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Intracellular trafficking of cancer-implicated proteins in women's cancers

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Declaration of originality

The work presented in this thesis was done by the author, Youngrock Jung, unless otherwise stated in the text. The research was carried out at Institute of Reproductive and Developmental Biology (IRDB), Hammersmith Campus, Imperial College, London. All external sources have been properly acknowledged.

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Abstract

Cancers from breast and ovary account for around 20% of women's death from malignant neoplasms in the UK. Although survival from cancer has been improved, about half of cancer patients are not able to survive for more than ten years. To the date, over 600 genes have been identified to be implicated in malignant transformation and these genes can be grouped into two broad classes: oncogenes and tumour suppressor genes. In addition to the genetic or epigenetic status of these genes, aberrant intracellular trafficking of these proteins is another contributing factor that also drives deregulation in cell homeostasis. In this Ph.D. study, the intracellular trafficking of two cancer-implicated proteins was investigated: the oncogenic protein membrane type-1 matrix metalloproteinase (MT1-MMP) in breast cancer and the tumour suppressor protein opioid-binding protein cell-adhesion molecule like (OPCML) in ovarian cancer.

MT1-MMP is an essential regulator of cancer cell invasion and metastasis, which are the major cause of human cancer death. The proteolytic activity of MT1-MMP promotes cellular invasion and requires this protein to be localised to invasive membrane protrusions called invadopodia. Here, we have explored the role of Rab27a in MT1-MMP secretion in breast cancer cell line MDA-MB-231. Depletion of this Rab GTPase silenced the effects of MT1-MMP-dependent gelatin degradation and 3D collagen invasion. Furthermore, Rab27a was found to be associated with MT1-MMP exocytosis-related proteins, such as IQGAP-1 and Exo84. Finally, we also showed that the regulation of MT1-MMP trafficking by Rab27a was not in biosynthetic pathway but through MT1-MMP recycling to the plasma membrane. In

conclusion, we suggest that Rab27a is a novel regulator of MT1-MMP recycling in breast cancer cells.

High grade serous ovarian cancer (HGSC) remains the most lethal gynaecological cancer despite advances in surgery and chemotherapy. OPCML is a glycosyl phosphatidylinositol (GPI)-anchored protein and it is downregulated in many types of cancers, including HGSC, where 84% of cases show loss of OPCML expression. Previously, our group identified that OPCML negatively regulates a specific repertoire of receptor tyrosine kinases (RTKs) by interacting with the extracellular domains of the RTKs causing a reduction in cell growth, migration and invasion. However, it has been poorly understood whether this happens on the cell surface (where OPCML is localised) or in endocytic vesicles after the two proteins are internalised together. In this study, using an ovarian cancer cell line SKOV3, we have identified that OPCML is internalised via a caveolin-1-dependent pathway, where dynamin and cholesterol were also found to be important. After the uptake, Rab5 and Rab7 appeared to be involved in the trafficking of OPCML. We could also observe active recycling and degradation of OPCML after the endocytosis. Finally, we tested the effect of OPCML on RTK distribution and trafficking. The result showed that activated AXL redistributes to caveolin-1-positive structures from clathrin-associated membranes in the presence of OPCML.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AJCC	American Join Committee on Cancer
Alix	ALG-2 interacting protein-X
aPKCι	atypical protein kinase C iota
ARF1	ADP-ribosylation factor 1
ATP	adenosine triphosphate
BAR	BIN1/amphiphysin/Rvs167
CAF	cancer-associated fibroblast
CD44	cluster of differentiation 44
Cdc42	cell division control protein 42 homolog
CLIC3	chloride intracellular channel 3
CLIC-GEEC	clathrin-independent carrier-GPI-anchored protein-enriched early endosomal compartment
COSMIC	Catalogue of Somatic Mutations In Cancer
CTxB	cholera toxin
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EEA1	early endosome antigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EHD2	Eps-15 homology domain-containing protein 2
EMT	epithelial to mesenchymal transition

ENTH	epsin N-terminal homology
Epi-A	epithelial-A
ER	endoplasmic reticulum
ER	oestrogen
ERK	(=MAPK1) mitogen-activated protein kinase 1
ESCRT	endosomal sorting complex required for transport
FCS	foetal calf serum
FEME	fast endophilin-mediated endocytosis
FGFR	fibroblast growth factor receptor
FIGO	Federation of Gynaecological Oncologists
GAP	GTPase-activating protein
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor
GGTase	geranylgeranyl transferase
GLUT1	glucose transporter type 1
GPI	glycosylphosphatidylinositol
GRAF1	GTPase regulator associated with focal adhesion kinase 1
GTP	guanosine triphosphate
HER2	Human epidermal growth factor receptor 2
HGSOC	high grade serous ovarian cancer
HIF	hypoxia-inducible factor

HOPS	homotypic fusion and vacuole protein sorting
HPX	hemopexin
Hsc70	heat shock cognate 70
Hsp90	heat shock protein 90
IGF-II	insulin-like growth factor-II
IL-2	Interleukin 2
IQGAP1	IQ motif containing GTPase activating protein 1
JIP3	JNK-interacting proteins 3
JNK	c-Jun N-terminal kinase
LAMP	lysosomal-associated membrane protein
LPS	lipopolysaccharide
MDR	multidrug resistance
MEF	mouse embryonic fibroblast
Mes	mesenchymal
MESNa	sodium 2-mercaptoethanesulfonate
MHC I	major histocompatibility complex I
MMP	matrix metalloproteinase
MT1-MMP	membrane type-1 MMP
MβCD	methyl-β-cyclodextrin
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NDRG1	N-myc-downregulated gene protein 1
NF-κB	nuclear factor kappa B
NST	no special type

N-WASP	neural WASP
OPCML	opioid-binding protein cell-adhesion molecule like
PACSIN2	PKC and casein kinase substrate in neurons 2
PAK1	p21-activated kinase
PBS	phosphate-buffered saline
PDGFR	platelet-derived growth factor receptor
PFA	paraformaldehyde
PgR	progesterone
PI(3)P	phosphatidylinositol 3-phosphate
PI(4,5)P_2	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphatidylinositol-3-OH kinase
PLA	proximity ligation assay
PTEN	phosphatase and tensin homolog
PTPRG	protein tyrosine phosphatase receptor type G
Rab	Ras-like proteins in brain
Ras	rat sarcomas
Rb	retinoblastoma
REP	Rab escort protein
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
Slp1	synaptotagmin-like protein 1

SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
STxB	shiga toxin
TGFα	transforming growth factor alpha
TGN	trans-Golgi network
THEM205	transmembrane protein 205
TIMP-2	tissue inhibitor of metalloproteinases-2
TNM	primary tumour, lymph node, metastasis
t-SNARE	target SNARE
VAMP-7	vesicle-associated membrane protein-7
VEGF	vascular endothelial growth factor
v-SNARE	vesicular SNARE
WASH	Wiskott-Aldrich syndrome protein and Scar homolog
WASP	Wiskott-Aldrich syndrome protein

CHAPTER 1. INTRODUCTION

1.1 CANCER

Cancer is one of the leading causes of death worldwide (1). In the UK, incidence rates for all cancer types have increased by almost 30% since the late 1970s, and over 350,000 cases were newly diagnosed in 2014. The increase of cancer incidence is larger in women, where the cases have increased by 37%, than in men, which show a 17% increase (2). The cancer types that show the highest mortality rates in both men and women are lung (61.4 per 100,000), bowel (27.1 per 100,000) and pancreas (15.0 per 100,000), which account for around 35-38% of all deaths from malignant neoplasms. In men, prostate cancer (13%) and oesophagus cancer (6%) have also high mortality, while cancers from breast (14.8%) and ovary (5.4%) kill many women (3). Although survival from cancer is improving and has doubled in the last 40 years in the UK, about 50% of people diagnosed with cancer cannot survive their disease for ten years or more (2010-11 in England and Wales) (4).

Cancer develops when normal cells in the body become “out-of-control”. There are different types of cancers but in all of them cancer cells continue to grow and divide and instead of dying they form new abnormal cells (5). Many cancer therapeutic candidates and treatments have been investigated to control this abnormality, but only a few have been left available in the clinic due to the complexity of cancer. According to the reviews written by Douglas Hanahan and Robert A. Weinberg (6), cancer acquires several biological capabilities during the multistep tumour development: resisting cell death, sustaining proliferative

signalling, evading growth suppressors, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics, and avoiding immune destruction. In addition, acquisition of these hallmarks is found to be facilitated by genome instability and/or tumour-promoting inflammation. These findings have led to the development of several drugs and therapies aimed at targeting specific molecules and pathways that are involved in one or or more of the above tumoral capabilities, as each of the hallmarks is crucial in the biology of human cancer (6).

1.1.1 Oncogenes and tumour suppressor genes

According to the Catalogue of Somatic Mutations In Cancer (COSMIC) (http://cancer.sanger.ac.uk/cancergenome/projects/census), which is a database system of somatic mutations that are collected from a variety of public sources and sorted into one standardised source, the number of genes for which mutations have been implicated in malignant transformation currently stands at 616, which is more than 1% of all human genes (7). Approximately 90% of these genes show somatic mutations, 20% contain germline mutations and 10% have both somatic and germline mutations. These cancer-implicated genes are normally essential for survival, growth and basic cellular activities in normal cells and tissues. However, their overexpression, loss of expression or mutation contribute to malignant tumour growth (8).

These genes are grouped into two broad classes, proto-oncogenes and tumour suppressor genes, depending on whether normal cells become cancerous from too much activity of the gene product or too little: a gain-of-function mutation in proto-oncogenes can

induce tumorigenesis, while a loss-of-function mutation in tumour suppressor genes enables transformation of normal cells into cancer (9). The functional products of these genes include signalling molecules, transmembrane receptors, cell-cell adhesion molecules, protein kinases, GTP-binding proteins, cell-cycle modulators, apoptosis regulators, DNA repair enzymes, transcription controllers, RNA splicing machinery components, chromatic modifiers, and metabolic enzymes (9). In other words, in each case the respective genetic mutation or epigenetic alteration can contribute to cancer development.

The mutation of a single copy of a proto-oncogene that converts it to an oncogene has a dominant, growth-promoting effect on a cell (9). Thus, we can identify the oncogene by its effect when it is added (e.g. by DNA transfection or through infection with a viral vector of the genome of a cell or an experimental animal). In the case of the tumour suppressor gene, on the other hand, the cancer-causing alleles produced by the change are generally recessive: often both copies of the normal gene must be removed or inactivated in the diploid somatic cell before an effect is seen (9).

The result of a gain-of-function mutation in an oncogene can appear either in the form of hyperactive-state or in the normal protein produced at a higher expression level (9). One example is a small GTPase family called *Ras* (10). They encode a group of proteins that transmit signals from cell surface receptors to the intracellular environment. Mutations in these genes are observed to block hydrolysis of bound GTP (active form) to GDP (inactive form) resulting in the sustained level of their activity and, therefore, uncontrolled transmission of signals. Another example is epidermal growth factor receptor (EGFR), which is involved in cell proliferation (9, 11). This receptor can be stimulated by its ligand EGF and then induce the downstream signal transduction. In human cancers, this gene is frequently

found to be overexpressed or to remain in its active form without EGF stimulation from deletion of its extracellular domain sequence or activating mutations in its kinase domain.

Tumour suppressor genes lose their functions upon genetic interruption or epigenetic downregulation of their expression (9). One of the most frequent forms of epigenetic changes in tumour suppressor genes is methylation of the C nucleotides in CG sequences, which prevents the recruitment of transcription factors on the site and then thereby silences the gene expression. *Rb* is a tumour suppressor gene and it encodes the Rb protein that regulates cell cycle in human cells (9). Thus, failure of the proper expression of this gene causes uncontrolled cell cycle progression, resulting in tumorigenic phenotypes.

The significant diversity originated from the numbers of cancer-implicated genes can be greatly simplified into key pathways/gene subsets that have the potential to be therapeutic targets (9). Some well-established pathway/gene subsets are for example the Rb pathway (regulation of cell-division cycle), the p53 pathway (control of responses to stress and DNA damage), the RTK/Ras/PI3K pathway (transduction of signal for cell growth and cell division) and the gene set that includes proteinases and cell adhesion molecules (regulation of cell invasion and metastasis) (9-12).

1.1.2 Intracellular trafficking and cancer

In addition to the genetic or epigenetic status of oncogenes and tumour suppressor genes, aberrant intracellular trafficking of these proteins is another contributing factor that

also drives deregulation in cell homeostasis leading to changes in cell proliferation and motility (13).

Misplacement of cancer-implicated proteins, affecting their levels at the plasma membrane, can be achieved by alterations of the transport of the translated forms of those genes. There are three main intracellular trafficking mechanisms that are involved in the regulation of protein levels at the plasma membrane: exocytosis, recycling, and endocytosis. These processes are the results of the coordination of specialised molecules that include Rab GTPases, the exocytic complex, and the endocytic machineries.

1.1.2.1 Rab GTPases

Eukaryotic cells are compartmentalised into chemically and functionally distinct subcellular domains. This endomembrane system is important for protein stability, regulated signal transduction, controlled cell polarity and motility, and programmed differentiation (13).

To achieve these, transport of content between organelles must happen in a specific and regulated manner and such transport can be described as a three-step process (14): vesicle budding from donor membranes, transport of vesicular carriers toward specific acceptor compartments, and docking and fusion of these structures with the acceptor membranes. There are sets of proteins involved in each step: first, cytosolic coat molecules that enable cargo sequestration and vesicle budding; second, motor proteins that allow vesicle movement along actin filaments or/and microtubules; last, SNARE (soluble N-

ethylmaleimide-sensitive factor attachment protein receptor) complexes that are the main mediators in membrane fusion (15). In addition to this, there is one more group of proteins that are essential for the coordination of these processes and these are called Rab (Ras-like proteins in brain) GTPases (14).

1.1.2.1.1 Rab GEFs, GAPs, GDIs and effectors

Rab GTPases, the largest subgroup of Ras small GTPases, are the key regulators of intracellular vesicle trafficking (16). Like other small GTPases, their activities are regulated upon GTP/GDP switching, which is followed by conformational changes leading to the recruitment of proper machineries on the cytoplasmic surface of intracellular membranes (16, 17). This reversible cycle between GTP-bound and GDP-bound forms was found to be the results of exchange and hydrolysis reactions stimulated by Guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) respectively (18). The major conformational changes occur in two variable regions of Rab proteins, called switch I and switch II (19). GEFs recognise specific residues in the variable regions and promote the release of GDP (20). GTP replaces GDP as soon as GDP is released due to the high cytosolic concentration of GTP (~1mM). Then the GTP-bound Rabs can interact with specific effector molecules through both switch and inter-switch regions (21).

Rab proteins can exist as either soluble forms or membrane-bound proteins, which is determined by a posttranslational modification called prenylation (22). This modification provides strong membrane association of Rabs by adding one or two lipophilic geranylgeranyl groups (20-carbon isoprenoid) at C-terminal cysteine residues. Prenylation,

geranylgeranylation in this case, is mediated by Rab geranylgeranyl transferase (GGTase II or RABGGTB) and once the modification is done, the Rab protein is chaperoned and localised to its correct membrane by molecules such as Rab escort protein (REP).

This process can be reversed by the activity of Rab GDP dissociation inhibitor (GDI), which dissociates geranylgeranylated Rab GTPases from membranes. Due to its specificity for GDP-bound Rabs, the Rab GDIs can mediate solubilisation of Rabs from membranes upon GTP hydrolysis (23). The Rab-GDI complex is recognised by a membrane-associated GDI displacement factor (GDF) and thereby GDI can also function to localise Rab GTPases to specific membranes (24). According to other studies, however, a membrane-bound GEF rather than GDI is found to be a main contributor in the precise membrane-targeting of Rab GTPases (25, 26).

One distinct feature that is observed in Rab proteins compared to the other small GTPases is the high degree of functional variety among Rab effectors (18). Effector proteins mediate the activities of Rabs by providing appropriate platforms for the additional recruitment of protein machineries. Rab effectors can also be GEFs for different Rab GTPases serving as gears that transmit the signal from one Rab to another in networked cascades (16, 17). Hence, this complexity of Rab effectors enables Rabs to seamlessly complete the series of steps required for membrane trafficking in a spatially and temporally regulated manner.

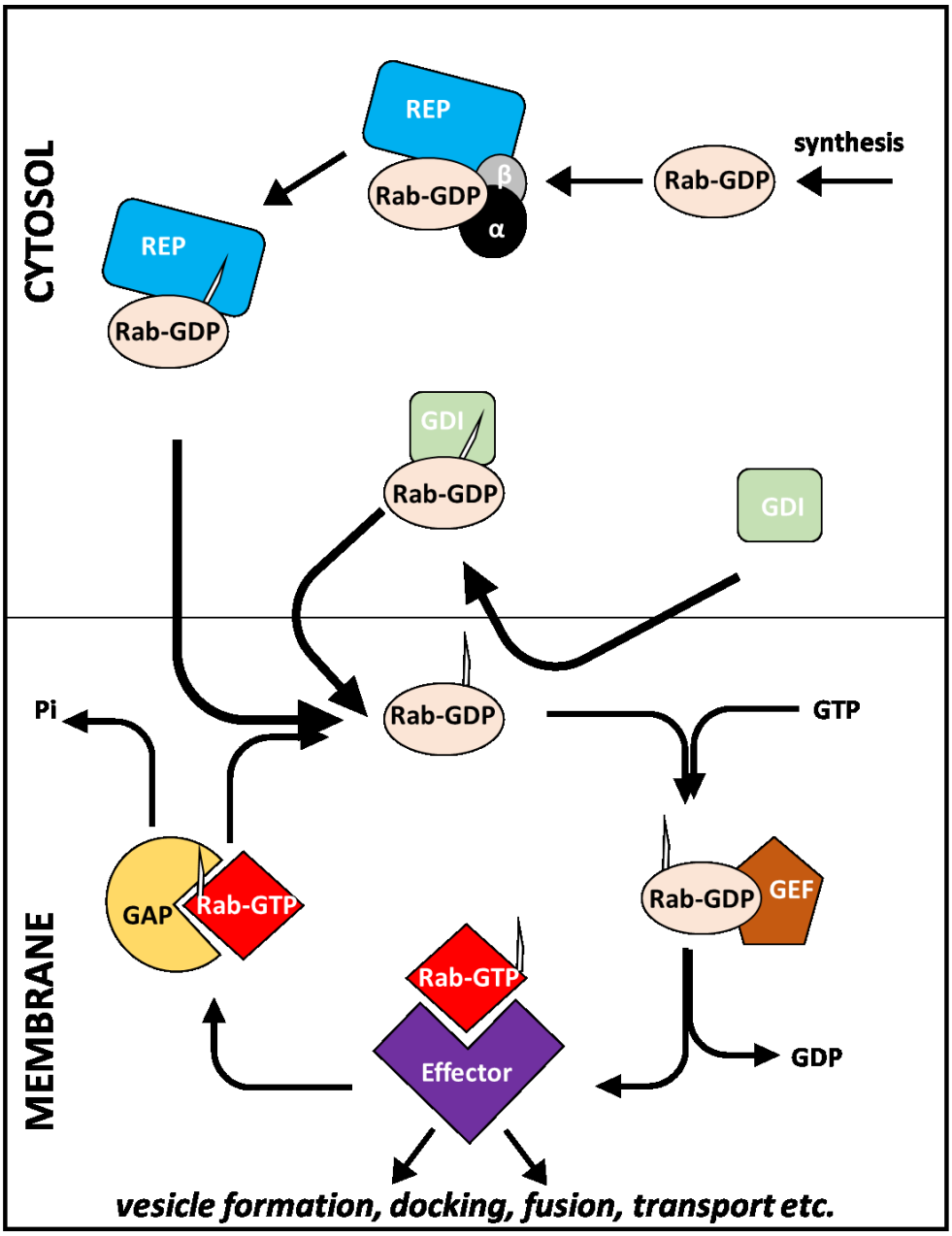


Figure 1. Rab GTPase cycle and intracellular localisation. The illustration is reproduced from Goody et al., 2005. Once synthesised, the Rab-GDP is recognised by REP (Rab escort protein) and associated with RabGGTase (α- and β-subunits). This process results in prenylation of Rab proteins, thereby enabling their localisation and anchoring to the target membranes. Rabs are activated by the action of GEF (guanosine nucleotide exchange factor) that replaces GDP with GTP. The active GTP-bound Rab interacts with effectors, which are required for the regulated Rab functions that include vesicle formation, docking, fusion, and transport. GTP hydrolysis in Rab is controlled by GAP (GTPase-activating protein). GDI (GDP dissociation inhibitor) activity is needed for the detachment of Rabs from the membrane.

1.1.2.1.2 Rab localisation and functions

Localisations of Rabs are listed in Table 1. Briefly, Rab1 is found to regulate ER-Golgi traffic whereas Rab2 is implicated in recycling, or retrograde traffic from Golgi to the ER. Rab6 controls intra-Golgi trafficking. Rab8, -10, and -14 are known to be involved in biosynthetic movements from the trans-Golgi network (TGN) to the plasma membrane. Other secretory vesicles are carried by Rab3, -26, -27, and -37 to secrete their cargo to the cell surface or extracellular environment. Rab27, together with Rab32 and Rab38, functions in the transport of lysosome-related organelles such as melanosomes. Rab5 regulates most of early endocytic transports and allows fusion of endocytosed vesicles with the early endosomes. Trafficking from the early endosomes towards the late endosomes/lysosomes is controlled by Rab7 while membrane transport from the early endosome to the recycling endosomes is directed by Rab15. Rab4 and Rab11 are involved in recycling process. There are some Rabs that show specialised functions. Rab18 mediates lipid droplet regulation whereas Rab24 and Rab33 are related to the formation of autophagosomal structures. Rab21 and Rab25 are involved in cell adhesion and cytokinesis by regulating integrin trafficking while Rab13 is linked to tight junction formation in polarised epithelial cells. Rab23 negatively affects the Sonic hedgehog (Shh) signalling pathway while Rab40 is involved in Wnt signalling pathway.

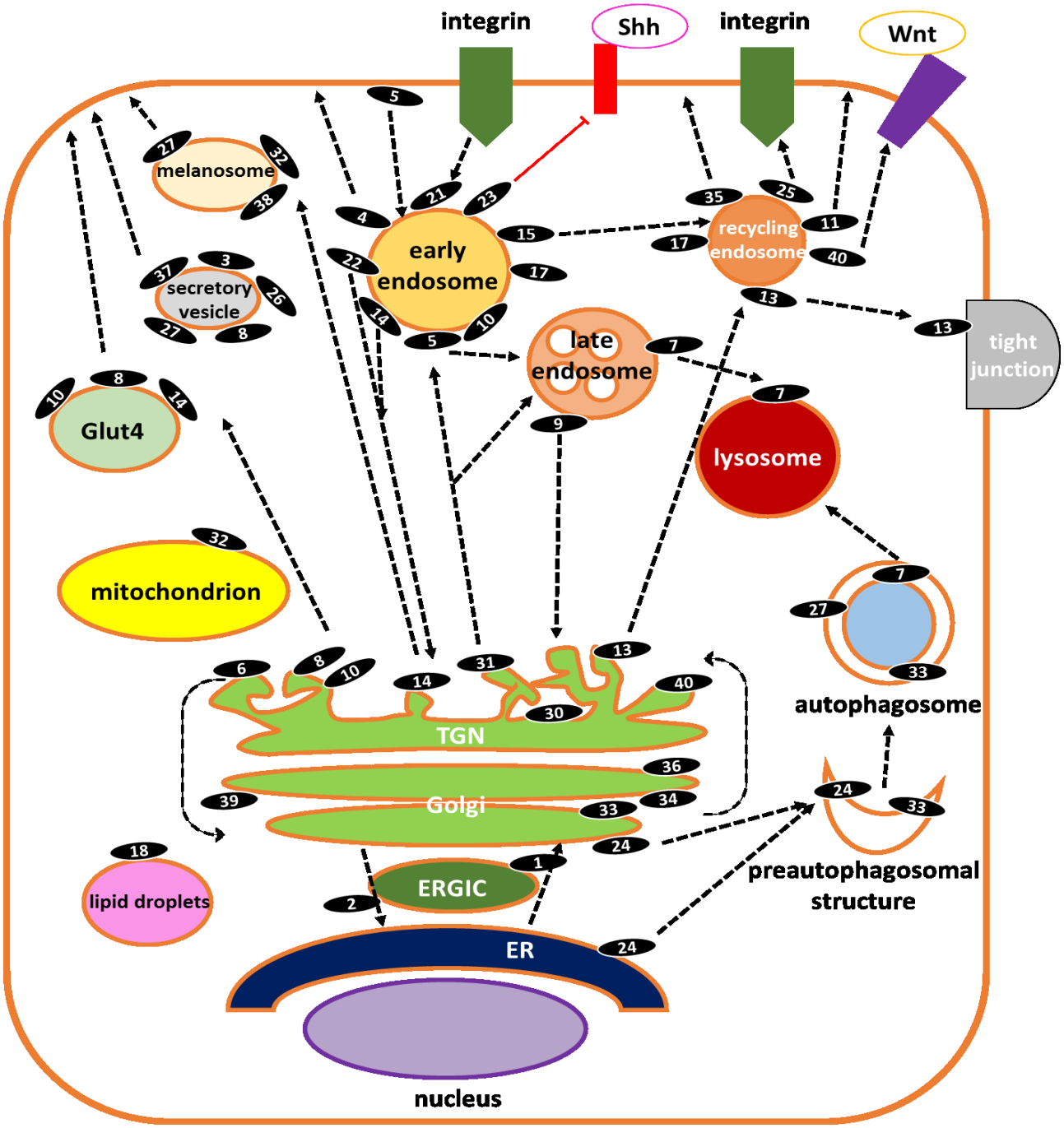


Figure 2. The intracellular localization of Rabs and their roles. The illustration is reproduced from Hutagalung et al., 2011. Rab1 controls ER-Golgi traffic whereas Rab2 regulates retrograde traffic from Golgi and the ERGIC back to the ER. Rab6 is involved in intra-Golgi traffic. Rab8, -10, and -14 are known to regulate biosynthetic pathway from the trans-Golgi network to the plasma membrane and the glucose transporter GLUT4 is one of the examples that use these Rabs to be secreted. Several secretory vesicles and granules are carried by Rab3, -26, -27, and -37. Rab27, -32, and -38 are found to regulate the melanosome transport. Rab5 is involved in fusion of endocytic vesicles with the early endosome and this transport can be directed towards the lysosome for degradation, which is mediated by Rab7, or to various recycling compartments to return factors to the plasma membrane. Rab15 directs membrane traffic from the early endosome to the recycling endosome. Rab4 and Rab11 regulate fast and slow recycling pathways, respectively. Rab18 is involved in lipid droplet regulation. Rab24 and Rab33 regulate formation of the preautophagosomal structure that functions by engulfing cellular components, subsequently generating the autophagosome. Rab21 and Rab25 control trafficking of integrins and therefore affect cell adhesion and cytokinesis. Rab13 is important in tight junction transport and formation in epithelial cells. Rab34 is localised on the Golgi and is involved in intra-Golgi transport. Rab35 regulates plasma membrane recycling of an essential factor during cytokinesis. Rab23 negatively regulates Sonic hedgehog (Shh) signalling by interacting with the transcription factors activated by the Shh pathway. Rab40 regulates Wnt signalling by recruiting components of the ubiquitination machinery. (ERGIC, ER-Golgi intermediate compartment; ER, endoplasmic reticulum; TGN, trans-golgi network; Shh, Sonic hedgehog)

Table 1 Rab proteins in intracellular pathways*

Rab	Intracellular localization	Function	Reference
Rab1A	ER, Golgi	ER to Golgi trafficking, intra–Golgi	(27-29)
Rab1B	ER, Golgi	ER to Golgi trafficking, intra–Golgi	
Rab2A	ER, ER–Golgi intermediate, Golgi	ER to Golgi trafficking	(30)
Rab2B	ER, ER–Golgi intermediate, Golgi	ER to Golgi trafficking	
Rab3A	Secretory vesicles, plasma membrane	Exocytosis of secretory vesicles (from TGN to the plasma membranes), neurotransmitter release	(31)
Rab3B	Secretory vesicles, plasma membrane	Exocytosis of secretory vesicles (from TGN to the plasma membranes), neurotransmitter release	
Rab3C	Secretory vesicles, plasma membrane	Exocytosis of secretory vesicles (from TGN to the plasma membranes), neurotransmitter release	
Rab3D (Rab16)	Secretory vesicles, plasma membrane	Regulated exocytosis in nonneuronal cells	
Rab4A	Early and recycling endosomes	Protein recycling to plasma membrane	(32, 33)
Rab4B	Early and recycling endosomes	Protein recycling to plasma membrane	
Rab5A	Plasma membrane, clathrin coated vesicles, early endosomes	Endocytic internalization and early endosome fusion	(30)
Rab5B	Plasma membrane, clathrin coated vesicles, early endosomes	Endocytic internalization and early endosome fusion	
Rab5C	Plasma membrane, clathrin coated vesicles, early endosomes	Endocytic internalization and early endosome fusion	

Rab6A	Golgi	Endosomes to Golgi, intra-Golgi transport, Golgi to ER	(34, 35)
Rab6A'	Golgi	Endosomes to Golgi, intra-Golgi transport, Golgi to ER	
Rab6B	Golgi	Intra-Golgi transport	
Rab7A	Late endosomes, lysosomes, melanosomes, phagosomes	Late endocytic trafficking (late endosomes to lysosomes)	(30)
Rab7B	Lysosomes	Late endocytic trafficking (late endosomes to lysosomes)	
Rab8A	Plasma membrane, vesicles, median Golgi, TGN	Exocytosis (TGN/recycling endosomes to plasma membrane)	(36)
Rab8B	Plasma membrane, vesicles, median Golgi, TGN	Exocytosis (TGN/recycling endosomes to plasma membrane)	
Rab9A	Late endosomes	late endosomes to TGN	(37, 38)
Rab9B	Late endosomes, Golgi	Unknown	
Rab10	Golgi, basolateral sorting endosomes	Exocytosis (TGN/recycling endosomes to plasma membrane)	(39-42)
Rab11A	Golgi, recycling endosomes, early endosomes	Protein recycling (TGN/recycling endosomes to plasma membrane)	(43-45)
Rab11B	Golgi, recycling endosome, early endosomes	Protein recycling (TGN/recycling endosomes to plasma membrane)	
Rab12	Golgi, plasma membrane	Retrograde transport (between plasma membrane and TGN)	(46, 47)
Rab13	Cell/tight junctions, TGN, recycling endosomes	Exocytosis (TGN/recycling endosomes to plasma membrane)	(48)
Rab14	Early endosomes, Golgi	Exocytosis (TGN/recycling endosomes to plasma membrane), transport between early endosomes and Golgi	(49, 50)
Rab15	Early/sorting endosomes, recycling endosomes	Exocytosis (sorting endosomes/recycling endosomes to plasma membrane)	(51)

Rab17	recycling endosomes	Apical recycling, transcytosis	(52-54)
Rab18	Golgi, ER, lipid droplets	ER–Golgi trafficking, lipid droplet formation	(55-58)
Rab19	Golgi	Unknown	(59, 60)
Rab20	Golgi, endosomes	Apical endocytosis/recycling	(56, 61)
Rab21	Early endosomes	Endosomal transport	(62)
Rab22A	Early endosomes	Endosomal transport, recycling	(47)
Rab23	Plasma membrane, endosomes, endocytic vesicles	Protein recycling to plasma membrane, endosomal transport	(63)
Rab24	ER/cis-Golgi, late endosomal structures	Autophagy-related processes	(47)
Rab25	Recycling endosomes	Apical recycling to plasma membrane	(64)
Rab26	Secretory granules	Exocytosis from TGN to plasma membrane	(65)
Rab27A	Melanosomes	Exocytosis	(66-69)
Rab27B	Plasma membrane, Golgi, cytosol	Exocytosis	
Rab28	Unknown	Unknown	
Rab28L	Unknown	Unknown	
Rab30	ER, Golgi	Maintaining structural integrity of Golgi, autophagosome formation	(60, 70-72)
Rab31 (Rab22B)	Early endosomes, TGN	Transport between TGN and early endosomes	(73)
Rab32	Mitochondria, melanosomes	Mitochondrial fission, TGN to melanosomes	(74-76)
Rab33A	Golgi	Autophagosome formation	(77)
Rab33B	Golgi	Autophagosome formation	

Rab34	Golgi, macropinosomes	Intra-Golgi transport	(78, 79)
Rab35	Recycling endosomes	Apical endocytic recycling	(80)
Rab36	Golgi	Unknown	(81)
Rab37	Secretory granules	Exocytosis from TGN to plasma membrane	(82)
Rab38	Melanosomes	TGN to melanosomes	(83, 84)
Rab39	Golgi	Unknown	(85)
Rab40A	Golgi, recycling endosomes	Endosome/intracellular transport	(86)
Rab40B	Golgi, recycling endosomes, secretory vesicles	Vesicle secretion	
Rab40C	Golgi, recycling endosomes	Endosome/intracellular transport	
Rab41	Golgi	Unknown	(87)
Rab42	Unknown	Unknown	
Rab43	ER–Golgi intermediate	ER–Golgi trafficking	(55, 88)
Rab44	Unknown	Unknown	
Rab45	Perinuclear region	Unknown	(89)

*Reproduced from (90)

1.1.2.2 The exocyst complex

The process that involves the delivery of intracellular vesicles to the cell edge, placing membrane proteins at the cell surface or releasing materials such as hormones or extracellular vesicles into the extracellular space, by the fusion of the vesicles with the plasma membrane is called exocytosis (91). In addition, the supply of lipid moieties required for membrane extension takes place via exocytosis and therefore it allows cellular polarity, growth and division, which are important in cell function and tissue development (91).

Exocytosis pathways can be classified into two types: a constitutive exocytosis and a regulated exocytosis (92, 93). The exocytic vesicles that take the route of the constitutive pathway undergo nonselective, constant secretion at the plasma membrane (92, 93). This system operates in all cells for secreting many soluble proteins into extracellular environments and also supplying the plasma membrane with lipids and proteins (92, 93). In contrast, the regulated exocytosis shows that the secretion of some materials such as hormones and neurotransmitters occur under a tightly controlled condition, specifically calcium influx to the cytoplasm caused by appropriate signals (92, 93). Some cells like neurons (94) and endocrine cells (95) are specialised in this mechanism of exocytosis releasing the cargo molecules that are originated from synaptic vesicles or secretory granules respectively. Many evidence have shown that Ca^{2+} is a key molecule in this mechanism and synaptotagmins are required for the process as the Ca^{2+}-sensors (94, 96). However, the exact mechanism is incomplete. Recently, Kreutzberger et al. used *in vitro* reconstituted system and suggested that calcium and calcium-dependent activator protein for secretion (CAPS) recruit secretory vesicles, purified from neuroendocrine cells, to the

target membrane where membrane fusion occurs (97). Synaptotagmin was found to catalyse membrane fusion in a calcium-dependent manner in this study (97).

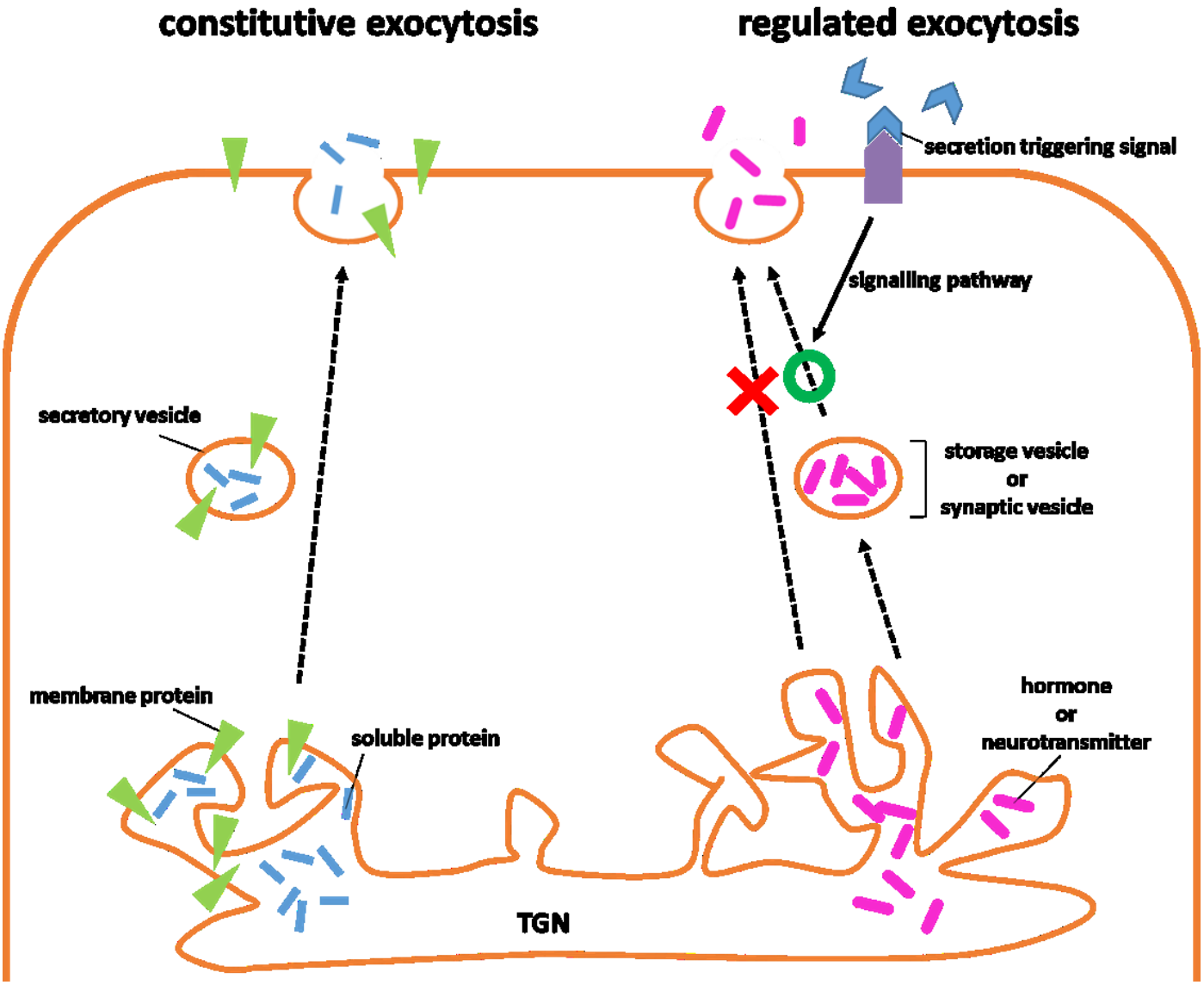


Figure 3. The constitutive exocytosis and the regulated exocytosis. This illustration is reproduced from Alberts et al., 2002. Proteins that are destined to be secreted are sorted in the TGN. In all eukaryotic cells, many proteins and lipids are transported in secretory vesicles to the plasma membrane in a nonselective, constitutive fashion (constitutive exocytosis). On the other hand, specialised secretory cells also show a regulated exocytic pathway for secretion of selected molecules. These molecules are generally kept in distinct storage compartments until an extrinsic signal that triggers the exocytosis of the molecules stimulates the cell.

1.1.2.2.1 Vesicle tethering and membrane fusion

In coordination with other proteins, such as Rab GTPases and motor proteins, cargoes that are originated from intracellular endomembranes travel along cytoskeletal filaments toward their destination (a spot at the inner leaflet of lipid bilayers of the cell surface) (91). The initial contact between the intracellular carriers and the plasma membrane, called tethering, is an essential step for proper exocytosis and it is mediated by the exocyst complex, which consists of 8 protein subunits, labelled as Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, Exo84 in yeast. In mammalian cells, they are also officially named EXOC1 (=Sec3), EXOC2 (=Sec5), EXOC3 (=Sec6), EXOC4 (=Sec8), EXOC5 (=Sec10), EXOC6 (=Sec15), EXOC7 (Exo70), EXOC8 (Exo84) and each subunit exists in several isoforms as the result of alternative splicing (98). Despite extensive studies on the exocyst complex using different model organisms including yeast, fungi, plant, and animal, unfortunately the mechanisms of assembly of the subunits and the modes of action are not fully established yet (99).

Most of the exocyst subunits are found to interact with multiple other subunits forming a complete complex. Several electron microscopy studies showed its variable structures, T-, Y-, or L-shaped, and these are thought to represent the functional structures of exocyst complexes undergoing vesicle membrane tethering (99).

Initially, it was proposed that the assembly of the exocyst complex was sequential as Sec3 and Exo70 were observed to directly bind to phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) and thus provide an assembly platform at the plasma membrane for the other subunits, which move to the plasma membrane on the secretory vesicle. This model was challenged by the evidence that showed the association of Sec3 and Exo70 with not only the

plasma membrane but vesicles (100). One recent investigation also contradicts this initial model by detecting only the fully assembled octameric complex and not any sub-complex isolated from yeast (101). From these results, all of the exocyst complex subunits seem to translocate on vesicles to the destination and upon their arrival, Sec3 and Exo70 might 'anchor' the whole structure, the exocyst complex and the vesicle, to the plasma membrane through their interaction with $PI(4,5)P_2$ (99, 102).

Once the secretory vesicles are tethered at the target membrane, membrane fusion step is followed to terminate the exocytic process. Membrane fusion between a secretory vesicle and the target plasma membrane takes place after vesicle tethering. Rothman and co-workers identified three key factors in membrane fusion: an ATPase that was sensitive to inactivation by *N*-ethylmaleimide (NEM) was named NEM-sensitive factor (NSF), adaptor proteins that are required for NSF function were named soluble NSF attachment proteins (SNAPs), and the SNAP receptors (SNAREs) (103-105). They also found that the SNAP receptor was a complex that is composed of three previously known proteins: the synaptic vesicle protein synaptobrevin II (VAMP2), the plasma membrane proteins syntaxin, and synaptosome-associated protein of 25kDa (SNAP-25) (106). In the following evidence, the SNARE complex emerged as the core machinery in membrane fusion that appears to be conserved throughout eukaryotic cells (15). The exocyst complex is found to interact with SNAREs, present on opposing membranes in two different forms, target t-SNARE and vesicular v-SNARE, to dock the vesicle to the acceptor membrane and end up inducing lipid fusion completing the exocytic process (107). In yeast, the exocyst subunit Sec6 was detected to interact with the t-SNARE protein Sec9 (the SNAP-25 homolog) (108). Sec6 also associates with the v-SNARE protein Snc2 and disruption of this binding by a mutation of

SNC2 causes mislocalisation of the exocyst complex and inhibition of exocytosis (109). In addition, it was also observed that the Sec1/Munc18 family protein Sec1, another SNARE regulator, is recruited by Sec6 to a vesicle secretion site proposing the importance of Sec6 in SNARE regulation (110). Exo84 exhibits its similar regulatory role on SNARE proteins by interacting with Sro7 and Sro77, which regulate polarised exocytosis via binding to the SNAREs (111).

There are three exocytic vesicle-membrane fusion models that have been proposed based on the microscopic observations: full-collapse fusion, kiss-and-run, and compound exocytosis. Full-collapse fusion has been suggested according to the observations of vesicle-like structures that are larger size (with diameters of ~60-120nm) than a regular vesicle (with diameters of ~30nm) (112). This is the result of fusion pore expansion and vesicle collapse during the complete release of secretory molecules (113). Kiss-and-run model represents the process of the partial release at the plasma membrane with a narrow fusion pore size (~0.5-3nm) in the absence of complete collapse of vesicular structures (114, 115). However, whether this form of exocytosis is an intermediate structure before collapse or pore closure is not clear. Lastly, some cells such as pancreatic acinar cells show multivesicular structures that undergo exocytosis (116, 117). This suggest that vesicles may attach to each other without collapsing. This mode of exocytosis is well established in nonneuronal cells containing large vesicles (~300-2000nm) while it was recently suggested that neuronal cells contain smaller vesicles (~20-50nm) (118).

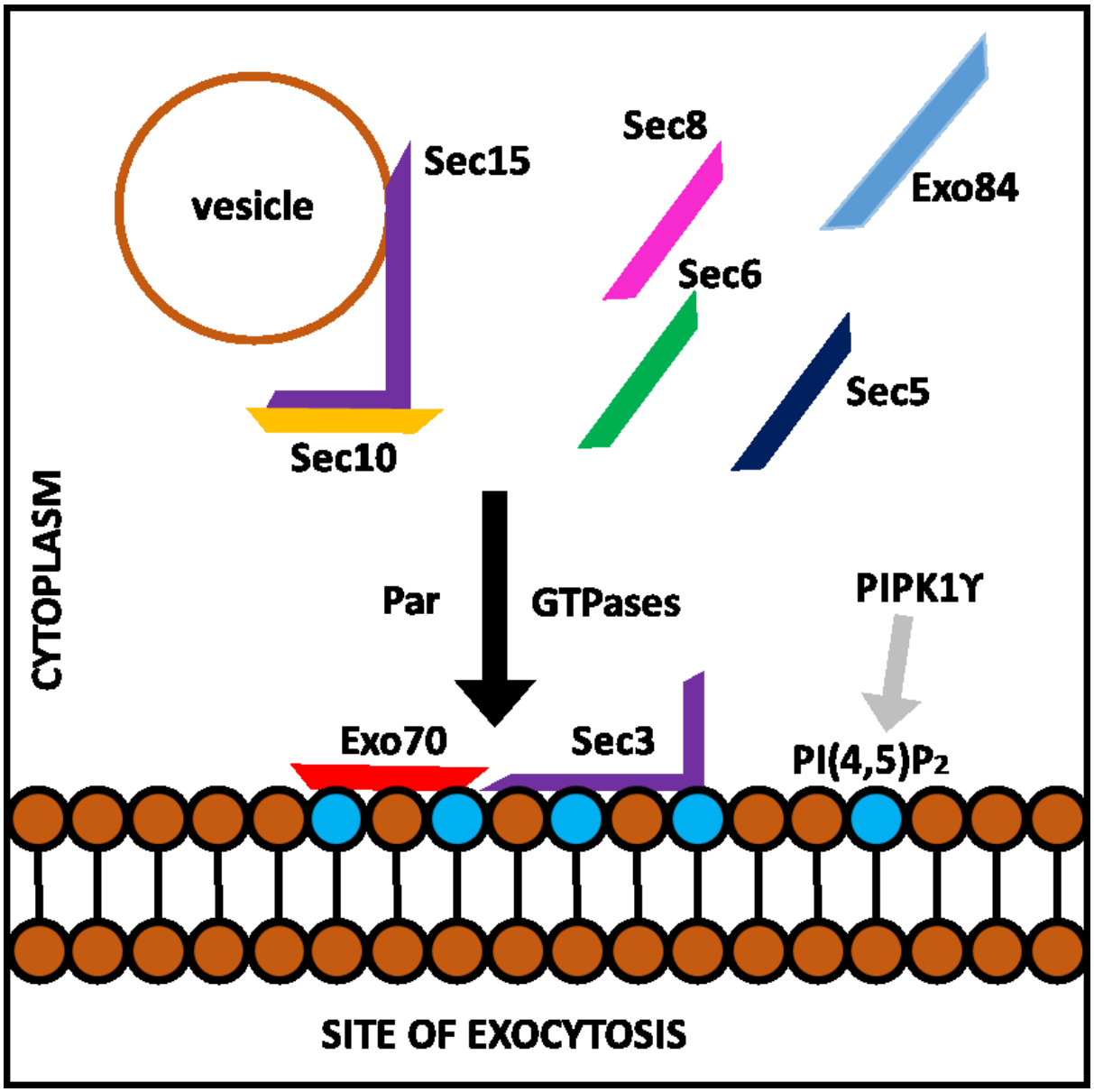


Figure 4. The exocyst complex subunits. The original illustration was produced by Noemi Polgar and Ben Fogelgren, 2017. Sec15 binds to the secretory vesicle, while Exo70 and Sec3 allow the exocyst complex and the target secretory vesicle to localise at the site of exocytosis by associating with $PI(4,5)P_2$, whose accumulation is induced by PIPKγ. In epithelial cells, the exocyst complex is found to regulate polarity establishment and maintenance by interacting with GTPases, polarity complexes (Par), and membrane phospholipids and subsequently regulating polarised secretion of intracellular vesicles.

1.1.2.2.2 The exocyst regulation and functions

Recent studies have identified that the exocyst mediates many signalling pathways that lead to exocytosis in a variety of different cellular processes. From the list of the

exocyst-interacting proteins (Table 2), it is possible to see how the exocyst is regulated and subsequently contributes to specific biological processes.

Table 2 The interactors of the exocyst complex subunits*

Exocyst subunit	Interacting partners	Species	Reference
Sec3	Rho1	Yeast	(119, 120)
	Cdc42	Yeast	(121)
	IQGAP1	Mammals	(122)
	$PI(4.5)P_2$	-	(123)
Sec5	RalA/B	Mammals	(124, 125)
	TBK1	Mammals	(126)
	GEF-H1	Mammals	(127)
Sec6	Sec9	Yeast	(108)
	Rtn1	Yeast	(128)
	Sec1	Yeast	(110)
	Snc2	Yeast	(109)
Sec8	PSD-95	Mammals	(129)
	SAP102	Mammals	(130)

	SAP97	Mammals	(131)
	IQGAP1	Mammals	(122)
	SH3BP1	Mammals	(132)
Sec10	ARF6	Mammals	(133)
	Par6	Mammals	(134)
Sec15	Sec4	Yeast	(135)
	Rab11	Mammals, Flies	(136, 137)
	Rab8	Mammals	(137)
	Bem1	Yeast	(138)
	Myo2	Yeast	(139)
	Rabin8	Mammals	(140)
Exo70	TC10	Mammals	(141)
	BIG2	Mammals	(142)
	ARPC1	Mammals	(143)
	$PI(4,5)P_2$	-	(144)
	Snapin	Mammals	(145)
	Rho3	Yeast	(146)

	Cdc42	Yeast, Mammals	(147)
	PIPKIγ	Mammals	(148)
	Bem1	Yeast	(149)
Exo84	RalA/B	Mammals	(150)
	Sro7, Sro77	Yeast	(111)
	SH3BP1	Mammals	(132)
	Par6	Mammals	(151)
Others	MycRIP	Mammals	(152)
	Paxillin	Mammals	(153)
	Dlg1	Mammals	(154)
	aPKC	Mammals	(155)
	Rab10	Mammals	(156)
	ULK	Mammals	(157)
	Beclin1	Mammals	(157)

*Reproduced from (99)

In yeast, post-Golgi secretory vesicles are found to accumulate in the cytoplasm upon mutation in the exocyst subunits (107, 144, 158-160). During budding of yeasts, the exocyst complex mediates exocytosis inducing the asymmetric expansion of the cell surface in the daughter cell. Polarised exocytosis by the exocyst can also be observed in other organisms. In *Arabidopsis thaliana*, the distribution of the exocyst is concentrated at growing pollen tubes and root tips, where the active membrane expansion takes place (161, 162). The exocyst complex plays an important role in the establishment of epithelial polarity in epithelial cells by mediating vesicle trafficking to the basolateral domain (148, 163-167). In neurons, similarly, the exocyst is observed at the growth cones, tips of neurites or branching points, where active exocytosis is necessary for surface membrane expansion (151, 168-170).

In addition to its basic role in polarised exocytosis, the exocyst, in coordination with actin remodelling and membrane reorganisation, can also contribute to directional cell migration and tumour invasion (99). Actin cytoskeleton remodelling at the cell leading edge is an essential molecular process for membrane protrusion prior to cell movement. Exo70 is known to promote actin polymerisation and branching through the direct association with the ARPC1 subunit of the Arp2/3 complex (171), which is the core machinery for actin branching forming a network that pushes the plasma membrane (172-174). Association between Exo70 subunit and the Arp2/3 complex is a downstream event of EGF signalling, which induces cell migration and tumour invasion (143, 175). Exo70 also causes high membrane curvature via the interaction with $PI(4,5)P_2$ and this leads to efficient membrane protrusion during cell migration (176).

To facilitate cell movement, several types of molecules, including signalling proteins, adhesion molecules and metalloproteinase, are required to be placed to the leading edge and the trafficking of these molecules is mediated by the exocyst and its binding partners, such as Rabs and Rals (153, 155, 177-179). Especially the exocytosis of matrix metalloproteinases (MMPs) is essential for forming invadopodia, which promote the degradation of extracellular matrix surrounding invasive tumour cells (122, 175, 180). ERK1/2 phosphorylates Exo70 in response to EGF stimulation and this in turn promotes MMP secretion by inducing the assembly of the exocyst complex (181). The exocyst is also known to bind the endosomal Wiskott-Aldrich syndrome protein and Scar homolog (WASH) complex and this interaction leads to the exocytosis of membrane type-1 MMP (MT1-MMP) from late endosomes (182).

1.1.2.3 The endocytic molecules

In contrast to exocytosis, endocytosis is the uptake process of extracellular materials, lipids and surface proteins. In eukaryotic cells, there are numerous portals available for these molecules and each endocytic entry has been extensively characterised depending on cargoes, molecules that are involved, and the scale of the process. Table 3 shows the different molecules that participate in the endocytic steps and their functions and regulatory mechanisms. In our discussion, only the smaller (<200nm) scale processes are examined due to the difference in the main roles between the large micrometre-scale pathways and the smaller scale pathways. Despite there are some shared machineries, for example the actin cytoskeleton is required for membrane remodelling in both cases, the

large-scale endocytic processes involve substantial uptake of membrane patches rather than the internalisation of specific cargoes (183-185).

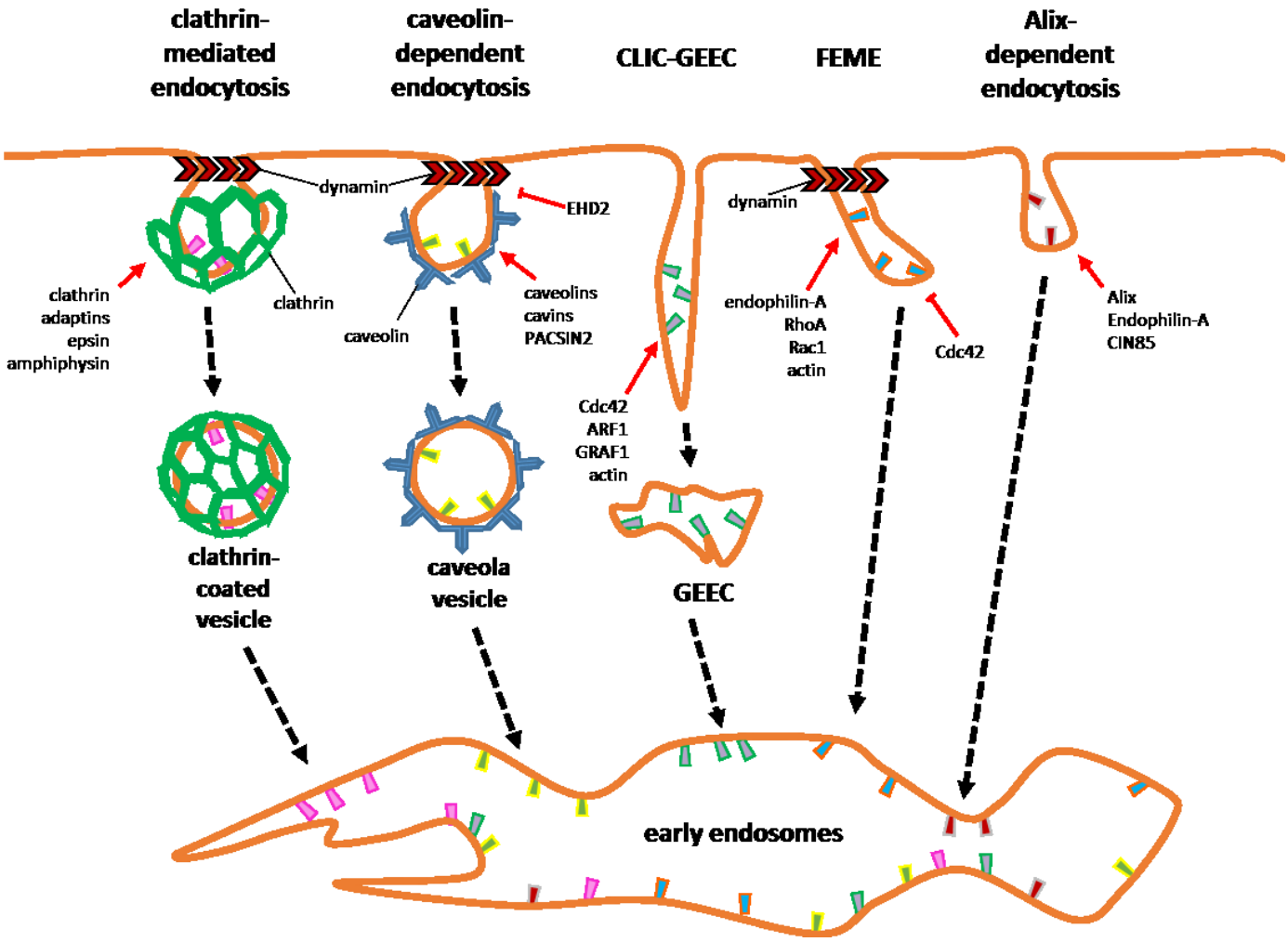


Figure 5. Endocytosis through clathrin-coated pits and caveolae, and clathrin-independent pathways. The illustration is reproduced from Johannes et al., 2015. The figure describes how different endocytic pathways work and the list of key molecules or negative regulators in each pathway. Clathrin-mediated endocytosis is known to require the activities of clathrin and other molecules such as adaptins, epsin and dynamin. Caveola vesicle is formed by the actions of caveolins, cavins, and PACSIN2. Dynamin also controls this pathway while EHD2 acts as a negative regulator. Clathrin-independent carrier (CLIC) GPI-anchored protein-enriched early endosomal compartments (GEEC) pathway is characterised by its tubular membrane invagination and the dynamin-independent uptake. This internalisation takes place as a result of the coordination of some key molecules, including Cdc42, ARF1, GRAF1, and actin. Fast endophilin-mediated endocytosis (FEME) is regulated by endophilin-A together with the activities of RhoA, Rac1, and actin. This pathway is negatively affected by dynamin inhibition, but Cdc42 inhibition promotes the internalisation. Recently, Alix-dependent pathway was identified as one of key clathrin-independent endocytic pathways for specific cargo uptake such as some GPI-anchored proteins and fluid-phase endocytosis. Endophilin is also involved in Alix-dependent cholera toxin internalisation.

Table 3 Overview of endocytic pathways*

Pathway	Machinery	Dynamin-dependence	Morphology	Known cargoes	References
clathrin-dependent endocytosis	clathrin adaptins epsin amphiphysin	+	~100 nm vesicles	transferrin LDL viruses EGF	(186)
caveolae-dependent endocytosis	caveolins cavins EHD2 PACSIN2 protein kinase C cholesterol actin	+	55-65 nm vesicles	cadherin occludin SV40?	(187)
fast endophilin-mediated endocytosis (FEME)	RhoA Rac1 PAK1 PAK2 cholesterol actin Endophilin-A	+	vesicular?	IL-2 receptor Iota toxin CTxB STxB	(188, 189)
CLIC/GEEC endocytosis	Cdc42 ARF1 GBF1 ARHGAP21 GRAF1 galectin-3 cholesterol actin	-	cisternal, tubular	GPI-anchored proteins CD44 CTxB	(190)

Other clathrin-or caveolin-independent endocytosis (1)	ARF6 CD59 cholesterol actin	+	?	MHC Class I	(191)
Other clathrin-or caveolin-independent endocytosis (2)	flotillin	-	vesicular?	CTxB CD59	(192)
Other clathrin-or caveolin-independent endocytosis (3)	Rac1 Src actin	-	tubular	AChR CD80/86	(190)
Other clathrin-or caveolin-independent endocytosis (4)	Alix Endophilin-A CIN85	?	?	EGFR IL-2 receptor GPI-anchored Proteins CTxB β1 integrin	(193)

*Reproduced from (193, 194)

1.1.2.3.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is the best characterised pathway to date among the internalisation processes at the plasma membrane. Essentially, all cell types utilise clathrin-mediated endocytosis for many different cargo transports as a “housekeeping” tool. These events include the regulation of surface proteins and lipids, internalisation of nutrients such

as low-density lipoproteins and iron-saturated transferrin, and uptake of growth factor receptors upon their activation for downstream signalling (195-197). In addition, its ubiquitous nature is exploited by some pathogens, such as the influenza virus and toxins, to gain entry into cells via this pathway (198, 199). The size of carriers (endocytic vesicles) are dependent on the species, cell types and the size of cargoes, and it has been observed to vary from 30 to 200 nm external diameter (200). Loss of function of the essential components (clathrin, adaptor proteins, epsin and dynamin, which are described below) of clathrin-mediated endocytosis appears to cause embryonic lethality (201-203). However, there have been some cases reported of human diseases such as cancer, myopathies, neuropathies, psychiatric diseases, neurodegenerative diseases, metabolic and genetic syndromes to be the result of perturbed clathrin-mediated uptake (200).

Clathrin-dependent endocytosis can be described in a mechanistic way as a three-step process (204). Firstly, endocytosis initiates with the regulated recruitment of clathrin coat proteins and adaptor proteins that are needed for cargo selection at the plasma membrane. Secondly, accessory proteins and dynamin function together to complete formation of endocytic vesicles that are coated with clathrin. Lastly, the ATPase chaperone Hsc70 and auxilin uncoat the clathrin-coated vesicles to facilitate the fusion of the vesicles with endosomes.

Initially, the selection of cargo proteins for internalisation requires the recognition of endocytic signals, located in the cytosolic domains of the cargo, by adaptor molecules (205). Adaptors and clathrin must be assembled at the plasma membrane, inducing the formation of clathrin-coated pits and the concentration of cargos (204, 205). The triskelion, composed of three copies of clathrin, is the assembly unit of the clathrin coat and the triskelia form a

lattice that provides a scaffold for recruitment of various clathrin-associated proteins. According to electron cryomicroscopy analysis, the clathrin heavy chain is proposed to provide critical stability to the lattice by making contacts between a trimerisation domain at its C-terminal and segments at the N-terminal domains of three additional triskelia, whereas the clathrin light chain functions as scaffolding protein by interacting with cytosolic regulatory proteins (206, 207). As triskelia do not directly bind to the plasma membrane, adaptors are required to support and stabilise the interactions between the clathrin coats and the membranes (208). A key adaptor molecule in clathrin-mediated endocytosis is adaptor protein 2 (AP-2) and its appropriate placement requires high local levels of $PI(4,5)P_2$ (208-211). Upon activation of phospholipid kinases on the inner leaflet, $PI(4,5)P_2$ levels increase transiently, leading to association of AP-2 with the membranes. Subsequently, the affinity of other factors for clathrin-coated pit nucleation also increases and this is followed by the formation of the clathrin lattice (212-215).

During the assembly of the clathrin machinery and the nucleation process at the plasma membrane, the generation of highly curved membranes from flat bilayers is also required to take place (204). There are four main factors known to promote clathrin-coated vesicle budding and assembly and these are important components of clathrin-mediated uptake. Membranes can be deformed by insertion of amphipathic helices into the cytosolic leaflet causing an asymmetry between the outer and the inner leaflets of the lipid bilayer (216). Proteins that contain epsin N-terminal homology (ENTH) domains are examples of this mechanism (217, 218). Another protein module that induces membrane curvature is the BIN1/amphiphysin/Rvs167 (BAR) domain. With its unique dimeric shape (an elongated crescent) and a positively charged concave surface allowing the direct interaction with

negatively charged membranes, the BAR domain has been proposed to function as a mould (scaffold) achieving a certain degree of membrane curvature and also recruiting other components (219). Some BAR domains are found to have an N-terminal amphipathic helix (N-BAR domain), which shows the combination of an ENTH domain and a BAR domain whose activity results in membrane curvature by both scaffolding and insertion of helices into the inner leaflet (220, 221). The last module that participates in clathrin-mediated endocytosis by facilitating membrane deformation is the Fes/CIP4 homology and BAR (F-BAR) domain. This domain is known to interact with dynamin and contributes to actin reorganisation, both of which are crucial factors for membrane fission (222, 223).

Dynamin is well-known to function as a mechanochemical molecule that generates GTP-dependent force to sever the membrane tube producing membrane vesicles (204). This GTPase has several modules that are necessary for its activity as a main mediator of membrane scission. There are a GTPase domain, a GTPase effector domain (that functions as a GAP in an intramolecular manner), a PH domain (for binding to $PI(4,5)P_2$), and a proline-rich domain (for binding to SH3 domain-containing proteins) (224-226). One of the binding partners of the proline-rich domain of dynamin is cortactin, which has an SH3 domain (227). Cortactin interacts with actin and also activates the Arp2/3 complex, which promotes the formation of branched actin filaments (227). Recent studies observed the recruitment of dynamin and actin assembly during the invagination of clathrin-coated pits and membrane fission reactions (228). This suggests that actin reorganisation contributes to complete membrane scission and clathrin-coated vesicle formation in coordination with dynamin (204).

Prior to transport to appropriate intracellular components, the clathrin-coated vesicles that are detached from the plasma membrane must dissociate their own clathrin coats. This uncoating process requires energy as clathrin assembly and lattice formation takes place spontaneously (204). One of the crucial factors in clathrin-coated vesicle uncoating is the heat shock cognate 70 (Hsc70), which is an ATPase (229). Another key protein is auxilin, which can directly bind to clathrin, AP-2, and Hsc70, thus enabling the recruitment of the ATP-bound ATPase to clathrin-coated vesicles (230, 231).

1.1.2.3.2 Caveolar endocytic pathway

Caveola, another well-known endocytic structure, also has a special coat surrounding its flask-shaped invagination of the plasma membrane. After the caveolar structure was first identified by electron microscopy 50 years ago, researchers have found several biological roles of caveolae and yet their functions are not fully elucidated. To date, caveolae are known to be implicated in vesicle trafficking, signal transductions, lipid regulation, and cell membrane protection from mechanical stress (232). A caveolar structure is characterised by oligomeric integral membrane proteins called caveolins and peripheral membrane proteins termed cavins. The size of caveolae varies from 50 to 100 nm and, in the case of non-muscle cells, there are approximately 140 molecules of caveolin-1, 30 – 70 molecules of caveolin-2 and a complex of cavins per cell, whereas caveolin-3 replaces the caveolin-1/2 complex in skeletal muscle cells (187, 233-238). According to the mutation analysis of caveolins and cavins in patients, caveola dysfunction is found to have a relation to several types of human

diseases such as muscular dystrophies, cardiac disease, infection, osteoporosis, lipodystrophy and cancer (239, 240).

The formation of mature caveolar structures requires three major components (234). First, caveolins are needed to play a "scaffolding" role. Among all the three caveolins, only caveolin-1 and caveolin-3 are able to induce caveola formation because caveolin-2 cannot oligomerise by itself (241-245). The basic scaffold unit, formed in the ER, consists of 15-25 caveolin oligomers (the ratio of caveolin-1 to caveolin-2 is 2:1 to 4:1 (244)), which have the capacity to interact with each other in the Golgi to build larger stable assemblies that are equivalent to functional caveolae. Second, a lipid bilayer enriched in cholesterol and sphingolipids is also necessary for caveolae formation. The Golgi is the place where the smaller scaffold subunits oligomerise and this polymerisation step is dependent on cholesterol. The strong association between caveolins and cholesterols makes the assemblies immobile in a special form of lipid microdomain where many different proteins are known to be functional and subsequently numerous biological events take place. Finally, cavins are also key factors in caveolae formation and their function is to stabilise the Golgi-derived caveolar structure through the association with phosphatidylserine, which is abundant in the inner leaflet of the regions rich in caveolae (246, 247). Other roles of cavins include induction of membrane deformation and budding of caveolar membranes.

The machinery required when caveolae undergo endocytosis includes dynamin, Eps-15 homology domain-containing protein 2 (EHD2) and PKC and casein kinase substrate in neurons 2 (PACSIN2), which perform the similar "pinch-off" mechanism as clathrin-coated pits show. Dynamin regulates caveolar budding from the plasma membrane by localising and functioning at the neck of caveolar invagination (248-250). Like dynamin, the ATPase

EHD2 also localises to the neck but it is known to act as a negative regulator of endocytosis as its depletion leads to increased caveolae budding (248, 249). It associates with caveolae by its affinity for $PI(4,5)P_2$, which is enriched around the caveolar opening (251). The actin cytoskeleton is also involved in this inhibitory regulation (252). Results obtained by modifying the expression levels of EHD2 suggest that EHD2 directly or indirectly links caveolae to actin in an ATPase cycle-dependent way. The membrane sculpting BAR domain protein PACSIN2 modulates membrane curvature and it can directly interact with EHD2, dynamin, and caveolin-1 (253-255).

There are some distinct features that can be seen in caveolar uptake in contrast to clathrin-mediated endocytosis. Caveolae show significant heterogeneity depending on different cell and tissue types, suggesting their cell/tissue-specific functions (256, 257). Also, unlike the ubiquitously distributed clathrin-coated pits, caveolar density is uneven on the plasma membrane and frequently shows higher abundance at the rear part of migrating cells (244, 258). In addition, caveolins, the main components required during the endocytic process, can directly bind cholesterols in the plasma membrane (259), while clathrin needs the assistance of adaptor molecules to be linked to the lipid bilayer. Furthermore, caveolins contain a scaffolding domain, which signalling proteins can bind to, making caveolae play a regulatory role in signal transduction (260). Lastly, caveolins, which are the caveolar coat proteins, do not go through cycles of assembly and disassembly once they are assembled, which is opposite to clathrin and coat proteins (COPs) (261). Instead, cavins, which are peripherally attached to the cytosolic side of caveolae, induce membrane curvature and regulate membrane budding by interacting with or being released from the membranes (238, 262, 263). Especially cavin-1 is known as a good marker of functional caveolae due to

its exclusion from structures such as those from the biosynthetic secretory pathway of caveolae or disassembled caveolar domains (for example, if cholesterol is depleted) (246).

1.1.2.3.3 Other clathrin-independent endocytosis

To date, unlike clathrin-mediated or caveolar endocytosis, which can be easily conceptualised with specific coat protein(s), morphologies, cargo types and membrane pinching machineries, several internalisation pathways have been identified that lack such specialised components required in classical endocytic steps (190).

The Interleukin 2 (IL-2) receptor is one of the cargoes that is internalised via a clathrin- and caveolin-independent and dynamin-dependent endocytic pathway (188, 264). The IL-2 receptor uptake is observed to take place within non-coated (or coated with as-yet-unidentified proteins) invaginations. In addition to dynamin, cholesterol is another crucial component for the IL-2 receptor endocytosis and it allows IL-2 receptor and many other IL receptors that share the conserved subunits to associate to lipid raft microdomains (188, 264). Rho A and Rac1 are also known important molecules for the activation of actin reorganisation, which facilitates IL-2 receptor internalisation (265).

Another example of cargo that uses a dynamin-dependent route, which is independent of clathrin and caveolin, is EGFR, whose known uptake mechanism at low dose is via the clathrin-dependent pathway. Sigismund et al. (266) found that high concentrations of EGF switched EGFR internalisation to a cholesterol-dependent, clathrin-independent pathway. Recently, Caldieri et al. described a type of EGFR endocytosis that is clathrin- and

caveolin-independent but requires plasma membrane-ER contact sites, where the endocytic invagination is formed and matured (267).

Fast endophilin-mediated endocytosis (FEME) is another recently characterised pathway (189). The BAR domain protein endophilin, which has been known as one of the peripheral components of clathrin-mediated endocytosis, was found to regulate this distinct endocytic pathway by playing central roles as an adaptor protein for cargo recruitment, a membrane curvature modulator, and a membrane scission protein. This mechanism is sensitive to inhibition of dynamin, phosphatidylinositol-3-OH kinase (PI3K), Rac, p21-activated kinase 1 (PAK1), and actin polymerisation but it is activated upon Cdc42 inhibition. The endocytic structures are distributed at the leading edges of migrating cells where $PI(4,5)P_2$ is present. The cargoes that are internalised, once stimulated by ligands, through this pathway include IL-2 receptors, cholera and shiga toxins (CTxB and STxB), several G-protein-coupled receptors (β_1- and α_{2a}-adrenergic receptor, dopaminergic receptors 3 and 4, themuscarinic acetylcholine receptor 4), and a group of RTKs (EGFR, VEGFR, HGFR, PDGFR, NGFR, and IGF1R) (189, 268).

Another endocytic pathway, which was recognised as an independent pathway specific for glycosylphosphatidylinositol (GPI)-anchored proteins, is called the clathrin-independent carrier (CLIC)-GPI-anchored protein-enriched early endosomal compartment (GEEC) pathway (269, 270). Internalisation is not affected by the inhibition of clathrin or dynamin and its morphology is elongated tubular forms rather than vesicular structures. The key factors in this pathway include Cdc42, whose inhibition negatively affects the uptake process, ADP-ribosylation factor 1 (ARF1), BAR domain protein GTPase regulator associated with focal adhesion kinase 1 (GRAF1), cholesterol and actin (269, 271-273). The distribution

of the endocytic structures of the CLIC-GEEC pathway is highly polarised to the leading edge of migrating cells, which is another distinct feature from clathrin- or caveolin-dependent pathways (271, 274).

Another endocytic process involves the activity of ARF6, which is present at the plasma membrane and on the endosomal membranes where specific cargoes are delivered to (190). Those cargoes that enter cells via this ARF6-associated pathway include glucose transporter type 1 (GLUT1; nutrient transport), cluster of differentiation 44 (CD44; matrix adhesion and cell-cell interaction), major histocompatibility complex I (MHC I; immune function), and some GPI-anchored proteins (191, 275). Once they are internalised independently of clathrin, the endosomes containing these cargoes were observed to converge with endosomal components containing the cargoes of classical clathrin-mediated endocytosis (276). Endosomal cargoes are sorted upon ARF6 inactivation and segregated cargo proteins can be transported toward the plasma membrane via a specific recycling pathway, which requires ARF6 activation (276).

Flotillin is another regulator that controls a distinct dynamin-independent endocytosis of specific cargoes such as CD59 and CTxB. Depletion of flotillin1 perturbes the uptake of these cargoes (277). However, this endocytic process has not been fully established as there are studies showing that dynamin may be implicated in the same cargo uptake that requires flotillin (278). Therefore, this pathway still needs further characterisation of, for example, the function of flotillin in endocytosis and the identification of additional machinery proteins required for endocytic vesicle formation (190).

Another internalisation pathway to be reviewed is the endocytosis of the acetylcholine receptor, CD80 and CD86 (190). When it is stimulated by its antagonist, α bungarotoxin, the acetylcholine receptor is internalised via a slow dynamin-independent endocytic pathway, which requires the activation of the non-receptor tyrosine kinase c-Src and subsequent activation of Rac1, which in turn results in actin polymerisation (279). HIV *Nef* protein also triggers a similar mechanism to downregulate the costimulatory proteins CD80 and CD86 by promoting their internalisation in macrophages to prevent the infected macrophages from activating naïve T cells (280).

Lastly, a recent study reported that ALG-2 interacting protein-X (Alix) is selectively necessary for clathrin-independent endocytosis (193). This protein is known to be involved in membrane bending and fission by binding and recruiting endosomal sorting complex required for transport (ESCRT) complex proteins, consequently affecting several biological processes such as neuronal apoptosis (281-283), cytokinesis (284, 285), cell spreading (286), membrane repair (287, 288), and virus egress (289). In this study, Alix was found to regulate fluid phase endocytosis, GPI-anchored protein endocytosis, cholera toxin uptake, and surface receptor internalisation in mouse embryonic fibroblasts (MEFs) (193). Alix is a major binding partner of endophilin-A and it requires the association with endophilin-A for the regulation of the cholera toxin endocytic pathway. However, GPI-anchored receptor or fluid phase endocytosis was not co-dependent on endophilin-A. In addition, endophilin-A has a dual role in both clathrin-mediated and clathrin-independent endocytosis (189) whereas Alix regulates only clathrin-independent pathways without affecting the clathrin-dependent process.

1.1.2.4 Aberrant intracellular trafficking in cancers

1.1.2.4.1 Rabs in cancers

According to integrative genomic studies, aberrations in Rabs were found to contribute to the tumorigenesis and progression of cancers. Associations between Rabs and specific cancer types include: Rab1A with tongue cancer (290), Rab2B with colon cancer (291), Rab5a and Rab7b with thyroid-associated adenomas (292), Rab23 with invasive gastric cancer (293), Rab27a with prostate carcinoma (294, 295), breast cancer (296-298), and hepatocellular carcinoma (299), Rab38 with melanoma (83). In addition, Rab1B, Rab4B, Rab10, Rab22A, Rab24 and Rab25 are upregulated in hepatocellular carcinomas (300). Rab27 isoforms (297, 298) and Rab31 (301) are linked with poor outcome in invasive breast cancer. Systemic study on ovarian cancers showed that almost half of Rab or Rab-associated gene expressions were upregulated (302).

There is a specific group of Rabs (Rab11A, Rab11B, Rab25/Rab11C) and their effectors that are responsible for intracellular recycling pathways and several studies demonstrated that they are strongly associated with multiple cancer lineages, such as breast, ovarian, lung, colon, renal, prostate, endometrial, bladder and carcinoid types (303). Many tumours appear to sustain elevated levels of incessant growth signals (e.g. RTK signalling) and migration signals (e.g. integrin signalling) at the plasma membrane by lowering the rates of endocytosis and promoting protein recycling. In addition, there are some active signalling complexes that localise to endosomes and their signalling takes place intracellularly or on return to the cell surface (304, 305). For example, transforming growth factor alpha (TGFα) is a ligand that is recycled back to the plasma membrane after

dissociating from its receptor EGFR in the acidic late endosomes resulting in sustained signalling, whereas EGF, another ligand of EGFR, forms a stable EGF/EGFR complex and it is subsequently delivered to lysosomes leading to attenuation of signalling (306). According to a genomic and transcriptomic analysis, the frequent 1q22 amplicon in breast and ovarian cancer was revealed to be associated with Rab25 (307-309). Ectopic expression of Rab25 is closely linked to tumorigenicity and metastatic potential in ovarian cancer cells (309). Abnormal recycling of growth factors and integrins in 3D cultures of ovarian cancer cells is also related to Rab25/Rab11 (305).

One of the well-known pathways that are implicated in a wide range of cancers is the PI3K pathway and aberrations in Rab-associated proteins appear to affect this pathway contributing to tumorigenic phenotypes. P85α-encoding molecule PIK3R1, which is mutated in many cancers, regulates the p110 catalytic subunit of PI3K and also functions as GAP to Rab4 and Rab5 (310). Once PIK3R1 is mutated to lose GAP activity, it results in sustained levels of activated platelet-derived growth factor receptors (PDGFRs) and induces the upregulation of its downstream signalling (310). Rab4 regulates E-cadherin recycling and stability by interacting with the N-myc-downregulated gene protein 1 (NDRG1) and downregulation of NDRG1 is linked to poor prognosis in metastatic breast and prostate cancers (311). Rab5 plays a key role in Rac-dependent actin assembly and cell migration by controlling Rac delivery to the plasma membrane and also by mediating the endocytosis and recycling of integrins (312, 313). Therefore, the mutation of PIK3R1 and its consequences show how abnormal Rab-associated molecules can cause malignant effects in cells.

Some Rabs are known to contribute to cancer cell invasion by promoting protease secretion (314). The secretion of MT1-MMP at peripheral invasive structures in breast

cancer cells is promoted by Rab8 subsequently increasing cell invasiveness (315). Rab7 inhibition causes impaired cell migration and invasion as it is also associated with MT1-MMP secretion (316). MMP9 secretion is found to require Rab27a in mammary carcinoma cells of mice promoting tumour invasion (317). Rab27B promotes proliferation, tumour growth, and metastasis by activating heat shock protein 90 (Hsp90) and matrix MMP2 (298).

The tumour–stromal cell communication can be mediated by some Rab proteins resulting in the modification of the tumour microenvironment (314). The change in cancer cell surroundings tends to subsequently facilitate cancer cell growth and metastasis (314). Important regulators of this process are cancer-associated fibroblasts (CAFs) (318). In squamous cell carcinoma, Rab21 contributes to cancer cell invasion by stimulating CAFs to remodel the extracellular matrix by inducing elevated integrin accumulation and actomyosin contractility in CAFs, which then cause collagen contraction (319). Other factors involved in tumour–stromal cell communication include tumour-derived exosomes and microvesicles (320). Rab27a has been shown to be an important regulator of exosome secretion in both *in vitro* and *in vivo* conditions. It is required for the exocytosis of exosomes in HeLa cells (321). *In vivo*, Rab27a inhibition in melanoma cell lines downregulated not only primary tumour growth but also the numbers of lung metastases due to impaired exosome secretion, which is supposed to recruit bone-marrow-derived cells to form a pro-metastatic niche (322).

The abnormal expression of some Rabs have been shown to have an impact on cell-cycle process (314). The overexpression of Rab6c, mainly localised in the centrosome, causes G_1-phase arrest, and its depletion results in tetraploid cells (323). Rab21-mediated integrin accumulation is required for cytokinesis and depletion of Rab21, therefore, induces

multinuclei and genomic instability (324). In the breast cancer cell line MCF-7, Rab27b overexpression contributes to cell-cycle progression and cell proliferation (298).

There are some Rabs that are linked to anticancer drug resistance in human cancers (314). Multidrug resistance (MDR) is frequently associated with the P-glycoprotein overexpression (325). This transmembrane protein is known to extrude anticancer drugs lowering their toxicity in the cells. When Rab4, whose expression is downregulated in MDR cells, is overexpressed, P-glycoprotein level is reduced, increasing drug sensitivity of MDR cells (326). Rab6c also shows a similar pattern, its expression is low in MDR cells and its increased expression level results in anticancer drug accumulation inside cells (327). On the other hand, Rab8 is found to be upregulated in cisplatin-resistant cancer cells (328). Its overexpression enables cisplatin-sensitive cells to acquire resistance to the chemotherapeutic molecule by promoting the exocytosis of a cisplatin-resistance-associated protein called transmembrane protein 205 (THEM205).

1.1.2.4.2 The exocysts in cancers

From the canonical role of the exocyst, including secretion, transport of membrane proteins and membrane expansion, it can be assumed that the exocyst complex would be directly or indirectly involved in cancer (91). In a third of all human cancers, oncogenic mutations in the gene that encodes Ral induce the production of this GTPase in its constitutive state (329). Exo84 and Sec5 are Ral effectors and their reduced expressions (knock-down) in human embryonic kidney cells result in a downregulated Ral-driven tumorigenicity, confirming that Ral acts in tumorigenicity in part through the exocyst (329).

The Ral-exocyst complex may play a scaffolding role by recruiting the IkappaB kinase, which in turn activates Akt and allows inhibition of apoptosis in cancer cells (330). A Sec8 knock-down study using flies found that JIP4, a JNK related protein, could interact with components in growth signalling pathways and this led to immortalisation of cells (331). In addition, another study using human cell lines found that Ral and Exo84 are key molecules for stress-induced autophagy, whose deregulation can cause cancer (157).

The invadopodium is a “finger-like” extension of the plasma membrane, which shows high density of actin filaments, and it is a well-known structure for breaching the extracellular matrix. The invadopodial degradative activity is required for cancer invasion and metastasis. The components of the invadopodium structure include cell-matrix adhesion molecules, extracellular signalling receptors (e.g. tyrosine kinases), actin-assembly mediators, and matrix proteases (332-335). The exocyst complex is involved in the secretion of MMPs and the formation of invadopodia by delivering the key molecules to the site where active extracellular matrix degradation takes place (182). Another role of the exocyst is actin regulation through the interaction with the Arp2/3 complex and its activator WASH (175, 182). In hepatocellular carcinoma cells, the exocyst complex also mediates the positioning of the G-coupled chemokine receptors CXCR4 at the plasma membrane allowing the receptor to trigger the signalling for tumour growth and metastasis (336).

Filopodia, similar architecture to invadopodia, are also actin-rich “finger-like” protrusions and they are rather linked to cell adhesion and migration. A study using mice skin melanoma cells found that Exo70 promoted the formation of filopodia by generating negative membrane curvature, in a similar manner to BAR domain proteins, and subsequently inducing Arp2/3-mediated actin polymerisation, which will drive the growth of

filopodia (176). The rest of the exocyst subunits are not related to membrane deformation nor filopodia establishment and, therefore, the activity described above may be of a free pool of Exo70 rather than of the exocyst as a whole complex (176).

1.1.2.4.3 The endocytic molecules in cancers

It has long been suspected that endocytosis is another physiological process that is modified in cancer. Not only do cancer cells show alterations in the overall appearance and dynamics of the plasma membrane, but also the common inability of cancer cells to properly regulate the function of several types of receptors, such as many RTKs, strongly suggests an inability to internalise, recycle, or degrade these key cancer drivers (337). As described earlier in this chapter, the canonical way of removing the activated forms of RTKs with their bound ligands (338) from the cell surface is often avoided by either oncogenic mutations in the growth factor receptors or their aberrant expressions at the cell surface (303, 339).

The ErbB family consist of four transmembrane RTKs and EGFR is known as the prototype of this family. Brain, head and neck, and other several tumours frequently show overexpression of ErbB family members through gene amplification or other mechanisms (340). These mechanisms include limited internalisation capacity of clathrin-coated pits, derailed ratio of cargoes to endocytic components, inhibited uptake of EGFRs, and recycling following the receptor endocytosis (341). At the plasma membrane, overexpression of ErbB family members cause the formation of dimers, primed for ligand binding and signalling, by increasing the association frequency of EGFR monomers (342). Dimerisation of ErbB protein

monomers produces both homodimer and heterodimers, and each combination results in different consequences depending on the intrinsic endocytic signals of the receptors, interaction with ubiquitin ligases, and sensitivity of the ligand-receptor complex to the endosomal pH. Human epidermal growth factor receptor 2 (HER2) or ErbB2 is the closest member to EGFR and its overexpression is frequently observed in some tumours such as breast and gastric cancers. Importantly, HER2 can effectively avoid the internalisation process due to the absence of known ligands for this receptor, the lack of internalisation signals, or its association with heparin sulfate proteoglycans or microdomains that retain HER2 at the plasma membrane (343, 344). In addition, internalised HER2 rapidly recycles back to the cell surface (345). Upon overexpression, HER2 can form heterodimers with EGFR promoting recycling of both molecules, which is partially caused by reduced receptor ubiquitination (346, 347).

The tumour microenvironment also enables enhanced and sustained RTK signalling by altering endocytic pathways. Hypoxia is one of the favourable conditions for cancers (6) and EGFR overexpression can be induced by the activation of hypoxia-inducible factor (HIF) in the core of solid tumours by the hypoxic microenvironment (348). The Rab5 effector rabaptin-5 is downregulated at the transcriptional level by HIF and this causes the attenuation of Rab5-mediated endocytic pathway leading to the delay of endocytosis-mediated deactivation of receptors (349).

There are known negative regulators of RTKs that affects their endocytosis and these suppressors are found to be weakened in tumours. According to a DNA sequencing analysis of leukemic bone marrow, a point mutation in Cbl, a main controller of RTK ubiquitination, appears to inhibit endocytosis and ubiquitination of Flt receptor, another type of RTKs (350).

Mig6/RALT is another example that negatively regulates relevant downstream signalling by interacting with all ErbB members and other RTKs. It targets EGFR to internalisation and degradation via the recruitment of endocytic adaptors, such as AP-2 and intersectins (351). From high-resolution genomic profiles of glial tumours, a highly recurrent focal deletion (1p36) was found for Mig6 (352).

1.2 BREAST CANCER AND MT1-MMP

1.2.1 Breast cancer

Breast cancer was the most common cancer registered in 2015, accounting for around one third (31.2%) of female cancer patients in England (353). According to the latest analysis, the five-year net survival rate for breast cancer patients is 86.0% (354). Breast cancer is a disease well known for its clinical and molecular heterogeneity (355-358). The most common subtype is invasive breast cancer of no special type (NST), which accounts for 9 out of 10 of breast cancers (359). Around 15% of these cancers are triple-negative breast cancer (360). There are also other types of breast cancer that are rare cases in clinic, such as invasive lobular carcinoma (10%), medullary breast cancer (5%), mucinous breast cancer (2%), tubular breast cancer (1%), inflammatory breast cancer (0.02%), and basal type breast cancer (361). In spite of this complexity, the principal clinical approach is the assessment of three biological markers: the expression of the endocrine receptors for oestrogen (ER) and progesterone (PgR) and the abnormal expression of HER2 (362).

Staging cancer is an essential step for accurate prognosis and appropriate treatment planning by determining how extensive the cancer is and where it is located in the body. The most commonly used staging system is the TNM Staging System (363), established by the American Join Committee on Cancer (AJCC). It is based on the description of the primary tumour (T), the extent of cancer spread to the lymph nodes (N), and the presence of distant metastasis (M). Once the T, N, and M are determined, they are commonly translated into an overall stage of I, II, III, IV. In general, earliest stage of breast cancer is stage I, where a small cancer is found only in the breast tissue. Stage II, which is still grouped as an early stage, indicates the condition where the cancer is either in the breast tissue or in the lymph nodes near the breast or in both sites. Stage III, which belongs to locally advanced breast cancer, refers to the state when the cancer has spread out of its original location to lymph nodes or to the skin of breast or to the chest wall. Lastly, stage IV breast cancer, called advanced cancer, shows the metastatic spread of cancer to other distant parts of the body. Although survival is generally good for breast cancer patients, the development of the disease toward more advanced status results in a significant decrease in survival rate (364): most women in stage I (99%) and stage II (90%), 60% of patients in stage III, and only 15% of women with stage IV breast cancer could survive for five years or more after they were initially diagnosed.

There are several therapeutic methods currently available for breast cancer treatment. These include surgery, radiotherapy, chemotherapy, hormonal therapy, and biological therapy, and patients are treated with tailored combinations of them to make therapies more precise with less side effects. However, tumour heterogeneity has been causing therapeutic challenges such as acquisition of drug resistance. To avoid this,

clinicians have been adopting a few strategies: combinations of targeted therapies for tackling driver mutations, therapies for targeting passenger mutations that bring deleterious effects, approaches based on identification and eradication of the lethal clones, targeting the tumour microenvironment with immunotherapeutics, and adaptive therapy, which handles the disease in a 'treat-to-stabilise' manner and not in the conventional 'treat-to-eradicate' manner (365).

1.2.2 MT1-MMP

The spread of tumour cells to distant sites of the body via metastasis is the major cause of human cancer death (366) and this process was classified as one of the acquired capabilities required for cancerous progression (367). With the capability to metastasise, cancer cells are able to "leave" the primary tumour and "disseminate" to new areas where nutrients and space are sufficient (367). The initiation of the metastatic cascade takes place with the cancer cells breaching and navigating through the surrounding tissue (invasion); then, once entered the microvasculature (intravasation), they circulate through the bloodstream and reach distant tissues where they exit (extravasation); here, they survive at the new site by adapting to the surrounding microenvironment and form secondary tumours (colonisation) (368). Among these steps, invasion, achieved by proteolysis and remodelling of the extracellular matrix, is the process where MT1-MMP is essential (369).

MT1-MMP belongs to the MMP family, which has 25 members in humans and is composed of multifunctional zinc-dependent endopeptidases that degrade the extracellular matrix (370). These proteases share a conserved structure that contains a signal peptide, a

propeptide and a catalytic domain and some MMPs also carry a hemopexin (HPX) domain that is involved in substrate recognition/degradation and protein-protein interaction (371, 372). Among the MMPs, which are mostly secreted enzymes, MT1-MMP is one of six membrane-anchored MMPs (MT1-MMP to MT6-MMP, also named as MMP-14, MMP-15, MMP-16, MMP-17, MMP-24 and MMP-25, respectively) and it is produced as zymogen (proMT1-MMP, ~64 kDa), which can be proteolytically cleaved by furin in the TGN to be catalytically active (~55 kDa) and transported to the cell surface (373, 374).

It has been reported that there are strong associations between protein and mRNA levels of MMPs and different human cancers (375) and the retrospective analysis of MMP expression levels in samples from cancer patients showed that the expression of several MMPs, including MT1-MMP, in primary tumours and/or metastases is positively correlated to progression, invasive stage and metastasis of cancer (375, 376).

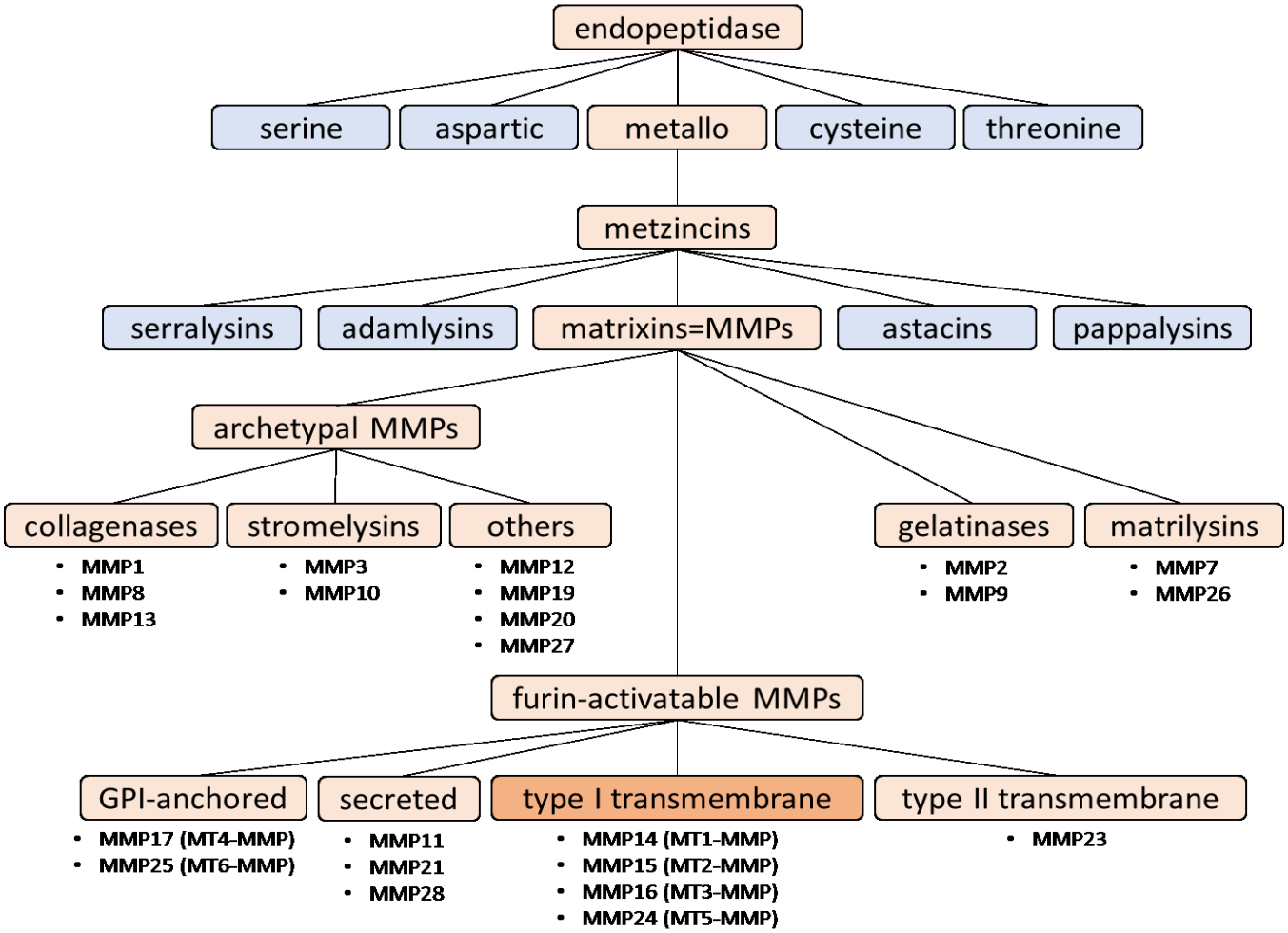


Figure 6. Schematic overview of MMP family members and other metzincin superfamily members. This is reproduced from Vandenbroucke et al., 2014. Matrix metalloproteinases (MMPs) share a common domain structure: a pre-domain, a pro-domain, a catalytic domain, and a haemopexin domain. The pre-domain that has a signal peptide that is required for secretion and the pro-domain keeps the enzyme inactive by the interaction between a cysteine residue and the Zn2+ ion group from the catalytic domain. The haemopexin-like carboxy-terminal domain is linked to the catalytic domain via a flexible hinge region. MMPs that belong to matrilysins lack the hinge region and the haemopexin domain. Gelatinases have a fibronectin type II motif added into the catalytic domain. Membrane-type MMPs (MT-MMPs), which MT1-MMP belongs to, have a transmembrane domain or a glycosylphospatidylinositol (GPI) anchor at the C terminus.

In complex with tissue inhibitor of metalloproteinases-2 (TIMP-2), MT1-MMP facilitates proteolytical activation of some secreted MMPs (proMMP-2 and proMMP-13) (377, 378), and it also has its own capability to cleave a variety of extracellular matrix components that include type-I, -II and -III collagen, gelatin, and cell-surface proteins such as CD44 and αv integrins (379). The basement membrane is the 50 to 100μm-thick structure that separates epithelial cells from connective tissues. This structure is highly cross-linked

and contains several components including type-IV collagen, laminin and heparin-sulphate proteoglycans (380). In spite of its toughness as a barrier to cell migration, degradation of the basement membrane by cancer cells takes place when the cells leave the primary tumour and during intravasation or extravasation (381). From the studies that tested the basement membrane degradation using either the reconstituted model named as Matrigel (382, 383), or the native basement membrane itself (384), MT1-MMP emerged to be, among all pericellular MMPs, the MMP mainly responsible for basement membrane breaching.

MT1-MMP is also known to play a crucial role in degrading and remodelling interstitial collagen networks. Invasive cells in these matrices have two possible migratory modes depending on the degree of cross-linking within the extracellular matrix and the conversion between the two modes is also possible (385, 386): i) amoeboid mode, which is a non-proteolytic migration where cells squeeze themselves between gaps in the 3D matrix (387) and ii) mesenchymal migration, which requires matrix degradation mediated by MT1-MMP and shows directional motility with adherent β1-integrin-dependent migration and Rac1-dependent actin rearrangement (388, 389). Therefore, cells that migrate through native, highly cross-linked matrices predominantly adopt a mesenchymal migratory mode using the proteolytic activity of MT1-MMP (372, 390-393). Although cells within certain interstitial tissues where the networks are of low degree of cross-link can adopt amoeboid motion (389), it is clear that degradation of basement membranes by MT1-MMP and/or other MMPs must occur prior to further invasion and metastatic processes.

Other studies on a molecular level found that MT1-MMP is enriched at invadopodia, which are selectively found in invasive cancer cells (394, 395). These actin-based membrane

protrusions are assembled at the focal sites of extracellular matrix degradation (332). Invadopodia share overall architecture, functions, morphological and molecular features with podosomes, which are produced in cells such as monocytes, macrophages and oesteoclasts or in cells transformed with Src kinase (332, 396). For example, both of them have an actin-rich core, surrounded by a ring of actin-associated and signalling proteins, and contain branched and unbranched actin filaments. In addition, both are located at the ventral cell surface but invadopodia are often located under the nucleus whereas podosomes are often situated behind the leading edge of the cell (333, 397, 398). Microtubules are required for invadopodial elongation, not formation (399), while podosomes assemble microtubules for elongation and/or formation (400, 401). Invadopodia, moreover, protrude longer into the extracellular matrix than the protrusions of podosomes and invadopodial structures are stable for hours while those of podosomes are maintained for minutes (397).

The assembly of invadopodial core components, including F-actin and cytoskeletal proteins, into precursor structures is induced by the activation of integrins or stimulation of cells through the treatment with growth factors or the interaction with extracellular matrix (402-404). Then the accumulation of MT1-MMP at nascent sites of these precursors causes maturation and stabilisation of invadopodia at the plasma membrane (405-407). Fundamentally, invadopodial MT1-MMP is originated from the recycling pathway and/or the biosynthetic, secretory pathway. It has been reported that cell surface MT1-MMP is endocytosed (408-410) and recycled to the surface (411, 412) in stationary cells. In addition to this, the Rab8-dependent pathway was proposed to deliver MT1-MMP from storage compartments to the plasma membrane in invasive cancer cells (315). According to the

work of Zhu et al. (413), endocytic/exocytic fluxes, rather than the biosynthetic pathway, are considered to be the main mechanism in constant supply of active MT1-MMP to the plasma membrane, where MMP's proteolytic ability becomes downregulated by tissue inhibitor of metalloproteases-2 that is enriched in cancer as a protective factor.

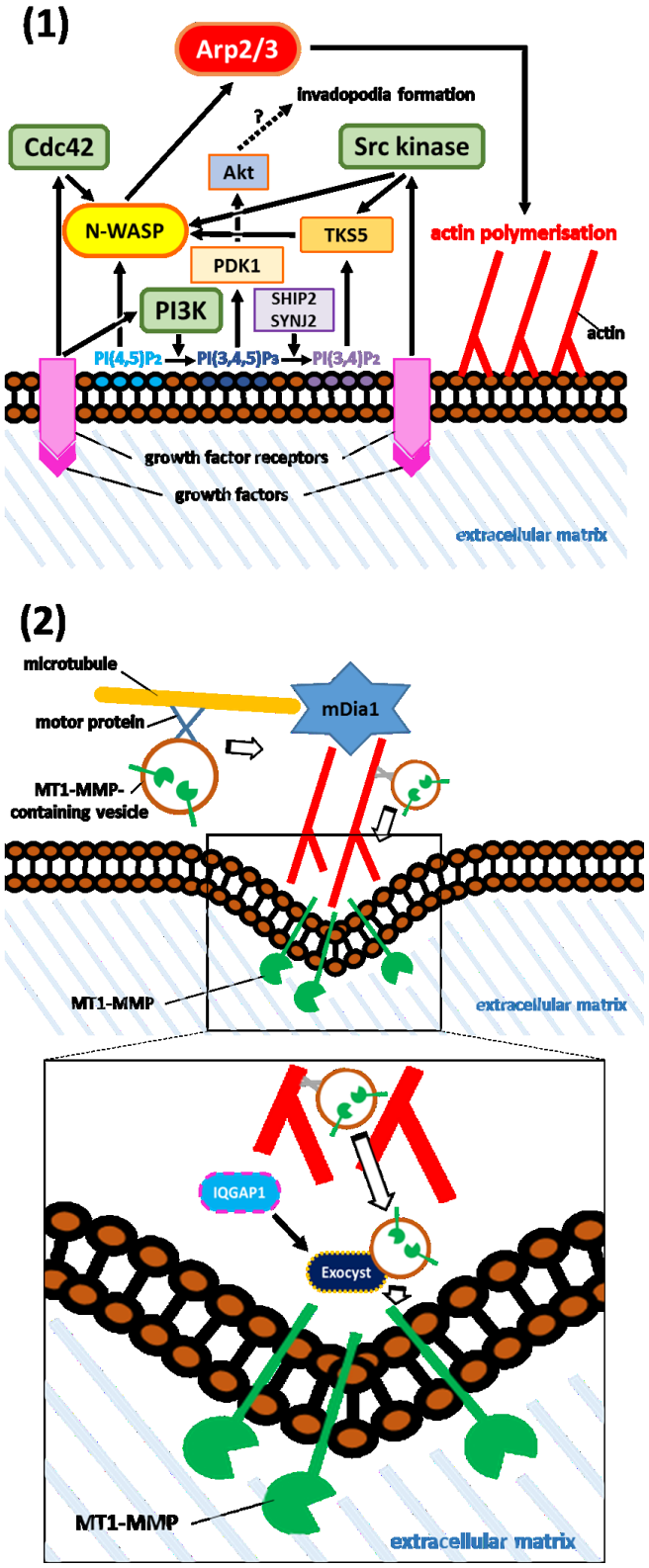


Figure 7. The formation of invadopodia. This illustration is reproduced from Hoshino et al., 2013. (1)Invadopodial signalling. Growth factor signalling via growth factor receptors results in invadopodia formation and actin polymerisation. Signals from protein kinases (Src kinase), lipid ($PI(4,5)P_2$ and $PI(3,4)P_2$), and Rho GTPase (Cdc42) converge on N-WASP, which in turn activates the Arp2/3 complex. $PI(4,5)P_2$ has been shown to be crucial for actin polymerisation by affecting a wide range of acting regulators including N-WASP and cofilin. PI3Ks phosphorylates $PI(4,5)P_2$ generating $PI(3,4,5)P_3$ and phosphoinositide-dependent protein kinase 1 (PDK1) mediates Akt activation at the downstream of $PI(3,4,5)P_3$. Both PDK1 and Akt have been found to be important factors for invadopodia formation, but the downstream effectors in this pathway are unclear. $PI(3,4)P_2$ is formed by SH2-domain containing $PI(3,4,5)P_3$ 5-phosphatase 2 (SHIP2) or synaptojanin-2 (SYNJ2) through dephosphorylation of $PI(3,4,5)P_3$. TKS5, a scaffolding protein, is recruited to the region of $PI(3,4)P_2$ accumulation and it can bind to N-WASP in response to Src activity, promoting actin polymerisation at invadopodia site. (2)MT1-MMP delivery to invadopodia and degradation of extracellular matrix. Proteinases such as MT1-MMP are central players in the extracellular matrix degradation. MT1-MMP-containing intracellular vesicles are transported along microtubules by motor proteins. Microtubule- and actin-binding protein mDia1 is required for the localisation of the vesicles at the invadopodial actin filaments. mDia1-interacting protein IQGAP1 is also known to interact with the exocyst complex, resulting in the exocytosis of MT1-MMP at invadopodial structure for the extracellular matrix digestion.

There is an emerging model that describes MT1-MMP recycling from the late endosomal/lysosomal compartments in triple-negative breast cancer cells (414, 415). An important factor in this model is a submicrometric actin dot on MT1-MMP-positive structures. The formation of the endosomal actin dots is dependent on the Arp2/3 complex, WASH, and cortactin. WASH depletion is shown to inhibit the endosomal actin assembly and MT1-MMP trafficking to invadopodia, consequently preventing pericellular matrix digestion and cancer cell invasion (415). Atypical protein kinase C iota (aPKCι) regulates endosomal actin via cortactin phosphorylation (414). Co-overexpression of aPKCι and MT1-MMP correlates with malignancy and poor prognosis of hormone receptor-negative breast cancers.

The interaction of WASH with the exocyst complex has been identified recently, and silencing WASH prevented proper recruitment of the exocyst complex and c-Jun N-terminal kinase (JNK)-interacting proteins 3 and 4 (JIP3 and JIP4) on MT1-MMP-positive late endosomes/lysosomes (415, 416). JIP3 and JIP4, known interacting partners of several motor proteins, are required for the association of MT1-MMP-positive endosomes with plus end-directed kinesin-1 and minus end-directed dynein/dynactin in MDA-MB-231 (416-418). The interaction of JIP3 and JIP4 with kinesin-1 and dynein/dynactin is regulated by ARF6 whose expression is frequently upregulated in cancer (416, 419-421). Based on these findings, Marchesin et al. proposed a mechanism illustrating that active ARF6 in the invadopodial domain at the plasma membrane is connected to the motors, mobile on microtubules, through its effectors JIP3 and JIP4, which are recruited on MT1-MMP-contraining endosomes (416).

There are several intracellular components that are closely involved in MT1-MMP secretion to invadopodia. The interaction of IQGAP1 with the exocyst complex is required for invadopodial structure formation and activity (407). IQGAP1, a key cell-polarity regulator, coordinates cytoskeletal assembly and exocytosis by linking the microtubule and actin cytoskeleton (422, 423). The exocyst vesicle-docking complex regulates the tethering of vesicles from TGN or recycling endosomes at the cell surface to facilitate exocytosis of MT1-MMP (407, 424, 425). It was reported that the exocytosis of MT1-MMP is controlled by a plasma membrane SNARE called Syntaxin-4 and a vesicular SNARE protein VAMP-7 (vesicle-associated membrane protein-7), which are SNAREs that mediate membrane fusion between transport vesicles and the plasma membrane (426, 427).

Recently, Rab2a has been reported to be a novel regulator in breast cancer cell dissemination by playing a role in MT1-MMP trafficking (428). Despite it has been known to mainly control ER-to-Golgi transport (291, 429), Rab2a was identified to be critically involved in MT1-MMP-dependent extracellular matrix degradation by affecting MT1-MMP trafficking from the late endosomes to the plasma membrane. VPS39, an important subunit of the late endosomal homotypic fusion and vacuole protein sorting (HOPS) complex (430), controls post-endocytic MT1-MMP recycling and it was found to directly interact with Rab2a in this study. The result that VPS39 silencing abrogated elevated proteolytic activity induced by ectopic Rab2a expression indicated that VPS39 plays a key role as a downstream effector of Rab2a in MT1-MMP trafficking. Accordingly, Rab2a was shown to be upregulated in human breast cancer and correlated to the disease recurrence in patients.

Although there have been many studies that proposed possible mechanisms for MT1-MMP delivery and targeting to invadopodia, it is still essential to find a clear answer

about MT1-MMP transport and activity at invadopodia in invasion and metastasis of human cancer cells.

1.2.3 Rab27a

Whilst some Rab proteins, such as Rab1 and Rab5, are ubiquitous and play housekeeping roles in the pathways of exocytosis and endocytosis, other Rabs, including Rab27a, are restricted to specific tissues: Rab27a is enriched in skin, eye, platelets, lung, spleen, pancreas, and intestine (431). Rab27a deficiency is associated with Griscelli syndrome, which results in immunodeficiency and pigmentary dilution of skin and hair with accumulation of melanosomes in melanocytes (432).

Rab27a is a secretory Rab that is known to function in the transport and docking of secretory vesicles to the plasma membrane (433) and it contributes to the transport and secretion of late endosomal/lysosomal compartments (431). In particular, Rab27a is known to be associated to lysosome-related organelles such as secretory granules in some immune cells and melanosomes in melanocytes, exosomal secretion, and other cancer-implicated protein exocytosis in cancers.

Rab27a was found to be necessary for granule secretion in cytotoxic T lymphocytes. In Rab27a-deficient ashen mice, cytotoxic T lymphocytes could not kill target cells or secret lytic granules that contain granzyme, which trigger rapid destruction of target due to the absence of Rab27a, whose function enables membrane docking of the granules at the immunological synapse (434). Jancic et al. found that dendritic cells originated from Rab27a-

lacking ashen mice have increased phagosomal pH and antigen degradation (435). This is the result of the delayed recruitment of the NADPH oxidase NOX2, which prevents phagosome acidification and antigen degradation, by Rab27a that regulates the trafficking of lysosome-related organelles to phagosomes.

Many researchers have been reporting the associations of Rab27a and neutrophil functions (436). Neutrophils are critical controllers of the innate immune response and they also modulate the adaptive immune response (437). Diseases in which neutrophils play important roles include acute and chronic inflammation (438), autoimmunity (439), metabolic disease (440), and cancer (441). There are four types of mobilizable organelles in mature human neutrophils: azurophilic granules, specific granules, gelatinase granules, and secretory vesicles (442, 443). In addition, multivesicular bodies/late endosomes in neutrophils are known to contain independent subcellular compartments, which can undergo exocytosis and fusion with the phagosomes (444). Each type of these organelles is characterised by different protein content and the way they are secreted (443). Considering the importance of their roles in human health, vesicular trafficking and exocytosis must remain tightly regulated and Rab27a plays a key role in the regulation of azurophilic, gelatinase and specific granules.

Azurophilic granules are known to contain prooxidative haemo-protein myeloperoxidase (445), the antimicrobial bactericidal/permeability-increasing protein defensins (446), and the proteases cathpsin G and elastase (447). These are associated with the neutrophil innate immune response to infections, and two effector molecules, synaptotagmin-like protein 1 (Slp1/JFC1) and Munc13-4, are required for Rab27a function in the regulation of azurophilic granule exocytosis (448). JFC1 is responsible for dismantling

polymerised actin trap and facilitating granule transport and exocytosis (449). Munc13-4 is a key factor in lipopolysaccharide (LPS)-dependent priming of exocytosis by regulating azurophilic granule docking at the plasma membrane and limiting movement of Rab27a-positive vesicles (450). It also plays an important role in NADPH oxidase activation by regulating docking and fusion of $p22^{phox}$-positive granules (451). Specific and gelatinase granules carry the membrane component of the NADPH oxidase, cytochrome b_{558}, and modulators of the immune and inflammatory responses such as gelatinase B (MMP9) (452) and lactoferrin (453). In addition, Rab27a is reported to be involved in adhesion molecule (CD44) presentation (454), required for systemic inflammation by mediating neutrophil infiltration at liver sinusoids (455), and in the chemotactic phenotype of neutrophils, which requires cell detachment and is facilitated by azurophilic granule protease release (456, 457).

The function of Rab27a as a secretory GTPase is well described in the studies of melanocytes. Melanocytes are generally found in the basal level of the epidermis at the hair follicle base (458). They contain many long dendrites for intracellular trafficking of melanosome from the cell body to surrounding keratinocytes (458). Melanosomes are lysosome-related organelles containing lysosome-associated membrane protein (LAMP) family proteins (459). It is essential that melanosomes are delivered to, via long-range microtubule-based transfer, and retained at the tips of dendrites by interacting with the actin cytoskeleton through myosin Va to facilitate their transfer to keratinocytes and normal pigmentation (458, 460, 461). Rab27a was found to promote transport and peripheral retention of melanosomes through recruitment of myosin Va and melanophilin, which bridges Rab27a and myosin Va on actin filaments (431, 462, 463).

Rab27a has been found to be associated with cancer, and exosomal secretion is one of the processes where Rab27a participates in this context. Exosomes are nanometre-sized membrane vesicles that are secreted into the extracellular space by many types of cells (464-467). Protein composition in exosomes varies and some proteins are cell type specific, thus influencing their extracellular functions. Some *in vitro* studies have reported that exosomes are involved in immune response induction, the pathogenesis of neurodegenerative diseases, the dissemination of viruses, and communication between tumours and their surroundings (321). Exosomes are originated from multivesicular bodies in most cell types (467). Intraluminal vesicles are generated in the lumen of microvesicular endosomes by inward budding of the membrane (468). However, some exosomes are formed from endosome-like domains budding at the plasma membrane in certain cell types, including T-lymphocytes (469). Ostrowski et al. demonstrated Rab27a plays a role in multivesicular endosome docking at the plasma membrane (321). In both Rab27a- and Rab27b-knockdown HeLa cells, exosome secretion was reduced but different phenotypes were observed, confirming that Rab27b was found to exert the same function but have different role in the exosomal pathway. In the same RNAi study, there was no change in secretion of a soluble protein via the constitutive exocytosis. Bobrie et al. tested modification of Rab27a level using mouse models (317). They observed that Rab27a-depletion led to reduction of exosome release and also MMP9 secretion, which is not exported via exosomes. These subsequently result in the decrease of primary tumour growth and metastasis but do not have an effect on non-metastatic carcinoma.

Exosomes can be secreted not only by tumour cells but also by stromal cells, enhancing cancer phenotype. Zhang et al. found that the tumour suppressor PTEN becomes

downregulated in human and mouse tumour cells when these cells are disseminated to the brain, but not to other sites (470). This is the result of epigenetic regulation by brain astrocyte-derived microRNAs that target PTEN and that are contained in exosomes. Subsequently, PTEN loss in brain metastatic tumour cells promotes tumour cell proliferation and prevents apoptosis by inducing secretion of the chemokine CCL2. Silencing Rab27a blocked exosome secretion by astrocytes decreasing the brain metastatic tumour outgrowth.

In addition to its contribution to tumour phenotypes via regulating exosome secretion, Rab27a also has been reported to associate with cancer through other types of secretory pathways, such as exosome-independent MMP9 secretion as described above. Rab27A enhances the capabilities of human breast cancer cells to invade and metastasise by controlling the exocytosis of insulin-like growth factor II (297). A recent study identified the role of Rab27a in colon cancer stem cells (471). Cancer stem cells are known be an important class of cancer cell type that can be characterised by several properties: self-renewable, potential to differentiate into multiple cell lineages, responsible for tumour progression, metastasis, and the acquisition of anti-cancer drug resistance (472-475). Colon cancer stem cells are surrounded by fibroblasts, endothelia, gliocytes, and immune cells (476), and Rab27a was tested whether it mediates autocrine and paracrine effects facilitating the cross-talk between cancer cells and their surroundings (471). Feng et al. demonstrated that the overexpression of Rab27a, regulated by the NF-κB pathway, increases the secretion of VEGF and TGF-β in spheres of the colon cancer cell line HT29, promoting tumour growth in both *in vitro* and *in vivo* conditions (471).

Previously it was found that MT1-MMP predominantly associates with late endosomal/lysosomal compartments (426). Since MT1-MMP exposure to the cell surface

occurs in a regulated manner, Rab27a could be a possible regulator of MT1-MMP secretion in addition to Rab8 (315). Indeed, previously (unpublished) we found that Rab27a controls the levels of MT1-MMP exposed at the plasma membrane in the breast cancer cell line MDA-MB-231 (477). This corresponded to the level of invasiveness of the cells in both bi and tri dimensional assays. However, the exact mechanism of action of Rab27a in this context has not been completely elucidated.

In our previous study, mRNA expression profile of Rab27a in breast cancer patients treated at Curie Institute was examined using Affymetrix microarray, and the analysis showed that Rab27a levels were upregulated in tumour samples compared to normal tissues (477). The tumour samples of poor prognosis-associated triple-negative (ER-negative, PgR-negative, HER2-negative) cancers showed higher Rab27a expression levels compared to other subtypes of breast cancer such as luminal A (ER-positive, PgR-positive, HER2-negative) and luminal B tumours (ER-positive, PgR-positive, HER2-positive or negative). In addition, the expression levels of Rab27a were correlated with the prognosis of breast cancer. Another analysis of an independent cohort showed the upward trend in Rab27a expression upon higher grade tumours. These results were confirmed by the analysis of publicly available Affymetrix database of 6 cohorts with more than 1,100 breast cancer patients. Based on these findings, a series of *in vitro* and *in vivo* experiments were carried out to investigate the association of Rab27a with aggressive subtypes of breast cancer, in which MT1-MMP plays a central role in cancer cell invasion and metastasis and determines the degree of “aggressiveness”.

1.3 OVARIAN CANCER AND OPCML

1.3.1 Ovarian cancer

Despite its incidence is not as high as breast cancer, ovarian cancer is the most lethal gynaecological malignancy. In England, ovarian cancer accounts for 4.2% female cancer registrations in 2015 (353) and the five-year net survival rate for patients diagnosed with ovarian cancer reaches only 42.9% (354). Like breast cancer, ovarian cancer is known as a complex disease containing a variety of histological subtypes. The aggressive subtype that accounts for 70-80% of ovarian cancer deaths is high grade serous ovarian cancer (HGSOC), which appears to share significant molecular features with aggressive basal-like breast cancer (358). HGSOC originates from the inactivation of tumour suppressor genes including *p53* (97% of cases) (478) and *BRCA1/2* (28%) (479). In addition, oncogenic mutations in PI3K/AKT pathway (480) and growth factor/RTK pathway are also found to occur less frequently in HGSOC and in other subtypes of ovarian cancer (481).

There are three major reasons that make ovarian cancer such a lethal disease: its late presentation, acquisition of chemoresistance, and the molecular/cellular heterogeneity of the disease (482). Currently, the system called the Federation of Gynaecological Oncologists (FIGO) is being used to categorise ovarian cancer into four different stages (483). In brief, stage I indicates the status where cancer is only present in ovaries. In stage II, cancer has grown outside ovaries but still within the area of the pelvis. Stage III shows the dissemination of cancer cells beyond the pelvis and into the abdominal cavity area. In this stage, cancer cells may also distribute to the lymph nodes in the upper abdomen, groin, or

near the uterus. Finally, stage IV refers to the metastasis of cancer cells to distant organs, including breasts and lungs. Lethality of the disease, like other cancers, is largely attributed to the status at the time of initial diagnosis. The advanced stage, which corresponds to loco-regional dissemination of the cancer cells, drastically lowers five-year relative survival rates (aged 15-99 years old women) : 90% at stage I, 43% at stage II, 19% at stage III, and only 3% at stage IV could survive five years after diagnosis (484). Its asymptomatic but rapid progression toward an irreversible condition delays the early detection of the disease and subsequently leads to the unwanted outcome.

Currently, the standardised treatment for this disease is a combination of cytoreductive surgical operation and platinum/taxane-based chemotherapy (482). However, this approach frequently results in chemoresistance, which usually makes treatment options limited to palliative care (485, 486). In addition to this, the existence of different histological subtypes (heterogeneity) has been challenging the same therapeutic regimen for ovarian cancer patients (487). To deal with this problem, intense research in developing novel therapies has been carried out and now there are several other strategies available for better treatments. These include anti-angiogenic therapeutics (e.g. anti-VEGF antibodies), DNA damage inhibitors (e.g. poly ADP-ribose polymerase inhibitors), gene therapy and immunotherapy (487).

1.3.2 OPCML

Opioid-binding protein cell-adhesion molecule like (OPCML) is a glycosyl phosphatidylinositol (GPI)-anchored tumour suppressor protein, which is a member of the

IgLON family that shares conserved type-I immunoglobulin domains (488). Several studies showed that OPCML is frequently downregulated in common cancers including non-small cell lung carcinoma, brain, bladder, gastric, colorectal, breast, and cervical cancers (489-492), confirming its broad tumour suppressor activity. In particular, the expression of this tumour suppressor gene is observed to be silenced in approximately 83% of epithelial ovarian cancer (EOC) cases as a result of epigenetic modifications and loss of heterozygosity (LOH) (493), and this is associated with poor survival of the patients (491).

The mammalian RTK superfamily has about 60 members that share a conserved architecture (494). After the first RTK, EGFR, was discovered, early studies identified how EGFR and other RTKs are activated and trigger signalling cascades: dimerisation of the receptors is induced by ligands, and this is followed by trans-phosphorylation of the cytoplasmic domain, which allows docking of phosphotyrosine-binding effectors that induce corresponding intracellular signalling (495). Currently, it is thought that there are not only a wide variety of RTK signalling input but also numerous outputs, including cell survival, proliferation, differentiation, migration, adhesion, and metabolism, depending on the temporal and spatial RTK activation (495). Investigations on how ErbB receptors are activated have yielded many findings on structural and molecular aspects of RTKs' function (342, 496-500). In addition, there have been other studies on how those RTKs can be pharmacologically inhibited (501). These studies have expanded our knowledge of ErbB signalling and targeting, but have also showed some limitations, such as the relatively less focus on other RTKs than the ErbB family (495). This can slow down the development of cancer therapeutics against RTKs considering the broad range of RTK signalling input and output, and also their role in the acquisition of resistance to inhibitor therapy due to

pathway redundancy and cross-talk, which help to maintain active the pathways downstream of the targeted RTKs (502).

Sellar et. al demonstrated from *in vitro* assays using OPCML-overexpressing cell lines that OPCML plays a role as a tumour suppressor in cancer cells by negatively affecting cell proliferation (493). McKie et al. then identified that OPCML in fact functions to downregulate not EGFR but several other RTKs such as HER2, HER4, EPHA2, FGFR1, and FGFR3 (503). The distribution of HER2 was further analysed compared to that of EGFR and this showed that OPCML-regulated RTKs (listed above) tend to redistribute toward lipid rafts in the plasma membrane in the presence of OPCML. These findings were supported by additional tests with exogenous recombinant OPCML *in vitro* and *in vivo*.

Recently Antony et al. (unpublished) added another RTK, AXL, to the RTK repertoire, whose functions and activities are downregulated by OPCML in cancer cells (504). AXL is known to promote epithelial to mesenchymal transition (EMT) of cells, cell migration, invasion, anti-apoptotic phenotype, cell survival, and cell proliferation in a wide range of cancers including ovarian cancer (505). Moreover, AXL is associated with poor prognosis in ovarian cancer (506) and its expression levels are higher in metastases compared to the primary tumours (507). Antony et al. found that OPCML also alters the distribution of AXL from detergent soluble membrane domains to insoluble domains (lipid rafts) via the interaction with ligand-bound AXL (504). This sequestration causes the inactivation of the stimulated receptor by the action of a phosphatase, protein tyrosine phosphatase receptor type G (PTPRG), which localises to lipid rafts. As a result, OPCML abrogates AXL downstream signalling and subsequent phenotypes such as cell motility and invasion.

CHAPTER 2. HYPOTHESIS AND AIM

2.1 MT1-MMP SECRETION AND RAB27A IN BREAST CANCER

Based on the findings regarding MT1-MMP and its regulated intracellular transport in cancer cells, we hypothesised that a secretory Rab GTPase such as Rab27a could be a candidate molecule involved in MT1-MMP secretion. In fact, we have identified a strong association between Rab27a and MT1-MMP transport by conducting the relevant *in vivo* and *in vitro* experiments.

Thus, the aim of this project is to further characterise the role of Rab27a in the trafficking of MT1-MMP. Firstly, we repeated some key experiments in another breast cancer cell line, BT549, to verify the more general nature of the previous findings. Secondly, in order to find an indication of the nature of the MT1-MMP compartment whose secretion is possibly controlled by Rab27a, we investigated if the two proteins interact with each other and at which step of the trafficking of MT1-MMP Rab27a is involved.

2.2 OPCML ENDOCYTOSIS

In a previous study from our group (503), OPCML was found to localise within the detergent-insoluble fraction, where cholesterol is enriched and many caveolin-1 molecules associate with the cytoplasmic side of the domain. The authors identified that OPCML co-localises with caveolin-1 and the early endosomal marker protein called Early Endosome

Antigen1 (EEA1) and also showed the shift of HER2 distribution from EEA1/clathrin-associated cellular compartments to caveolin-1-associated structures in the presence of OPCML. However, the exact mechanism of the OPCML endocytosis and the trafficking shift of RTK in OPCML-overexpressing cells remain poorly understood. Therefore, we hypothesised that OPCML is internalised via a caveolin-1-dependent endocytic pathway and its association with RTKs leads to the modified intracellular trafficking of the RTKs.

Our primary aim is to examine the candidate endocytosis pathways for OPCML uptake. Here we showed how we tested and confirmed that OPCML internalises via a clathrin-independent, caveolin-1-dependent pathway. Also, we demonstrated how OPCML intracellularly traffics once it is endocytosed and when OPCML recycling or degradation occurs. The second aim is to investigate how OPCML affects RTK, AXL in particular, trafficking.

CHAPTER 3. MATERIALS AND METHODS

Cell culture

MDA-MB-231 breast adenocarcinoma cells and BT549 cells were purchased from ATCC (American Type Culture Collection) and grown and maintained in DMEM-medium (Sigma) at 37C. The medium was supplemented with 10% foetal calf serum (FCS) (First Link (UK) Ltd.), 2mM L-Glutamine (Gibco) and 1% Penicillin-streptomycin (Gibco). MDA-MT1ch cells are MDA-MB-231 cells that stably express MT1-MMP-mCherry. MDA-MB-231 cells containing Str-KDEL MT1-MMP-SBP-mCherry (MDA-RUSH) were kindly provided by Dr Gaelle Boncompain and Dr Frank Perez from the Institut Curie in Paris, France. These cells were cultured in DMEM+GlutaMAX-I (Gibco) supplemented with 10% FCS.

SKOV3 ovarian adenocarcinoma cells and SKOV3-derived cell lines were grown and maintained at 37°C in RPMI-medium (Sigma), which was supplemented with 10% FCS (First Link, UK, Ltd.), 2mM L-Glutamine (Gibco) and 10µg/ml Puromycin (Gibco). Antibiotics was removed before experiments. 0.02% EDTA solution (Sigma) with or without Trypsin solution from porcine (Sigma) was used to detach and collect the cells for some experiments.

siRNA transfection and inhibitor treatment

Cells were transfected with ON-TARGETplus siRNA (Dharmacon) plus Oligofectamine (Invitrogen) based on manufacturer's instructions. All cells were studied after 3 days. Control cells were transfected with non-targeting siRNA. The siRNA oligo sequences are

siRab27a_1: 5'-GGAGAGGUUUCGUAGCUUA-3'; siRab27a_2: 5'-CCAGUGUACUUUACCAAUA-3'; siMT1-MMP: 5'-GGAAACAAGUACUACCGUU-3'.

SKOV3 OPCML were transfected with non-targeting siRNA (Dharmacon) or siRNAs to transiently knock down clathrin heavy chain (5'-UAAUCCAAUUCGAAGACCAAUUU-3', ON-TARGETplus, Dharmacon), Cdc42 (5'-UCAGAUUUGAAAUAUUUAATT-3', FlexiTube, QIAGEN), endophilin A2 (5'-UGCUCAACACGGUGUCCAATT-3', FlexiTube, QIAGEN), or Alix (5'-GCCGCUGGUGAAGUUCAUUU-3', ON-TARGETplus, Dharmacon) in complex with Oligofectamin (Invitrogen). To knock down caveolin-1, two oligo sequences were used to target two different regions of caveolin-1 (caveolin-1_#7 targets 5'-GCAAAUACGUAGACUCGGA-3', and caveolin-1_#9 targets 5'-GCAUCAACUUGCAGAAAGA-3'). According to the provided protocol guidelines, cells were incubated for 72 hours after siRNA treatment and then analysed.

Dyngo4a (ab120689, Abcam), a highly potent dynamin inhibitor, was used to see the effect of dynamin inhibition on the OPCML endocytosis. The inhibitor was tested by blocking the transferrin (T23364, Thermo Fisher) uptake. Cells were pre-incubated in serum free medium containing 100µM of Dyngo4a before the internalisation assay.

Lentiviral transduction

To obtain stable cell lines, cells were transduced with a lentivirus that encodes for a GFP-ratRab27a fusion (generous gift from C. Wasmeier) for Rab27a overexpression or that contains the following sequences for Rab27a knockdown: mi27a_1: human GIPZ lentiviral shRNAmir, clone V2LHS_47482 (Open Biosystems), 5'-

CTCGAGAAGGTATATTGCTGTTGACAGTG

AGCGcggctgcagctttattagcttaTAGTGAAGCCACAGATGTAtaagctaataaagctgcagcctTGCCTACTGC

CTCGGAATTC-3' (generous gift from Molly Strom); mi27a_2: 5'-TGCTGAAACTTT GCTCATTTGTCAGGGTTTTGGCCACTGACTGACCCTGACAAGAGCAAAGTTT-3'; both virus contained also the gfp gene.

SKOV3 ovarian adenocarcinoma cells were stably transduced with an empty lentivirus (SKOV3 EMPTY) or a lentivirus overexpressing OPCML, (SKOV3 OPCML), which were already available and routinely used in the lab.

Antibodies

Mouse α-Rab27a (4B12, homemade), mouse α-MT1-MMP (LEM 2/15.8, Millipore), rabbit α-calnexin (ADI-SPA-860-D, Stressgen, Enzo Life Sciences), goat α-Exo84 (C-16, Santa Cruz Biotechnology), rabbit α-Rab7 (9367, Cell Signaling Technology), rabbit α-IQGAP-1 (H-190, Santa Cruz), mouse α-Sec8 (610658, BD Biosciences), rabbit α-GAPDH (5174, Cell Signaling Technology), goat α-OPCML (AF2777, R&D), mouse α-Cdc42 (ab41429, Abcam), rabbit α-endophilin A2 (SC-25495, Santa Cruz Biotechnology), mouse α-Alix (AM01318PU-N, Acris GmbH), rabbit α-calnexin (ADI-SPA-860-D, Stressgen, Enzo Life Sciences), rabbit α-caveolin-1 (AB18199, ABCAM), rabbit α-AXL (8661, Cell Signaling Technology), mouse α-phospho-ERK1 pT202/pY204 + phospho-ERK2 pT185/pY187 (ab50011, Abcam), rabbit α-ERK1/2 (ab17942, Abcam), α-rabbit, α-mouse, and α-goat secondary antibodies conjugated to horseradish peroxidase (Dako) were used in immunoblotting.

Mouse α-MT1-MMP (LEM 2/15.8, Millipore), rabbit α-clathrin heavy chain (4796, Cell Signaling Technology), rabbit α-caveolin-1 (3267, Cell Signaling Technology), rabbit α-Rab5 (3547, Cell Signaling Technology), α-APPL1 (3858, Cell Signaling Technology), rabbit α-EEA1 (3288, Cell Signaling Technology), α-Rab7 (9367, Cell Signaling Technology), α-LAMP1 (9091, Cell Signaling Technology), rabbit α-AXL (8661, Cell Signaling Technology), phalloidin (Invitrogen), and secondary antibodies (Alexa Fluor ® 488, 546, and 633 Goat α-mouse secondary antibody, Life Technologies) were used in immunofluorescence microscopy.

Immunoblotting

MDA-MB-231 and BT549 cells were lysed with lysis buffer (Tris 50mM pH7.5, 137mM NaCl, 10% glycerol, 0.5% triton x100, 1mM EDTA), along with protease and phosphatase inhibitor cocktails (Calbiochem, Boston, MA). SKOV3 cells were lysed with with RIPA buffer (#R0278, Sigma–Aldrich) together with the same protease and phosphatase inhibitor cocktails. Lysates were spun at 13,000 g and the supernatant was quantified by using BCA assay kit (Thermo Fisher). Proteins in the supernatant were loaded into a SDS-PAGE gel to be separated and then transferred onto a PVDF (Millipore) or a nitrocellulose membrane (BioTrace^{TM} NT, Pall Corporation). Then proteins were probed with the required primary antibodies overnight in the cold room after saturation of the membrane in PBS + 0.1% Tween 20 + 10% semi-skimmed milk at room temperature. After washing with TBST (Tris-Buffered Saline + Tween20), the membrane was incubated with α-rabbit, α-mouse, and α-goat secondary antibodies conjugated to horseradish peroxidase (Dako), washed and then

incubated with ECL-TM Prime Western Blotting Reagent (GE Healthcare) to detect the antibodies.

Gelatin degradation assay

To prepare fluorescent gelatin coated coverslips as previously described (407), coverslips were incubated in 0.5 μg/ml poly-L-lysine (Sigma) and fixed with 0.5% glutaldehyde (Sigma-Aldrich) in PBS. Then coverslips were coated with fluorescent gelatin (FITC or TexasRed) in the dark and treated with 5mg/ml sodium borohydride (Aldrich) to quench the glutaldehyde. BT549 cells were placed on top of coverslips and let to degrade for 7 hours in the 37°C incubator before fixation using 4% paraformaldehyde (PFA) in PBS. Images were obtained with a Zeiss Axiovert 200M Widefield Microscope using a 63x objective. From two independent coverslips, at least 12 images per coverslip were taken for each cell line and each experiment was performed 3 times. The levels of degradation were assessed by ImageJ: the degradation index was defined by dividing areas of degradation (measured by thresholding the black pixels in the black areas of gelatin degradation) by the number of cells and then normalised to controls.

Spheroid invasion assay

Multicellular spheroids of BT549 cells were prepared as previously described (508). BT549 cells were suspended and counted to make 20 μl inverted droplets that contained 2 x 10^3 cells each. The droplets were incubated for 3-7 days until they formed proper sized

spheroids. Once their formation was completed, spheroids were embedded in a 2.2 mg/ml acid-extracted rat-tail collagen I matrix (Corning). Then spheroids were fixed with 4% PFA once the type-I collagen matrix polymerised (day 0) or after 2 days at 37°C (day 2). Spheroids were labelled with phalloidin and then at least 10-16 spheroids for each condition were analysed using a Leica TCS SP5 confocal microscope and ImageJ. The experiments were repeated 3 times.

Co-immunoprecipitation (co-IP)

The Crosslink IP kit (Thermo Fisher) was used to immunoprecipitate Rab27a (with goat α-Rab27a, C-20, Santa Cruz) or MT1-MMP (LEM 2/15.8, Millipore)) in MDA-MB-231 cells. Each antibody was cross-linked to Protein A/G Plus Agarose and non-cross-linked antibodies were removed. Cells were lysed in IP Lysis/Wash buffer and cell debris were removed by centrifugation at 13,000 g for 10 minutes at 4°C. After pre-clearing the lysate using Control Agarose resin, supernatants were incubated with the cross-linked antibody for 2 hours at 4°C. Beads were washed with IP Lysis/Wash Buffer and then 1X Conditioning Buffer before bound proteins were eluted in Elution Buffer. Eluates were analysed by immunoblotting analysis.

Retention Using Selective Hook (RUSH) and Surface staining

As shown by Boncompain et al. (509), MDA RUSH cells (5.0 x 10^4cells) were plated on sterile coverslips and incubated in DMEM+GlutaMAX-I (Gibco) supplemented with 10% FCS at 37°C.

Avidin (Sigma-Aldrich) was added into the medium to a final concentration of 10 µg/ml to minimise the effect of free biotin that may release MT1-MMP from the ER. After the treatment with 40 µM biotin (Sigma-Aldrich), at each time point cells were fixed with 2% PFA for 7 minutes at RT and stained with rabbit α-RFP (Rockland) without permeabilisation. Cells were then stained with goat-α-rabbit-AlexaFluor633 (Molecular Probes) for imaging by confocal microscopy (Leica TCS SP5).

Cell surface biotinylation and recycling assay

Recycling assay was performed as described previously (510). Cells were washed thrice with ice-cold PBS to suspend endocytosis and then were treated with 0.2 mg/ml of EZ-Link^{TM} sulfo-NHS-SS-biotin (Thermo Fisher) on ice. Cell surface biotinylation was allowed for 15 minutes and subsequently the reaction was quenched with cold 50 mM Tris buffer. After washing with cold PBS, cold culture medium was added to the dishes and then cells were incubated at 37°C for 15 minutes (for MT1-MMP recycling) or 5 minutes (for OPCML recycling). The surface-bound biotin was stripped by cold membrane-impermeant MESNa buffer (150 mM sodium 2-mercaptoethanesulfonate (MESNa), 50 mM Tris, 100 mM NaCl, 1 mM EDTA in PBS) on ice. Once cells were rinsed with cold PBS, additional incubation at 37°C was conducted. At each time-point cells were either treated with cold MESNa buffer (to remove surface bound biotin again) or PBS on ice. Difference in amounts of bioinylated protein of interest between MESNa-treated cells and PBS-treated cells is equivalent to the amount of the recycled protein at that time-point. Cells were lysed with IP lysis buffer (Thermo Fisher) and spun down. The supernatant was added to streptavidin sepharose

beads (Cell signalling) and mixed overnight at 4°C. After rinsing the beads five times, lysis buffer (Tris 50 mM pH7.5, 137 mM NaCl, 10% glycerol, 0.5% TritonX100, 1 mM EDTA) was added to the beads and then they were boiled with loading dye (5% β-mercaptoethanol, 5% SDS, 50% glycerol, 0.1% bromophenol blue in Tris 250mM pH6.8) for 10 minutes before immunoblotting analysis.

Immunofluorescence microscopy for internalisation assay

For immunofluorescence imaging, cells were seeded on 13 mm diameter glass cover slips at a seeding density of 50,000 cells per well in 4 well plates. For 5-minute internalisation assay, cells were kept on ice, stained with mouse α-OPCML primary antibody (MAB27771, R&D) in PBS and washed three times with PBS. Cells were then incubated in serum-free medium at 37 °C for the indicated length of time to allow the internalisation of the antibody-bound surface OPCML. At each time point in the internalisation assay, to visualise the internalised OPCML-positive vesicles only, some batches of the cells were washed with acid buffer (pH 2, 25mM sodium acetate in culture medium) for 1 minute 4°C to strip off the remaining antibodies that were bound to the surface OPCML molecules that had not been internalised yet by that time point. This acid-wash step was skipped for cells used to monitor the total surface OPCML. After neutralisation with alkaline buffer (pH 10, 25mM Tris in culture medium) and then rinse with PBS once, cells were fixed with 4% PFA for 20 minutes at room temperature, washed three times with PBS containing 1% FBS and saponin (final concentration 0.05 mg/mL) and stained with other primary antibodies. Then cells were

stained with secondary antibodies. Cells were washed 3 times with PBS/FCS/Saponin and 3 times with PBS.

For PLA DUOLink (OLink Biosceinces, Sigma-Aldrich) experiment, the protocol was followed as per manufacturer's instructions. Two different proteins were probed with corresponding antibodies. These antibodies are required to be different species for proper binding of PLA probes, which will carry out amplification of fluorescence signals if they are close to each other. For this reason, this technology is widely used to show associations or co-localisation of two proteins of interest.

To do the pulse-chase experiment, fluorescently labelled α-OPCML antibody was used. A fluorescent dye, ATTO488-AEDP-NHS-ester (ATTO488), contains a disulfide bond in the linker so that fluorescent signal can be removed by treatment of a reducing agent such as MESNa buffer. ATTO488-AEDP-NHS-ester-conjugated α-OPCML (ATTO488-α-OPCML) was prepared according to the manufacturer's instruction. After the pulse uptake, cells were treated with cold MESNa buffer on ice for tracking only the endocytosed ATTO488-α-OPCML during the chase step. Intracellular trafficking was arrested with 4% PFA treatment at each time-point.

Once cells were properly stained and fixed, coverslips were mounted on a glass slide using the mounting medium (ProLong Gold Antifade Mountant, Life Technologies), and imaged using the Leica TCS SP5 Confocal Laser Scanning Microscope. Quantification of the OPCML uptake, assessment of OPCML association with caveolin-1 or clathrin using PLA DUOLink, and quantification of the degree of co-localisation in pulse-chase experiment were performed using the Zeiss Cell Observer wide-field microscope and ImageJ (JACoP). Z-stack

images of whole body of a cell were acquired and then they underwent deconvolution process, carried out by a software called Huygens (Scientific Volume Imaging).

Membrane fractionation

Cells with or without α-OPCML treatment (at 37°C for 5 minutes) were harvested by using EDTA solution and rinsed with cold PBS. Then cells were osmotically lysed with a low salt buffer (20mM Tris-HCl, pH 7, containing the complete protease inhibitor cocktail, Roche) and the cell suspension was passed through a 21-gouge needle. Then the suspension was spun down at 110,000 g, 4°C for 1 hour in the Optima^{TM} MAX Ultracentrifuge (Beckman Coulter). The membrane pellet was washed with cold PBS and centrifuged once again at 110,000 g, 4°C for 30 minutes. Then the membrane pellet was resuspended and incubated in 1% Triton-X100 in cold PBS for 30 minutes at 4°C. The detergent treated membrane fraction was then centrifuged at 110,000 g at 4°C for 1 hour. The supernatant (detergent-soluble membrane fraction) was retained and the pellet (detergent-resistant membrane fraction) was solubilised in 1% SDS and then the fractions were analysed by immunoblotting. To probe the mouse α-OPCML, which indicates the presence of the internalised OPCML, the nitrocellulose membrane was incubated only with the α-mouse secondary antibody before the antibody detection step.

Cholesterol depletion

To see whether cholesterol is required for OPCML endocytosis, cholesterol in SKOV3 OPCML cells was depleted by methyl-β-cyclodextrin (MβCD, C4555, Sigma Aldrich). Cells were

grown on 13mm coverslips and pre-treated with 5mM or 10mM of MβCD for 30 minutes at 37°C before OPCML internalisation assay. Reduction of cholesterol level was confirmed with Filipin (C4767, Sigma Aldrich) staining, which binds cholesterol. The result of Filipin staining was imaged with non-DAPI mounting medium (P36930, Thermo Fisher) under a confocal microscope (405nm wavelength).

Statistical analysis

Statistical significance was tested using OriginPro 8 (OriginLab Corporation, One Roundhouse Plaza, Northampton, MA, USA). One-way ANOVA with Dunnett post hoc test was selected to calculate significance of differences between more than two groups. Data in plots are given as means ± standard error (SE) if n=3, and difference was considered significant when $p<0.05$.

CHAPTER 4. RESULTS

4.1 MT1-MMP SECRETION AND RAB27A IN BREAST CANCER

Molecular subtyping based on gene expression or copy-number variation has been used to show how clinical breast cancer specimens and cell lines can be characterised (511-513). To identify the role of Rab27a in MT1-MMP trafficking in breast cancer considering the importance of MT1-MMP in breast cancer invasion and metastatic phenotype, we tried to select a cell line that has high potential of invasiveness and metastasis for this study. In our previous data analysis of the tumour samples from breast cancer patients (477), Rab27a expression was upregulated in triple-negative cancers compared to the other types of breast cancers. Among the subtypes of triple-negative breast cancer cell lines, the cells that belong to "mesenchymal-like/claudin-low" subtype (512, 514) show stellate morphology in three-dimensional culture (515) and high invasiveness in chamber assays (511). Therefore, one cell line, named MDA-MB-231, from the subtype was selected and used in our study, and another cell line, BT549, from the same subtype was used for the two *in vitro* assays (gelatin degradation assay and collagen invasion assay) to support the findings from the experiments that used MDA-MB-231.

4.1.1 Gelatin degradation correlates with Rab27a expression in BT549

One of the *in vitro* experiments in our previous study (477) was to test whether Rab27a affects the degree of pericellular proteolysis in a triple negative breast cancer cell line, MDA-MB-231. Gelatin is composed of a mixture of extracellular matrix macromolecules

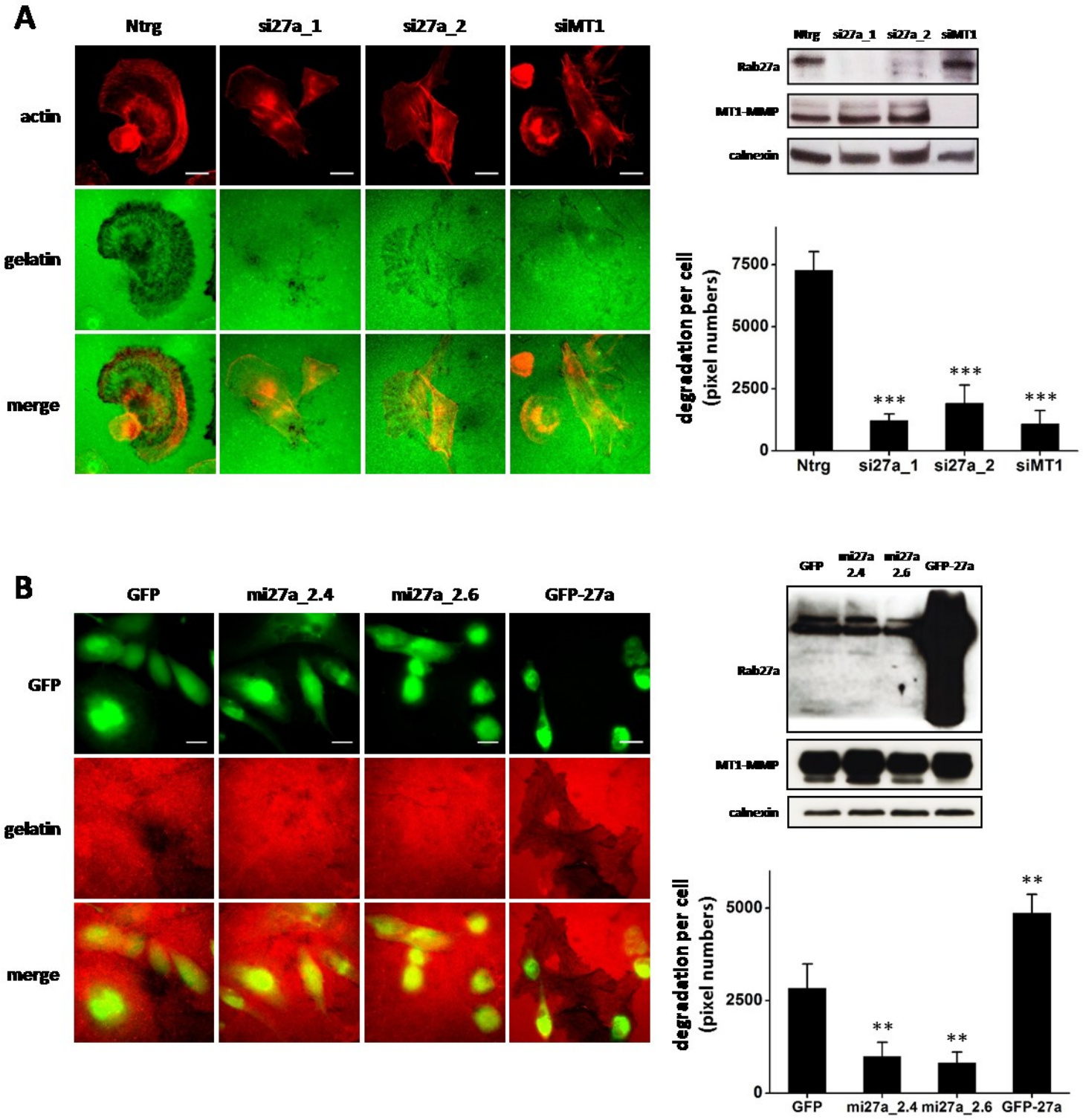


Figure 8. Gelatin degradation is affected by the levels of Rab27a expression in BT549. (A)BT549 cells were transfected with two independent siRNAs for Rab27a (si27a_1 and si27a_2) or MT1-MMP (siMT1) for 3 days and then placed onto fluorescent FITC-gelatin for 7 hours. Cells were labelled with Phalloidin-TexasRed and the areas of degradation are shown as black dots (in the Gelatin panel). Depletion levels of Rab27a and MT1-MMP were confirmed by immunoblot in the right panel (Calnexin was used as a loading control). (B)BT549 cells were stably transduced with a miRNA for Rab27a (two clones, mi27a_clone 4 and clone 6) that carried the gfp gene as well. Other BT549 cells were transduced with GFP (control) or GFP-Rab27a (overexpression). These cells were seeded onto TexasRed-gelatin-coated coverslips and assessed for their degradative ability as stated in A. Values represent mean ± SE of at least 3 independent experiments. 80-100 cells per condition were analysed in each experiment. The degrees of gelatin degradation by each type of cell were statistically analysed using one-way ANOVA and Dunnet post-test to calculate significance between control and different condition groups (***P<0.001, **P<0.01). Scale bar=10µm.

of the assay using MDA-MB-231 showed that modification in the expression level of Rab27a also changed the ability of cancer cells to degrade gelatin. To confirm this result, another triple negative breast cancer cell line called BT549 was tested in the current study. Cells that were transiently and efficiently depleted of Rab27a with two different siRNAs could degrade less gelatin than control cells (Figure 8A). The reduced capacity of gelatin digestion in the Rab27a-depleted cells was similar to that observed in cells with reduced MT1-MMP expression (Figure 3A). When the same cell line was stably transduced to express low levels (using a miRNA against Rab27a) or high levels (overexpressing GFP-Rab27a) of Rab27a, an inhibition or an increase of gelatin degradation respectively was observed (Figure 8B). Altogether, this indicates that expression levels of Rab27a are strongly associated with MT-MMP-dependent proteolytic activity of cancer cells.

4.1.2 Spheroid invasion in a tri-dimensional collagen model is downregulated upon Rab27a reduction in BT549

Culturing cells as spheroids has been widely used in tumour biology as it reproduces more accurately the 3D structure of the tumour microenvironment compared to monolayer cultures, by mimicking the initial dissemination of micrometastases, which are not yet vascularised (517, 518). In addition, spheroids in 3D microenvironments display nutrient and signal gradients, cell-cell interactions, and cell-ECM interactions that cause multicellular resistance phenomena such as stratification of layers and reduced drug permeability (519). As previously tested in MDA-MB-231, invasiveness of BT549 cells was assessed upon altered levels of Rab27a in a type-I collagen matrix, where cells require MT1-MMP for their invasion (516). For the assay, cells were grown to form spheroids and embedded into collagen

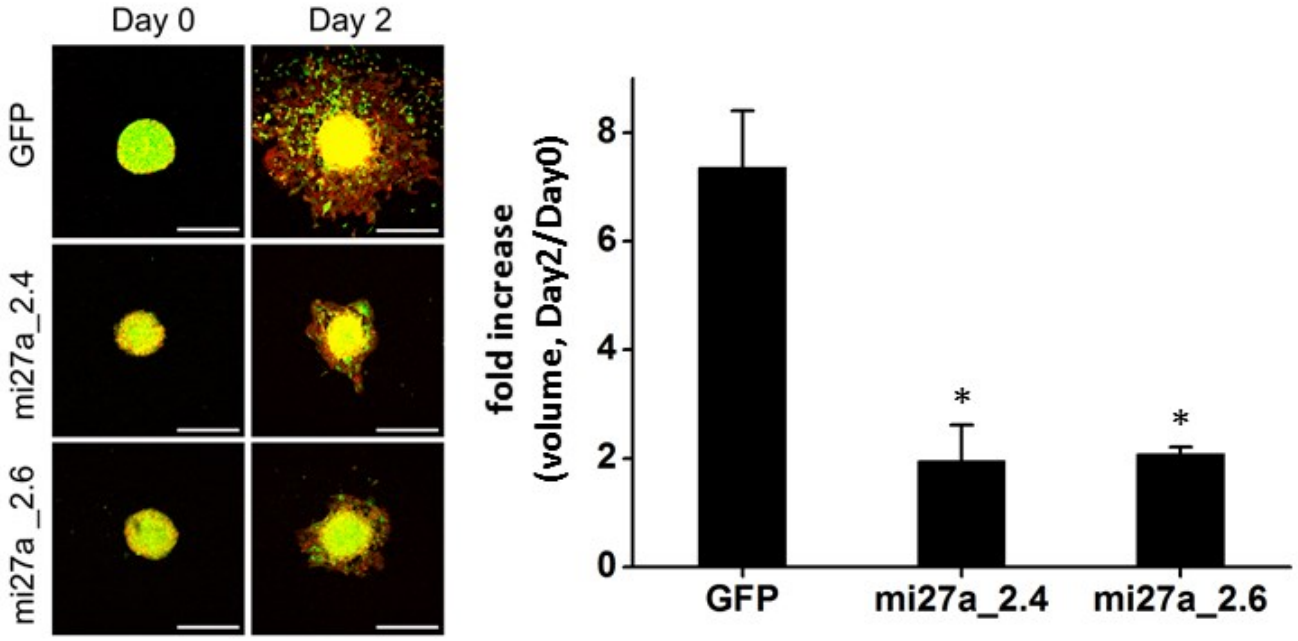


Figure 9. Rab27a depletion has an impact on breast cancer cell invasion. BT549 cells, stably depleted of Rab27a (mi27a clone4, mi27a clone 6) were grown as spheroids and embedded into type I collagen matrix for two days. Invasion of transduced cells (GFP+, green) into collagen was visualised by fluorescence microscopy after actin staining with Phalloidin-TexasRed (red) and quantified by ImageJ. Fold increase of volumes of spheroids were represented as mean ± SE from 3 independent experiments. In each experiment, 10-16 spheroids per condition were analysed for quantification. One-way ANOVA and Dunnet post-test was used to calculate significance of difference between control and the groups with reduced Rab27a (*P<0.05). Scale bar=500 µm.

matrices that were placed onto coverslips. Then they were incubated for two days at 37C before visualisation using confocal microscopy. A great reduction in their degradative ability was observed in Rab27a-depleted spheroids (Figure 9). Based on these observations and the previous data, it was further confirmed the impact of Rab27a on MT1-MMP-dependent breast cancer cell invasion in a 3D microenvironment.

4.1.3 Rab27a co-localises with MT1-MMP-positive vesicles in MDA-MB-231

The differential effects from modified expression level of Rab27a on the cellular phenotypes as described above led us to further investigate whether Rab27a associates with MT1-MMP-positive vesicles in cancer cells. For the fluorescence microscopy analysis,

MDA-MB-231 cells that overexpress both MT1-MMP-mCherry and GFP-Rab27a were placed on gelatin-coated coverslips. It was found that Rab27a co-localised with the small MT1-

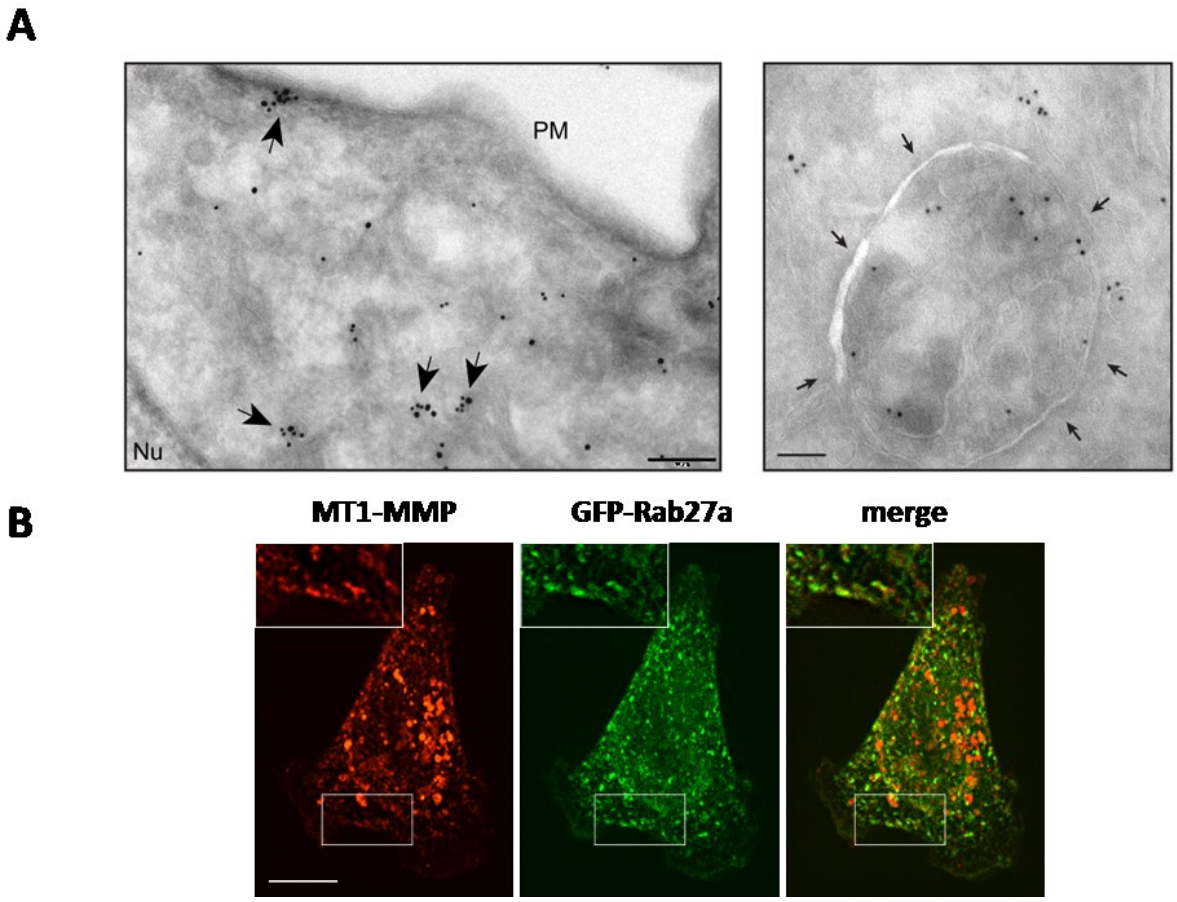


Figure 10. Rab27a preferentially associates with small MT1-MMP-positive vesicles. (A) Transmission electron microscopy (TEM) cryoimmunolabelling (Adapted from Recchi et al., unpublished): MT1-MMP is labelled with 10 nm gold particles, GFP is labelled with 15 nm gold particles. Arrows in the left panel indicate vesicular structures positive for both MT1-MMP and Rab27a. In the right panel small arrows specify the membrane of the multi-vesicular body, which is positive only for the smaller gold particles that are equivalent to MT1-MMP. PM: plasma membrane; Nu: nucleus. Scale bar= 200 nm. (B) Confocal fluorescence microscopy shows the co-localisation between Rab27a and MT1-MMP at the cell peripheral region (insert). Red: MT1-MMP-mCherry; Green: GFP-Rab27a. Scale bar= 10 µm.

MMP-positive peripheral structures at the lower planes of a cell body rather than the larger compartments, which generally locate at middle or high plane (Figure 10B). In the previous study, the co-localisation between Rab27a and MT1-MMP was observed by transmission electron microscopy (TEM) cryoimmunolabelling (Figure 10A).

Each protein was labelled with a different sized gold particle, 10nm particles with MT1-MMP and 15nm ones with GFP-Rab27a, so that images could provide information of whether the two proteins co-localise and where the co-localisation takes place. The co-localisation between MT1-MMP and GFP-Rab27a was mainly detected in the small vesicular structures in close proximity to the plasma membrane. However, it was not observed in large multi-vesicular bodies, which seem to be the larger compartments observed by fluorescence microscopy.

In the following confocal microscopy analysis, co-localisation of the two proteins was preferentially found in endosomal compartments rather than in the Golgi (Figure 11). Unlike the high degree of co-localisation of GFP-Rab27a with MT1-MMP-positive small vesicles, most of the signals of GFP-Rab27a were not fully but partially overlapped with larger endosomal structures such as early endosomes, late endosomes, or lysosomes. These findings led us to find out whether Rab27a and MT1-MMP physically interact with each other directly or indirectly. Furthermore, if there is detectable interaction between those two proteins, we hypothesised that Rab27a could play a role in MT1-MMP-dependent cancer cell behaviours by regulating MT1-MMP transport pathways, excluding the Golgi-related trafficking.

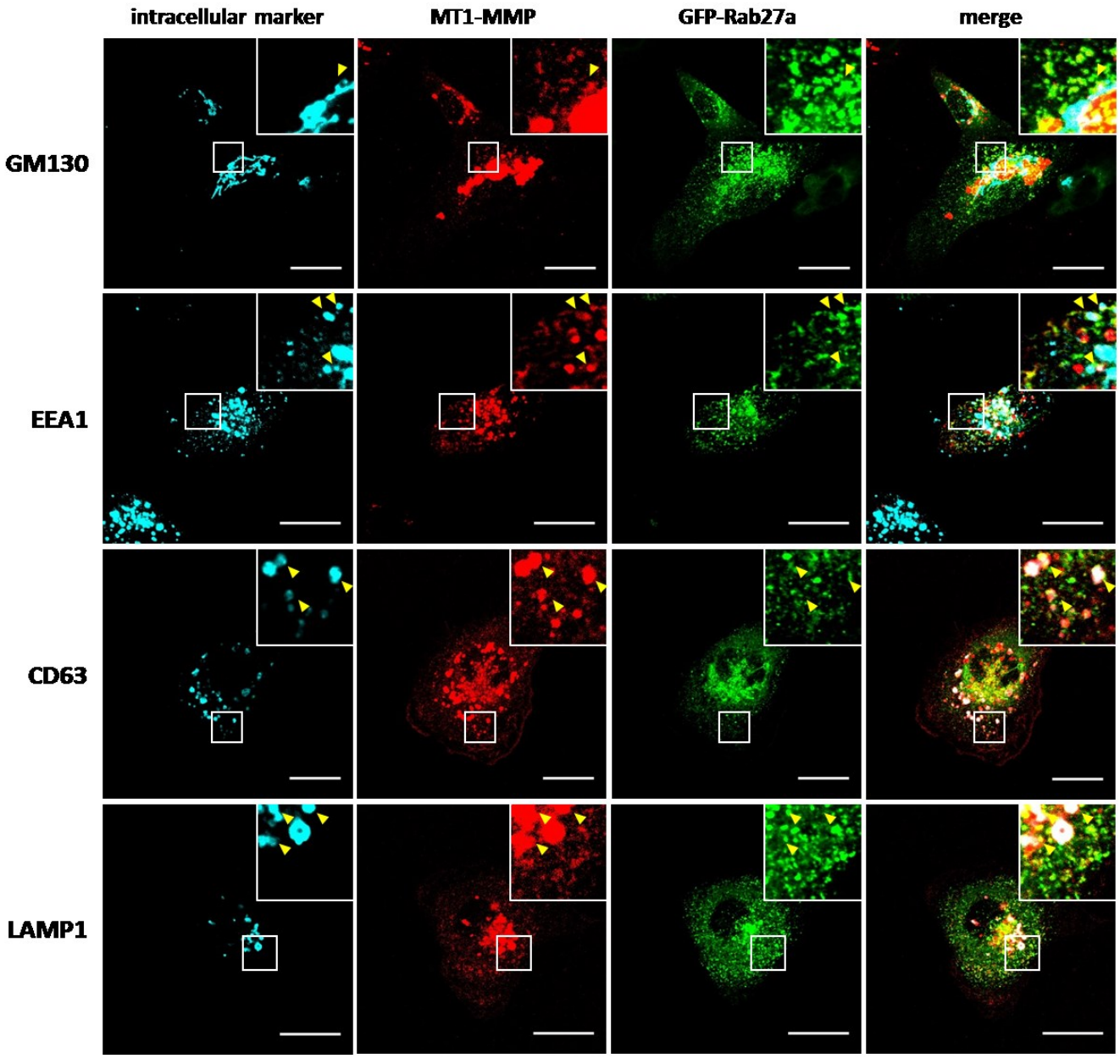


Figure 11. Rab27a partially co-localises with large MT1-MMP-positive endosomal compartments. Confocal microscopy analysis shows the types of intracellular compartments where the co-localisation of Rab27a and MT1-MMP is detected. Four different markers were selected to define the structures of the Golgi (GM130), the early endosomes (EEA1), or the late endosomes/lysosomes (CD63 or LAMP1) respectively. Inserts show higher magnification of the cell in the indicated area. Yellow arrows indicate the regions of co-localisation of three proteins; Cyan: intracellular marks; Red: MT1-MMP-mCherry, Green: GFP-Rab27a. High degrees of co-localisation between GFP-Rab27a and MT1-MMP are observed as shown previously whereas large MT1-MMP-positive endosomal structures are partially overlapped with GFP-Rab27a signals. Co-localisation between GFP-Rab27a and MT1-MMP-mCherry at the Golgi is rarely found. Scale bar= 10 µm. The levels of co-localisation between MT1-MMP and Rab27a at different cellular compartments was quantified using ImageJ. 4-6 cells per marker were analysed for this preliminary data (mean ± SE).

4.1.4 Rab27a interacts with MT1-MMP in MDA-MB-231

To identify the binding partners of MT1-MMP or Rab27a, we tested co-immunoprecipitation of each molecule with selected candidate proteins. Based on the results of the gelatin degradation assay and 3D invasion assay, we thought that Rab27a could be involved in MT1-MMP-dependent invadopodial extracellular matrix degradation and specifically in MT1-MMP delivery to invadopodial structures. In fact, previously, it was verified that the different levels of Rab27a expression modified the amount of the cell surface MT1-MMP at the ventral surface in close contact with the gelatin (unpublished data). The Rab27a-depleted cells showed substantially less MT1-MMP on the plasma membrane compared to control cells and, thus, this suggests that Rab27a contributes to MT1-MMP exposure at the cell surface. This directed us to narrow down our scope to the molecular machineries required for MT1-MMP exocytosis at the plasma membrane. In this experiment, a member of the exocyst complex, Exo84, and a key molecule for regulated exocytosis, IQGAP1, were selected.

We clearly showed that the immunoprecipitated Rab27a was associated to MT1-MMP (Figure 12). However, we could not detect Rab27a together with the immunoprecipitated MT1-MMP (data not shown). This could be due to the position of the epitopes the antibodies are binding to: in the case of the anti-MT1-MMP, the epitope might overlap with the area required for protein-protein interaction with Rab27a. Moreover, strikingly, the immunoprecipitated Rab27a was also associated to IQGAP1 and Exo84, which are essential components for invadopodia formation. This suggests that Rab27a might be one of the regulators in the assembly of invadopodial components or the maturation of the

structures by associating with MT1-MMP and the molecules that regulate MT1-MMP secretion.

To exclude that Rab27a levels may affect the expression levels of proteins involved in MT1-MMP trafficking such as IQGAP1, Exo84, Sec8 and Rab7 (an important factor in late endocytic pathway of MT1-MMP trafficking) (316), the proteins present in the lysates of cells with different levels of Rab27a were immunoblotted with the respective antibodies. However, no significant differences appeared, showing that the amount of Rab27a does not alter the levels of these proteins.

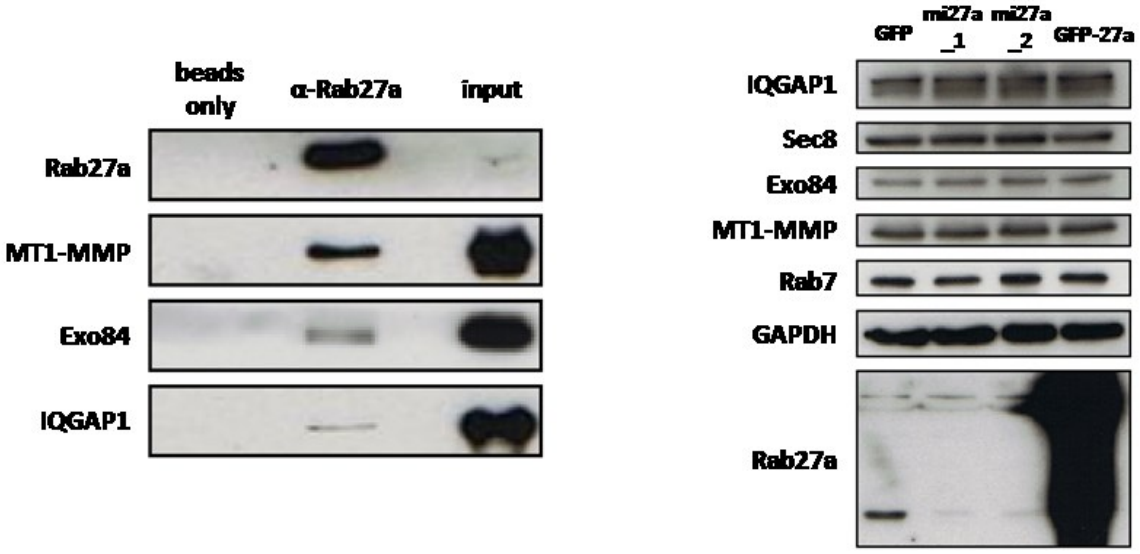


Figure 12. Rab27a interacts with MT1-MMP, IQGAP1, and Exo84. In the left panel, co-immunoprecipitation result shows that IQGAP1, Exo84, or MT1-MMP associates with the immunoprecipitated Rab27a in MDA-MB-231. Lysate of MDA-MB-231 cells was immunoprecipitated with control (no antibody; beads only), or anti-Rab27a, and interacting proteins were analysed by immunoblotting. (Input: 1/100 of the whole cell lysate). The right panel shows that the endogenous levels of IQGAP1, Sec8, Exo84, MT1-MMP and Rab7 do not change upon Rab27a depletion (mi27a_1 or mi27a_2) or overexpression (GFP-27a) in MDA-MB-231 cells.

4.1.5 Rab27a contributes to MT1-MMP exocytosis via the non-biosynthetic pathway in MDA-MB-231

To clarify whether Rab27a trafficks MT1-MMP to the cell surface from the Golgi via the biosynthetic route or Rab27a mediates MT1-MMP recycling between the endosomal compartments and the plasma membrane, we utilised the Retention Using Selective Hook (RUSH) system, which is a two-state secretion assay designed to synchronise the transport of the protein of interest (509). Basically, there are two proteins to be co-expressed: 1) the hook protein (KDEL in this experiment, located in the ER) that carries the core streptavidin, and 2) the reporter protein (MT1-MMP in this experiment), whose trafficking will be followed (Figure 13A). The reporter protein is fused to a fluorescent protein (mCherry in this experiment) and to the streptavidin-binding peptide (SBP), which binds to the streptavidin-hook fusion and causes the reporter protein to be retained in the ER. By adding biotin, whose affinity for streptavidin is high enough to out-compete the SBP, we could trigger the release of the reporter protein from the ER and visualise the trafficking of the reporter protein thanks to the fluorescent mCherry.

Using MDA-MB-231 cells that have been adapted to this system (MDA-RUSH), the early kinetics of MT1-MMP secretion were measured when cells were grown on glass coverslips (Figure 13C). The cells were previously modified to express different levels of Rab27a so that the comparison in the kinetics enabled us to analyse the effects of Rab27a on MT1-MMP trafficking in the biosynthetic pathway (Figure 13B). If Rab27a controls the trafficking of MT1-MMP from the ER to the plasma membrane, we would expect to see a shifted graph, depending on the expression levels of Rab27a, from the graph of the control cells. In our study, however, we found that MT1-MMP transport to the plasma membrane

did not seem to be significantly affected by depletion or overexpression of Rab27a (Figure 14). Also, the kinetics of cells that were placed on gelatin-coated coverslips were measured in the same way, and no dramatic shift of the graphs was observed. These results indicated that the possibility where Rab27a is involved in MT1-MMP secretion via the biosynthetic pathway could be excluded. Therefore, Rab27a might regulate MT1-MMP trafficking during the mobilisation of a pre-existing internal pool of functional MT1-MMP available for extracellular matrix degradation and invasion.

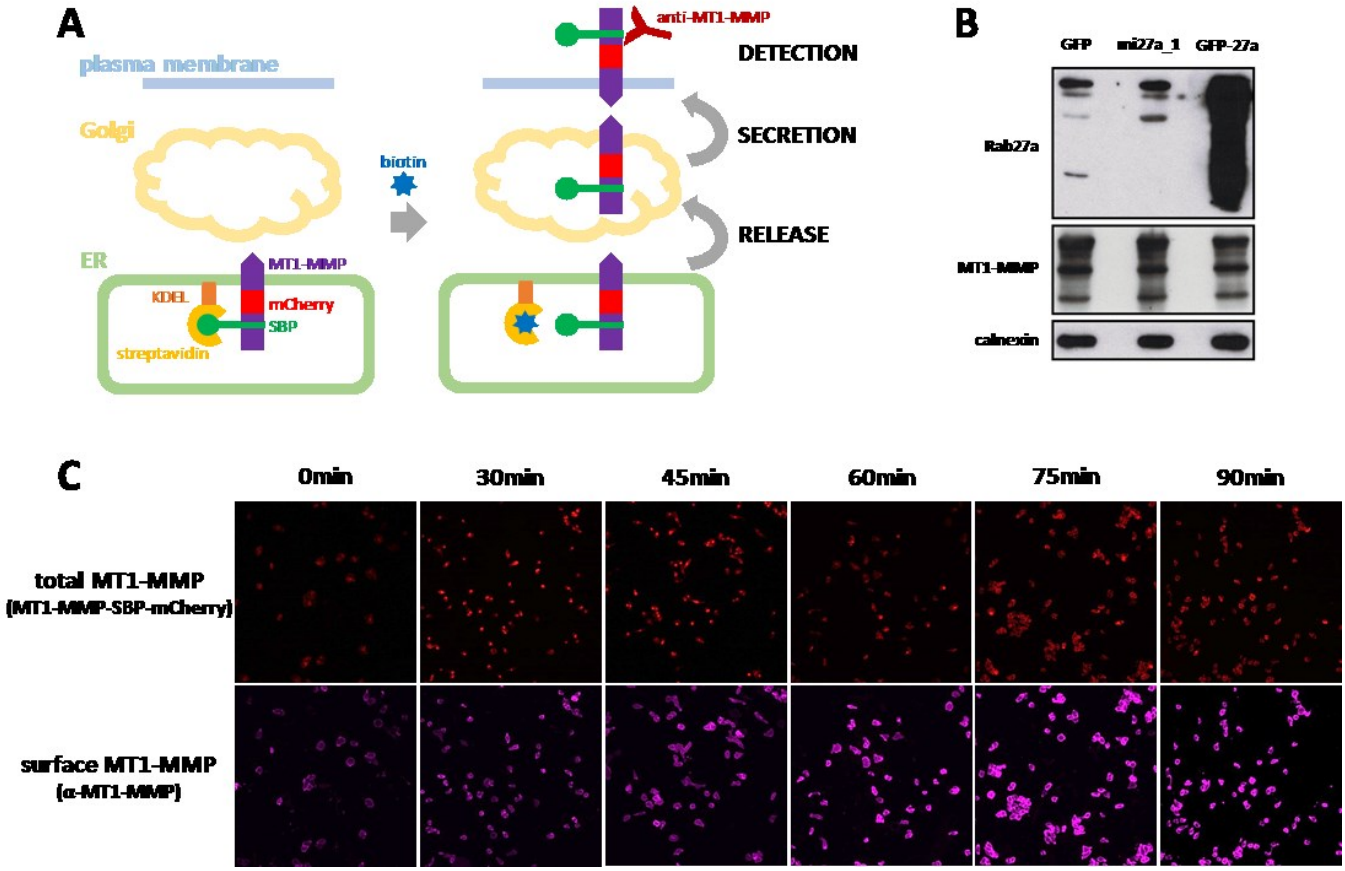


Figure 13. The RUSH system. (A) Principle of the RUSH system consisting of three key components: streptavidin fused to a KDEL (hook for the ER), MT1-MMP-SBP-mCh and biotin. MT1-MMP is retained in the ER due to the interaction between SBP (streptavidin-binding protein) and streptavidin. Biotin addition releases the hook by replacing SBP. The fluorescent protein mCherry enables to monitor the traffic of MT1-MMP easily (Adapted from Boncompain and Perez, 2012). (B) Depletion (mi27a_1) or overexpression (GFP-27a) of Rab27a in MDA-RUSH cell lines used in the experiments was confirmed by immunoblot. (C) Total (red) or surface (magenta) MT1-MMP were imaged at different time-points by a confocal microscope. Biotin was added at 0 min. The total MT1-MMP proteins were visualised with the signals of mCherry from MT1-MMP-SBP-mCherry, and the MT1-MMPs, released from ER and then exported to the cell surface were detected with an α-MT1-MMP antibody.

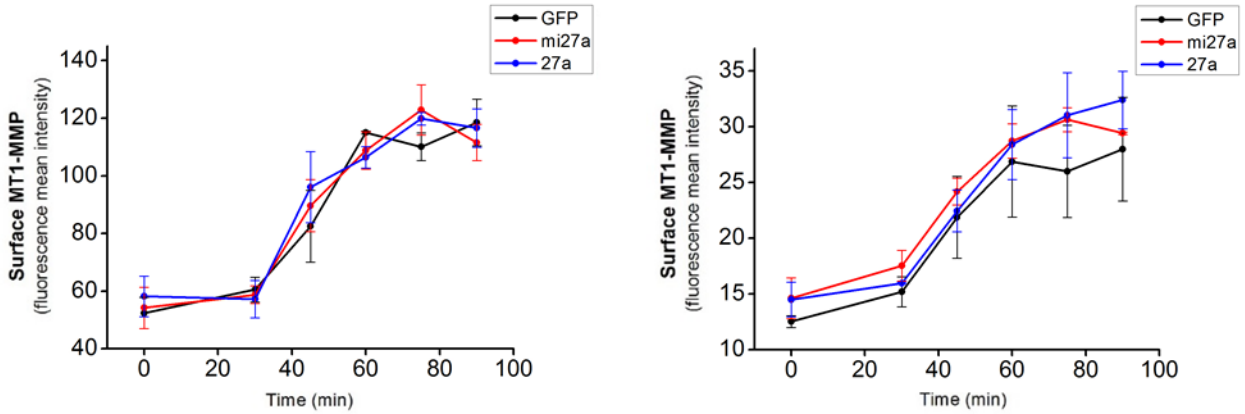


Figure 14. MT1-MMP delivery from the ER to the plasma membrane is not affected by Rab27a depletion or overexpression in MDA-MB-231 cells. Surface MT1-MMP levels in MDA-RUSH cells of control, Rab27a-depleted, or Rab27a-overexpression over time after biotin addition (at 0min) were quantified by ImageJ. The left panel shows the kinetics in the cells on glass coverslips while the right panel displays the trends in the cells on gelatin-coated coverslips. The results are expressed as fluorescence mean ± SE at each time-points from three independent experiments (n=3). 150-200 cells per condition were analysed in each experiment. One-way ANOVA and Dunnet post-test was used to test statistical difference between GFP, mi27a, and 27a cells at each time-point. In the both environments, with or without gelatin, there is no significant change in the degrees of the MT1-MMP secretion upon the modification of the intracellular amount of Rab27a.

4.1.6 Rab27a is involved in MT1-MMP recycling in MDA-MB-231

To investigate whether Rab27a affects MT1-MMP exocytosis through the recycling pathway, we used the biotin-streptavidin system to perform a pulse-chase experiment as described previously (411). The scheme of the assay is shown in Figure 15A. The surface MT1-MMP in MDA-MB-231 cells was firstly labelled with biotin at low temperature, and then the cells were kept at 37°C for 15 minutes to induce internalisation (pulse). Then the cells were chilled to be treated with a specific buffer for the purpose of removing the surface biotin. After biotin removal, any intercellular trafficking was allowed at 37°C for 30 minutes or 60 minutes. Cells were cleansed with the buffer at each time-point to get rid of any biotin (including from biotinylated MT1-MMP) that recycled back to the plasma membrane. Cells in each condition were lysed and the biotinylated proteins were collected with streptavidin beads. Finally, samples were analysed by immunoblotting MT1-MMP.

The result (Figure 15 C,D) clearly showed that the MT1-MMP recycling rate of Rab27a-depleted MDA-MB-231 cells for the first 30 minutes after the pulse (15+30min) was much lower than control and Rab27a-overexpressing cells. The degree of MT1-MMP recycling in Rab27a-depleted cells was recovered at the later time-point (15+60min) as high as the cells with high expression level of Rab27a, which showed sustained levels during the experiment. The MT1-MMP recycling rate in control cells peaked at the early time-point and decreased later. In summary, depletion of Rab27a appeared to delay the early recycling of MT1-MMP while Rab27a overexpression allowed constant MT1-MMP recycling at higher levels than in normal condition. Considering the experimental condition where we monitored (chase) the recycling of MT1-MMP right after its internalisation (pulse), this suggests that Rab27a is involved in the fast recycling process of MT1-MMP in MDA-MB-231 cells.

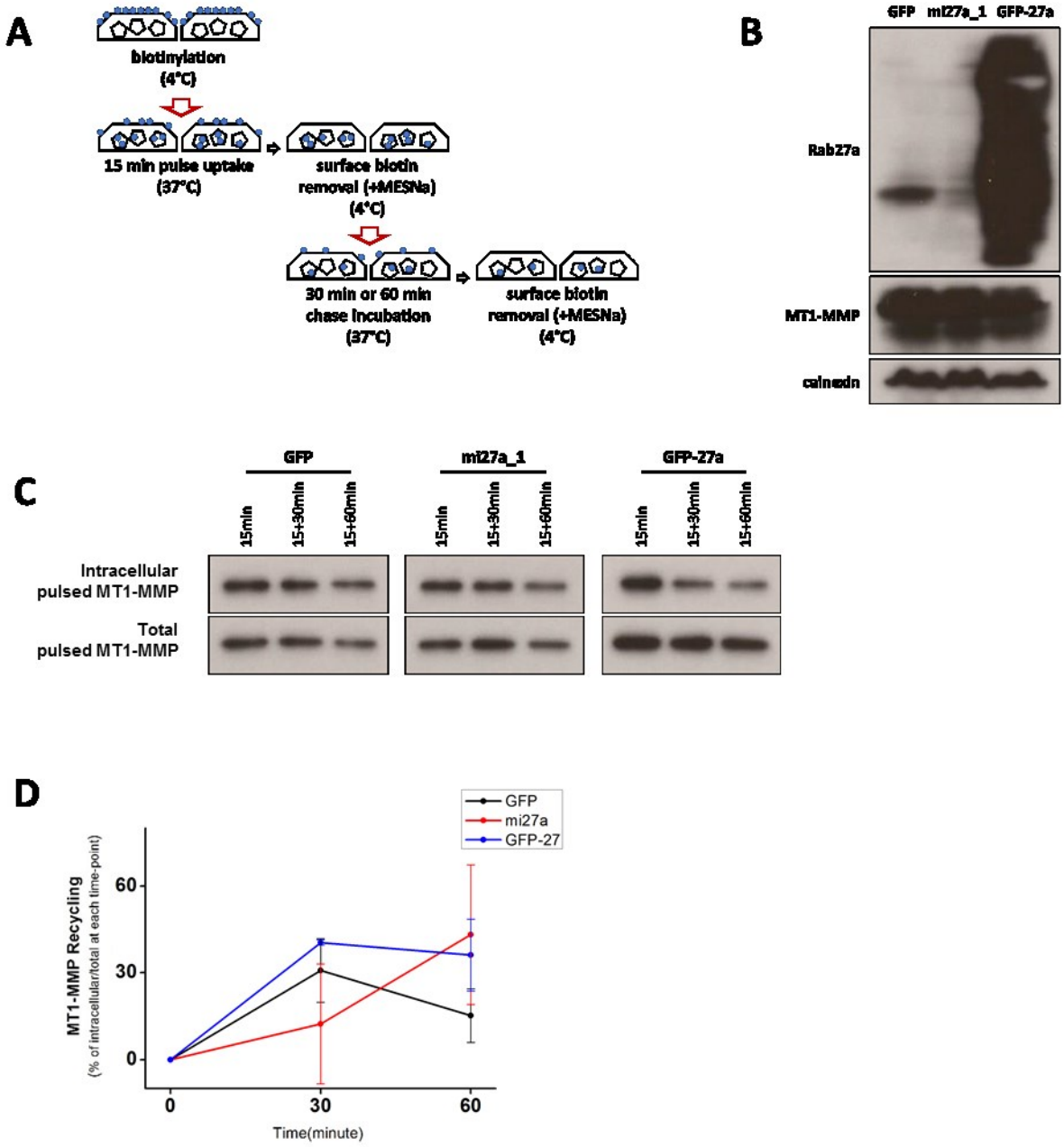


Figure 15. Rab27a regulates MT1-MMP recycling. (A) The design of the pulse-chase experiment. Initially, surface proteins, including MT1-MMP, were biotinylated on ice. Then the cells with the biotinylated surface proteins were moved to 37°C and kept for 15 minutes. A reducing agent, MESNa, was used to release the biotin from the biotinylated surface proteins at 4°C. The biotinylated proteins that were internally localised through endocytic process could avoid the effect of MESNa, and their intracellular movements were allowed for 30 or 60 minutes at 37°C. At the indicated time-points, cells were washed again with MESNa and only the biotin-labelled proteins that had recycled back to the cell surface lost biotins. As a result, the degree of reduction of the biotinylated proteins after MESNa treatment corresponds to the rate of their recycling. The biotinylated proteins were collected by streptavidin beads. (B) MDA-MB-231 cells were prepared to stably express different levels of Rab27a; control, Rab27a-depleted, or Rab27a-overexpressing. (C) The samples of the pulse-chase experiment were analysed by immunoblotting MT1-MMP. MT1-MMP among the biotinylated proteins was probed by an α-MT1-MMP antibody. Two different groups of samples, MESNa wash (Intracellular pulsed MT1-MMP) and no wash (Total pulsed MT1-MMP) respectively, were examined at three different time-points; 15 minute-pulse, 15 minute-pulse followed by 30 minute-incubation, or 15 minute-pulse followed by 60 minute-incubation. (D) The recycling rate of the pulsed MT1-MMP was assessed by calculating the amounts of the biotin-labelled MT1-MMP with MESNa treatment against the amounts without MESNa treatment at each time-point. The result graphs are presented as mean ± SE of two independent experiments (n=2).

4.2 OPCML ENDOCYTOSIS IN OVARIAN CANCER

4.2.1 OPCML endocytic vesicles co-localise with caveolin-1

For the study of the OPCML endocytosis in ovarian cancer cells, a cancer cell line called SKOV3, originated from the ovary (ascites) of an ovarian cancer patient and widely used for studying biology of ovarian cancer, was selected. Due to downregulated expressions of OPCML in cancer cells, including SKOV3, this cell line was genetically modified to overexpress OPCML (SKOV3 OPCML). Despite there were cells expressing fluorescent OPCML, such as GFP-fused or mCherry-fused OPCML, those cell lines were not used for this study as it had not been fully tested whether the fluorescent OPCML was functional as the original tumour suppressor protein. Also, there is no known ligand for this receptor. Alternatively, an α-OPCML antibody was used as a probing tool in our 3-step internalisation assay. Firstly, SKOV3 OPCML cells were kept at low temperature (4°C) before treatment with the α-OPCML antibody. Due to the inhibition of membrane dynamics by low temperature, α-OPCML antibody can only probe the surface OPCML. Secondly, the change of temperature, from 4°C to 37°C, allowed membrane trafficking, including the movement of the antibody bound OPCML. 5-minute incubation at 37°C provided traceable amounts of small endocytic vesicles and also excluded irrelevant post-endocytic vesicle transport. Third, cells were kept cold again and then the surface bound antibody molecules were removed by an appropriate buffer before fixation. This resulted in visualisation of the endocytosed OPCML (Figure 16B right).

In order to find out the type of endocytosis, a simple test was carried out utilising the biotin-streptavidin system. Cell surface proteins, including OPCML, were initially biotinylated and then their intracellular movement was allowed at 37°C. After 5-minute incubation, surface bound biotin molecules were removed before lysing the cells and the cell lysates were incubated with streptavidin beads. As a result, only the biotinylated proteins that had gone through endocytosis could be probed by streptavidin and therefore the amount of the protein of interest could be measured using an antibody that targets the protein. The amounts of the internalised OPCML with or without α-OPCML antibody treatment were analysed and it was shown that the α-OPCML antibody had no triggering effect on OPCML

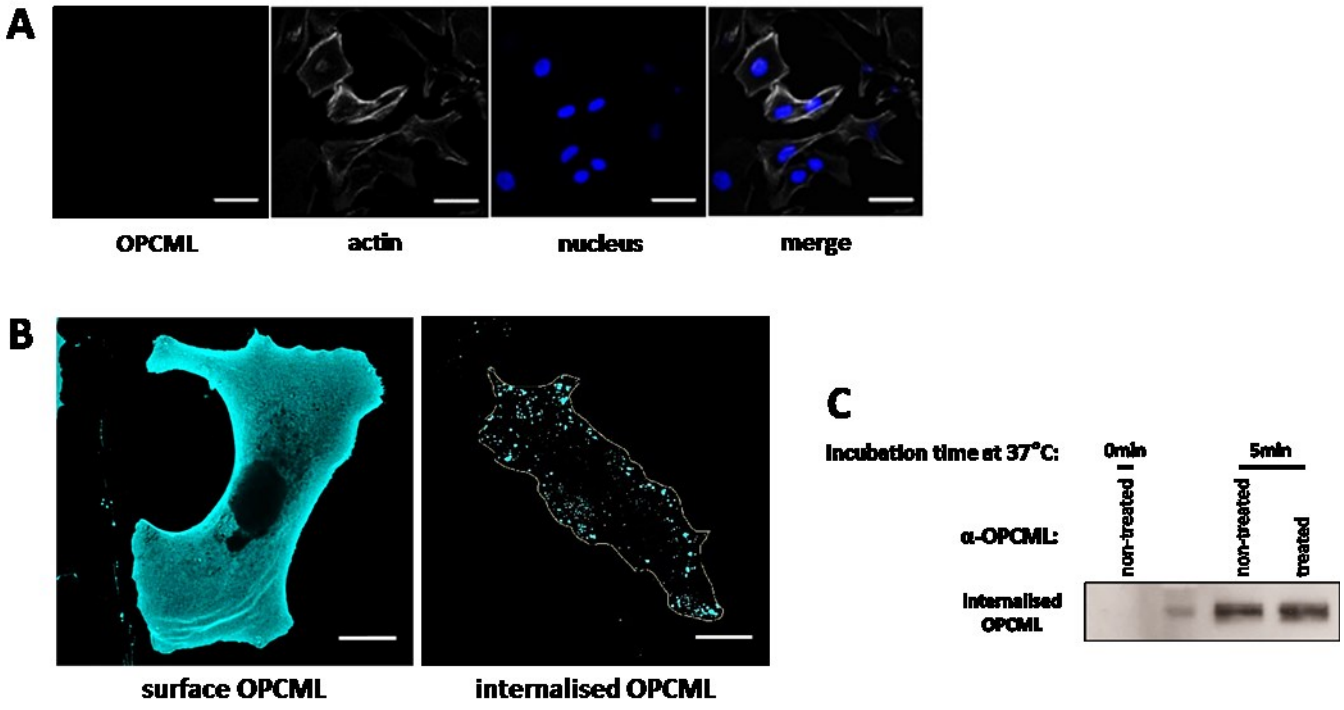


Figure 16. Experimental setup for this study. (A) SKOV3 EMPTY. This is equivalent to the original SKOV3 cell and expresses little OPCML. Scale bar=20 µm (B) SKOV3 OPCML, modified SKOV3 cell line to overexpress OPCML. The OPCML at the SKOV3 cell surface was stained with α-OPCML antibody (on the left). After the antibody staining, internalisation of the surface OPCML was allowed under temperature shift, from 4°C to 37°C. Removal of the surface bound α-OPCML antibodies enabled visualisation of the engulfed OPCML (on the right). Scale bar=20 µm. (C) Treatment of α-OPCML antibody does not induce the OPCML internalisation. This was confirmed by measuring the amounts of biotinylated surface OPCML uptake between with and without α-OPCML antibody treatment.

endocytosis, which in turn confirmed that this study deals with the constitutive endocytosis of OPCML (Figure 16C).

In the previous study (520), OPCML was observed to co-localise with caveolin-1, the marker of detergent insoluble membrane. Therefore, we hypothesised that the OPCML endocytosis also takes place in a caveolin-1 dependent manner. Immunofluorescence microscopy was used to examine the localisation of the internalised OPCML. In SKOV3 OPCML cells, high levels of co-localisation between the endocytosed OPCML and caveolin-1 were observed (Figure 17A). In contrast to clathrin, whose distribution seemed to be non-specific throughout the cytoplasm, caveolin-1 appeared to specifically localise at the areas where the endocytic vesicles of OPCML were found. To confirm this observation, SKOV3 OPCML cells were transfected with two different plasmids to overexpress fluorescent caveolin-1 (RFP) and clathrin (GFP). The result of the internalisation assay showed that OPCML positive vesicles co-localised with caveolar structure at the greater degree compared to with clathrin (Figure 17B). Altogether, these findings suggested that OPCML possibly takes the caveolin-1-dependent route during its endocytic process.

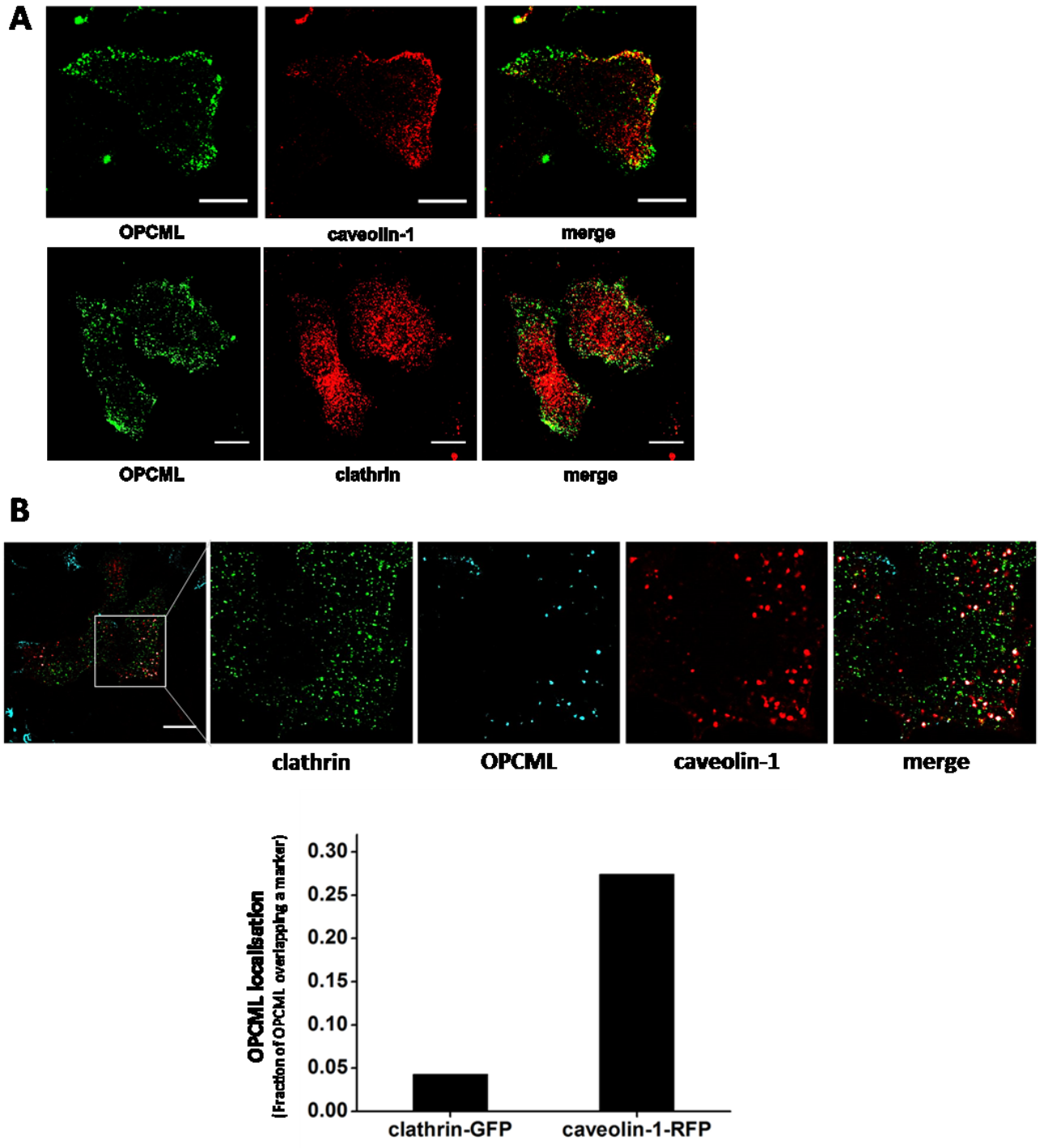
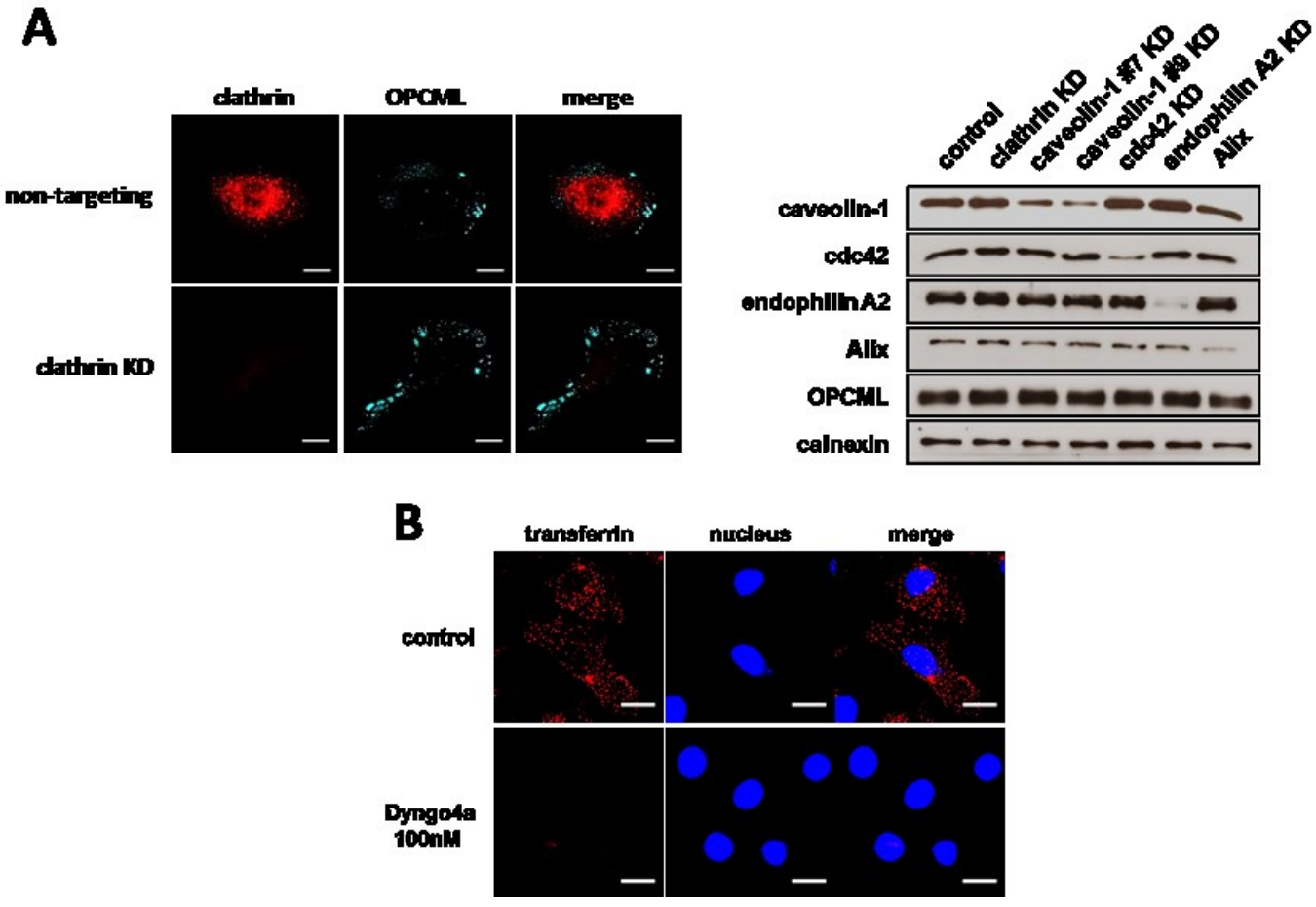


Figure 17. Endocytic vesicles of OPCML co-localise with caveolin-1 in SKOV3 cells. (A) SKOV3 OPCML cells were used for 5-minute internalisation assay. The endocytic vesicles of OPCML co-localise with the endogenous caveolin-1. (B) Plasmid transfection enabled SKOV3 OPCML to overexpress fluorescently tagged caveolin-1 (RFP) and clathrin (GFP). The result shows the clearer localisation of the internalised OPCML at the caveolar structures rather than in clathrin positive structures. Scale bar= 20 µm. The plot shows the levels of co-localisation between OPCML and clathrin (GFP) or caveolin-1 (RFP) that were quantified using ImageJ. 8 transfected cells were analysed for this preliminary data.

4.2.2 Caveolin-1 inhibition downregulates the OPCML endocytosis

To confirm via which pathway OPCML is endocytosed, we repeated the internalisation assay with SKOV3 OPCML cells with different expression levels of key endocytic molecules. In this study, five proteins including clathrin, caveolin-1, cdc42, endophilin A2 and Alix, which represent clathrin-mediated internalisation, caveolae-dependent endocytosis, CLIC/GEEC endocytosis, FEME pathway, and Alix-dependent pathway respectively, were selected to be depleted by siRNAs. Inhibition of dynamin, a key regulatory protein in several endocytic processes, was also tested in OPCML internalisation assay. Inhibition of the target molecules was confirmed by immunofluorescence microscopy or immunoblot (Figure 18A,B) and internalisation of OPCML in different conditions was assessed by wide-field microscopy. The result showed that siRNAs against caveolin-1 or an inhibitor to dynamin had a significant inhibitory impact on OPCML endocytosis whereas the



others did not seem to affect the process (Figure 18C,D). Based on the known role of dynamin in caveolar endocytosis (248-250), this result confirmed that OPCML is internalised via caveolin-1-dependent intracellular trafficking.

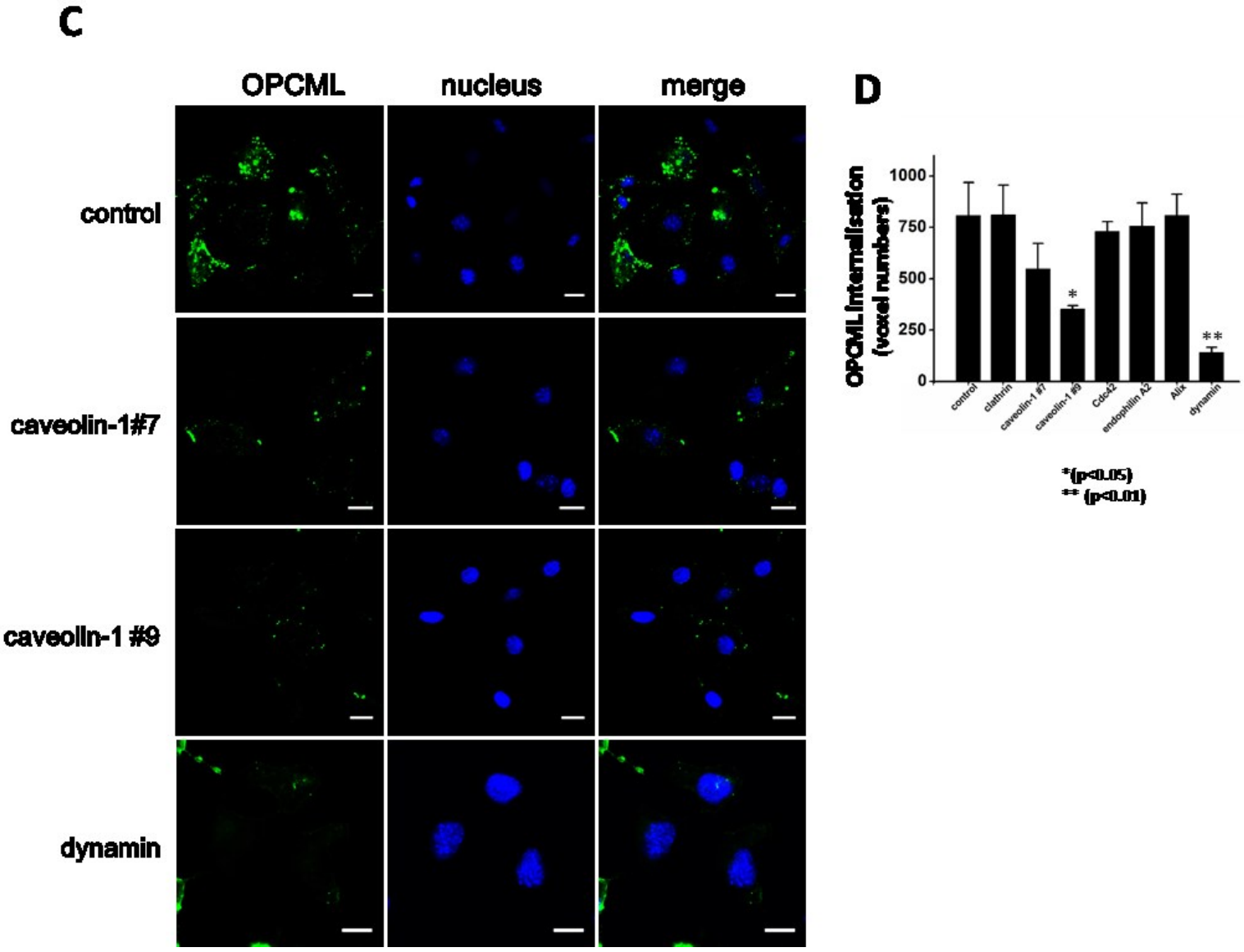


Figure 18. OPCML uptake decreases upon caveolin-1 depletion. (A)Knockdown of the selected endocytic regulators was confirmed by immunofluorescence (clathrin, on the left) or immunoblot (the rest, on the right). (B)Dyngo4a, a potent dynamin inhibitor, was used to examine the effect of dynamin inhibition on OPCML internalisation. Its action was confirmed by transferrin uptake, which requires dynamin. (C)Wide-field images of the internalisation assay after siRNA transfection or inhibitor treatment. Scale bar= 20 µm. (D)The amounts of the OPCML uptake were quantified showing that caveolin-1 knockdown and dynamin inhibition negatively affected the OPCML endocytosis. Values represent mean ± SE of 3 independent experiments (n=3). One-way ANOVA and Dunnet post-test was used to test statistical difference between control and other groups (**P<0.01, *P<0.05).

4.2.3 OPCML internalisation is inhibited upon cholesterol depletion

According to the previous work (504, 520), cholesterol was found to play a crucial role in GPI-anchored OPCML distribution by composing membrane lipid rafts and therefore facilitating its tumour suppressing activity against several RTKs. In addition, cholesterol is required for caveolae formation (234). Here, to examine the role of cholesterol in OPCML trafficking from the plasma membrane to intracellular compartments, cells from the internalisation assay were further analysed by membrane fractionation.

We found that endocytosed α-OPCML antibodies were mainly localised to the detergent (1% Triton X-100) resistant membrane, where GPI-anchored OPCML is normally distributed, rather than in the detergent soluble membrane (Figure 19A). Cholesterol depletion using Methyl-beta-cyclodextrin (MβCD) was also tested to see whether lowering the levels of cholesterol would disrupt the OPCML uptake. OPCML trafficking was negatively affected by MβCD, which confirmed that cholesterol is required not just for OPCML distribution and function but also for OPCML internalisation (Figure 19B).

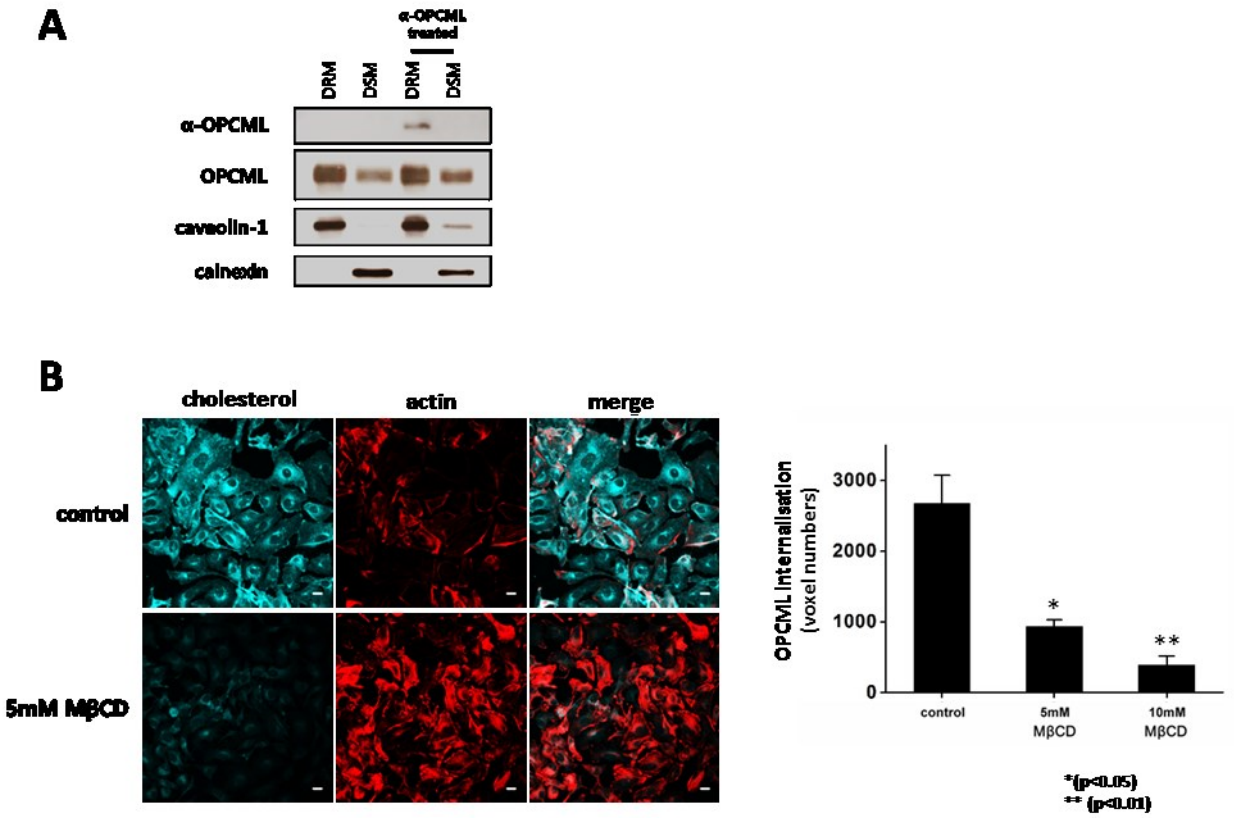


Figure 19. Cholesterol is another crucial component in the GPI-anchored OPCML internalisation. (A) 5-minute internalisation assay was followed by membrane fractionation assay. The result shows the distribution of OPCML and the membrane type where the internalising OPCML (α-OPCML) is located to. OPCML appears to be placed on the detergent insoluble membrane (DRM) during invagination and endocytosis. Caveolin-1 and calnexin are markers for the detergent insoluble membrane domain (DRM) and the detergent soluble membrane domain (DSM) respectively. (B) The effect of cholesterol depletion on OPCML internalisation was tested. When cholesterol is removed from cell membranes by methyl-beta-cyclodextrin (MβCD) (on the left), OPCML uptake is inhibited in a dose-dependent manner (on the right). Cholesterol depletion was confirmed by confocal microscopy. Scale bar= 20 μm. The amounts of the internalised OPCML in each condition were analysed by wide-field microscopy as described earlier. Values represent mean ± SE of 3 independent experiments (n=3). One-way ANOVA and Dunnet post-test was used for statistical analysis (**P<0.01, *P<0.05).

4.2.4 Rab5 and Rab7 are involved in OPCML intracellular trafficking

In order to further characterise the intracellular movements of OPCML beyond its endocytosis, in situ proximity ligation assay (PLA) DUOLink was carried out. This assay showed the strong proximity signals between OPCML and caveolin-1 during the early endocytic process (at the 5-minute time-point) (Figure 20B,C). The intensity of the fluorescent signals decreased at the later time-points strengthening our findings that caveolin-1 is the endocytic carrier molecule that regulate the OPCML internalisation. The signals between OPCML and EEA1 peaked at 30-minute time-point and the combination

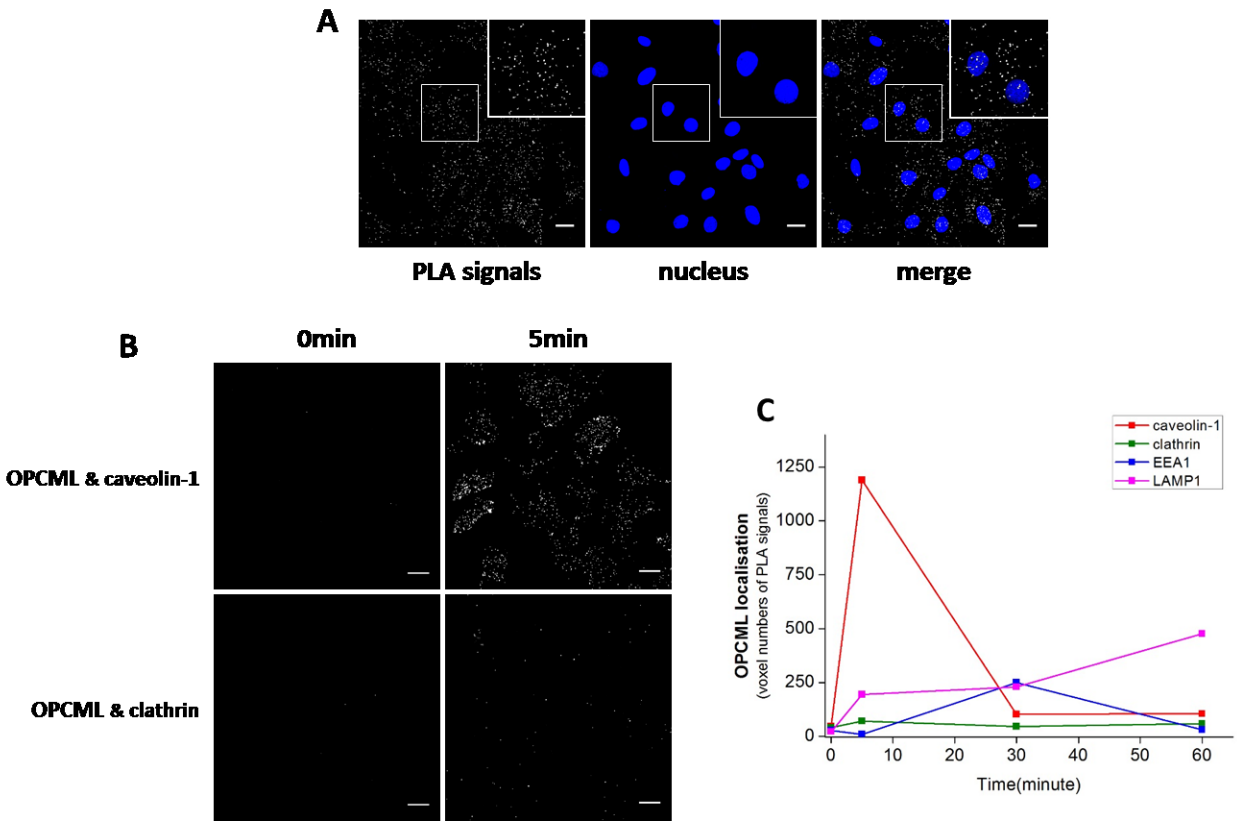
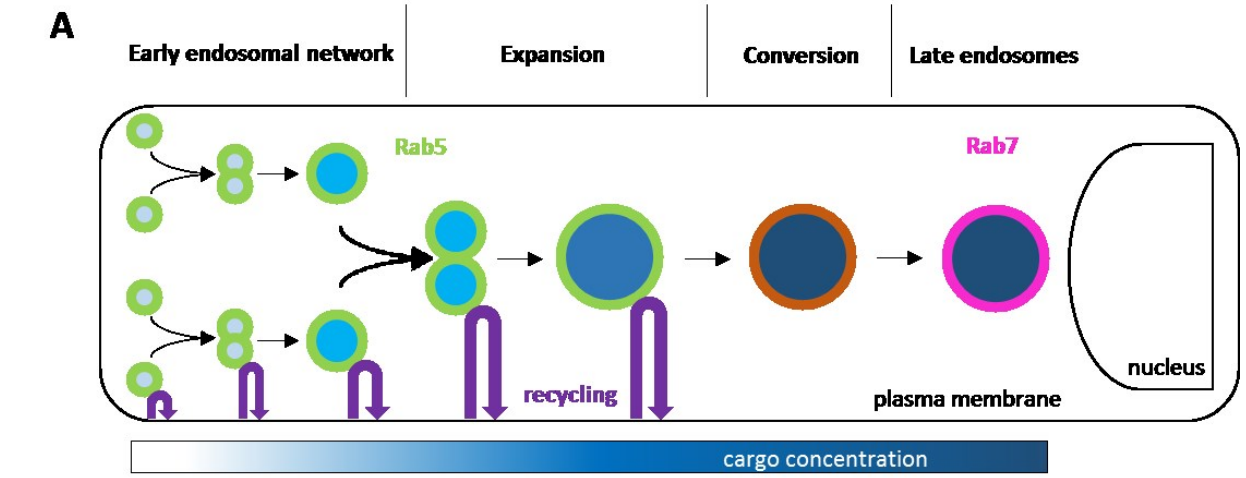
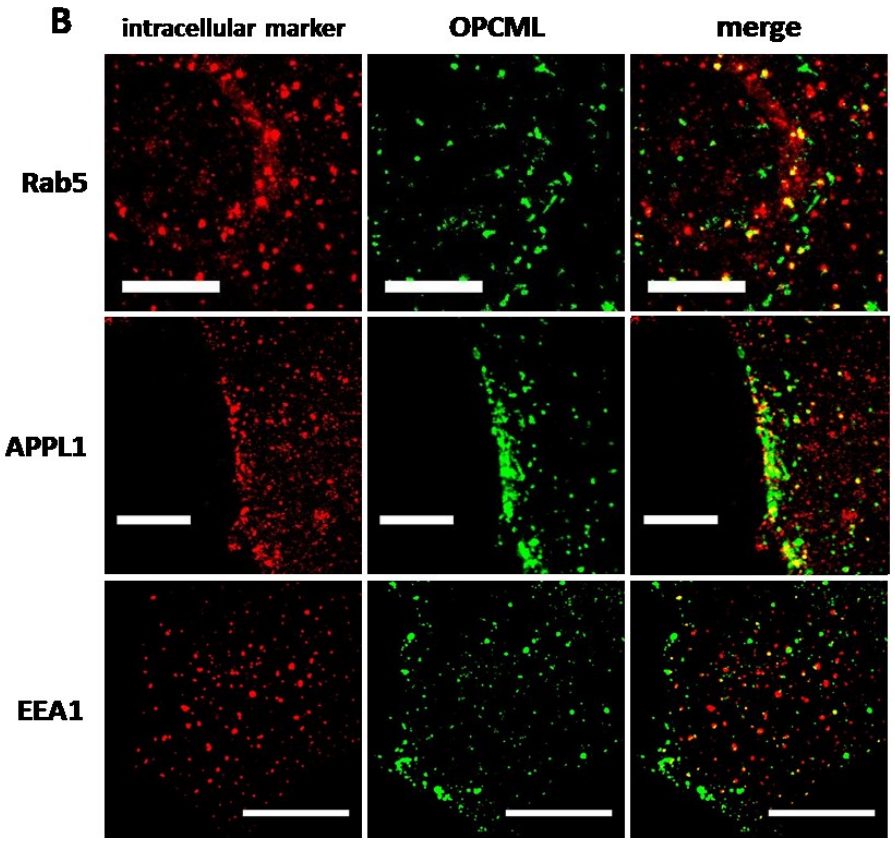


Figure 20. Proximity ligation assay (PLA) DUOLink data shows the strong association between OPCML and caveolin-1. (A) Example of immunofluorescence microscopy images of PLA signals (white dots) and nucleus (blue). Scale bar= 20 µm (B) Representative images from the internalisation assay show the drastic increase of PLA signals from the combination of OPCML and caveolin-1, at the early time-point (5 minute). Scale bar= 20 µm (C) Results of the internalisation assay that was analysed by PLA DUOLink show that OPCML seems to be internalised via the caveolin-1-dependent pathway and migrate to early endosomes (EEA1 as a marker) to then reach late endosomes (LAMP1 as a marker). The assay was performed once (n=1). For each condition, 80-100 cells were analysed to quantify the degrees of proximity between two proteins.

between OPCML and LAMP1 produced the higher signals over time. According to this, OPCML seemed to translocate from caveolin-1-enriched sites to early endosomes, and then to the late endosomes/lysosomes within this 1-hour time frame.

To investigate which proteins are involved during this OPCML trafficking, we proceeded to perform a pulse-chase experiment with more intracellular markers. After 5-minute pulse uptake, the fluorescently labelled α-OPCML antibodies that are bound to the surface OPCML lost their fluorescence by treatment with a reducing agent, which cleaves the disulfide-bond-containing linker that connects the antibody and the fluorophore. This allowed the follow-up detection of the internalised OPCML bound to the intact fluorescent antibody during the time-course experiment. This co-localisation analysis revealed sustained levels of co-localisation between Rab5 and OPCML over time (Figure 21B,C). There are two distinct types of Rab5-positive early endosomes, which can be distinguished by the Rab5 effector molecules APPL1 or EEA1. The canonical early endosomes are defined by Rab5 and EEA1, which are translocated to the target membrane by the presence of phosphatidylinositol 3-phosphate (PI(3)P) (18, 521). Other Rab5-positive early endosomes that are enriched in the sub-cortical area of the cell contain APPL1 and APPL2, and this type of early endosomes are devoid of EEA1 and PI(3)P (522, 523). Some early endosomes (~15%-30% of total) are found to contain both EEA1 and APPL1 (522, 523). This diversity in distinct classes of early endosomes provides additional platforms of signalling regulation by molecules such as RTKs before moving to late endosomes and lysosomes (524).


A
Early endosomal network
Expansion
Conversion
Late endosomes
Rab5
Rab7
recycling
plasma membrane
nucleus
cargo concentration


B
intracellular marker
OPCML
merge
Rab5
APPL1
EEA1

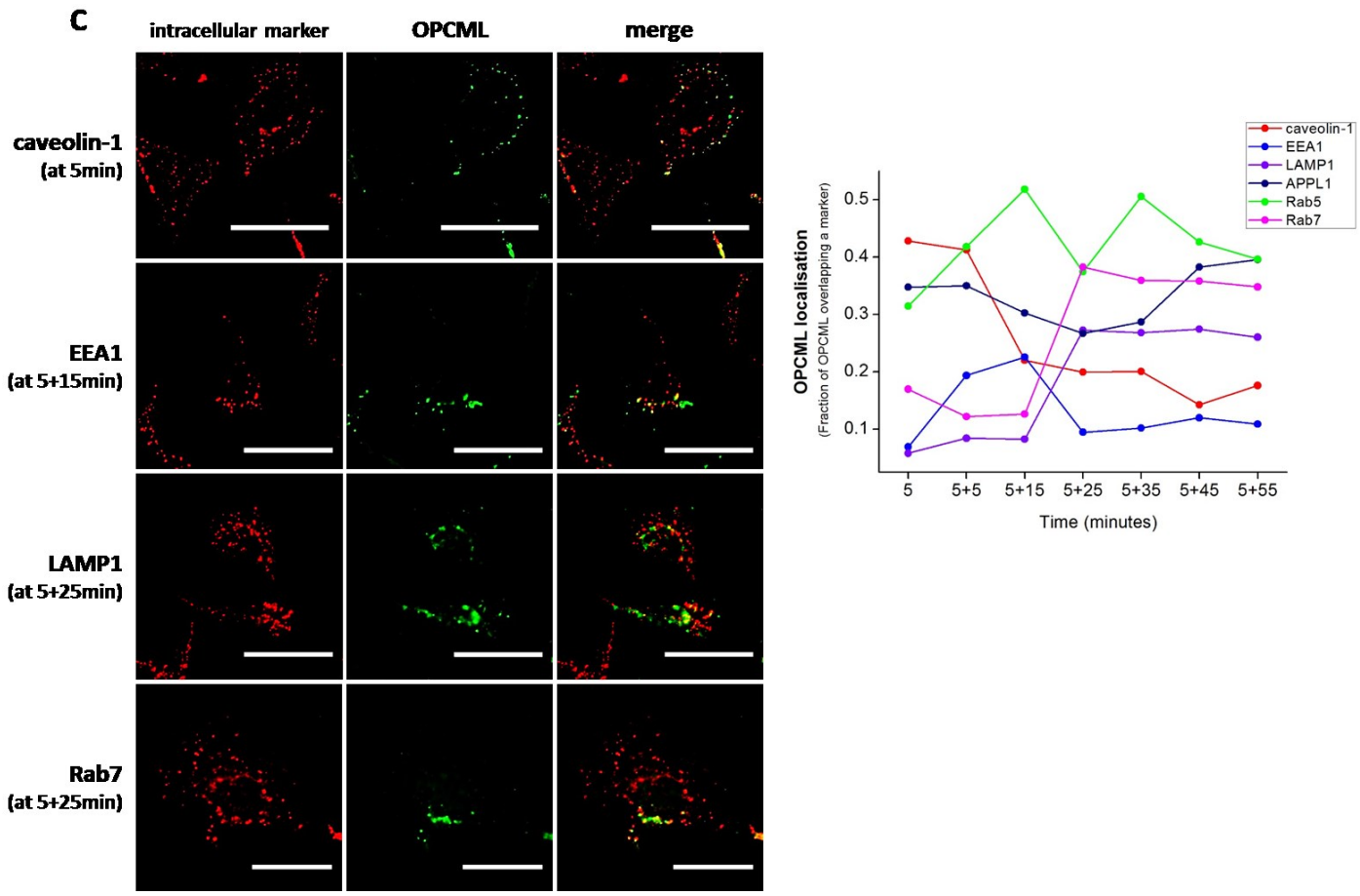


Figure 21. Rab5 and Rab7 are involved in OPCML intracellular trafficking. (A) The model developed by Rink et al. describes the endosomal dynamics. Rab5-positive early endosomal membranes are illustrated with green colour and Rab7-positive late endosomes are shown with magenta outline. The transient co-localisation of Rab5 and Rab7 is expressed as dark orange during the conversion stage. Purple arrows represent interactions of cargo vesicles with early endosomes and subsequent recycling. The gradient bar at the bottom displays the concentration of cargos. (B) The confocal images show the co-localisation between OPCML and Rab5 or the Rab5 effector APPL1 or EEA1 after the 5-minute uptake of OPCML in SKOV3 OPCML. Scale bar= 20 µm (C) The pulse-chase co-localisation analysis by wide-field microscopy was quantified (on the right) showing that OPCML sequentially migrates via caveolin-1 toward EEA1, and then LAMP1. Rab7 appears to be involved in the late OPCML transport to the late endosomes. Scale bar= 20 µm. The result is produced from a single experiment (n=1). 15-25 cells per each condition were analysed.

Once sorted during the sorting process in the Rab5-positive endosomes, degradative cargo molecules are moved to the late endosomal compartments where Rab7 plays a key role in the transport (Figure 21A). The mechanism that describes the replacement of Rab5 with Rab7 on the endosomal membranes located at the perinuclear region is called Rab5-to-Rab7 conversion (525). Rink et al. demonstrated that Rab5 levels on individual early endosomes fluctuates through continuous fusion and fission events, subsequently forming a large network of Rab5-positive endosomes. They found that some of these Rab5-positive

endosomes that are destined to translocate to other membrane domains rapidly lose their identity as the early endosomes by dissociating Rab5 and recruiting Rab7. Indeed, the movement of the internalised OPCML between the early endosomes and the late endosomes was observed to be through Rab7 (Figure 21B,C).

Recycling is another event in which Rab5 is strongly implicated (Figure 21A). However, other Rabs are also involved in recycling: Rab4 plays a role in fast recycling from the early endosomes to the cell surface (33, 526), and Rab11 is localised to recycling endosomes (45, 527) and the TGN (528) and it is known to regulate the recycling process of transferrin (527) and vesicular stomatitis virus glycoprotein (VSV-G) (529). Sönnichsen et al. identified that early endosomes and recycling endosomes consist of several Rab domains, including Rab4-, Rab5-, and Rab11-positive domains, whose relative amounts between endosomes differ (530). According to their model (Figure 22A), the early endosomes at the cell periphery area mainly contain Rab5- and Rab4-positive domains and only few Rab11. In contrast, recycling endosomes mostly carry Rab4- and Rab11-positive domains rather than Rab5.

In this context, the result of the pulse-chase co-localisation study that showed the sustained levels of co-localisation between OPCML and Rab5 could be explained by the fact that Rab5 is found to have regulatory roles in several endosomal dynamics, including endocytosis, conversion to late endosomes, and recycling.

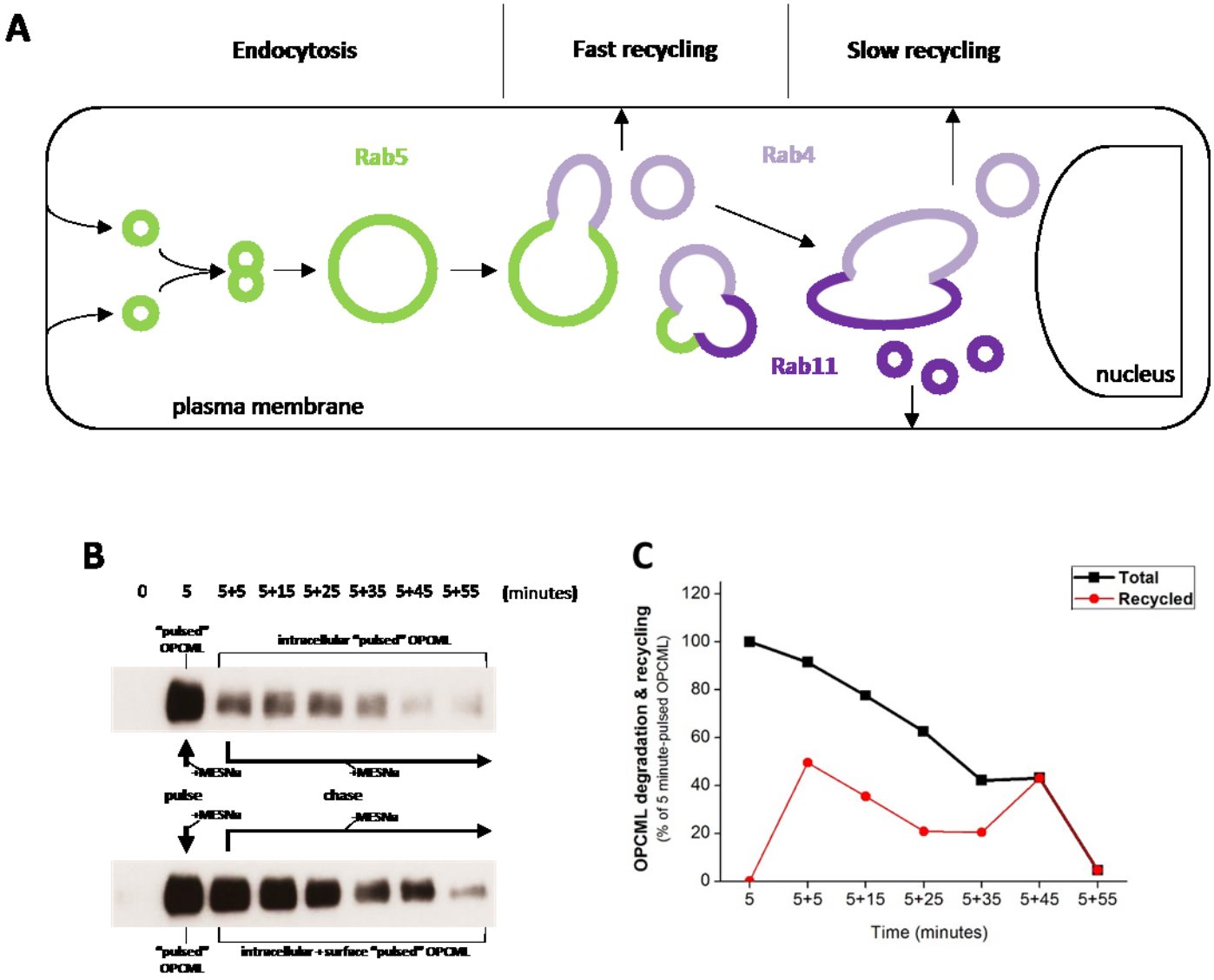


Figure 22. OPCML recycling. (A) A model, reproduced from Sönnichsen et al., 2000, of Rab distribution during transferrin recycling process. Intracellular compartmentalisation is determined by arrangements of functional domains. Here, two most abundant combinations are described, Rab5 (green) and Rab4 (light purple), and Rab4 and Rab11 (dark purple). Fast recycling mainly occurs from the result of rapid sorting, from Rab5-positive domains into Rab4 domains, on the same endosomes. Slow recycling is achieved when endosomal membranes are largely occupied by Rab4 and Rab11 domains (in the case of transferrin, this takes place at pericentriolar membranes). (B) Immunoblot result of internalisation assay using biotin-streptavidin system. Surface proteins were biotinylated before 5-minute internalisation 37°C, and the surface-bound biotins were removed by a washing buffer (MESNa) at low temperature (4°C). Incubation at 37°C resumed to analyse biotinylated proteins at different time-points. The upper blot shows that cells was treated with the washing buffer at each time-point representing the biotinylated proteins that were present intracellularly. The bottom blot indicates that cells were analysed without removal of the surface biotin at each time-point keeping the biotinylated proteins that had been internalised during the first 5 minutes. Samples were analysed by immunoblotting OPCML. (C) Quantification of the result of the internalisation assay described in (B). The result shows that OPCML recycling and degradation is actively achieved after endocytosis. The black line indicates the total amount changes of the biotinylated OPCML that had been engulfed during the 5-minute pulse uptake. The red line represents the amounts of recycled OPCML. The result presented was from a single experiment (n=1).

The recycling of OPCML was examined by carrying out internalisation assays using the biotin-streptavidin system (Figure 22). The endocytosis of the surface proteins after biotinylation (4°C) were allowed at 37°C for 5 minutes and then the engulfed biotinylated proteins were analysed by immunoblotting OPCML at different time-points. The amount of OPCML recycling was equivalent to the difference between the amounts of total biotin-labelled OPCML, without removal of the surface biotin, and the intracellular biotinylated OPCML, with washing buffer treatment to remove the extracellular biotin. The results showed that active fast recycling of biotinylated OPCML was occurring right after internalisation. The recycling rate decreased over time until there was a second peak at the later time-point (5+45 minutes). OPCML degradation was also detected in the experiment. The amount of the biotinylated OPCML that had internalised during the first 5-minute pulse incubation was gradually reduced throughout the assay and over 90% loss was observed at last time-point (5+55 minutes).

In summary, GPI-anchored OPCML is found to be internalised via a caveolin-1- and dynamin-dependent endocytic pathway and this process requires cholesterol. After the internalisation, OPCML seems to migrate to the early endosomes and Rab5 appears to be involved in this trafficking. Rab7 is strongly correlated to the OPCML movement from the early endosomes to the late endosomes. During this intracellular trafficking, OPCML recycling is observed, which seems to start taking place right after endocytosis. In addition, the endocytosed OPCML is steadily degraded and most of it is lost within an hour after the uptake. It is still not clear whether this fast OPCML degradation is due to the intrinsic characteristics of OPCML or the viral transduction which boosts up its expression continuously and might causes the fast replacement of pre-existing OPCML with new ones.

4.2.5 Activated AXL redistributes to caveolin-1 in the presence of OPCML

Previously, our group discovered that some RTKs were negatively regulated by OPCML through direct interaction with the extracellular domains of the receptors (503). We recently found that AXL is another RTK whose signalling pathway is suppressed by OPCML in an analogous manner. In the unpublished study by Antony et al., our group demonstrated that PTPRG, which localises in lipid rafts, is the key molecule that causes the abolition of AXL downstream signalling. However, it is not known whether this happens on the cell surface or in intracellular endocytic vesicles after OPCML and AXL are internalised together.

According to the literatures, AXL distribution pattern seems to vary in different cellular conditions. Valverde (531) showed that AXL internalisation and following degradation in human lens epithelial cells was prevented by the treatment of monensin, an inhibitor of clathrin-dependent endocytosis. However, Laurance (532) et al. found that Gas6-AXL signalling in human umbilical vein endothelial cells is dependent on its localisation to lipid rafts/caveolae. TGFβ is an example that takes different endocytic routes, subsequently inducing different cellular responses (533-535). Clathrin-dependent TGFβ endocytosis promotes downstream signalling and cellular responses, while caveolin-1-dependent uptake of TGFβ causes rapid degradation of TGFβ-stimulated TGFβ receptors and attenuation of TGFβ signalling.

In order to identify whether and how OPCML affects the trafficking of AXL, first the stimulation of AXL was tested as described in the previous study (Antony et al.). SKOV3 EMPTY and SKOV3 OPCML cells were serum-starved overnight before Gas6 treatment. AXL activation was assess by the phosphorylation levels of ERK, which is a Gas6-AXL downstream

signalling molecule (Figure 23A). Once SKOV3 EMPTY cells are treated with Gas6 (400ng/ml), the level of phospho-ERK is upregulated after 30 minutes and this level is sustained over time. The peak of phospho-ERK at t=30 minutes can also be found in SKOV3 OPCML, but its loss is seen from t=60 minutes.

After confirming that AXL is stimulated by Gas6 and its effect on the signalling pathway is suppressed by OPCML, AXL distribution pattern during stimulation assay was examined by using PLA DUOLink (Figure 23B-E). Upon stimulation by its ligand Gas6, the change of association of AXL with clathrin or caveolin-1 was monitored at different time-points and the effect of the presence of OPCML on the association was also evaluated. When SKOV3 EMPTY cells were treated with Gas6, AXL was translocated to clathrin-positive membrane domains right after stimulation (t=5 minutes) and then dissociated over time. However, this increase in AXL-clathrin co-localisation was not seen in SKOV3 OPCML. Conversely, the association between AXL and caveolin-1 massively rose in the presence of OPCML, while this effect was lost in SKOV3 EMPTY. This result indicates that Gas6-stimulated AXL is probably internalised via clathrin-dependent endocytosis in the absence of OPCML in SKOV3 cells, but its distribution and trafficking pattern appears to change toward a caveolin-1-dependent mode when OPCML is expressed in the cells. In the future work, in order to design a time-course experiment that can visualise the AXL-Gas6 internalisation, it will be necessary to examine the exact timing of AXL stimulation after Gas6 treatment (e.g. between 0 and 30 minutes) by looking at the level of phosphorylation of AXL itself, which is upstream of ERK phosphorylation, as a result of Gas6 binding to the receptor.

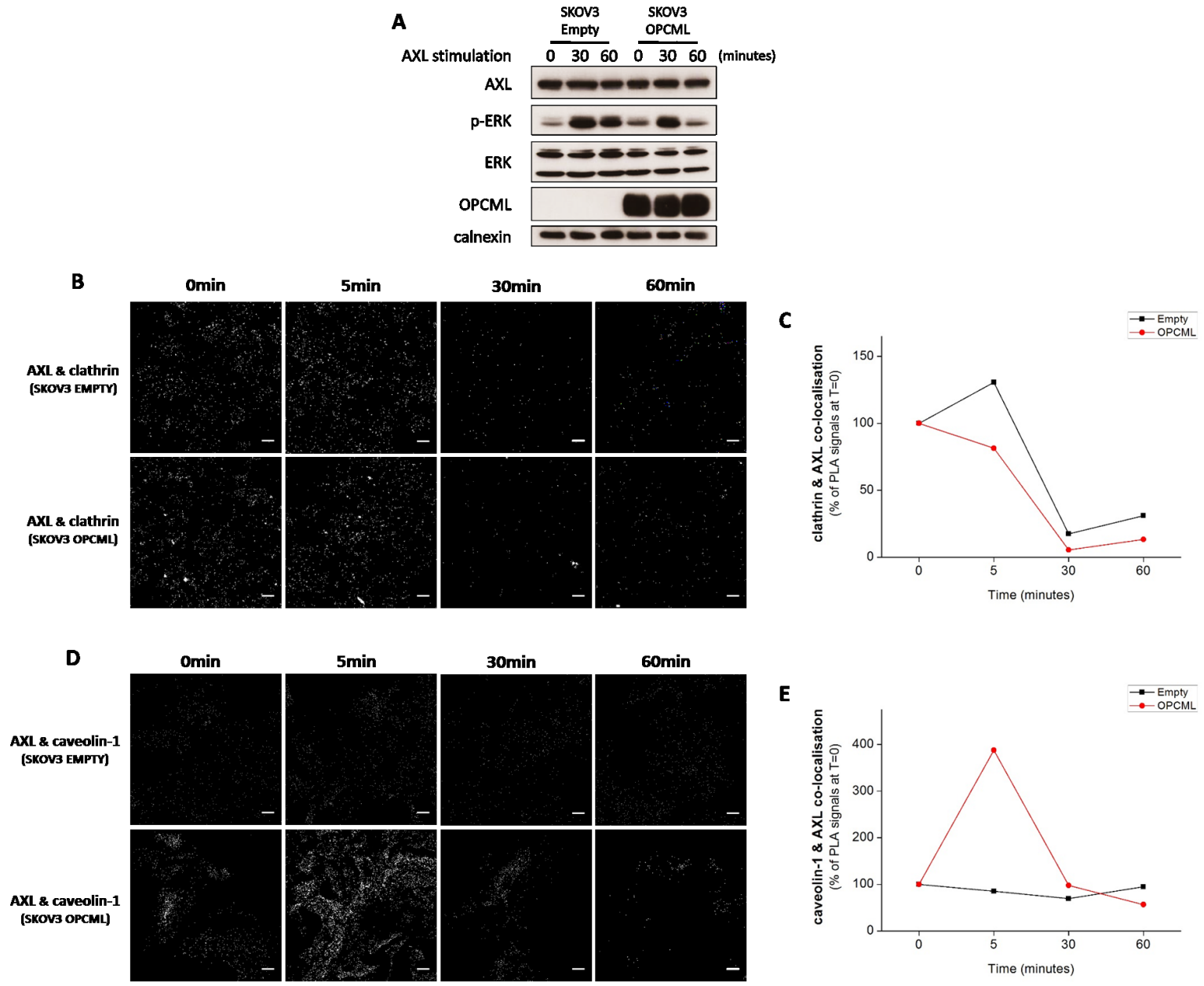


Figure 23. AXL redistribution upon Gas6 treatment in SKOV3 cells. (A)Immunoblot result shows that AXL downstream signalling is attenuated in the presence of OPCML in SKOV3 cells. Gas6 was treated on starved SKOV3 cells at 0 minute. Phospho-ERK (p-ERK) expression levels are sustained at 30 minutes and 60 minutes after stimulation in SKOV3 EMPTY. Its expression is lowered at 60 minutes in SKOV3 OPCML cells. (B-E)Results of the AXL stimulation assay that was analysed by PLA DUOLink. Associations between AXL and clathrin, and AXL and caveolin-1 were imaged by confocal microscopy and assessed by ImageJ. Results were respectively normalised to the PLA signals at T=0 minute of each condition. (B,C)Translocation of AXL to clathrin positive structures was observed at T=5 minutes in SKOV3 EMPTY cells whereas effect disappeared in SKOV3 OPCML cells. In both cell types, the levels of co-localisations between AXL and clathrin greatly decreased at the later time-points. (D,E)Massive redistribution of AXL to caveolin-1-positive compartments was detected at T=5 minutes in SKOV3 OPCML cells, while this was not found in SKOV3 EMPTY cells. Scale bar= 20 µm. The assay was performed once (n=1). In this experiment, 90-100 cells at each time-point were analysed.

CHAPTER 5. DISCUSSION

5.1 MT1-MMP SECRETION AND RAB27A IN BREAST CANCER

MMPs have been known as important regulators of many physiological processes, such as tissue remodelling, embryogenesis, and angiogenesis (536). Based on their central role in extracellular matrix degradation, which subsequently facilitates cell migration and the release of signalling molecules, there has been evidence suggesting that MMPs are also closely linked to the immune system response (537). As described earlier, they have been known to have strong associations with many cancers. Among them, MT1-MMP has been an attractive target of studies for its critical role in cancer biology, such as tumour progression, invasion, and metastasis. However, the complexity of the disease and the lack of knowledge and understanding of the general mechanisms involved in MT1-MMP trafficking and functioning has delayed the development of a clinically approved MT1-MMP inhibitor until today (536). Initially, MMP inhibitors were developed to contain the catalytic zinc-binding group and a backbone that mimics the structure of the natural peptide substrate of MMPs (538). In spite of its high potency, these hydroxamate-based inhibitors were not successful in several clinical studies as they were often metabolically labile and caused unexpected toxicity from broad MMP inhibition for the high structural homology between the different MMPs (539). Recent research has changed the strategy switching the target sites from the highly conserved, catalytic sites to less conserved and more specific sites. Using functional blocking antibodies is another method that was recently highlighted for its high selectivity and potency (540). Some research groups have tested some selective antibody-based MT1-

MMP inhibitors *in vitro* and *in vivo* and the results were promising (541-543). At the same time, there have been numerous contributions to improve the basic knowledge of MT1-MMP in cancer cells in recent years.

In this study, we have explored the novel role of Rab27a in MT1-MMP transport and secretion in breast cancer cells. To investigate whether the degree of intracellular Rab27a expression affects the metastatic phenotypes of cancer cells including digestion of the extracellular matrix component and 3D invasion through a collagen matrix that mimics the tumour microenvironment, gelatin degradation assay and 3D collagen invasion assay were conducted. The native extracellular matrix is a key intercellular factor that contains many biochemical signals like a reservoir and thereby mediates numerous cellular function (544, 545). For this reason, the extracellular matrix has been considered as a natural scaffold that needs to be studied and mimicked for the generation of artificial scaffolds that would be an essential biomaterial in several research fields, such as drug delivery application (546). Gelatin is a mixture of collagen and collagen binding proteins, which are the main component of the native extracellular matrix and are largely present in some of the tissues including bone, cartilage, connective tissue, and skin (547). In gelatin degradation assay, cells are plated on a thin layer of fluorescent gelatin and a result indicates the degree of invasiveness. However, this matrix proteolysis is restricted to the ventral cell surface (405). Type-I collagen is widely used as a model of interstitial matrix, and its fibrillar network, through which cells can migrate exerting proteolytic remodelling, enables the analysis of 3D invasion of cells (370). Breast cancer cells were modified with siRNAs or virus to express reduced levels or elevated levels of Rab27a before the functional assays. The results showed that breast cancer cells became less aggressive in terms of matrix digestion along with

reduced expression levels of Rab27a. On the other hand, the overexpression of Rab27a led to the more degradation of gelatin by cancer cells. In the spheroid 3D invasion assay, which mimics micrometastasis dissemination, a significant decrease in 3D migration from Rab27a-depleted spheroids was observed. In line with observing higher amount of surface MT1-MMP in Rab27a-overexpressing cancer cells, we confirmed that Rab27a plays a key role in matrix degradation and 3D invasion of breast cancer cells by controlling MT1-MMP secretion to the cell surface.

According to microscopy analysis, Rab27a was found to be associated with MT1-MMP positive vesicles and also to other proteins related to MT1-MMP secretion. From TEM cyroimmunolabelling, labelling MT1-MMP and Rab27a with different size gold particles allowed visualisation of co-localisation of these two proteins and of the cellular compartment of the co-localisation. The work showed that the co-localisation between MT1-MMP and Rab27a was found in the small vesicular structures near the plasma membrane. This observation was followed by a fluorescent microscopy study that also showed co-localisation of GFP-Rab27a with MT1-MMP-mCherry-positive small vesicles. From this, the goal of our work was to identify whether MT1-MMP and Rab27a physically interact with each other and in which step of MT1-MMP secretion mechanism Rab27a is involved.

To test whether there is a molecular association between MT1-MMP and Rab27a, co-immunoprecipitation was carried out. Exo84, a subunit of the exocyst complex, and IQGAP1 were selected to widen the range of the association to the extent of the known machineries required for MT1-MMP secretion. We could clearly show that the immunoprecipitated Rab27a was associated with MT1-MMP, Exo84, and IQGAP1 confirming

the potential role of Rab27a in the regulation of MT1-MMP exocytosis. Then, our next question was how Rab27a affects the MT1-MMP trafficking to the plasma membrane. To answer this, the RUSH system was adapted to test the effect of differential expression levels of Rab27a on the biosynthetic pathway of MT1-MMP. After the synthesis of the protein form at the ER, fluorescently labelled MT1-MMP molecules were simultaneously released upon the treatment with biotin, which displaces the MT1-MMP fusion protein from the streptavidin in the ER. Neither lower or higher expression level of Rab27a could significant change MT1-MMP delivery to the plasma membrane. It was the same result when the cells were placed on a gelatin layer that causes the cells to supply functional MT1-MMP to the contacts between the cells and the gelatin. This indicated that Rab27a might be involved in MT1-MMP trafficking from a pre-existing batch of functional MT1-MMP to the cell surface.

To test this hypothesis, we designed a pulse-chase experiment using a biotin-streptavidin system. Surface proteins were initially biotinylated at low temperature before the pulse at 37°C for 15 minutes. The biotin still bound to surface molecules was then removed by a buffer that can cleave the chemical bond between the biotin and the biotinylated proteins, and then the cells were kept at 37°C again. As only the biotinylated proteins were collected by streptavidin beads, the amount of the recycled MT1-MMP could be monitored by immunoblotting analysis. The rate of the MT1-MMP recycling was dependent on the expression level of Rab27a: for the first 30 minutes after the pulse, Rab27a-depleted cells showed a much lower amount of recycled MT1-MMP compared to control and Rab27a-overexpressing cells. And this low recycling rate as a result of silencing Rab27a was shown to recover as high as the cells overexpressing Rab27a at the later time-point (60 minutes after the pulse). While Rab27a-overexpressing cells showed sustained

levels of MT1-MMP recycling, the rate in control cells peaked at the early time-point and lowered later. This work, together with the result from the work using the RUSH system, clearly show that Rab27a is involved in the early recycling process of MT1-MMP in breast cancer cells and not in the biosynthetic pathway.

Rab8 was reported to regulate polarised delivery of MT1-MMP to the invasive structures at the plasma membrane via the biosynthetic pathway in a 3D collagen matrix (315). The authors stressed that MT1-MMP found in the recycling compartment was not involved in the extracellular matrix degradation process. However, there have been several studies on functional MT1-MMP recycling to focal sites of degradation in breast cancer cells. A membrane fusion machinery, the v-SNARE TI-VAMP/VAMP7, was found to regulate MT1-MMP-dependent matrix degradation by mediating the transport of MT1-MMP vesicles from the late endosomal compartments (426). Another study has described the regulatory role of the actin nucleation-promoting protein N-WASP in MT1-MMP trafficking from Rab7-positive late endosomal structures (548). Monterio et al. reported that the endosomal Arp2/3 activator WASH and the exocyst complex interact on MT1-MMP-containing late endosomes and this interaction is required for tubular connections between the late endosomes and the plasma membrane enabling focal delivery of MT1-MMP (415). Macpherson et al. suggested that chloride intracellular channel 3 (CLIC3) controls MT1-MMP trafficking from the late endosomal/lysosomal compartments above the plane of cell-matrix adhesion in ER-negative breast cancer cell lines (549). Rab2A and its effector VPS39 are other regulators of MT1-MMP recycling from late endosomes to the plasma membrane and this subsequently controls MT1-MMP-dependent matrix degradation activity and invasion though the extracellular matrix (428).

Rab27a has been known as a regulator of secretory vesicle trafficking and secretion from late endosomes/lysosomes (431, 433). In particular, exosome secretion is one of the crucial molecular processes that Rab27a is involved in (321). However, this aspect was not dealt with in this study as our previous work found that there was no change in the amount of exosomes purified from cells with different expression levels of Rab27a (477). There is evidence that Ra27a is involved in the exosome-independent secretion of MMP9 in mammary carcinoma cells inducing a prometastatic environment for tumour cells (317). We excluded MMP9 secretion topic from our study as MT1-MMP-dependent cancer cell behaviour was the main focus of our investigation and we also observed that typical cancer phenotypes, such as gelatin degradation, disappeared with the silencing of MT1-MMP, which in turn indicated that the effect of other MMPs, including MMP9, would be negligible in those assays.

To further characterise the role of Rab27a in MT1-MMP export in the context of cancer cell invasion, there are several things that can be done. Firstly, it is necessary to identify the binding partners of Rab27a or MT1-MMP in stationary state or in invasive conditions (e.g. when cells are embedded into 3D matrices for example). This could be done by doing mass spectrometry of the samples obtained following co-immunoprecipitation or other types of pull-down assays. Acquisition of information about respective binding partners of Rab27a and MT1-MMP in different experimental conditions would provide a better understanding of the step where Rab27a plays its role in MT1-MMP recycling and also elucidate unknown subunits in the machinery required for MT1-MMP exocytosis.

A second approach would be more specific to the known regulators of MT1-MMP secretion in breast cancer cells. Those regulators, including Rab8, VAMP7, N-WASP, WASH,

CLIC3 and Rab2a, could be selectively examined to establish whether Rab27a can cooperatively work with any of them. In fact, like Rab27a, Rab8 was found to mediate the actin-based movement of melanosomes in melanocytes (550). In addition to Rab27a, which shows a reversible association with the motor protein myosin V for the trafficking of melanosomes via effectors called melanophilin and MyRIP, Rab8 appeared to co-localise with Rab27a on melanosomes and contribute to the redistribution of the organelle. From further analysis, this coordination between Rab27a and Rab8 was not the result of the presence of a common effector molecule but it was suggested that it might be achieved by a common nucleotide exchange factor. Matsunaga et al. has reported recently that Rab2a regulates secretory granule exocytosis via Noc2 (also known as RPH3AL), an effector molecule in common with Rab27a (551). It was demonstrated that Noc2 bridges Rab2a, functioning in granule biogenesis and maturation, and Rab27a, controlling the trafficking to the plasma membrane. Rab5 and Rab4 are other candidates with which Rab27a might coordinate to achieve MT1-MMP-dependent invasive phenotypes. Rab5A is a known regulator of invadopodial structures and this protein is required to promote endo/exocytic cycles of crucial cargoes such as β3 integrin and MT1-MMP (552). This cycle is Rab4- and RABENOSYN-5-dependent and plays a central role also in HGF/MET signalling, which drives cancer cell invasion *in vitro* and *in vivo*. As we have found that Rab27a seems to play a role in MT1-MMP fast recycling, it can be hypothesised that Rab27a is involved in this MT1-MMP recycling circuitry inducing a proteolytic invasive program in cancer cells.

Thirdly, the development of a Rab27a inhibitor would be useful to define the roles of Rab27a in cancer cells. Considering its high expression in breast cancer cells and our findings from this study, the use of an inhibitor for Rab27a could provide additional control in the

regulation of MT1-MMP trafficking in breast cancer instead of directly targeting MT1-MMP. Furthermore, the inhibition of Rab27a in cancer cells could have a potential as an anti-cancer therapeutic method. Wang et al. has published a work about the association of Rab27a with the invasive and metastatic abilities of breast cancer cell (297). Rab27a was found to promote the secretion of insulin-like growth factor-II (IGF-II), which in turn regulates the expressions of other molecules, including VEGF, MMP9, cyclin D1, p16, and cathepsin D, that are linked to invasive and metastatic phenotypes. Therefore, it was suggested that the blockage of Rab27a-mediated IGF-II secretion would be an effective strategy to prevent dissemination of breast cancer cells. A recent work by Li et al. has shown that silencing Rab27a decreased the rates of cell proliferation, migration and invasion of lung cancer cells in both *in vitro* and *in vivo* conditions (553). This was the result of the reduction of the anti-apoptotic protein Bcl-2 and the upregulated expression of apoptotic proteins caspase-3, caspase-9 and Bax. Also, they found that the inhibition of Rab27a can increase drug sensitivity of lung cancer cells to a chemotherapeutic agent. Although the mechanism of this finding is not clear, this study suggested a new application of Rab27a inhibition in cancer treatment in combination with the currently available therapies.

Forth, the modulation of the immune system via exosome secretion is one of the multiple biological aspects that Rab27a affects. Exosomes from heat-shocked lymphoma cells were found to contain elevated levels of immunogenicity-related molecules such as CD40, CD86, Hsp60, Hsp90, MHC class I and II, and induce efficient anti-tumour T-cell immunity (554). Another study has reported that the anti-tumour response of effective natural killer cells could be induced by exosomes derived from anti-cancer drug-treated cancer cells (555). Li et al. have found that exosomes from Rab27a-overexpressing cells

promoted a stronger immune response and induced maturation of dendritic cells (556). A lung cancer cell line was transfected to overexpress Rab27a and higher levels of exosomal marker proteins, including CD9, CD63, Hsp70 and Hsp90, were shown without any change in their intracellular levels. In addition, after the treatment on dendritic cells, the exosomes containing upregulated levels of MHC and co-stimulatory molecules such as CD80 and CD86 enhanced dendritic cell maturation, which subsequently led to $CD4^+$ T cell proliferation. Elevated levels of cytokines also enable Rab27a-overexpressing cells to trigger more potent immune-stimulatory activity. In the following work, the effect of immunisation with exosomes derived from Rab27a-overexpressing cells was tested. The results showed significant inhibition of tumour growth in mice by the action of the exosomes from Rab27a-overexpressing cells compared to normal exosomes. Upregulation of type I cytokine, an important factor in tumour suppression through stimulation of macrophages and cytotoxic T-lymphocytes, was also seen in exosomes derived from Rab27a-overexpressing cells. Along with this, there have been many studies proposing exosomes to be a useful source of tumour antigen and therefore they could be utilised to design cancer vaccines (557-562). On the other hand, attention to the role of tumor-derived exosomes as immunosuppressive and favouring tumour progression also has been growing (563). Thus, it will be essential to further identify the possible consequences of Rab27a modification in the context of tumour-associated immunity for the successful development of cancer vaccines that would promote anti-tumoural immune responses and Rab27a inhibitors that would reduce tumorigenic exosomal secretion.

5.2 OPCML ENDOCYTOSIS IN OVARIAN CANCER

OPCML is a tumour suppressor protein, which is frequently downregulated in several cancers including ovarian cancer (489-492). Most cases of epithelial ovarian cancer, a lethal malignancy that begins with nonspecific symptoms, exhibit reduced expression of OPCML due to epigenetic modification and loss of heterozygosity (493). This appears to be associated with poor survival of cancer patients (491). The current standardised treatment of ovarian cancer is a combination of debulking surgery and platinum/taxane-based chemotherapy (482). However, due to the complexity and heterogeneity of the disease, the result of this approach frequently becomes chemoresistance acquisition (485-487). For this reason, there has been much effort in diversifying strategies, which now include anti-angiogenic therapeutics, DNA damage inhibitors, gene therapy and immunotherapy (487).

RTKs are one of the main targets of common anti-cancer therapeutics as they control a wide variety of signalling pathways controlling general cellular processes, including cell survival, proliferation, migration, adhesion, differentiation, and metabolism (495). The different steps leading to RTK activation and signal transduction have been widely characterised: interaction between ligands and receptors, dimerisation of the receptors induced by ligands, trans-phosphorylation of the cytoplasmic domains of the receptors, and association of phosphotyrosine-binding effectors with the receptors that then transduce intracellular signals toward downstream signalling molecules (495). Most of these studies have focussed on the characterisation of the activation of the ErbB family (495). Previously, our group has shown that OPCML inhibits a panel of RTKs, including HER2, HER4, EPHA2, FGFR1 and FGFR3, and their signalling pathways in ovarian cancer cells (520). AXL was

recently added to this list of RTKs whose functions and activities are negatively regulated by OPCML (504). From this study on AXL, the localisation of AXL was found to be redistributed by OPCML to detergent-insoluble membrane domains and it was identified that the downregulation of signalling by OPCML is the result of the action of a phosphatase, which resides in these membrane domains (504).

McKie et al. observed that exogenous recombinant OPCML exerted a similar RTK suppressing function as that detected in OPCML-overexpressing cells *in vitro* and *in vivo*, confirming the potential of OPCML as an anti-cancer therapeutic (520). For this reason, it was important to understand more about what determines the concentration of OPCML on the cell surface, i.e. how OPCML is internalised and transported intracellularly. Although McKie et al. identified that OPCML co-localises with caveolin-1 and EEA1 and also showed the redistribution of HER2 from from EEA1/clathrin-associated cellular compartments to caveolin-1-associated structures in the presence of OPCML (520), these experiments were performed at the steady state on fixed cells, with no previous induction of internalisation, and showed caveolin mostly localised in the Golgi compartment. Therefore, the mechanism of OPCML uptake and the shift in RTK distribution by OPCML remained to be clearly established. Hence, the aim of our study was to characterise OPCML endocytosis and the effect of OPCML on RTK, AXL in this project, intracellular trafficking in OPCML-overexpressing ovarian cancer cells.

To study OPCML trafficking, SKOV3 cells, a cell line widely used for research in ovarian cancer biology, were genetically modified to overexpress OPCML (SKOV3 OPCML). A commercially available α-OPCML antibody was used as a detection tool in our 3-step internalisation assay: SKOV3 OPCML cells were kept at low temperature (4°C) during the

treatment with the α-OPCML antibody, then the incubation at 37°C for 5 minutes allowed membrane trafficking to form traceable amounts of small endocytic vesicles and then cells were chilled down again to exclude post-endocytic vesicle transport and also to be stripped of the surface bound antibody molecules by acid-wash before fixation.

In order to identify the endocytic pathway of OPCML, immunofluorescence microscopy was used to examine the localisation of the internalised OPCML in SKOV3 OPCML. We observed co-localisation of OPCML with endogenous caveolin-1 but not with endogenous clathrin. And this trend was consistent in SKOV3 OPCML cells that were transfected to overexpress fluorescent caveolin-1 (RFP) and clathrin (GFP). To confirm through which endocytic route OPCML was internalised, the internalisation assay was conducted in SKOV3 OPCML cells with different expression levels of key endocytic molecules. Clathrin, caveolin-1, cdc42, endophilin A2, and Alix, which represent clathrin-mediated internalisation, caveolae-dependent endocytosis, CLIC/GEEC endocytosis, FEME pathway, and Alix-dependent pathway respectively, were selected to be depleted by appropriate siRNAs. Inhibition of dynamin, a key regulator of multiple endocytic processes, was also included in the test. The result showed that silencing caveolin-1 or inhibiting dynamin significantly downregulated OPCML endocytosis. As dynamin is one of the known regulators of caveolar endocytosis, this result could confirm that OPCML internalisation takes place via the caveolin-1-dependent pathway. In addition, the effect of cholesterol depletion on the OPCML endocytosis was also examined. Cholesterol is a main component of detergent insoluble membrane domains and the vast majority of OPCML is distributed to these membrane domains, which was confirmed by membrane fractionation assay. For cholesterol depletion, MβCD was used to extract cholesterol from cell membranes before

the internalisation assay. The result confirmed that cholesterol is crucial for OPCML endocytosis and this supported the finding that OPCML internalises via caveolin-1-mediated pathway as cholesterol is also required for caveolae formation (234).

Once confirming the key molecules for OPCML endocytosis, we proceeded to investigate the post-endocytic trafficking of the internalised OPCML. Firstly, in situ proximity ligation assay (PLA) DUOLink was carried out to see whether this system reflects our earlier findings. Two primary antibodies prepared from different species recognise two distinct target proteins. PLA probes are used as secondary antibodies to recognise the two primary antibodies. Each of these probes is coupled to an oligonucleotide plus (PLA +) or an oligonucleotide minus (PLA −). An additional probe, which is circular, targets PLA+ and PLA− only if the distance between these probes is less than 40 nm. A DNA polymerase will then amplify the circular DNA, which is finally targeted by fluorescent detection probes. As a result, the formation of fluorescent signals is specific to the close association between the two proteins. The strongest proximity signals between OPCML and caveolin-1 were detected during the early endocytic process (at the 5-minute time-point) while the signals between OPCML and clathrin remained at basal levels. The signals between OPCML and EEA1 reached a peak after OPCML trafficking was allowed for 30 minutes, whereas OPCML and LAMP1 produced higher signals over time. This result strengthened our findings and also showed OPCML translocation from caveolin-1 positive vesicles to late endosomes/lysosomes through early endosomes.

In order to identify the molecules that are involved in this OPCML translocation, a pulse-chase experiment was carried out. For this experiment, we used a fluorescently labelled α-OPCML antibody which contains a disulfide bond between the antibody and a

fluorescent dye molecule. This allowed the removal of the signal from the surface OPCML-bound antibody with the treatment of a reducing agent after 5-minute pulse uptake. Then the signals of the internalised OPCML-bound antibodies could remain intact and were monitored during the time-course experiment. This analysis showed sustained levels of co-localisation between Rab5 and OPCML. Rab5-positive early endosomes can be defined as APPL1-positive endosomes and EEA1-positive compartments (18, 521-523). This diversity of early endosomes is known to enable additional regulation of signalling (524). Once sorted in Rab5-positive endosomes, degradative cargo molecules are moved to the late endosomal compartments where the replacement of Rab5 with Rab7 on the endosomal membranes takes place. In this experiment, indeed, the movement of the internalised OPCML between the early endosomes and the late endosomes was observed to be through Rab7. Recycling pathway is another process in which Rab5 is strongly implicated. Other Rabs that are also involved in recycling are Rab4 and Rab11. Sönnichsen et al. suggested that early endosomes and recycling endosomes consist of several Rab domains, including Rab4-, Rab5-, and Rab11-positive domains, whose relative amounts between endosomes differ (530). Altogether, the sustained levels of co-localisation between OPCML and Rab5 could be due to the regulatory roles Rab5 in multiple endosomal processes, including endocytosis, conversion to late endosomes, and recycling.

The recycling of OPCML was further examined by internalisation assays using the biotin-streptavidin system. The endocytosis of the surface proteins after biotinylation (4°C) was allowed at 37°C for 5 minutes, the surface biotin was removed and then the amount of internalised biotinylated proteins was monitored by immunoblotting OPCML at different time-points. The amount of OPCML recycling was calculated by deducting the amounts of

intracellular biotinylated OPCML (the extracellular biotin is removed again at the indicated time-points) from the amounts of total biotin-labelled OPCML (without removal of the surface biotin). The results showed that OPCML recycling was actively occurring especially at the early time-point right after internalisation. The steady degradation of the engulfed OPCML was also observed until over 90% was lost at the last time-point compared to the beginning. This fast OPCML degradation could be due to the intrinsic characteristics of OPCML and/or to the OPCML-overexpressing system, which might cause a massive supply of new OPCML and the fast replacement of pre-existing OPCML. It will be necessary to further characterise how Rab5 regulates the OPCML transport, which molecules are required to control OPCML recycling, and which mechanisms are involved in OPCML degradation (e.g. ubiquitin-proteasome and/or lysosome system).

To expand our knowledge of RTKs, the Gas6-AXL signalling pathway was selected for our study and we designed the PLA DUOLink assay to see whether the distribution and trafficking of this RTK would be modified by the presence of OPCML upon AXL stimulation. The degree of AXL stimulation was assessed by the level of phosphorylation of ERK, a Gas6-AXL downstream signalling molecule. The results showed that the level of phospho-ERK was elevated at 30 minutes after Gas6 treatment and that this level was sustained over time in SKOV3 EMPTY. The stimulation showed the same trend in the beginning in SKOV3 OPCML, but the level of ERK phosphorylation dropped after 60 minutes. After confirming that AXL is activated by Gas6 and its downstream signalling is suppressed by OPCML in SKOV3 OPCML, AXL distribution pattern during stimulation assay was examined by using PLA DUOLink. When SKOV3 EMPTY cells were treated with Gas6, AXL was observed to localise to clathrin-positive membrane domains right after stimulation (t=5 minutes) and then dissociated over

time. However, this signal increase of AXL-clathrin proximity was not seen in SKOV3 OPCML. Conversely, the proximity signal between AXL and caveolin-1 significantly increased in the presence of OPCML, whereas this trend was not detected in SKOV3 EMPTY. This result seems to indicate that Gas6-stimulated AXL might internalise via clathrin-mediated endocytosis in SKOV3 EMPTY cells, but its distribution and trafficking pattern appeared to switch to a caveolin-1-dependent mode in the presence of OPCML. To confirm this finding and test the hypothesis further, it will be primarily required to develop proper detection tools such as fluorescently labelled Gas6 for tracking Gas6-bound stimulated AXL. This would allow visualisation of the actual receptors that are associated with the ligands instead of all the receptors, which might include non-stimulated receptors, blunting the genuine result.

According to data analysis using transcriptomic microarray CSIOVDB dataset (564) of 3431 human ovarian tumour samples, patients of early-stage or low-grade cancer appear to have significantly higher expression levels of OPCML compared to aggressive late-stage or high-grade cancer patients (Figure 24). This information can provide a guide to a future treatment regimen using OPCML-based therapeutics, which will have higher impact on more advanced disease.

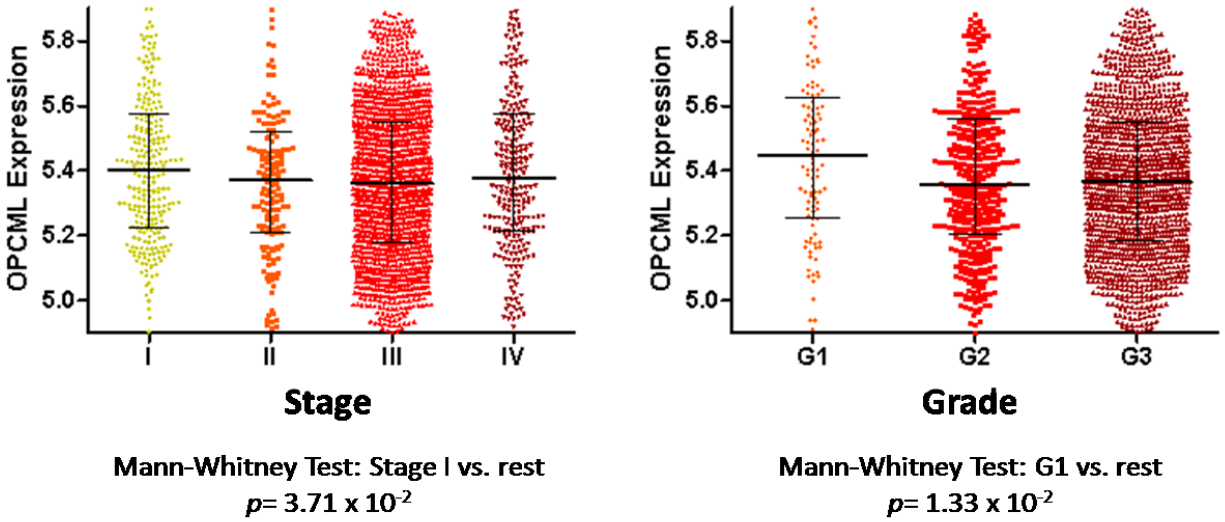


Figure 24. OPCML expression in clinical data. OPCML gene expression patterns depending on cancer stage (left panel) or grade (right panel) in 3431 ovarian tumour samples from CSIOVDB dataset. Early stage (Stage I) or lower grade (G1) shows higher expressions of OPCML compared to the advanced states of disease.

To develop a new anti-cancer therapeutic strategy using OPCML, one of the things to be examined is how stably this GPI-anchored protein would remain present at the plasma membrane, where it is functional as a tumour suppressor. First, it would be ideal to have cell lines that express endogenous OPCML at a detectable level. However, quantitative RT–PCR assay results showed abolition of OPCML expression in 17 ovarian cancer cell lines and 14 non-ovarian cancer cell lines compared to normal ovary expression (493). For this reason, in our study we were obliged to genetically modify cells to overexpress OPCML, but this system would not feasible for clinical applications. Our group has been working on a pathway that upregulates OPCML expression in fallopian tube cell lines, but the increased amount of OPCML could be confirmed mainly at mRNA levels not at protein levels. As a result, microscopic visualisation of the endogenous protein for intracellular trafficking research with the material currently available was not successful. This fundamental change

in the experimental system from the genetically modified to the normal cellular environment would produce more valuable data in terms of therapeutic development.

Secondly, electron microscopy studies will be able to find out more about not only OPCML and RTK intracellular trafficking but also the effects of the presence of OPCML in cells. Basically, it will be possible to check whether the association of OPCML with a target RTK takes place on the cell surface or in intracellular endocytic vesicles. Also, if OPCML and the RTK are internalised together, it is necessary to answer the question whether the RTK is internalised via its own endocytic route or via the caveolin-1-mediated pathway. Möbius et al. has proposed a structural classification that categorises five types of cellular structures, from type 1 structures corresponding to invaginations at the plasma membrane (early endocytic structures) through to type 5 structures corresponding to lysosomes (post endocytic structures) (565). This classification will allow us to track the proteins of interest, determine in which compartments the cargoes are transported to and measure the rate of the movements. In addition, it will be interesting to see any morphological changes of cytosolic vesicles and compartments in the presence of OPCML, which might be also be important in other cellular phenotypes of OPCML-expressing cells.

Thirdly, it is necessary to confirm our findings in other types of cells. Epithelial ovarian cancer shows a high level of heterogeneity and this complex biological diversity has been classified into distinct molecular subtypes based on gene expression profiling (566, 567). Tan et al. has reported that there are five distinct subtypes of epithelial ovarian cancer: epithelial-A (Epi-A), epithelial-B (Epi-B), mesenchymal (Mes), Stem-A, and Stem-B (568). This scheme was developed based on distinct clinicopathological characteristics, deregulated pathways and patient prognosis. This heterogenous molecular background of epithelial

ovarian cancer requires multiple validations of experimental findings. For example, the SKOV3 cells that were used in our study belong to Mes-subtype and they exhibit a sustained phospho-ERK response upon Gas6-AXL activation, whereas another ovarian cancer cell line called PEO1, which is of the Epi-A subtype, shows a transient response to the stimulation (506). Considering this diversity of cellular networks between the subtypes, it will be essential to test at least one cell line from each subtype to see any modifications in the endocytic pathways of RTKs induced by OPCML.

Finally, the intracellular trafficking of recombinant OPCML must be examined. Previously, our group purified a recombinant OPCML consisting of soluble human OPCML domain 1-3, excluding the signal peptide and the GPI anchor, and this was tested on ovarian cancer cells *in vitro* and *in vivo* (520). Although the study concluded that this recombinant OPCML uses the same mechanism of action as the full-length OPCML re-expressed in cells, the mechanism of endocytosis of recombinant OPCML is not understood yet. To calculate the accurate dose in future clinical application, the trafficking pathways of the recombinant protein need to be examined so that the desired amount of OPCML can be available at the plasma membrane, where OPCML functions.

CHAPTER 6. CONCLUSION

This thesis presents the studies of two cancer-implicated proteins, an oncogenic protein, MT1-MMP, and a tumour suppressor protein, OPCML. Breast cancer cells for MT1-MMP and ovarian cancer cells for OPCML were used in each study to investigate how these proteins traffic intracellularly.

From the first study, we discovered that Rab27a plays a central role in MT1-MMP exocytosis, thereby affecting MT1-MMP-dependent breast cancer cell behaviours. Rab27a has been known as a tumour microenvironment regulator by secreting molecules such as growth factors and cytokines in an exosome-mediated or exosome-independent manner (297, 317, 321, 322). However, our *in vitro* data showed that Rab27a promotes the remodelling of the matrix independently of the association with stromal or immune cells. In addition, other results suggest that Rab27a is involved in MT1-MMP recycling pathway not biosynthetic pathway. This work is the first of its kind to describe these novel aspects of Rab27a functioning in breast cancer cells which is distinct from its canonical roles.

In the second study, we examined the possible mechanisms for the OPCML endocytosis in ovarian cancer cells and found crucial factors, including caveolin-1, dynamin, and cholesterol, for the internalisation process. Also, our findings expanded our understanding in the post-endocytic intracellular trafficking of OPCML which was not dealt with in the previous study (520). The importance of the role of OPCML in AXL redistribution on the plasma membrane was highlighted by Antony et al. (504). Although further investigation is required, the result of our AXL stimulation assay using PLA DUOLink suggests that the role of OPCML in the RTK regulation might not be limited to the repositioning the RTKs on the cell surface but might

be involved in redirecting the AXL endocytic pathways and the following intracellular transport.

In line with these findings, future work will focus on mapping the exact mechanisms of how MT1-MMP is secreted by Rab27a with other molecules and how OPCML modifies the distribution and the trafficking of RTKs. Considering the negative results of the clinical trial with the MT1-MMP inhibitor, addition of an alternative molecule to be therapeutically targeted will allow us to have a more systemic control over MT1-MMP function reducing the side-effects that can be caused by direct targeting. Also, development of a therapeutic that mimics OPCML structure and functions will be able to diversify the methods of RTK inhibition and therefore it will be useful to prevent the development of resistance against inhibitors.

CHAPTER 7. REFERENCES

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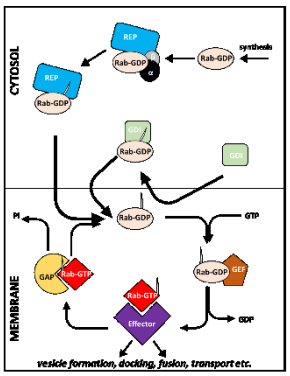
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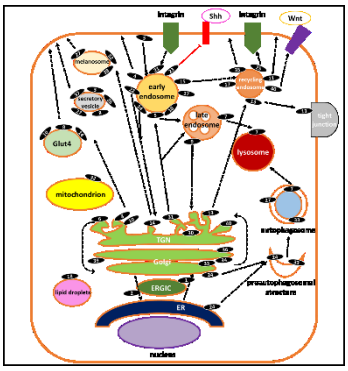
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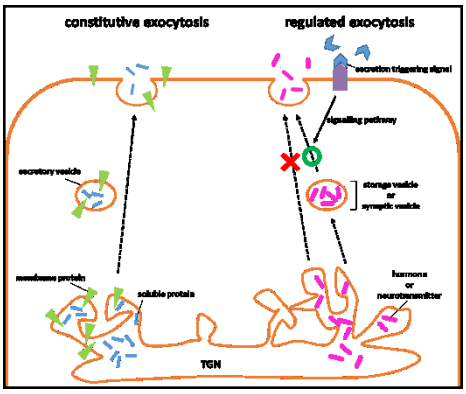
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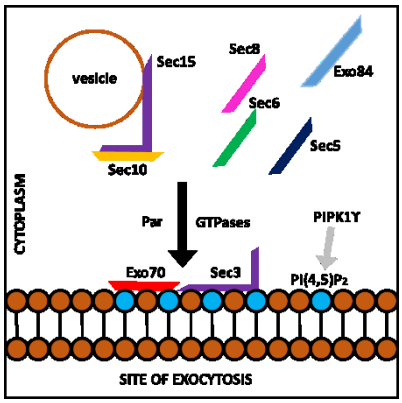
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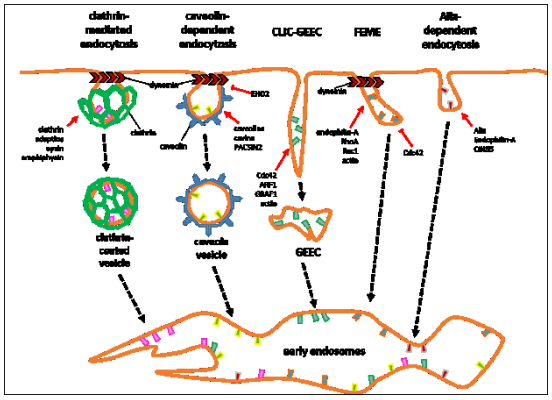
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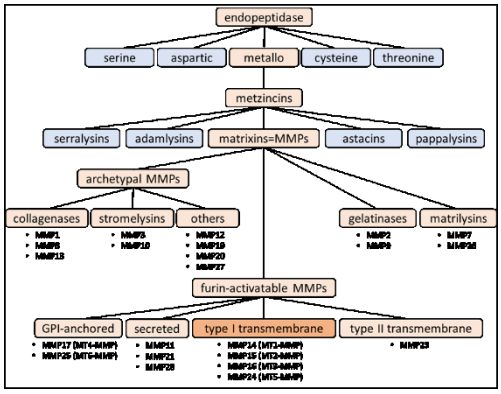
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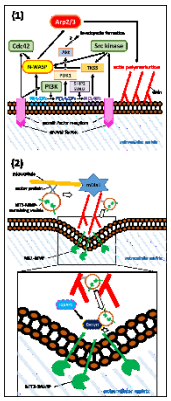
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