde vesicles (VPS35) and recycling endosomes (Rab11), but not to late endosomal (Rab7) or lysosomal (LAMP-1) compartments (I, Figs. 1d, S1c-d). These experiments also indicated that the subcellular distribution of HER2 correlated with SORLA levels, as HER2 was mainly restricted to the plasma membrane in SORLA-high cell lines, but was distributed to both plasma membrane and endosomes in SORLA-intermediate and SORLA-low cell lines. We validated this further in these three cell lines with flow cytometry measurements, showing that HER2 cell surface levels increase with higher SORLA expression (I, Fig. S1e).

Next, we performed co-immunoprecipitation experiments to study the association between SORLA and HER2 in cells, and found that the two proteins co-precipitate (I, Fig. 1e). We studied the association further by examining the binding and localisation of truncated versions of the different domains of the SORLA-GFP

fusion protein using pulldown experiments and microscopy, and found that while the extracellular domain of SORLA is required for association with HER2, the cytosolic domain of SORLA is needed for correct endosomal localisation in cells (I, Figs. 1f-g, S2a-c).

As the level of SORLA expression and the amount of intracellular HER2 seemed to be inversely correlated in our cell lines, we suspected that SORLA could be regulating the distribution of HER2 between the cell surface and the cytoplasm. Knockdown of SORLA in the SORLA-high BT-474 cell line with HER2 predominantly on the plasma membrane resulted in a substantial decrease in both cell surface and total HER2 protein levels. Conversely, overexpression of SORLA in the SORLA-intermediate MDA-MB-361 and the SORLA-low JIMT-1 cell lines with more intracellularly distributed HER2 resulted in a significant increase in both cell surface and total HER2 protein levels (I, Figs. 2a-c). These effects were due to protein-level changes, as *ERBB2* mRNA levels remained unchanged (I, Fig. S3a).

Perhaps unsurprisingly, with HER2 being a driver of proliferation, these loss- and gain-of-function experiments also affected cell proliferation. Loss of SORLA and the subsequent reduction in cell surface HER2 levels resulted in decreased BT-474 cell proliferation, while SORLA overexpression and the subsequent increase in cell surface HER2 levels stimulated the growth of JIMT-1 cells (I, Figs. 2d-e, S3d-f). These effects seemed to be restricted to HER2-amplified cells, as SORLA knockdown in the non-HER2-amplified MFM-223 breast cancer cell line had no effect on cell proliferation (I, Fig. S3g).

In order to better understand the underlying signalling behind these changes in cell proliferation, we looked for potential changes in activity in the PI3K/Akt and MAPK pathways, and found that SORLA knockdown resulted in reduced phosphorylation of both Akt and its downstream target 4E-BP1, as well as a decrease in cyclin D1 protein levels. In contrast, no changes in Erk1/2 phosphorylation were detected (I, Figs. 2f, S3h). This indicated that the loss of SORLA negatively affects HER2-dependent signalling via the PI3K/Akt pathway, leading to reduced cell proliferation in these HER2-amplified breast cancer cell lines. We further validated the role of SORLA in cell proliferation by performing SORLA knockdown followed by rescue using either full length SORLA or the truncated SORLA fragments. Only the full length SORLA construct was able to fully rescue cell proliferation to control levels (I, Figs. 2g-h, S3i). To test the effect of SORLA knockdown in an in vivo setting, we injected control and shSORLA-silenced MDA-MB-361 cells into the mammary ducts of NOD.SCID mice. Multiple ductal carcinoma in situ (DCIS) tumours formed in the mice injected with control cells, whereas the formation of DCIS tumours in the mice injected with shSORLA-silenced cells was almost fully repressed (I, Fig. 2i).

The changes in cell surface HER2 levels upon SORLA knockdown or overexpression led us to hypothesise that SORLA could be regulating the trafficking of HER2 by promoting its recycling to the plasma membrane. To test this, we utilised microscopy to examine HER2 localisation after SORLA knockdown in BT-474 cells, which normally retain their HER2 on the cell surface, and observed a significant increase in intracellular HER2 (I, Figs. 3a-b). Treating the cells with primaquine to inhibit endosomal recycling mirrored this effect (I, Figs. 3c-d), which suggested that these cells actively and rapidly recycle their intracellular pool of endocytosed HER2. A HER2 internalisation assay utilising fluorophore-conjugated trastuzumab showed an accumulation of intracellular HER2 in SORLA-silenced MDA-MB-361 cells compared to control cells after 30 minutes of internalisation (I, Figs. 3e-f). We then measured the ratio of HER2 that was trafficked back to the cell surface, and observed a significant reduction in HER2 recycling back to the cell surface in the SORLA-silenced cells (I, Fig. 3g). Conversely, when we overexpressed SORLA-GFP in the SORLA-low JIMT-1 cell line, which has a higher ratio of intracellular HER2 at steady state, there was an increase in rapid recycling of HER2 to the cell surface (I, Figs. 3h-i, S4a). Combined, these results indicated that SORLA promotes the recycling of HER2 to the plasma membrane.

5.1.2 SORLA knockdown results in HER2 lysosomal aggregation, defective HER2 signalling and drug sensitivity

The intracellular accumulation of HER2 in SORLA-silenced cells prompted us to investigate whether loss of SORLA leads to altered trafficking of the intracellular pool of HER2. Co-staining of HER2 and LAMP-1 revealed that HER2 accumulated in late endosomal/lysosomal compartments in SORLA-silenced cells (I, Fig. 4a). Interestingly, this was not accompanied by efficient HER2 degradation, as HER2 protein levels remained almost unchanged (I, Fig. S5a). The SORLA-silenced cells displayed a perinuclear aggregation of enlarged LAMP-1- and CD63- (LAMP-3) positive lysosomal compartments, which was absent in control cells (I, Figs. 4b-c, S5b-d). Interestingly, simultaneous knockdown of both SORLA and HER2 partially alleviated the lysosomal aggregation, suggesting that the phenotype is partially caused by altered trafficking causing HER2 rerouting into lysosomes (I, Fig. 4d).

After further validation of our observation of lysosomal enlargement using transmission electron microscopy (I, Fig. 4e), which suggested potential maturation defects, we set out to measure the activity of the lysosomes. To test this, we utilised a DQ Red BSA kit to monitor the loss of quenching of a fluorogenic BSA substrate upon proteolysis, and found that SORLA-silenced cells displayed significantly lower signal intensity, indicative of deficient proteolytic activity (I, Figs. 4f, S5f).

Cationic amphiphilic drugs (CADs) can be used to target the lysosomal instability in cancer cells, causing them to aggregate and making their membranes more permeable (Petersen et al., 2013; Ellegaard et al., 2016). We hypothesised that we could utilise CADs to target the already dysfunctional lysosomes of SORLA-silenced cells. To test this, we treated the anti-HER2 therapy-sensitive BT-474 and the anti-HER2 therapy-resistant MDA-MB-361 cell lines with the CAD ebastine. SORLA knockdown combined with ebastine treatment had an additive negative effect on cell viability in both cell lines, and caused a strong increase of the apoptosis marker PARP1 (I, Figs. 4g-i). Taken together, the loss of SORLA renders both anti-HER2 therapy-sensitive and resistant HER2-amplified breast cancer cell lines vulnerable to CAD treatment, presumably due to a loss in lysosomal integrity.

Next, we investigated the potential contribution of SORLA to the clinical outcome in patients. Cancer tissue microarray (TMA) data showed that 38% of HER2-amplified breast cancers have moderate or high expression of SORLA (I, Fig 5a). Moreover, analysis of large datasets (including The Cancer Genome Atlas, The European Genome Phenotype Archive and the Gene Expression Omnibus) using the online survival analysis tool Kaplan-Meier plotter indicated that high *SORL1* predicts poor overall survival for patients with HER2-amplified breast cancer (I, Fig. 5b). We then expanded our investigation to include bladder cancer, where HER2 overexpression is a common occurrence (Yan et al., 2015; Zhao et al., 2015), and analysed a TMA of 199 bladder cancer patients. Interestingly, SORLA and HER2 correlated significantly in this cancer type (I, Fig. 6a), which prompted us to investigate the relationship between SORLA and HER2 in the 5637 bladder carcinoma cell line, harbouring a HER2-activating mutation (de Martino et al., 2014). SORLA knockdown in this cell line caused a decrease in cell proliferation compared to control cells (I, Figs. 6b-c), and SORLA-silenced 5637 cells subcutaneously injected into nude mice formed significantly smaller tumours, displaying lower levels of cell proliferation, but not apoptosis, compared to control cells (I, Figs. 6d-e, S6c). Finally, we performed a colony formation assay, showing that, similarly to HER2-amplified breast cancer cells, SORLA knockdown sensitises 5637 cells to ebastine treatment (I, Fig. 6f). These results suggests that SORLA could be a viable therapeutical target not only in HER2-amplified breast cancer, but in other HER2-driven cancer types as well.

5.2 Selective uptake of integrins via the CG pathway (II)

5.2.1 Swip1 interacts with the integrin endocytic machinery and CG components

The trafficking of proteins between different subcellular compartments is an essential cellular process and a prerequisite for life. In the field of integrin research, we have been interested in understanding how cells regulate the distribution of integrins between the cell surface and the cytoplasm, enabling extracellular matrix (ECM) attachment, integrin signalling, cell survival and migration (Paul, Jacquemet and Caswell, 2015; Moreno-Layseca et al., 2019). One of the identified key controllers of integrin traffic is the small GTPase Rab21 (Pellinen et al., 2006; Alanko et al., 2015). While Rab21 binds to integrins regardless of its GDP/GTP activation status, it needs to be active in order to facilitate integrin endocytosis (Pellinen et al., 2006). However, we lacked mechanistic insight into how this occurs. In order to expand our understanding of this process, we set out to identify new interactors of Rab21 that could play a role in co-regulating integrin endocytosis.

We performed a mass spectrometry screen utilising stable isotope labelling with amino acids in cell culture (SILAC) (Hubner et al., 2010; Meyer and Selbach, 2015) in MDA-MB-231 triple-negative breast cancer (TNBC) cells expressing wild type Rab21 (WT-Rab21), a constitutively active $Rab21^{Q76L}$ mutant (CA-Rab21), or an inactive $Rab21^{T33N}$ mutant (DN-Rab21), and identified the actin-binding protein Swip1 as a potential interactor of active Rab21 (II, Figs. 1a, S1a-d). We validated this finding with both GFP and GST pulldowns, confirming that Swip1 bound to wild type and active Rab21, but not to inactive Rab21 or the closely related Rab5 (II, Figs. 1b-c, S1e). Using microscopy, we observed that Swip1 localised to Rab21-positive endosomes in MDA-MB-231 cells (II, Fig. 2a), and a proximity ligation assay (PLA) showed that endogenous Swip1 and Rab21 exist in close proximity to each other, suggesting that they interact in cells (II, Fig. 2b). Bimolecular fluorescence complementation experiments (BiFC) indicated that the two proteins colocalised mainly on EEA1- and Rab5-positive early endosomes and VPS35-positive retrograde vesicles (II, Figs. S2a-c), similarly to previous reports of Rab21 localisation (Pellinen et al., 2006; Alanko et al., 2015; Del Olmo et al., 2019). Moreover, immunostaining of active β1-integrin showed overlap between integrins, Swip1 and Rab21 on endosomes (II, Fig. S3a), suggesting that Swip1 interacts with integrin cargo together with Rab21.

We then performed live total internal reflection fluorescence (TIRF) microscopy, a technique that improves signal-to-noise ratio by restricting the excitation of fluorophores to a thin section at the interface between specimen and coverslip, and

observed Swip1-Rab21 BiFC complexes moving close to the ECM-plasma membrane interface (II, Fig. 2c). To get a more detailed view of this, we utilised structured illumination microscopy (SIM), a super-resolution technique that combines data from moiré patterns of different angles to reconstruct an image with improved resolution, and were able to distinguish Swip1, Rab21- and β1-integrin-positive structures close to the plasma membrane (II, Figs. 2d-e). To get a better idea of the nature of these structures, we co-stained for other Rab proteins, showing that these structures are specifically enriched in Rab21 compared to Rab5, Rab7 or Rab11 (II, Fig. S3c).

We hypothesised that these structures could be forming as parts of endocytic events, and went back to the list of proteins detected in our mass spectrometry screen (II, Figs. 1a, S1a-d) to look for any relevant hits. Interestingly, we found that Arf1, a regulator of CG endocytosis (Kumari and Mayor, 2008), was detected as a putative interactor of active Rab21. Indeed, we found that GFP-Swip1 and GFP-Rab21 co-immunoprecipitated with both Arf1 and IRSp53, another component of the CG endocytic machinery (Thottacherry et al., 2019) (II, Fig. 3a). Moreover, endogenous IRSp53 co-immunoprecipitated with both Swip1 and Arf1, and PLA confirmed that IRSp53 existed in close proximity to active β1-integrin in cells (II, Figs. 3b-c). Next, we used SIM microscopy to compare Swip1 colocalisation with markers of CG-, clathrin- and caveolin-mediated endocytic pathways, and found that Swip1 showed a significant preference for the CG pathway compared to the other endocytic pathways (II, Figs. 3d-e). Bimolecular complementation affinity purification (BiCAP) experiments, where interactors of two proteins of interest, tagged with complementary halves of the Venus fluorescent protein, can be isolated and identified (Croucher et al., 2016), supported this finding, as the Rab21-Swip1 complex pulled down both β1-integrin and the CG component IRSp53, but not dynamin, a component of both clathrin- and caveolin-mediated endocytic pathways (Thottacherry et al., 2019) (II, Fig. 3f). Live TIRF imaging close to the plasma membrane showed the Rab21-Swip1 complexes moving towards structures containing IRSp53, before disappearing from view (II, Fig. 3g). Taken together, these findings suggested that Swip1 interacts with the CG machinery together with Rab21.

5.2.2 Swip1 mediates integrin cargo-specific uptake via the CG pathway in an actin-dependent manner

Our findings suggested that there was a link between Swip1, Rab21 and the CG endocytic machinery. We hypothesised, that these components could be working together to facilitate integrin endocytosis through the CG pathway. To test this, we first performed a series of integrin trafficking assays in MDA-MB-231 cells. Swip1

knockdown resulted in significantly reduced intracellular active β1-integrin signal after 5, 15 and 30 minutes of internalisation (II, Fig. 4a). This effect could be fully rescued by re-expression of GFP-Swip1, and GFP-Swip1 overexpression caused a substantial increase in internalised active β1-integrin in control siRNA-transfected cells (II, Fig. 4b). The reduced intracellular integrin signal after Swip1 knockdown was not a result of decreased β1-integrin protein levels, as both total and cell surface β1-integrin expression levels remained unchanged after Swip1-silencing (II, Figs. S4b-c). We then investigated whether knockdown of Swip1 or Rab21 affected the uptake of inactive β1-integrins, and found no significant changes between the control and silenced conditions (II, Fig. 4c), indicating that Swip1 and Rab21 specifically promote the endocytosis of active β1-integrins.

In order to get a better understanding of the relationship between Swip1 and Rab21 during integrin internalisation, we wanted to see if uncoupling Rab21-integrin binding would affect the association between integrins and Swip1. To test this, we created a version of GFP-α2-integrin where the Rab21 binding motif (Pellinen et al., 2006; Alanko et al., 2015) was mutated (KR1160/1161AA). SIM imaging of the Rab21-binding-deficient α2-integrin together with mScarlet-Swip1 showed a substantial decrease in colocalisation (II, Fig. 4d). Moreover, Rab21 knockdown significantly reduced the colocalisation between Swip1 and β1-integrins (II, Fig. 4e). These results indicated that Swip1 is dependent on an intact Rab21-integrin interaction in order to mediate integrin endocytosis.

Next, we wanted to know whether Swip1 and Rab21 associated with one or several integrin heterodimers. To test this, we performed GFP pulldowns with different integrin α-subunits. Both Swip1 and Rab21 co-precipitated with many different α-subunits (II, Fig. 4f), in line with previous reports of Rab21 interacting with several α-subunits (Pellinen et al., 2006). In addition, silencing Swip1 resulted in decreased internalisation of both β1-integrin and several integrin α-subunits compared to control cells (II, Figs. 4g, S4d). When both control and Swip1-silenced cells were treated with primaquine, which blocks receptor recycling, this difference in intracellular integrin signal increased further, indicating that Swip1 silencing results in defective integrin internalisation (II, Figs. 4g, S4d).

If Swip1 and Rab21 mediate integrin endocytosis via the CG pathway, it would be reasonable to assume that a loss of a CG machinery component would impair integrin internalisation. To test this, we studied the role of IRSp53 and Arf1 in integrin internalisation. Silencing of either IRSp53 or Arf1 resulted in a substantial reduction in integrin uptake compared to control cells (II, Figs. 4h, S5a). Importantly, overexpression of GFP-Swip1, which normally boosts integrin uptake (II, Fig. S5c), was unable to increase integrin uptake in IRSp53-silenced cells (II, Fig. 4h), indicating that IRSp53 function is crucial for integrin uptake via this pathway. Moreover, overexpression of GFP-Swip1 resulted in an increase in PLA signal

between IRSp53 and active β1-integrin (II, Figs. 4i, S5d), suggesting that an increase in Swip1 activity leads to more integrins being fed into the CG pathway.

To further validate that Swip1 is endocytosed via the CG pathway, we observed the uptake of active β1-integrin together with the CG cargo dextran. A double-uptake assay confirmed colocalisation between endocytosed dextran and integrins (II, Fig. 5a). Circa 50% of the internalised active β1-integrin colocalised with dextran, while 20% colocalised with transferrin, a cargo of clathrin-mediated endocytosis, indicating that integrin endocytosis is actively occurring via both pathways in MDA-MB-231 cells (II, Fig. S5e). In order to determine whether Swip1 mediates integrin uptake specifically, or whether it regulates the uptake of a larger selection of cargos through the CG pathway, we examined the effect of Swip1 knockdown on the two CG cargos dextran and major histocompatibility complex I (MHCI). Silencing Swip1 had no effect on the uptake of these cargos, while silencing of IRSp53 impaired the uptake of both dextran and MHCI, showing that, unlike IRSp53, Swip1 is not part of the core CG machinery (II, Figs. 5b-c). Rather, this suggested that Swip1 could be the first identified cargo adaptor for the CG pathway, enabling cargo-specific uptake of integrins through a pathway that has been considered to internalise cargo in a non-specific manner.

To validate these findings further, we studied integrin and MHCI uptake in two other TNBC cell lines, MDA-MB-468 and BT-20. Silencing Swip1 had the same effects in these cell lines, with integrin uptake being impaired, while MHCI uptake remained unaffected (II, Figs. 5d, S6a). Moreover, we tested the role of Swip1 in the uptake of transferrin, which is internalised via clathrin-mediated endocytosis, and EGFR, which is internalised via both clathrin-mediated and clathrin-independent but dynamin-dependent endocytic pathways (McMahon and Boucrot, 2011; Caldieri et al., 2017), and found these pathways to be unaffected by Swip1 knockdown (II, Figs. S6c-d). Taken together, these findings suggest that Swip1 specifically promotes integrin uptake via the CG pathway by acting as a cargo-specific adaptor, while leaving the uptake of other cargo unaffected.

We then investigated the effect of CG pathway activation on integrin uptake and whether Swip1 is required for this process. To test this, we treated MDA-MB-231 cells with a hypotonic medium, before switching back to an isotonic medium, resulting in relaxation of membrane tension, which is known to activate CG endocytosis (Thottacherry et al., 2018). This treatment stimulated CG endocytic activity in both control and Swip1-silenced cells, as dextran uptake was significantly increased without being affected by Swip1-silencing. However, Swip1 knockdown blocked the increase in integrin uptake upon CG pathway activation (II, Fig. 5e), validating that Swip1 is needed specifically for CG-mediated integrin uptake.

Next, we investigated the mechanism of cargo specificity further. Knockdown of Rab21 decreased the colocalisation between Swip1 and IRSp53, indicating that

Rab21 is required both for integrin binding (II, Fig. 4e) and for correct recruitment to the CG pathway (II, Fig. 5f). Interestingly, Swip1 interacted with the inactive form of Arf1, but not with active Arf1 (II, Fig. 5g). As Arf1 has been shown to localise to the plasma membrane before CG endocytic events occur (Lundmark et al., 2008; Sathe et al., 2018), it is feasible to assume that inactive Arf1 binds to Swip1, which, in turn, binds active Rab21-bound integrin cargo, forming a pre-assembled complex prior to internalisation at sites of CG endocytosis.

Considering that Swip1 is an actin-binding protein (Kwon et al., 2013), and that actin has been shown to be important for CG endocytosis (Hemalatha and Mayor, 2019), we asked whether Swip1 requires actin binding to facilitate integrin endocytosis. To test this, we created two deletion constructs lacking either the first or the second EF-hand domain of Swip1 (EF1 or EF2), which have been suggested to mediate actin binding (Park et al., 2016), and performed GFP pulldown assays. The lack of EF1 resulted in both loss of actin binding and an inability to stimulate integrin uptake, while full length Swip1 and Swip1 lacking EF2 retained both of these functions (II, Figs. 6a-c). Moreover, Swip1 and β1-integrin were found together with actin on structures close to the cell surface (II, Fig. 6d). These results indicate that Swip1 requires its actin binding function in order to facilitate integrin internalisation.

We then turned our focus to intracellular events, where Swip1 and F-actin localised around Rab21-positive endosomes in a punctate distribution (II, Fig. 6e). After validating the endosomal distribution of Swip1 using electron microscopy (II, Fig. 6f), we set out to investigate the effects of Swip1-silencing. Swip1 knockdown reduced vesicle migration speed, and caused Rab21-positive endosomes to localise mostly to the cell periphery (II, Fig. 6g). The vesicle movement was actin-dependent, as inhibition of actin polymerisation using cytochalasin D reduced vesicle speed (II, Fig. 6h). Moreover, while re-expression of full-length Swip1 fully rescued vesicle movement speed, the EF1 deletion construct was unable to do so (II, Fig. 6i). Thus, Swip1 requires its actin binding function to enable both selective integrin uptake via the CG pathway and intracellular traffic of Rab21-positive endosomes (II, Fig. 6j).

5.2.3 Swip1 regulates adhesion turnover, migration and invasion and is a prognostic factor in TNBC

Cells rely on a constant and dynamic balance between integrin adhesion formation and disassembly in order to migrate and invade through their surroundings (Paul, Jacquemet and Caswell, 2015; Moreno-Layseca et al., 2019). It seemed likely to us that the internalisation of integrins through the CG pathway would play a role in maintaining this balance. Therefore, we utilised microscopy to examine vinculin-positive focal adhesions after knockdown of either Swip1, Arf1 or IRSp53. We

found that silencing Swip1 or the CG pathway components resulted in focal adhesion build-up, indicative of defective focal adhesion disassembly (II, Figs. 7a, S8b). We then followed the dynamics of paxillin-positive adhesions in live cells, and observed significantly slower focal adhesion turnover rates in Swip1-silenced cells (II, Fig. 7b). Live-cell imaging of Swip1-Rab21 BiFC complexes together with paxillin revealed a pattern to their localisation, with a significant proportion of the BiFC complexes localising close to focal adhesions (II, Fig. 7c). These results suggest that the Swip1-Rab21 interaction occurs at focal adhesion sites, where Swip1 regulates focal adhesion turnover by mediating integrin internalisation through the CG pathway.

We then investigated how the slower adhesion dynamics in Swip1-silenced cells affected cell migration and invasion. Wound-healing experiments showed significantly slower migration speeds for both Swip1- and Rab21-silenced MDA-MB-231 cells (II, Figs. 7d, S8c), and a random migration assay showed that both MDA-MB-231 and MDA-MB-468 cells migrated more slowly after Swip1 knockdown (II, Fig. S9). Cell invasion was also affected, as MDA-MB-231 cells displayed reduced invasion through a three-dimensional collagen matrix after Swip1 knockdown (II, Fig. 7e).

Finally, we studied the potential clinical relevance of Swip1 in breast cancer. Analysis of Swip1 mRNA levels in a cohort of 192 breast cancer samples showed that Swip1 was highly expressed in 65% of HER2+ and 70% of TNBC samples, subtypes which are seen as particularly aggressive (II, Fig. S10a). Swip1 staining of HER2+ and TNBC tissue microarrays also displayed high Swip1 expression (75% and 65% of samples, respectively), and revealed a significant association between high Swip1 expression and worse clinical outcome specifically in TNBC (II, Fig. 7f). Keeping in mind that Swip1 facilitated integrin uptake from the cell surface, we quantified the amount of Swip1 at the membrane in these samples, and found that samples with high levels of Swip1 at the cell membrane had substantially worse overall survival compared to samples with medium or low Swip1 membrane levels (II, Figs. S10c-d). This remained true after correcting for Ki67-positivity, tumour size, lymph node metastasis and tumour grade. Moreover, TNBC samples with high membranal Swip1 expression had significantly more lymph node metastases (II, Fig. S10e). Our results show that high Swip1 expression is an independent prognostic factor for lymph node metastasis and worse overall survival among TNBC patients.

5.3 EPLINα promotes integrin recycling and breast cancer cell migration (III)

5.3.1 EPLINα regulates integrin recycling from Rab21-positive early endosomes in an actin-dependent manner

Cell adhesion and migration is dependent on the tightly regulated trafficking of integrins (Paul, Jacquemet and Caswell, 2015; Moreno-Layseca et al., 2019). In our previous study (original publication II), we found that Swip1 acts as the link between active Rab21-bound integrins and the CG endocytic pathway, facilitating integrin internalisation via this route. However, the fate of this internalised, Rab21-bound integrin cargo remained unexplored.

The same mass spectrometry screen that identified Swip1 in our previous study, also identified the actin binding protein EPLIN as a putative interactor of active GTP-bound Rab21 in MDA-MB-231 TNBC cells. EPLIN has two isoforms (α and β) with identical amino acid sequences apart from a unique N-terminal stretch of amino acids in the β-isoform (III, Fig. 1a). MDA-MB-231 cells expressed both isoforms of EPLIN, with EPLINα being the predominant, strongly expressed one (III, Fig. 1b). We used microscopy to examine the localisation of endogenous total EPLIN in cells, and found that it overlapped with the actin cytoskeleton, namely the actin cortex, lamellipodia and stress fibres, as has been reported previously (Maul et al., 2003; Linklater et al., 2021), but was also found to overlap with actin puncta on endosome-like structures (III, Fig. 1c). We then co-expressed both isoforms in cells and compared their localisation. Both isoforms localised to lamellipodia and the cortex, but while EPLINβ localised strongly to stress fibres, EPLINα was found to overlap with structures resembling vesicles (III, Fig. 1d). To get a closer look at these endosome-looking structures, we used SIM imaging to visualise EPLINα together with F-actin and Rab21. Interestingly, EPLINα overlapped clearly with F-actin puncta around the Rab21-positive endosomes (III, Fig. 1e). Airyscan super-resolution imaging, which utilises 32 detector elements that act as separate pinholes with their own point spread functions to enhance resolution, validated this finding, showing overlap and a similar intensity distribution between endogenous EPLIN and F-actin on Rab21-positive endosomes containing active β1-integrin cargo (III, Fig. 1f). Moreover, Rab21-EPLINα BiFC complexes localised to endosomes together with F-actin (III, Fig. 1g). These data suggested that EPLINα interacts with Rab21 and F-actin on endosomes.

To confirm that EPLIN and Rab21 interact, we first performed GFP-pulldowns, showing that the two proteins associate in cells (III, Fig. 1h). We then studied the direct binding between GST-EPLINα and either GTP-Rab21 (active) or GDP-Rab21

(inactive) using microscale thermophoresis, a technique that measures the interaction of two molecules by detecting changes in the fluorescent signal of a fluorescently labelled target after a change in temperature is induced by an infrared laser, as well as the movement of the molecules along the temperature gradient created by the laser. These experiments showed a strong direct binding between EPLINα and active Rab21, while binding between EPLINα and inactive Rab21 was observed only faintly (III, Figs. 1i-j, S1a-b).

Our observation of EPLIN localising to Rab21-positive endosomes containing integrin cargo (III, Fig. 1f), combined with Rab21 being an established regulator of integrin traffic, led us to hypothesise that EPLINα could be playing a role in the regulation of integrin traffic. To test this, we compared the amount of internalised cell surface-labelled active β1-integrin in control- and EPLIN-silenced cells. Silencing of total EPLIN (both isoforms) resulted in a substantial accumulation of active β1-integrin inside the cells (III, Fig. 2a). In contrast, silencing only EPLINβ did not have any effect on intracellular integrin levels, suggesting that EPLINα was the isoform regulating integrin traffic. We suspected that the intracellular accumulation of integrins upon EPLIN knockdown could be caused by impaired integrin recycling, and investigated this possibility by performing a previously established fluorescence-quenching-based integrin recycling assay (Mai et al., 2011), where we measured the remaining signal from internalised cell surface-labelled active β1-integrins after a recycling step. In this assay, the internalisation step is performed in the absence of serum to prevent constitutive recycling, followed by a recycling step in the presence of serum (Powelka et al., 2004). Significantly more intracellular integrin signal remained in EPLIN-silenced cells after recycling, confirming a defect in recycling (III, Fig. 2b).

Further examination of EPLINα and F-actin on Rab21-positive endosomes using both Airyscan imaging and live-cell imaging revealed that EPLINα and F-actin localised to the base of tubular structures forming from the endosomes (III, Figs. 2c-d). These endosomes colocalised with the early endosomal markers EEA1 and Rab5 in the perinuclear region (III, Fig. 2e), in line with previous reports of Rab21 localisation (Pellinen et al., 2006; Alanko et al., 2015; Del Olmo et al., 2019). Taken together, our findings suggested that EPLINα facilitates integrin recycling from early endosomal compartments, potentially by regulating endosomal tubulation events that enable recycling from early endosomal compartments (Grant and Donaldson, 2009).

In order to increase our understanding of how EPLINα is recruited to endosomes, we first examined the effect of Rab21 knockdown on EPLINα localisation, and found that loss of Rab21 did not affect the localisation of EPLINα to EEA1-positive endosomes (III, Fig. S3). We then studied the role of F-actin in the recruitment process, and started by determining the location of two actin binding sites in the

EPLIN amino acid sequence, based on previous reports (Maul et al., 2003). Based on analyses of the EPLIN sequence and predicted structure using the eukaryotic linear motif (ELM) prediction tool (Kumar et al., 2024) and AlphaFold3 (Abramson et al., 2024), we created three deletion mutants of EPLINα lacking either (1) a C-terminal WH2 actin binding motif, (2) a predicted actin binding motif in a short N-terminal helix, or (3) lacking both motifs (III, Figs. 3a-b). While both single-deletion mutants showed reduced actin binding, the double-deletion mutant (ΔΔ) resulted in almost complete loss of actin binding (III, Figs. 3c-d). Moreover, while WT-EPLINα co-precipitated with the Arp2/3 subunit ARPC1B, this interaction was lost with the ΔΔ-mutant (III, Fig. 3e), suggesting that EPLINα associates with F-actin at branched actin nucleation sites.

Live imaging of the EPLINα-ΔΔ-mutant in cells showed reduced endosomal localisation compared to EPLINα-WT, suggesting that EPLINα is recruited to endosomes by F-actin (III, Fig. 3f). To test whether loss of actin binding affected the ability of EPLINα to promote integrin recycling, we re-expressed either EPLINα-WT or EPLINα-ΔΔ in EPLIN-silenced cells, and performed integrin recycling experiments. EPLINα-WT fully rescued active β1-integrin recycling back to control levels, whereas EPLINα-ΔΔ was unable to rescue integrin recycling (III, Fig. 3g). Importantly, re-expression of EPLINβ did not rescue integrin recycling in EPLIN-silenced cells, further validating that, out of the two isoforms, EPLINα is responsible for facilitating integrin recycling (III, Fig. S2).

5.3.2 BioID identifies EPLIN isoform-specific interactors

BioID utilises a promiscuous biotin ligase (BirA*), which biotinylates proteins in its immediate vicinity. Fusing the biotin ligase to a protein of interest and expressing it in cells results in biotinylation of proximal proteins, allowing affinity-based isolation and detection of putative interactors of the protein of interest (Roux et al., 2018). In order to better understand the differences between the isoforms of EPLIN, we created BirA*-tagged constructs of EPLINα and EPLINβ (III, Figs. 4a, S4a), and performed proximity-dependent biotinylation followed by mass spectrometry to identify the isoform-specific interactomes in MDA-MB-231 and HCC1937 TNBC cells. The screen identified 91 proximity interactors, many of which were linked to cellular processes related to the actin cytoskeleton and cell adhesion, based on Gene Ontology (GO) analyses (III, Figs. 4b, S4b-c), in line with the known functions of EPLIN (Collins et al., 2015). The screen also identified putative interactors that preferred one isoform over the other in either one or both of the cell lines (III, Figs. 4c, S4d). In fact, even shared interactors showed differential enrichment scores between the two isoforms (III, Fig. 4d). Thus, the two EPLIN isoforms may influence their shared interactors in different ways, depending on e.g. the duration, distance

and cellular location of the interactions. For EPLINα, the preferred interactors included proteins such as the cytoskeletal protein zyxin (ZYX) (Wu et al., 2024), the early endosomal protein SYNJ1 (Fasano et al., 2018), and cortactin (CTTN), which is an Arp2/3 activator implicated in endosomal cargo recycling (Puthenveedu et al., 2010). Proximity interactors that preferred EPLINβ included the actin crosslinker filamins (FLNB and FLNC) (Zhou, Hartwig and Akyürek, 2010) and calponins (CNN2 and CNN3), which are linked to various actin regulatory functions (Liu and Jin, 2016).

Besides CTTN, the shared interactors supervillin (SVIL) and coronin-1c (CORO1C) have also been reported to regulate endosomal traffic. SVIL is a known EPLIN interactor (Oakes et al., 2009; Smith, Fang and Luna, 2010), and has been shown to promote integrin recycling (Fang et al., 2010). CORO1C, is an actin binding protein that localises to both lamellipodia and the actin cortex, as well as to sorting endosomes, where it regulates endosome fission (Puthenveedu et al., 2010; Hoyer et al., 2018; King et al., 2022; Striepen and Voeltz, 2022). Based on the literature, EPLIN and CORO1C localised to many of the same cellular structures, including endosomes, where EPLINα localised in our experiments. As the potential role of CORO1C in integrin trafficking had not been studied, we chose to explore this avenue.

5.3.3 CORO1C regulates integrin recycling downstream of EPLINα

While the WASH complex and Arp2/3-mediated actin branching initiate endosome fission and constrict the endosomal membrane (Duleh and Welch, 2010), recent findings suggest that the later steps require halting of actin branching to allow access for other components in the fission machinery (Dhawan, Naslavsky and Caplan, 2022). With both CORO1C and EPLIN being known inhibitors of Arp2/3 (Maul et al., 2003), we reasoned that the two could act together to regulate Arp2/3 during fission of recycling endosomes.

Confocal and super-resolution microscopy revealed that CORO1C and EPLINα overlapped at F-actin puncta on Rab21-positive endosomes (III, Figs. 5a-b). We then performed integrin trafficking assays, showing that loss of CORO1C impaired integrin recycling (III, Fig. 5c). GFP pulldown experiments showed that CORO1C and EPLIN associated with Rab21 in cells (III, Fig. S5a), and PLA between CORO1C and EPLIN confirmed that these proteins exist in close proximity to each other, with PLA signal close to the plasma membrane as well as on EEA1-positive early endosomes (III, Figs. 5d, S5c). Interestingly, the association on early endosomes was lost upon knockdown of Rab21 (III, Fig. 5d), implying that Rab21 is required for CORO1C-EPLIN endosomal association. Silencing of EPLIN

resulted in reduced CORO1C localisation to endosomes without affecting CORO1C protein levels (III, Fig. 5e), whereas silencing of CORO1C had no significant effect on the endosomal localisation of EPLIN (III, Fig. S5d). This indicated an order of events where EPLINα is first recruited to endosomal F-actin, where it interacts with active Rab21, followed by recruitment of CORO1C and the subsequent promotion of integrin recycling.

5.3.4 EPLINα promotes cell migration and invasion and correlates with a mesenchymal phenotype

Next, we assessed the potential clinical relevance of our findings. First, we analysed EPLIN protein expression in lysates from a cohort of 105 breast cancer patients, and categorised them into EPLINα-high, EPLINβ-high, equal expression, or no expression, based on their EPLIN isoform expression pattern (III, Figs. 6a-b). Interestingly, EPLINα was highly expressed in a large fraction of ER-negative and triple-negative breast cancer tumours (III, Figs. 6c-d), and survival analysis showed a trend of poorer overall survival, albeit not reaching statistical significance (III, Fig. 6e). EPLINα-high samples also expressed higher mRNA-levels of vimentin (III, Fig. 6f), suggesting a potentially more mesenchymal phenotype for these tumours. In addition, EPLINα-high samples had high mRNA-levels of CORO1C (III, Fig. 6g), in line with previous reports of a pro-invasive role in TNBC (Castagnino et al., 2018).

To test whether the potential correlation between high levels of EPLINα and a more mesenchymal phenotype remained true in our cell lines, we examined the EPLIN isoform expression pattern in the breast cancer cell lines HCC1937, T47-D, MCF7, MDA-MB-231 and BT549. Interestingly, the cell lines that expressed predominantly EPLINα (MDA-MB-231 and BT549) also had high vimentin protein levels, while the cell lines that expressed predominantly EPLINβ displayed low vimentin but high E-cadherin levels, an epithelial marker (III, Fig. 7a). Analysis of cell morphology showed a distinct difference between the EPLINα- and EPLINβ-high cell lines, with EPLINα-high cell lines being more elongated and EPLINβ-high cell lines being more circular in shape (III, Figs. 7b-c).

To evaluate whether these differing phenotypes correlated with differences in motility, we performed random migration experiments using all five cell lines, and found that the EPLINα-high cell lines migrated significantly faster compared to the EPLINβ-high cell lines (III, Figs. 7d, S6b). To confirm that EPLINα promotes cell migration, we studied the motility of MDA-MB-231 cells after EPLIN-silencing and re-expression of its isoforms. Total EPLIN-silencing resulted in substantially lower migration speed, and re-expression of EPLINα-WT fully rescued cell migration back to control levels (III, Fig. 7e). Importantly, re-expression of EPLINβ-WT, or the

actin binding-deficient versions of the isoforms (EPLINα-ΔΔ or EPLINβ-ΔΔ) did not rescue cell migration speed. This indicated that EPLINα promotes cell migration in an isoform-specific manner, and requires its actin binding function to do so. To assess the impact of CORO1C on cell migration, we measured cell migration speed after CORO1C knockdown, and found that CORO1C-silencing also resulted in decreased cell motility (III, Fig. 7f). Finally, we used a Matrigel invasion assay to test if EPLIN plays a role in cancer cell invasion through a three-dimensional matrix, and found cell invasion to be significantly impaired after EPLIN knockdown (III, Fig. 7g).

Taken together, our findings suggest that EPLINα promotes breast cancer cell migration by binding to endosomal actin and, in cooperation with CORO1C, facilitating the recycling of early endosomal integrin cargo back to the cell surface.

6 Discussion

6.1 SORLA and its role in HER2 traffic and promotion of oncogenicity (I)

The human epidermal growth factor receptor (HER) family of receptors include EGFR (HER1), HER2, HER3 and HER4. They reside on the cell surface, where they form homo- and heterodimers upon growth factor or ligand-independent binding, resulting in growth- and survival signalling through activation of MAPK and/or PI3K/Akt pathways (Junttila et al., 2009; Yarden and Pines, 2012). While HER2 is the only HER receptor that is unable to bind a ligand, it is the preferred dimerisation partner of the other family members. HER3 on the other hand, has impaired kinase activity, and forms heterodimers to produce signals. A HER2-amplification is found in 15-20% of breast cancers, and is an established driver of oncogenicity (Yarden and Pines, 2012). The prevailing view has been that HER2 is found primarily on the cell surface, where it binds its partners to facilitate growth factor signalling. Whether HER2 resists internalisation and resides solely on the plasma membrane, or whether the cell employs recycling machinery to retrieve it from the cytoplasm after endocytosis, has been an open question in the field. Our research (original publication I) established that HER2 undergoes endocytosis and recycling, and that HER2 recycling is mediated by SORLA, which is highly expressed in HER2-amplified breast cancer cells and associates with HER2 on endosomes to direct the receptor back to the cell surface. In doing so, SORLA promotes sustained HER2-mediated oncogenic signalling through the activation of the PI3K/Akt signalling pathway. Moreover, we show that loss of SORLA reroutes HER2 to defective lysosomes, resulting in lysosomal accumulation of the receptor. Taking advantage of the compromised lysosomal integrity, we demonstrated that loss of SORLA sensitises HER2-amplified breast cancer cells to the lysosome-targeting cationic amphiphilic drug ebastine, resulting in cancer cell death.

An interesting observation made during the study was that HER2-therapy-sensitive breast cancer cell lines had higher SORLA expression and maintained most of their HER2 on the cell surface, whereas HER2-therapy-resistant cell lines had comparatively lower SORLA expression and displayed a larger fraction of intracellular HER2. This is in line with the role of SORLA as a facilitator of HER2

recycling, and the regulation of cell surface receptor availability represents a mechanism for how cancer cells can control their cell surface signalling. Indeed, the concept of growth factor receptor trafficking as a means to regulate signalling is not a new one, and examples of this phenomenon include trafficking of EGFR and MET (Hammond et al., 2003; Sigismund et al., 2013). Additionally, keeping more of their HER2 inside of the cell could act as a mechanism of resistance against therapeutical targeting of the cell surface receptors. Indeed, caveolin-1-mediated uptake of HER2 has been shown to redistribute HER2 from the cell surface to an intracellular pool. This hides the receptor from the antibody drug trastuzumab, which requires the extracellular domain of HER2 for binding (Pereira et al., 2018). As high intratumor variability in HER2-levels contributes to anti-HER2 therapy resistance, a recent study suggests that caveolin-1 inhibition could improve anti-HER2 therapy by anchoring more HER2 receptors on the plasma membrane (Pereira et al., 2022).

While our study established that SORLA and HER2 associate on endosomes, it did not address whether this binding occurs directly or via an intermediary. This open question was answered in a follow-up study (Al-Akhrass et al., 2021), where experiments using surface plasmon resonance determined that the SORLA ectodomain binds directly to the ectodomains of both HER2 and HER3. The follow-up study demonstrated that, upon HER3 binding of the growth factor heregulin, the HER2-HER3 dimer stimulates MAPK signalling, resulting in increased transcription and protein expression of SORLA. By maintaining SORLA levels in this manner, breast cancer cells stimulate the recycling of the HER2-HER3 dimer to the cell surface, enabling continued growth factor signalling.

Our study found that SORLA and HER2 associated on EEA1- and Rab5-positive early endosomes, together with the retromer component VPS35. Since then, the trafficking route of the SORLA-HER2-HER3 complex has been characterised further (Al-Akhrass et al., 2021), revealing that the complex is recycled in a Rab4-dependent manner. The SNX27-retromer-WASH pathway is one of the main pathways of endosomal cargo retrieval (Wang et al., 2018), and the PDZ domain of SNX27 has been shown to mediate binding with the SORLA cytoplasmic tail (Huang et al., 2016). The tail of SORLA also interacts with the GGA family of proteins (Jacobsen et al., 2002), and GGA3 has been shown to mediate the recycling of the MET receptor via the Rab4-route to the plasma membrane (Parachoniak et al., 2011). Moreover, during cargo retrieval, SNX27 has been shown to mediate sequence-directed sorting to Rab4-positive tubules, facilitating rapid, direct recycling from early endosomes to the plasma membrane (Temkin et al., 2011). In our study, we found that the cytoplasmic tail domain of SORLA is required for its correct localisation, a finding that fits well with the established importance of cytoplasmic tail sorting motifs for cargo retrieval (Carosi et al., 2023). Taken together, while SORLA has been shown to mediate the trafficking of amyloid precursor protein

(APP) between early endosomes and the Golgi in neurons, it is tempting to speculate that the SORLA-HER2-HER3 complex could be recycled directly to the plasma membrane via the SNX27-retromer-WASH-Rab4 pathway in a rapid fashion in breast cancer cells.

It is well-established that HER3 signals through the PI3K/Akt pathway and mediates resistance to HER2-targeted treatments (Junttila et al., 2009; Kodack et al., 2017; Mishra et al., 2018). Targeting the trafficking pathway that promotes HER2 and HER3 recycling to the cell surface could therefore be a way to combat treatment-resistance. Following this line of thinking, further work has been put into assessing the potential of SORLA as a therapeutical target in HER2-amplified breast cancer since the publication of our study. Loss of SORLA sensitises breast cancer cells to the HER2/EGFR dual tyrosine kinase inhibitor neratinib in an in vivo zebrafish model (Al-Akhrass et al., 2021). Moreover, disrupting the ability of SORLA to support HER2-HER3 trafficking using antibody-based inhibition of SORLA on the cell surface, reverts breast cancer cell resistance to trastuzumab in 3D organoids and a mouse xenograft model (Al-Akhrass et al., 2022). Thus, SORLA is a potentially impactful target in the treatment of HER2-amplified breast cancer.

6.2 Swip1 as an integrin-specific cargo adaptor in CG endocytosis (II)

The selection of specific cargo for endocytosis is thought to be mediated by adaptor proteins that bind to cargoes at the plasma membrane, recruiting them to the endocytic site. While it is evident that this mechanism is utilised during clathrin-mediated endocytosis, the existence of mechanisms for cargo-selectivity has remained an open question in less well-characterised routes of endocytosis such as the CG pathway. In fact, the CG pathway has been considered to be a non-specific route through which the cell can internalise cargoes, such as GPI-anchored proteins, in bulk (Sabharanjak et al., 2002; Rioux and Prosser, 2023). The pathway is thought to have high endocytic capacity, and has been linked to membrane turnover during cell migration (Howes et al., 2010), as well as to the regulation of membrane tension (Thottacherry et al., 2018). Our discovery of Swip1 as the first identified cargo adaptor for the CG pathway (original publication II) offers a new dimension to CG endocytosis, and implies that other, as-yet-unknown, CG adaptors could exist as well, enabling cargo-selective uptake through the pathway. We found that Swip1 mediates the selective uptake of active β1-integrins through the CG pathway. It achieves this by bringing integrin-bound Rab21-GTP to inactive Arf1 at the site of CG endocytosis, resulting in uptake of the cargo. Through this action, Swip1 regulates integrin endocytosis, focal adhesion dynamics, and integrin-mediated cell migration and invasion. Moreover, increased Swip1 expression independently

correlates with increased metastasis and worse survival in triple-negative breast cancer.

Previous work from our laboratory established Rab21 as a regulator of integrin traffic, and showed that both active (GTP-bound) and inactive (GDP-bound) forms of Rab21 bind to integrins, but that only the active form facilitates integrin endocytosis (Pellinen et al., 2006). The question remained how the switch from inactive to active Rab21 leads to integrin internalisation. Our study answers this question, showing that Swip1 binds preferentially to active Rab21, providing a selective mechanism whereby the Rab21-bound integrin cargo is recruited for internalisation only upon Rab21 activation. More work is needed to characterise the mechanism further, e.g. related to the mechanism of Rab21 activation.

We showed that Swip1 associates with both Arf1 and IRSp53, which are established components of the CG endocytic machinery. Perhaps somewhat surprisingly, Swip1 bound directly to the inactive, but not the active form of Arf1. In the current model of CG endocytosis, Arf1 localises to the endocytic site in its inactive form, before being activated by its GEF GBF1 (Sathe et al., 2018). Therefore, we propose a model where integrin-bound active Rab21 is first recruited to the site of uptake by inactive Arf1, followed by cargo release into the CG pathway upon Arf1 activation. Furthermore, we found that Swip1-actin binding is necessary for CG-mediated integrin endocytosis. It is possible that the burst of Arp2/3-mediated actin polymerisation that occurs upon CG pathway activation is involved in funnelling the integrin-Rab21-Swip1 complex through the pathway.

Rab21 binds integrins via a GFFKR motif in the integrin α-subunit cytoplasmic tail (Pellinen et al., 2006). Swip1 associated with several different α-integrins and regulated their uptake, highlighting that Swip1 mediates the endocytosis of multiple different integrin αβ1-combinations, and implying that CG endocytosis of integrins can be active and promote cell migration on different ECM substrates. In line with this, we found that Swip1 regulates adhesion turnover on collagen I-, fibronectin- and laminin-coated surfaces, and that the Swip1-Rab21 interaction occurs in the vicinity of focal adhesions. Importantly, we showed that while Swip1 mediates integrin uptake, it does not affect the uptake of other known CG cargoes, including the fluid phase cargo dextran and the major histocompatibility complex I. Moreover, inhibiting the CG pathway itself blocked integrin endocytosis, in support of its role in internalising integrins.

Previous work has reported the interplay between IRSp53 and Cdc42, which are recruited to discrete foci at the leading edge of cells, where they promote actin polymerisation and filopodia formation to facilitate cell migration (Disanza et al., 2013). Our findings add another layer to the IRSp53-dependent regulation of cell motility, showing that the CG-mediated uptake of active integrins is dependent on IRSp53. Interestingly, in addition to regulating cell motility through integrin uptake,

we also discovered a role for Swip1 in endosomal vesicle transport. Loss of Swip1, or mutation of its actin-binding site, resulted in slower movement of Rab21-positive endosomes. Swip1 has been shown to mediate crosslinking of actin filaments (Lehne et al., 2022), and regulates actin network remodelling to prevent cofilin-mediated actin depolymerisation (Huh et al., 2013). Thus, Swip1 may act as a mediator of endomembrane-actin association, and could potentially aid in the local maintenance of actin tracks used by endosomal vesicles during locomotion, which is an interesting unexplored avenue.

Swip1 has been shown to modulate lamellipodial dynamics at the leading edge, promoting cell migration (Huh et al., 2013; Lehne et al., 2022). Thus, it is possible that, in addition to decreased CG-mediated integrin uptake and adhesion dynamics, Swip1 loss also affects cell migration due to its role in lamellipodia. Moreover, in macrophages, Swip1 has been reported to regulate the expression of NPFs such as N-WASP and WAVE2, promoting actin polymerisation (Tu et al., 2018). Whether this is also true in other cell types is an open question, but it could potentially represent another Swip1-mediated mechanism for increased cell migration. Regardless, we established that integrins are selectively internalised through the CG pathway in a Swip1- and Rab21-GTP-dependent manner, and showed that both Swip1 and Rab21 knockdown hampers the migration of triple-negative breast cancer cells. Swip1 regulates integrin-mediated adhesion dynamics, as well as invasion through a 3D collagen matrix. Finally, we found that Swip1 is highly expressed in triple-negative breast cancer patient samples, and high Swip1 expression at the cell membrane correlated strongly and independently with increased metastasis and worse overall survival, indicating that Swip1 could be a useful negative prognostic marker in triple-negative breast cancer.

6.3 Regulation of integrin recycling and cell migration by EPLINα (III)

The adhesion and motility of cells is controlled by a constant cycle of integrin endocytosis and recycling. Following our study on the role of Swip1 as a regulator of CG-mediated integrin endocytosis, we were left with open questions: What happens to the Rab21-bound active integrin cargo after it enters the cell? Is it recycled? If so, how is that regulated? To find clues that could guide us forward, we revisited the list of Rab21-interactors from the same mass spectrometry screen that identified Swip1 as an interactor of Rab21. One interesting hit was the actin crosslinking protein EPLIN. The two isoforms of EPLIN (α/β), have been shown to localise to different structures of the actin cytoskeleton. In endothelial cells, EPLINα has been shown to localise to lamellipodia, where it negatively regulates Arp2/3-mediated actin polymerisation, whereas EPLINβ localises to stress fibres and

increases their stability (Taha et al., 2019). We examined EPLIN isoform localisation in breast cancer cells, and discovered that, in addition to the aforementioned locations, EPLINα was recruited to discrete actin puncta on active β1-integrin-containing Rab21-positive early endosomes (original publication III). We found that EPLINα binds directly to Rab21-GTP on endosomes, and our trafficking assays established that EPLINα facilitates the recycling of integrins back to the cell surface. In doing so, EPLINα promotes cell motility in an actin-binding-dependent manner. To increase our understanding of EPLIN and its isoforms, we used BioID to identify the isoform-specific interactomes of EPLIN. We followed up one of the hits, coronin 1C, and showed that it localises to actin puncta on Rab21-positive endosomes together with EPLINα. Moreover, mimicking the function of EPLINα, coronin 1C promoted integrin recycling and cell migration.

EPLIN was originally named a tumour suppressor after its discovery, due to its reduced expression in a collection of epithelial cancer cell lines, including oral, prostate and breast cancer cells, compared to immortalised epithelial cells (Maul and Chang, 1999). Since then, EPLIN has been implicated in the maintenance of epithelial integrity by associating with cell-cell junctions (Abe and Takeichi, 2008; Taguchi, Ishiuchi and Takeichi, 2011), and has been reported to be downregulated in certain cancers (Liu et al., 2016; Collins et al., 2018; Gong et al., 2021). However, several recent reports have implicated EPLIN in promoting cancer progression, showing elevated EPLIN levels in head and neck, oesophageal, bile duct and primary liver cancers (Huang et al., 2022; Ma et al., 2022; Liu et al., 2024; Obulkasim et al., 2024; Zhang et al., 2024; You et al., 2025). The seemingly contradictory results in different cancers suggest that the role of EPLIN may vary depending on the cancer type and context. Moreover, further research into the individual roles of the two EPLIN isoforms is warranted.

Regulators of integrin traffic, such as Swip1 (original publication II) and Rab21, have been shown to be upregulated in breast cancer (Ye et al., 2014), and we found prominent EPLINα expression in triple-negative breast cancer cells, correlating with a mesenchymal, motile phenotype. We discovered that both EPLINα and coronin 1C localised to the base of forming endosomal tubular compartments together with actin. WASH- and Arp2/3-mediated endosomal branched actin polymerisation is an important mediator of the formation of tubular subdomains where cargo is segregated for recycling, and coronins have been shown to control tubule dynamics during cargo sorting (Puthenveedu et al., 2010; Dhawan, Naslavsky and Caplan, 2022; Striepen and Voeltz, 2022) by negatively regulating branched actin formation. The fission of endosomal tubules is thought to require local restriction of branched actin polymerisation at the site of scission, allowing the scission machinery (EHD1) to perform its task. As EPLIN has been shown to inhibit Arp2/3-mediated actin polymerisation, we propose a model where EPLINα and coronin 1C cooperate to

orchestrate actin dynamics at tubules on Rab21-positive endosomes, resulting in scission and cargo recycling. Interestingly, Rab21 has been shown to associate with the WASH and retromer complexes to ensure efficient endosomal sorting of clathrin-independent cargo (Del Olmo et al., 2019). Moreover, Rab21 has been suggested to regulate endosomal tubule fission, as Rab21-knockout HeLa cells display enlarged endosomal tubules and disrupted retromer-dependent recycling of the GLUT1 receptor (Pei et al., 2023). Taken together, these findings point towards a mechanism of integrin retrieval and recycling where Rab21 brings together integrin cargo with cargo retrieval machinery on the early endosome, leading to the partitioning of integrins in tubular subdomains. The formation of these tubules is powered by local branched actin network polymerisation, which recruits EPLINα. EPLINα acts by both selecting the Rab21-bound integrin cargo for retrieval and by regulating branched actin network dynamics at the tubule together with coronin 1C, finally allowing EHD1-mediated tubule scission. The net result is the promotion of integrin recycling, which facilitates integrin-mediated adhesion and sustained cell motility.

Our BioID data revealed a range of putative interactors associated with the actin cytoskeleton, and while many interactors were shared between the two EPLIN isoforms, we also found isoform-dependent differences in the two cell lines. Moreover, even shared interactors showed differences in enrichment, possibly reflecting the differential localisation of the two isoforms. In other words, even though the two isoforms can bind the same interactor, they can potentially serve different functions due to the interaction occurring in different parts of the cell. The early endosomal protein SYNJ1 is an interesting putative EPLINα-interactor, as it has been reported to mediate cargo recycling from early endosomes (Fasano et al., 2018). Thus, it could be implicated in EPLINα-mediated integrin recycling. Cortactin, which interacted preferably with EPLINα in MDA-MB-231 cells, is an established regulator of endosomal branched actin dynamics, and has been reported to localise to the base of endosomal tubules together with WASH and Arp2/3 (Chakrabarti, Lee and Higgs, 2021), akin to the endosomal localisation pattern of EPLINα in our study. Thus, while cortactin also plays a role in the regulation of lamellipodia, where EPLINα also localises, it is tempting to speculate that the two proteins could coordinate branched actin dynamics on endosomes. EPLINβ has been linked to the maintenance of actin cytoskeleton stability (Taha et al., 2019), and localises strongly to stress fibres in our study. In line with this proposed role as an actin stabiliser, many putative interactors of EPLINβ function by increasing actin cytoskeleton resistance and stability, such as calponins (CNN2 and CNN3) and filamins (FLNB and FLNC), or by regulating the stability of epithelial cell junctions (SHROOM3) (Nishimura and Takeichi, 2008; Hsieh and Jin, 2023; Katsuta, Sokabe and Hirata, 2024).

Lastly, while we show that EPLINα localises to integrin-containing Rab21-positive early endosomes, and establish that it facilitates integrin recycling and cell migration, we also observed EPLINα in other structures of the actin cytoskeleton, such as the actin cortex and lamellipodia. The regulation of lamellipodial actin dynamics at the leading edge is important for cell migration, and it is therefore possible that our loss- and gain-of function experiments could have affected cell migration through other mechanisms at the leading edge. In the future, an increase in our understanding of Rab21-EPLIN binding could allow us to create EPLIN constructs that are unable to bind Rab21, which could aid us in pinpointing the contribution of EPLIN on endosomes.

7 Conclusions and summary

The purpose of this thesis was to identify and characterise new mechanisms that regulate the trafficking of integrins and the HER2 receptor in breast cancer cells. We solidified the importance of HER2 recycling for its oncogenic potential, and built on previous work to discover how the small GTPase Rab21 mediates integrin endocytosis. Our discovery of Swiprosin-1 as the first cargo-specific adaptor for the CLIC/GEEC pathway sheds new light on the function of the pathway, and indicates the possible existence of other cargo adaptors that could enable specific uptake of other cargoes through the pathway. Moreover, the identification of EPLINα as a mediator of integrin recycling adds a new player to the list of regulators in the cargo retrieval machinery. Importantly, the work in this thesis underlines the importance of the concerted interplay between integrin endocytosis and recycling for cancer cell adhesion and migration, increases our understanding of how breast cancer cells control receptor trafficking to promote their growth and motility, and highlights how cancer cells take advantage of endosomal trafficking mechanisms to promote their malignancy.

Original publication I

We found that HER2 undergoes cycles of internalisation and recycling in HER2-amplified breast cancer cells. HER2 associates with the sorting receptor SORLA on endosomes, and SORLA promotes HER2 recycling back to the cell surface. By directing HER2 to the cell surface, SORLA supports HER2 activity, allowing HER2 oncogenic signalling through the PI3K/Akt pathway to support cell proliferation (Figure 6). Loss of SORLA reroutes the HER2 receptor to lysosomes, disrupts HER2-driven signalling and compromises lysosomal integrity. Taking advantage of the reduced lysosomal integrity, we showed that SORLA knockdown sensitises HER2-amplified breast cancer cells to the cationic amphiphilic drug ebastine. Taken together, our findings highlight the central role of growth factor receptor trafficking, utilised by cancer cells to promote growth, survival and therapy-resistance through the control of growth factor receptor presence on the cell surface, and presents a new potential avenue for the development of therapies against HER2-amplified cancer.

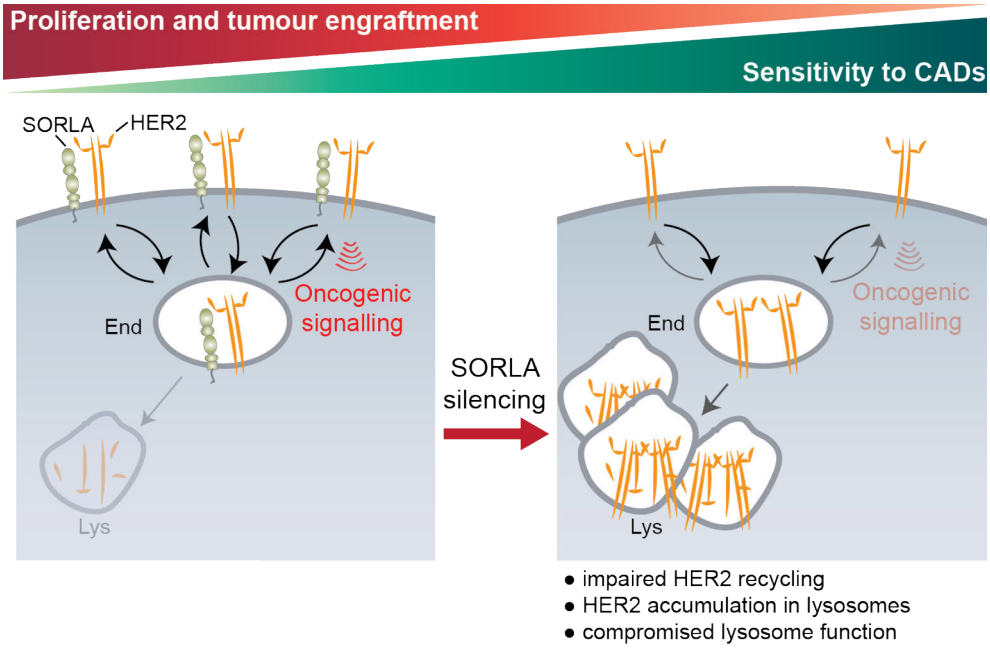

Figure 6. Summary of original publication I. SORLA co-traffics with HER2 and directs intracellular HER2 back to the cell surface to maintain HER2 oncogenic signalling. Loss of SORLA diverts HER2 to lysosomes, compromises lysosome function and sensitises the cells to the cationic amphiphilic drug (CAD) ebastine. Image from original publication I, fig. 7. End = Endosome, Lys = Lysosome.

Original publication II

We identified the actin-binding protein Swiprosin-1 as the first cargo adaptor for the clathrin- and dynamin-independent CLIC/GEEC endocytic pathway. Swiprosin-1 interacts with active Rab21, an established regulator of integrin traffic, and recruits Rab21-bound active integrins to the CLIC/GEEC pathway by binding to inactive Arf1, a component of the CLIC/GEEC endocytic machinery (Figure 7). Loss of Swiprosin-1 or the CLIC/GEEC component IRSp53 resulted in inhibition of integrin endocytosis and disrupted focal adhesion turnover. The uptake of integrins through the CLIC/GEEC pathway is dependent on Swiprosin-1-actin binding, as disruption of the EF1-hand domain of Swiprosin-1, responsible for actin binding, rendered Swiprosin-1 unable to mediate integrin endocytosis. We discovered that Swiprosin-1 is highly expressed in triple-negative breast cancer, and its knockdown decreased both cell motility and invasion. Finally, we found Swiprosin-1 to be an independent negative prognostic marker for triple-negative breast cancer. Our work provides important new insights into integrin endocytosis and the clathrin- and dynamin-independent mechanism utilised in the CLIC/GEEC pathway for integrin uptake.

The identification of a cargo-specific adaptor for the CLIC/GEEC pathway challenges the view of the pathway being a non-specific way for cells to internalise cargoes in bulk. Moreover, our findings underline the importance of integrin adhesion turnover and integrin trafficking for the migratory potential of cancer cells.

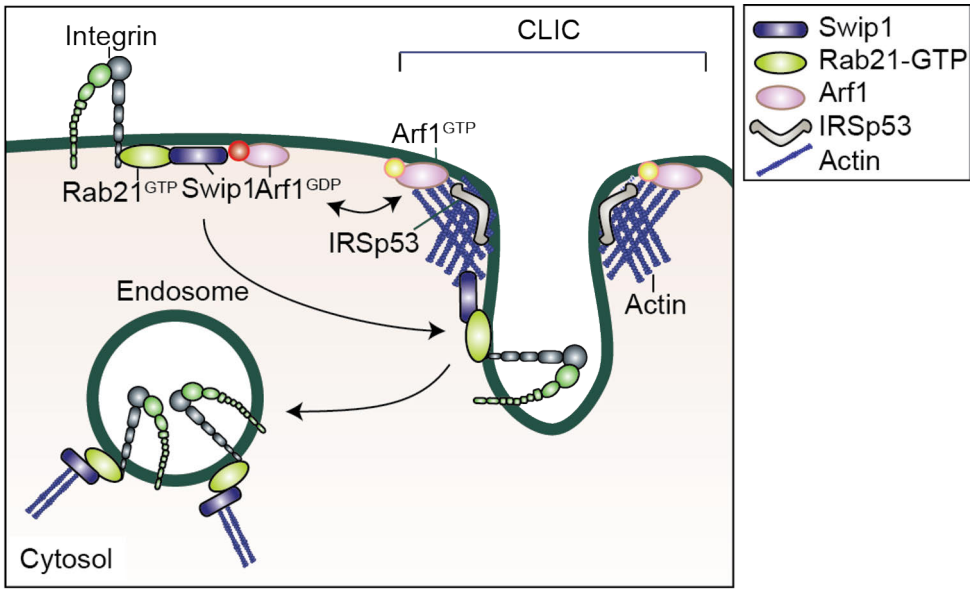

Figure 7. Summary of the mechanism of integrin uptake through the CLIC/GEEC endocytic pathway. Swiprosin-1 (Swip1) couples active Rab21-bound integrins to the CLIC/GEEC machinery, promoting integrin endocytosis. Image from original publication II, fig. 6j. CLIC = Clathrin-independent carrier.

Original publication III

Here, we found that EPLINα binds directly to active Rab21, and localises to integrin-containing Rab21-positive early endosomes, where it mediates integrin recycling back to the plasma membrane (Figure 8). We revealed that EPLINα requires its actin binding sites for proper endosomal localisation and mediation of integrin recycling. We used BioID to identify putative EPLIN isoform-specific interactors, and characterised the role of the interactor coronin 1C further, showing that it localises to actin puncta on Rab21-positive endosomes together with EPLINα. Like EPLINα, coronin 1C also facilitated integrin recycling. Moreover, both EPLINα and coronin 1C promoted breast cancer cell migration, and EPLINα expression correlated with a more mesenchymal and motile phenotype in breast cancer cells. Our findings shed further light on the fate of Rab21-bound internalised integrins and identifies new players promoting integrin recycling back to the plasma membrane. The proposed

role of EPLINα as a regulator of endosomal actin dynamics opens up the possibility that EPLINα could impact the sorting and retrieval of other endosomal cargoes, in addition to integrins. Combined with our findings in original publication II, our work describes a cycle of integrin trafficking, from endocytosis to sorting and subsequent recycling back to the cell surface, and emphasises the importance of this multi-step process for breast cancer cell migration.

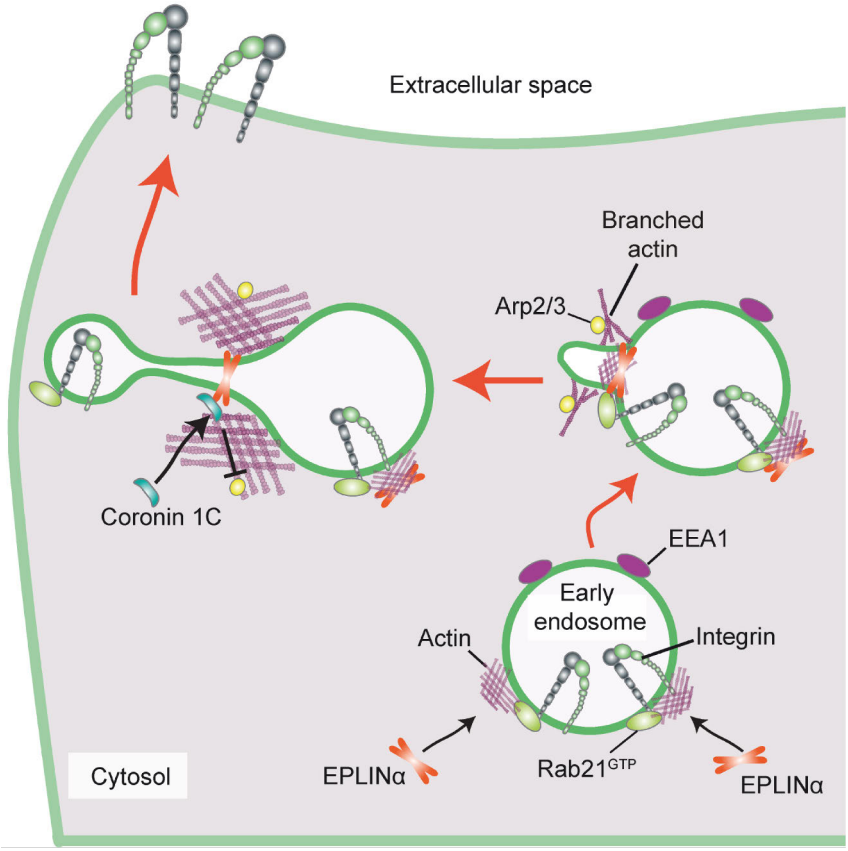

Figure 8. Summary of the proposed mechanism of EPLINα-mediated integrin recycling back to the cell surface. Together with coronin 1C, EPLINα regulates Arp2/3-mediated branched actin polymerisation on Rab21-positive early endosomes to promote endosomal fission and integrin recycling back to the cell surface. Through this mechanism, EPLINα facilitates integrin-mediated adhesion and migration of triple-negative breast cancer cells. Image from original publication III, graphical abstract. EEA1 = Early endosome antigen 1.

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