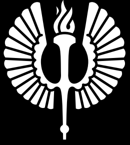

UNIVERSITY OF TURKU

NEW INSIGHTS INTO RECEPTOR TRAFFICKING IN HUMAN CANCER

Pranshu Sahgal

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University of Turku

Faculty of Medicine
Cell Biology and Anatomy
Turku Doctoral Programme of Molecular Medicine – TuDMM
Turku Centre for Biotechnology
Turku, Finland

Supervised by

Johanna Ivaska, Ph.D.
Academy Professor
Professor of Molecular Cell Biology
Cell Adhesion and Cancer Laboratory
Turku Centre for Biotechnology
University of Turku, Turku
Finland

Reviewed by

Dr. Stéphanie Miserey-lenkei, Ph.D.
Institut Curie, Paris
France

Prof. Stefan Veltel, Ph.D.
Hochschule Bremen, Bremen
Germany

Opponent

Guido Serini, M.D. Ph.D.
Assistant Professor of Clinical Biochemistry and Molecular Biology
Head, Laboratory of Cell Adhesion Dynamics
Candiolo Cancer Institute – FPO, IRCCS and
Department of Oncology
University of Torino School of Medicine, Torino
Italy

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin Originality Check service.

Front cover by Emma Kivi
ISBN 978-951-29-7472-6 (PRINT)
ISBN 978-951-29-7473-3 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Grano Oy - Turku, Finland 2018

Con el tiempo todo se consigue

To my Family

Pranshu Sahgal
New insights into receptor trafficking in human cancer

University of Turku, Faculty of Medicine, Cell Biology and Anatomy, Turku Doctoral Programme of Molecular Medicine-TuDMM, Turku Centre for Biotechnology, Turku, Finland

ABSTRACT

Plasma membrane receptors play an essential role in cancer by regulating cancer-related processes such as cell proliferation and migration in response to cues from the extracellular ligands. Integrins are cell-matrix adhesion receptors which connect the exterior of the cells to the interior facilitating adhesion and regulating many aspects of cell biology including proliferation and survival signaling. Receptor tyrosine kinases (RTKs) are cell surface receptors responding to soluble growth-factors to activate cellular signaling pathways that can be distinct or overlapping with integrins. The extent and strength of signaling by both receptor types depends on the time they engage themselves on the plasma membrane before being endocytosed inside the cell. The endocytosed receptors can either be degraded or recycle back to the plasma membrane. Therefore, the trafficking of receptors is a decisive factor for maintaining the proper cellular functions. Any dysregulation in ther trafficking may contribute to pathological conditions such as cancer. In this thesis, the role of new proteins in regulating the trafficking of integrins and two RTKs, MET and HER2 in human cancer was explored.

To investigate integrin traffic, we employed high throughput RNAi screening and proximity-dependent biotinylation (BioID) to find new regulators of integrin trafficking. These studies allowed me to establish a critical role for Golgi-localized, gamma adaptin ear-containing, ARF-binding (GGA2) and small GTPases RAB13 in promoting recycling of active but not inactive β1-integrin to the plasma membrane to facilitate breast carcinoma cell migration and *in-vivo* invasion. Furthermore, I also established the role of a related adaptor, GGA3, in promoting recycling of total pool of β1-integrin to the plasma membrane.

In addition, I made important discoveries about oncogenic RTK traffic in cancer. A yeast two-hybrid screen identified TNS4 as the direct interactor of MET oncogene. This interaction coupled MET to β1-integrin and stabilized active MET on the plasma membrane thus promoting its oncogenic signaling. We also identified a novel regulator of HER2 traffic in breast cancer. Sortilin related receptor 1, a sorting protein previously implicated in Alzheimer's disease and obesity, was found to be a key regulator of HER2 oncogenic fitness in breast and bladder cancer cells. My results show that SORLA supports HER2 stability and signaling through supporting HER2 recycling to the plasma membrane.

In summary, in this thesis, the new functions of GGA2, GGA3, MET and SORLA have been demonstrated in regulating trafficking of different plasma membranes receptors, therefore, influencing signaling and various cancer-related processes linked with these receptors in different cancel models.

Keywords: Integrin, RTK, Integrin traffic, Receptor traffic, GGA2, MET, TNS4, HER2, SORLA

Pranshu Sahgal
Uusia havaintoja reseptoriliikennöinnistä ihmisen syövässä

Turun yliopisto, Lääketieteellinen tiedekunta, Solubiologia ja anatomia, Molekyylilääketieteen tohtoriohjelma-TuDMM, Turun biotekniikan keskus, Turku, Suomi

TIIVISTELMÄ

Solukalvon reseptoreilla on tärkeä rooli syövissä. Ne säätelevät syövän kannalta tärkeitä prosesseja, kuten solujakautumista ja soluliikettä. Integriinit ovat solun pinnan tarttumisreseptoreja, jotka liittävät solunväliaineen ja solun ulkoisen ympäristön solun tukirankaan. Integriinit säätelevät solujen tarttumista ympäristöönsä ja monia solun toimintoja, kuten soluliikettä ja solujen eloonjäämistä. Solun pinnan reseptorityrosiinikinaasit (RTK) ovat reseptoreja, jotka reagoivat solun ulkoisiin kasvutekijöihin ja välittävät kasvua lisääviä signaaleja solun tumaan. Monet reseptorityrosiinikinaasien käynnistämistä solunsisäisistä viestintäpoluista risteävät integriinireseptoreiden viestipolkujen kanssa. Reseptorien signalointiaktiivisuus on riippuvainen reseptorien sijainnista solukalvolla. Reseptoreja otetaan solun sisään endosytoosilla ja ne joko palautuvat solukalvoille kierrätyksen kautta tai ne kohdennetaan hajotukseen solun lysosomeissa. Näin ollen endosytoosin ja kierrätyksen tasapaino säätelee reseptorien toimintaa soluissa. Tämän väitöskirjan tarkoituksena on ollut tutkia integriinien ja kahden RTKn, MET ja HER2, liikennöintiä syöpäsoluissa ja niiden roolia syöpäbiologiassa.

Integriinien liikennöintiä soluissa tutkittiin käyttämällä RNAi seulontaa ja läheisyys-biotinylaatioon perustuvaa menetelmää. Yhdessä nämä paljastivat, että sovitinproteiini GGA2 and pieni GTPaasi RAB13 muodostavat yhdessä proteiinikompleksin, joka tarvitaan aktiivisten integriinien tehokkaaseen kierrätyksen takaisin solukalvolle. Näin ollen tämä proteiinikompleksi lisää solujen liikkumista ja syöpäsolujen invaasiota *in vivo*. Myös GGA2:n proteiinin sukulaisproteiinin GGA3:n havaittiin säätelevän integriinien kierrätystä rintasyöpäsoluissa ja lisäävän integriinien määrää soluissa.

Tässä väitöskirjassa havaittiin myös, että reseptoriliikenne on tärkeä säätelijä syövän kasvua lisäävien MET- ja HER2-reseptorien toiminnalle syöpäsoluissa. MET sitoutuu aktiivisuusriippuvaisesti Tensiini-4 proteiiniin ja tämä edesauttaa MET-reseptorin stabilisuutta suojaten sitä lysosomihajoitukselta. Toisaalta, havaittiin myös, että SORLA-niminen proteiini säätelee HER2-reseptorin liikennettä ja on välttämätön HER2:n syövän kasvua lisäävälle signaloinnille. Yhteenvetona voidaan todeta, että tässä väitöskirjassa kuvataan uusia toimintoja GGA2, GGA3, MET ja SORLA proteiineille syövässä. Erityisesti väitöskirjassa kuvataan syöpäsoluille keskeisiä reseptorikierrätysmekanismeja eri syöpämalleissa.

Avainsanat: integriini, reseptorityrosiinikinaasi (RTK), integriiniliikennöinti, reseptoriliikennöinti, GGA2, MET, TNS4, HER2, SORLA

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ABBREVIATIONS

AC-LL/ DXXLL	Dileucine acidic motif
ADAM	A disintegrin and metalloproteinase
AMPK	AMP-activated protein kinase
AP-1/2/3	Adaptor Protein-1/2/3
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ARF1/3/6	ADP ribosylation factor 1/3/6
C16orf62	Chromosome 16 Open Reading Frame 62
CAD	Cationic amphiphilic drugs
C-Cbl	Cbl Proto-Oncogene
CCP	Clathrin-coated pit
Cdc37	Cell Division Cycle 37
CDE	Clathrin-dependent endocytosis
CDR	Circular dorsal ruffles
CIE	Clathrin-independent endocytosis
CLIC	Clathrin-independent carriers
DFGXϕ	Asp-Phe-Gly-any residue-hydrophobic residue
Dok-1	Docking Protein 1
DSCR3	Down syndrome critical region gene 3
ECD+CD	Extracellular domain+cytoplasmic domain
ECM	Extracellular matrix
EEA1	Early Endosome Antigen 1
EGF	Epidermal growth factor
EPS15	Epidermal growth factor receptor substrate 15
ERK	Extracellular signal-regulated kinase
FAK	Focal Adhesion Kinase
FCHo1/2	Fer/Cip4 homology domain-only proteins 1 and 2
FERM	F for 4.1 protein, E for ezrin, R for radixin and M for moesin
GAE	Gamma-adaptin ear
GAP	GTPase activating protein
GAT	GGA and Tom1
GEF	Guanine nucleotide exchange factor
GFFKR	(Gly-Phe-Phe-Lys-Arg)
GFP	Green fluorescent protein
Grb2	Growth factor receptor-bound protein 2
HER2/3/4	Human epidermal growth factor receptor 2/3/4
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
Hsp90	Heat shock protein 90
ICAP-1	Integrin Cytoplasmic domain-Associated Protein-1
IGFR2	Insulin-like growth factor 2
ILK	Integrin-Linked Kinase

IP	Immunoprecipitation
JAK/STAT	Janus kinase/ signal transducer and activator of transcription
Lamp1/2	Lysosomal-Associated Membrane Protein 1/2
LDL	Low-density lipoprotein
LDL-R	Low-density lipoprotein receptor
LFA-1	Lymphocyte function-associated antigen 1
LRP-1	LDL Receptor-Related Protein 1
M6PR	Mannose-6-Phosphate Receptor
MAPK	Mitogen-activated protein kinase
MDC	Mono-dansyl-cadaverin
MET	Mesenchymal-epithelial transition factor
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NPxY	Asn-Pro-X-Tyr
PACS1	Phosphofurin acidic cluster sorting protein 1
PDGF	Platelet-derived growth factor
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphoinositide 3-kinase
PKCα/ε	Protein kinase C α/ε
PKD1	Polycystic Kidney Disease 1
PNRC	Perinuclear recycling compartment
PSM	Peptide spectral matches
PTB	Phosphotyrosine-binding
RAB4/5/6/7/10/11/13/25	Ras-related protein Rab 4/5/6/7/10/11/13/25
RASA1	RAS P21 Protein Activator 1
RGD	Arginine-Glycine-Aspartic acid motif
RIAM	Rap1-GTP-interacting adaptor molecule
Rin2	Ras and Rab interactor 2
RTK	Receptor tyrosine kinase
SH2	Src homology region 2
Shank1/2/3	SH3 And Multiple Ankyrin Repeat Domains 1/2/3
SNX17	Sorting Nexin 17
SORL1/ SORLA	Sortilin related receptor 1
STAT3	Signal transducer and activator of transcription 3
TBD	Tyrosine binding domain
T-DM1	Ado-trastuzumab emtansine (Kadcyla^{TM})
TfnR	Transferrin receptors
TGF	Transforming growth factor
TGN	Trans Golgi network
TM+CD	Transmembrane domain+cytoplasmic domain
TNS1/2/3/4	Tensin 1/2/3/4
VHS	Vps27, Hrs, and STAM
VPS29/35	Vacuolar protein sorting-associated protein 29/35

LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerical:

I. GGA2 and RAB13 regulate activity-dependent β1-integrin recycling.
Sahgal P, Alanko J, Paatero I, Arjonen A, Pietilä M, Rokka A, Ivaska J.
bioRxiv 353086

II. Regulation of cell migration and β1-integrin trafficking by the endosomal adaptor GGA3.
Ratcliffe CD*, Sahgal P*, Parachoniak CA, Ivaska J, Park M.
Traffic. 2016 Jun;17(6):670-88

III. SORLA-driven endosomal trafficking regulates the oncogenic fitness of HER2.
Pietilä M*, Sahgal P*, Peuhu E, Jäntti N, Paatero I, Andersen OM, Padzik A, Blomqvist M, Saarinen I, Boström P, Taimen P and Ivaska J.
bioRxiv 299586

IV. Tensin-4-dependent MET stabilization is essential for survival and proliferation in carcinoma cells.
Muharram G, Sahgal P, Korpela T, De Franceschi N, Kaukonen R, Clark K, Tulasne D, Carpén O, Ivaska J.
Dev Cell. 2014 May 27;29(4):421-36

* Equal contribution

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1. INTRODUCTION

Unlike unicellular prokaryotes like bacteria, eukaryotes such as human beings and all higher order animals are composed of membrane-bound millions of complex cells. These cells house a variety of structures called organelles. Organelles are distinct both functionally and structurally. Organelles coordinate with each other to keep a balance between various cellular activities hence maintaining cellular homeostasis and optimal cellular function. The nucleus is the most crucial organelle of a cell. It contains the heritable DNA in a highly condensed form called as chromosome. Humans have 23 pairs of the chromosome and each chromosome has unique sequences of DNA called as genes. Genes are responsible for making the proteins. These proteins control every single function in an organism. Proteins have different structures and functions and can even interact with each other to alter their function. Any abnormal change in the genes or proteins can eventually lead to the diseased state including cancer.

One such protein category is ‘plasma membrane receptors’. They reside on the plasma membrane of the cell and are responsible for establishing either physical contact or just mediating the exchange of information between a cell and its surrounding or among cells. This characteristic of plasma membrane receptors makes them control every aspect of a cell’s life. Deregulation in the expression or function of these receptors can change a normal cell to cancer cell by altering its properties, e.g. attachment to the extracellular substrates, spreading, migration or invasion potential to far reached places in the body where it should typically not supposed to go. The article “Hallmarks of cancer: the next generation” by (Hanahan, Weinberg 2011) showcases such properties of a cell which deem it cancerous.

Integrins are plasma membrane receptors which establish both the physical contact (adhesion) and the communication (signaling) between cells and also between cells and extracellular habitat. On the contrary, plasma membrane receptors called receptor tyrosine kinases (RTKs) usually are involved only in signaling. Both integrins and RTKs undergo dynamic spatiotemporal regulation and shuttle between plasma membrane and interior of the cell. This shuttling known as receptor trafficking determines the stability, expression and activity of these receptors hence the way they control cellular processes. A plethora of other proteins (regulators) interact directly or indirectly with plasma membrane receptors and regulate the receptor trafficking. Therefore, any deregulation in this trafficking can lead to an abnormal cellular function which can lead to cancer.

In this thesis, the new mechanisms and novel regulators for trafficking of integrins and RTks were explored, along with evaluating the functional consequences of their deregulated trafficking on cancer-related processes.

2. REVIEW OF THE LITERATURE

2.1 Plasma membrane and receptors

Cell is a fundamental unit of life. Higher living organisms consist of millions of cells. A cell comprises of inner mass called cytoplasm which harbors many organelles and an outer lipid bilayer called plasma membrane. Plasma membrane acts as an interface between the interior and exterior of the cell and houses the proteins called “plasma membrane receptors” (Alberts 2008). These receptors are embedded in the plasma membrane and can be either peripheral or extend all across the plasma membrane. The receptors which extend all across the plasma membrane are called as transmembrane receptors and consist of the outer extracellular domain, a transmembrane domain which traverses the lipid bilayer and the inner cytoplasmic domain. These transmembrane receptors are implicated in many cellular functions which ultimately decide the fate of the cell. Dysregulation in the function or in the structural integrity of transmembrane receptors often leads to the diseased state and linked with classical “hallmarks of cancer” (Blazek, Paleo et al. 2015, Hanahan, Weinberg 2011).

There have been numerous transmembrane receptors discovered so far. They have different modes of operation leading to their distinct function and outcome for the cell. For example, Receptor tyrosine kinase (RTKs) are such receptors which are activated by a small extracellular ligand which causes phosphorylation and structural changes in the cytoplasmic domain of the receptor and eventually recruitment of intracellular signaling proteins. Two major types of RTKs are c-MET or HGFR whose ligand is HGF; and ERBB family consisting of EFGR, HER2, HER3 and HER4. The ligand for EGFR is EGF. The second example of the transmembrane receptor is cell-matrix adhesion receptors, integrins. Integrins usually bind matrix associated ligands like collagen and fibronectin but can also recognize cell-surface counter receptors and soluble ligands. The third example of the transmembrane receptor is low-density lipoprotein receptor (LDL-R) whose ligand is LDL. LDL-R orchestrates cholesterol homeostasis (Yeagle 2016, Watson, Arey et al. 2014).

All the three receptors mentioned above are dynamic and undergo constant endocytosis and recycling back to the plasma membrane. The extent of time receptors engage themselves before being endocytosed determine the strength of the signaling linked with them hence cellular processes. Moreover, there are reports now that some of the plasma membrane receptors can even signal from the endosomes. Also, these receptors can also cross-talk with each other. In this thesis, new insights of trafficking of these receptors and their implication on human cancer have been studied hence opening new doors for a better understanding of these receptors and new drug discovery and development opportunities (Lemmon, Schlessinger 2010, Wiley, Burke 2001).

2.2 Integrins

2.2.1 Integrin superfamily

Integrins are heterodimeric transmembrane glycoprotein adhesion receptors which connect the exterior of the cell to the inner cytoplasm and are evolutionarily conserved in mammals. Integrins consist of 18α and 8β subunits resulting in 24 possible heterodimers. They are present in almost all cell types except blood cells in vertebrates (Hynes 2002a). Integrins are divided into four families based on the ligand they bind: collagen, laminin, RGD (Arginine-Glycine-Aspartic acid motif) or leukocyte restricted heterodimers (Figure 1). Integrins comprise of large globular N-terminal which interacts with the ECM ligand, a transmembrane domain and a much shorter cytoplasmic C-terminal tail. The tail consists of various overlapping and non-overlapping binding sites for proteins responsible for integrins association with the cytoskeleton, their trafficking and signal transduction (Luo, Springer 2006, Campbell, Humphries 2011a). Integrins are implicated in all major cellular functions like adhesion, survival motility, polarity and cytoskeleton reorganization. Role of integrins in various human maladies including cancer is also identified, but the precise mechanisms how integrins and its interactors affect cancer and cancer-related processes are still to be well elucidated (Hamidi, Ivaska 2018, Xiong, Balcioglu et al. 2013).

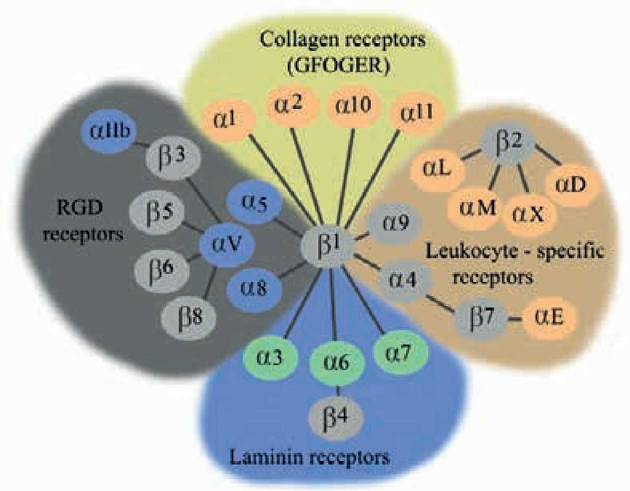

Figure 1: Schematics of integrin family in vertebrates representing different integrin subunits and their ligands. Modified from (Hynes 2002b).

2.2.2 Integrin Activity

The activation and inactivation of integrins govern their function. Unlike other adhesion receptors, e.g., cadherins, integrins are bidirectional signaling

molecules. Vast structural changes, especially in the extracellular domain of integrins, determine their activity. During outside-in signaling, the ligand binds the extracellular domain causing the conformational changes and separation of the cytoplasmic tails leading to the binding of the regulatory proteins to the tail. Tail separation upon ligand binding involves disruption of a salt bridge which keeps the α and β cytoplasmic tails together. Moreover, ligand binding also incurs the integrin heterodimers to cluster and form the multimeric complexes. Contrarily, during the inside-out signaling, binding of the activator molecules to the cytoplasmic tail, e.g., talin and kindlin causes the structural changes and increased affinity of the extracellular domain for the ligands (Gough, Goult 2018, Shattil, Kim et al. 2010).

Switch-blade model of integrin activation is widely accepted. According to this model, integrins exist in three conformations: 1) Bent conformation- the ligand binding domain is closed and bent towards the plasma membrane having low affinity and room for ligand binding. 2) Primed conformation- the ligand binding domain is still closed but extended and having an intermediate affinity for ligand binding. 3) Extended conformation- the ligand binding domain is open ligand-bound (Campbell, Humphries 2011b, Luo, Carman et al. 2007) (Figure 2). It has been recently reported that between the low affinity inactive and high-affinity active conformation, six states of intermediate integrin conformations may exist (Zhu, J., Zhu et al. 2013).

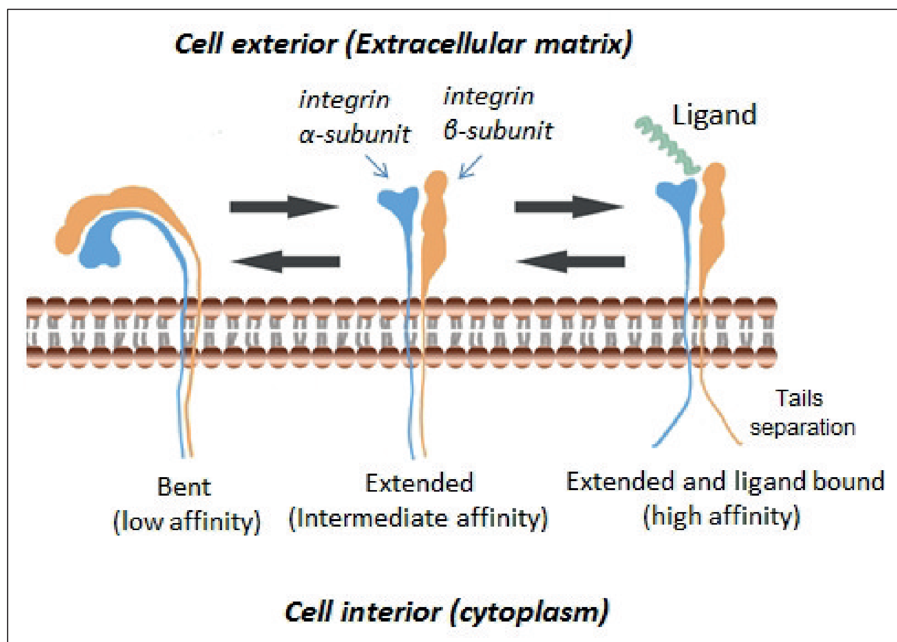

Figure 2: Representation of switchblade model of integrin activation.

The cytoplasmic tail of β-integrin consists of two conserved Asn-Pro-X-Tyr (NPxY) motifs. These motifs interact directly with the proteins contain the Phosphotyrosine-binding (PTB) domain, eg. talin (1/2) and kindlin (1/2/3) are two activators of integrins which bind the NPxY motif through their FERM (F for 4.1 protein, E for ezrin, R for radixin and M for moesin) domain. The proximal NPxY motif binds talin and distal NPxY motif binds kindlin together with talin for integrin activation (Ye, Snider et al. 2014). Apart from the activators, there are also inactivating proteins known to bind not only β tail but also α tail. Inactivators like Dok-1, ICAP-1 and Filamins (A/B/C) bind the NPxY motif of β tail and prevents the binding of the activators (Bouvard, Pouwels et al. 2013). On the other hand, inactivator Sharpin binds to the GFFKR (*Gly*-Phe-Phe-Lys-Arg) motif in the α tail and results in steric hindrance for the binding of activators to the β tail (Rantala, Pouwels et al. 2011). Sometimes integrin activity can also be influenced by regulators which may not directly bind integrin tail, e.g., Shank family of scaffold proteins bind Rap1 and reduces its availability for binding RIAM a key protein involved in recruiting talin to the plasma membrane. Rap1 is a small GTPases which activate integrin through talin binding (Zhu, L., Yang et al. 2017, Lilja, Zacharchenko et al. 2017). In many pathological conditions, the activity of these activators and inactivators has been compromised such as skin disorders (Winograd-Katz, Fassler et al. 2014a, Hegde, Raghavan 2013). In cancer, elevated integrin activity is linked with increased cell migration and proliferation which is the result of either high expression of integrin activators or low expression of integrin inactivators (Rantala, Pouwels et al. 2011, Calderwood, Campbell et al. 2013, Jin, Tien et al. 2015).

2.2.3 Focal Adhesion

As the name suggests, focal adhesions help the cells to adhere to the substrate. Cell adhesion is necessary for cell growth, survival and migration. Focal adhesions are dynamic in nature and integrin activation and clustering are primarily responsible for their formation, function and disassembly (Jin, Tien et al. 2015). Focal adhesions connect the cytoskeleton of the cell with the extracellular matrix and in this way also responsible for mechanosensing and mechanotransduction (Wolfenson, Bershadsky et al. 2011). Nascent adhesions are formed at the contact site of active integrins and ECM at the cell's protruding edge. Nascent adhesions either disassemble or mature in focal complexes with the engagement of more active integrins. Focal complexes grow in size to form focal adhesions (Zaidel-Bar, Itzkovitz et al. 2007). Focal adhesions also serve as the site for signal transduction and cytoskeleton organization, but integrins lack any enzymatic or catalytic activity by themselves. Hence, the recruitment of regulatory proteins along with scaffold and adaptor proteins to the cytoplasmic tail of integrins is necessary (Burridge 2017). Cancer cells often display remarkably transformed focal adhesions dynamics (Maziveyi, Alahari 2017).

Focal adhesions consist of hundreds of proteins. The function of some of them is still not known (Winograd-Katz, Fassler et al. 2014b). Proteins like paxillin and vinculin are present at the adhesion sites from the beginning of their formation. Vinculin is an essential component of focal adhesions and links integrin bound talin with the actin. Later during focal complexes maturation, new proteins get recruited. Some of these are signaling proteins like Integrin-Linked Kinase (ILK) and Focal Adhesion Kinase (FAK); and others are scaffolding proteins like α-actinin and Tensins. Alpha-actinin reinforces the integrin-cytoskeleton interface (Bays, DeMali 2017, Chen, Alonso et al. 2003).

Tensins (1-3) crosslink integrin with the actin cytoskeleton. Through their PTB domain, tensins bind the NPxY motif in β-integrin and interact with tyrosine phosphorylated proteins such as Src, FAK and PI3K through their SH2 domain. Tensins play a role in cellular growth, adhesion, migration and RhoGTPase signaling (Blangy 2017). Recently, AMP-activated protein kinase (AMPK), an important energy-sensing enzyme, was shown to suppress expression of tensin1 and tensin3 expression resulting in reduced β1-integrin activity (Georgiadou, Lilja et al. 2017). Tensin4, also known as Cten does not have N-terminal domain unlike other tensins for binding actin (Figure 3) and does not activate b1-integrin similarly to tensin-1 and tensin-3. Tensin4 has been emerging as a promising biomarker for cancer and its high expression is observed in lung, breast, ovarian and gastric cancers (Lo 2014a, Sakashita, Mimori et al. 2008, Chan, Chiu et al. 2015). Our new mechanistic finding on the role of tensin4 in cancer is described in results and discussions.

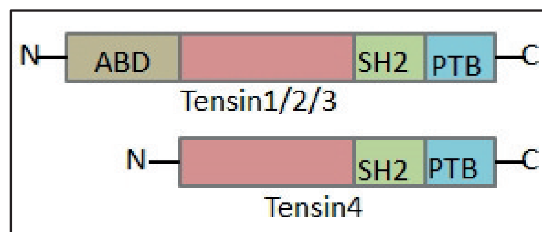

Figure 3: The Structural difference between Tensin 4 and Tensin1/2/3. Tensin4 lacks N-terminal actin-binding domain (ABD).

2.3 Integrin Trafficking

The expression level and activity of the plasma membrane receptors including integrins determine their function. Receptors undergo endocytosis from the cell surface hence reducing their expression on the cell. Integrin endocytosis was first proposed in 1989 (Bretscher 1989). Integrin endocytosis is crucial for the disassembly of focal adhesions and downregulation of integrin-mediated signaling. Once endocytosed, integrins go either to the lysosomes for degradation or recycle back to the plasma membrane where they form new adhesion sites. This process of endocytosis and recycling is very dynamic and

known as integrin trafficking. Hence integrin trafficking is a vital phenomenon for proper cell migration (De Franceschi, Hamidi et al. 2015, Valdembri, Serini 2012, Chao, Kunz 2009).

Protein Kinase C family plays an important role in integrin trafficking by phosphorylating different substrates. While PKCα induces integrin endocytosis, PKCε supports b1-integrin recycling (Wang, Y., Arjonen et al. 2015, Ivaska, Vuoriluoto et al. 2005). Deregulation of integrin traffic is involved in impaired cell migration and cancer-related processes which are discussed later. How integrin endocytosis is different for the active and inactive integrins is not well known apart from the fact that the net endocytosis rate of the active β1-integrin is more than that of inactive β1 integrin (Arjonen, Alanko et al. 2012a).

2.3.1 Brief introduction to Golgi, endosomes and endosomal trafficking

Golgi, Golgi body or Golgi apparatus is an important cellular organelle in eukaryotes and forms the component of the endomembrane system and is responsible for the processing and secretion of the proteins among its diverse cellular functions. Mammals on the one hand have of a single Golgi near nucleus while yeast has several Golgi dispersed across cytoplasm. Golgi consists of several flattened disc-like structures known as cisternae which are stacked together. The cisternae closest to the nucleus/endoplasmic reticulum (ER) represent the first or cis face of Golgi (forms Cis Golgi Network or CGN), and the one away from nucleus represent the last or Trans face of Golgi (forms Trans Golgi Network or TGN) (Boncompain, Perez 2013, Day, Staehelin et al. 2013). The newly synthesized proteins from ER enter the Golgi through its cis face and exist through its trans face. Post-translation modifications of the proteins by different enzymes occur majorly at the Golgi (Potelle, Klein et al. 2015) e.g. the β1-integrin partially get glycosylated in ER and termed as immature β1-integrin, which then moves to Golgi where it further gets glycosylated and termed as mature β1-integrin (Bellis 2004, Akiyama, Yamada et al. 1989).

Endosomes are also an important component of the endomembrane transport system and represent the membrane-bound vesicular structures with a size of 50-500 nm. Endosomes are responsible for sorting and transport of different proteins including the membrane receptors like integrins and RTKs between different compartments in the cell (Chi, Harrison et al. 2015, Scott, C. C., Vacca et al. 2014). Phosphatidylinositol phosphates (PIPs) and small GTPases RABs give these endosomes their varied functional identity (van Meer, Voelker et al. 2008, Stenmark 2009).

There are three major intracellular pathways mediated by different endosomes:

1.) Bi-directional transport between TGN and cytoplasm. Movement of endosomes with their cargo from TGN is called anterograde transport and towards TGN is called retrograde transport (Doray, Ghosh et al. 2002, Chia, Gleeson 2011).
2.) Bi-directional transport between plasma membrane and cytoplasm. From plasma membrane by endocytosis, early endosomes transport the cargo to cytoplasm and from cytoplasm by recycling, recycling endosomes transport the cargo back to the plasma membrane (Goldenring 2015, Grant, Donaldson 2009).
3.) Uni-direction transport of cargo from late endosomes to lysosomes where the cargo gets degraded (Ng, Gan et al. 2012).

2.3.2 Small GTPases RAB and ARF play a role in receptor trafficking

RABs and ARFs are the members of Ras superfamily of small GTPases. In mammals, about 70 RAB and 6 ARF proteins are involved in various steps of endocytic traffic including vesicle formation, maturation, docking and undocking. RABs and ARFs also influence lipid metabolism and cytoskeleton structure. They oscillate between the GTP-bound active state and GDP-bound inactive state. Guanine nucleotide factors (GEFs) catalyze the exchange of GDP to GTP. On the other hand, the co-enzymatic activity of GTPase activating proteins (GAPs) assist the small GTPases for the hydrolysis of GTP to GDP (Vetter, Wittinghofer 2001). While RABs function by recruiting the effector proteins, e.g. Rabaptin by RAB5; ARFs recruit mainly coat proteins, e.g. AP-2 by ARF6. Effector protein for small GTPases preferentially binds the GTP-form of the GTPase (and that it is not a GAP/GEF). Notably, coat proteins might also be effector proteins for the ARFs. These small GTPases act as the identity tags for the endosomes and work in close association with each other (Kjos, Vestre et al. 2018, Langemeyer, Frohlich et al. 2018). RAB5, for example, is implicated in early endosome formation/function but the exchange of RAB5 on endosomes to RAB7 achieved by VPS/HOPS complex (GEF for RAB7), predestine the integrins loaded endosomes for degradation (Rink, Ghigo et al. 2005). Ras and Rab5 interacting protein RIN2 is an effector protein of RAB5 which associates with RAB5 and R-Ras in the focal adhesions of endothelial cells and promote active β1-integrin endocytosis and Rac signaling (Sandri, Caccavari et al. 2012).

Owing to the essential functions of RABs and ARFs for the cell, deregulation in their expression or activity is linked with many pathophysiological conditions including cancer (Tzeng, Wang 2016, Zhen, Stenmark 2015). In the forthcoming topics, their important roles will be discussed including new functions into membrane trafficking in results and discussion section.

2.3.3 Routes of integrin endocytosis

Integrins can be internalized by all four known routes of internalization described below (Figure 4) and the specific route employed by the cell is very

context dependent and can also be specific of certain integrin heterodimers. The selectivity of the cell to choose one of these routes remains incompletely understood and is dependent on different factors, e.g. the cell type, cell-cycle stage, cell to cell interaction, extracellular environment, receptors crosstalk, etc (Maritzen, Schachtner et al. 2015, Margadant, Monsuur et al. 2011). These pathways are briefly discussed below.

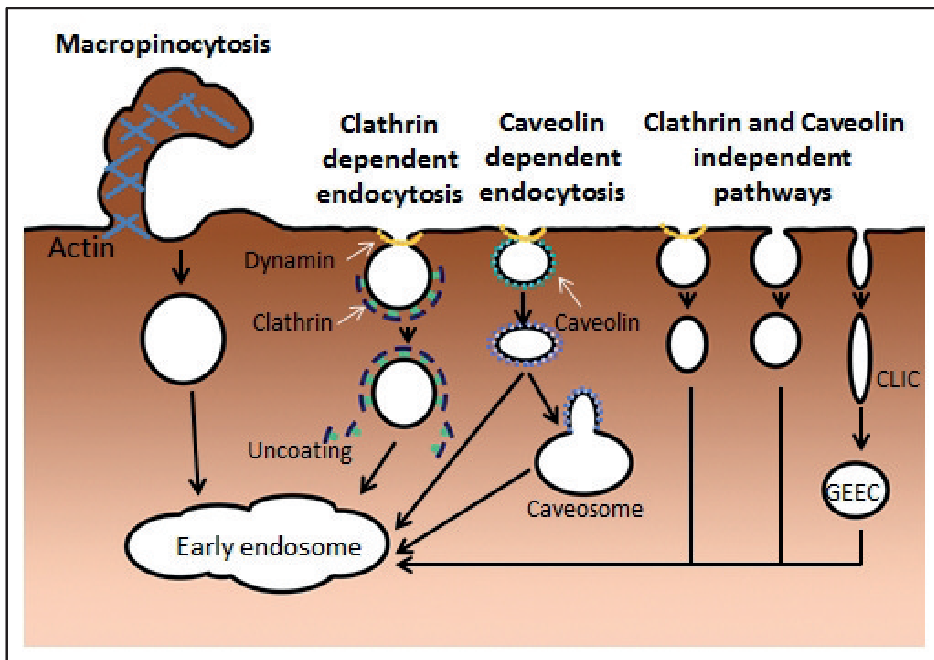

Figure 4: Representation of different routes of receptor/integrin endocytosis. Modified from (Mayor, Pagano 2007a).

Clathrin-dependent endocytosis (CDE)

Receptors for endocytosis are sorted at the plasma membrane by clathrin-coated pits (CCP). Clathrin-coated pits are formed by a coat protein called clathrin. Three heavy clathrin chains and three clathrin light chains form together a triskelion. These triskelia assemble to form a pentagonal 3D lattice which causes the pulling of the plasma membrane and formation of invagination engulfing the endocytosed cargo. The nucleation step necessary for CCP formation initiates when lipid PI(4,5)P2 on the plasma membrane recruits the nucleation protein F-BAR domain-containing Fer/Cip4 homology domain-only proteins 1 and 2 (FCHo1/2) and scaffold proteins EPS15 and intersectin (Maib, Smythe et al. 2017, Simonsen, Wurmser et al. 2001). This complex further engages the adaptor protein AP-2. AP-2 with the help of other specific adaptors like Numb and Dab2 employ the receptors destined for internalization. Finally, the CCP pinches off by the GTPase dynamin which hydrolyzes GTP at the neck.

Clathrin, in the end, dissociates from free clathrin-coated vesicles called endosomes. Clathrin then moves to the new site at the plasma membrane for invagination formation (Lampe, Vassilopoulos et al. 2016, de Leon, Valdivieso 2016). Numb and Dab2 contain the PTB domain and interact directly with the NPxY motif of the β-integrin cytoplasmic tail. Knockdown of Numb and Dab2 hampers the β1-integrin endocytosis and cancer cell migration (Calderwood, Fujioka et al. 2003).

Clathrin-independent endocytosis (CIE)
Caveolae are the pear-shaped invaginations about 50-100 nm in diameter rich in lipids. They are composed of oligomeric caveolin-1 protein along with cholesterol and glycosphingolipids. Caveolae abscission from the membrane requires dynamin activity similar to CCP abscission (Mayor, Pagano 2007b). In the caveolae membrane, PKCα (a serine-threonine kinase) is present which interacts directly with β1-integrin tail and responsible for its endocytosis (Melnikov, Sagi-Eisenberg 2009). Knockdown of caveolin-1 affects the endocytosis of α2β1 in osteosarcoma and α5β1 endocytosis in myofibroblast. It also affects β2 (leukocytes) and β3 (endothelial cells) integrin endocytosis. In mesenchymal stem cells of bone marrow, active β1-integrins are endocytosed by CIE together with CDE (Howes, Kirkham et al. 2010, Shi, Sottile 2008, Echarri, Del Pozo 2006). Interestingly, in some cells types, if CDE is blocked, CIE pathways are activated, e.g., upon overexpression of RAB21 in mouse embryonic fibroblasts expressing b1-integrin NPxY-motif mutants defective for binding to clathrin-adaptors CIE dominates over CDE. Similar results are also obtained when cells are treated with mono-dansyl-cadaverin (MDC), the inhibitor of clathrin (Pellinen, Tuomi et al. 2008).

Clathrin and dynamin-independent endocytosis
Clathrin and dynamin-independent endocytosis have been observed in the migratory cells at their leading edge highlighting the importance of this pathway in cell migration. Clathrin-independent carriers (CLICs) and glycosylphosphatidylinositol enriched early endosomal compartments (GEECs) are the routes of clathrin and dynamin-independent endocytosis and are dependent on cholesterol and actin. CLIC noticeably can endocytose a much larger load of cargo when compared with CME especially in fibroblasts (Howes, Kirkham et al. 2010, Chadda, Howes et al. 2007). Curvature formation and abscission for CLIC involves mainly RhoGTPase activating proteins family, e.g. ARHGAP26. Small GTPases RAB and ARF play a role in receptor trafficking like β1-integrin along with other plasma membrane receptors such as CD44, CD109 and LRP-1 which are also present on CLIC. CD44 is the receptor for matrix metalloproteinases (MMPs) and collagens and plays an essential role in cell adhesion and migration (Sathe, Muthukrishnan et al. 2018, Chaudhary, Gomez et al. 2014).

Circular dorsal ruffles and macropinocytosis

Circular dorsal ruffles (CDR) are the membrane protrusions rich in F-actin and are formed at the apical side of the cells mainly as a result of growth factors stimulation. CDRs employ macropinocytosis to endocytose the membrane receptors, i.e., integrins which play a role in cell spreading, adhesion and migration. During macropinocytosis of integrins in fibroblasts, integrins are endocytosed by the macropinocytic cups which are approx. 500nm in diameter to the EEA1 and RAB5 positive early endosomes followed by their recycling and new adhesions formation at the rear surface of the cell (Lim, Gleeson 2011, Gu, Noss et al. 2011).

2.3.4 Integrin recycling

Once the integrins are endocytosed, they are inside the cells in endosomes. Every endosome has a different function and destiny for the endocytosed integrin. RABs give endosomes their identity. ARFs cooperate with RABs in the process of endosomes formation, transport and fusion. Endocytosed integrins either get degraded in lysosomes or get recycled back to the cell membrane. Integrin recycling is an essential step for the endocytosed integrins to recruit themselves back to the plasma membrane and forms adhesion sites between the cell and extracellular environment for cell polarity and migration (Bridgewater, Norman et al. 2012, Caswell, Vadrevu et al. 2009). The precise molecular mechanisms and factors which determine the fate of integrins for degradation or recycling are still to be well understood, although it is known that Sorting Nexin 17 (Snx17) binds the distal NPxY motif in the β1-integrin tail and prevents the integrins from getting degraded by facilitating their recycling back to the cell membrane (Bottcher, Stremmel et al. 2012). A new study has shown that Snx17 is the part of a multiprotein complex (DSCR3, C16orf62 and VPS29 are other members) called retriever (McNally, Faulkner et al. 2017). Retriever is functionally and composition wise different from the classical recycling traffic complex, retromer which comprises Snx17 and VPS35 (Osborne, Phillips-Krawczak et al. 2015). Active β1-integrins are also reported to localize in the talin and FAK positive endosomes where they are involved in signaling and recycle to the leading edge of the plasma membrane for forming new focal adhesion (Nader, Ezratty et al. 2016, Alanko, Mai et al. 2015).

There are mainly two pathways by which integrins recycle. “Short loop” recycling pathway which is RAB4 dependent and “long loop” recycling pathway across perinuclear recycling compartment (PNRC) which is RAB11 and/or ARF6 dependent. It is known that active integrins prefer the RAB11- dependent long loop recycling and inactive integrins recycles by RAB4- dependent short loop and F-actin dependent pathways or go through Trans Golgi Network (TGN) mediated retrograde traffic (Arjonen, Alanko et al. 2012b, Caswell, Norman 2006). The selectivity of these pathways based explicitly on the active or inactive conformation of integrins is poorly understood and the function of

molecules elucidating this regulation is yet to be determined. Though both of these pathways are also involved in the recycling of other receptors like transferrin receptors (TfnR) and RTKs, there are several proteins which are uniquely linked with integrin recycling only, e.g. RAB11 effector called Rab coupling protein (RCP) affects integrin recycling but not TfnR recycling (Paul, Jacquemet et al. 2015, Powelka, Sun et al. 2004a).

Recycling of integrins is also affected by the growth factors stimulation. e.g., major pool of endocytosed β1-integrins recycles back to the plasma membrane after 5 minutes of growth factors treatment through RAB11 and ARF6 dependent long loop pathway. Interestingly, αvβ3 but not α5β1 under platelet-derived growth factor (PDGF) stimuli circumvent the long loop recycling pathway and recycle through the RAB4 dependent shorter pathway (Roberts, Barry et al. 2001). This process is dependent on the binding of phosphorylated polycystin1 (PKD1) with the cytoplasmic tail of β3-integrin (Woods, White et al. 2004). This highlights that not all integrin heterodimers recycle similarly under growth factor stimulation and preference of integrins to adopt a particular recycling pathway may change under different condition both in-vitro and under physiological conditions. In breast cancer cells, RAB21 binds the α-tail of integrin heterodimer and supports its endocytosis. Inside the cells, RASA1 (p120RasGap), a negative regulator of RAS-mediated signaling competes with RAB21 for binding to integrins. If RASA1 replaces RAB21, the integrin cargo recycle back to the plasma membrane perhaps by RAB11 dependent pathway (Mai, Veltel et al. 2011). Figure 5 illustrates the trafficking routes of integrins and the involvement of important RABs in these routes.

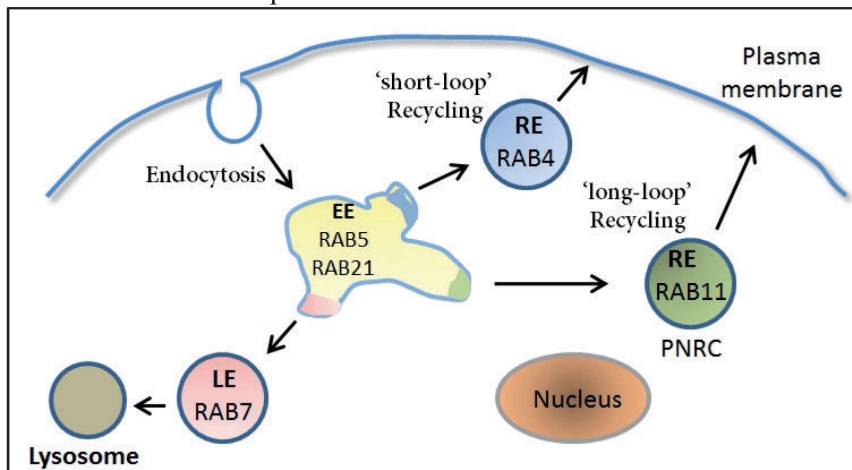

Figure 5: Simplistic schematics of pathways of integrin trafficking with associated endosomes and RABs. EE: early endosomes, RE: recycling endosomes, LE: late endosomes.

2.3.5 Deregulated integrin traffic in cancer cell migration and Invasion

Endocytic traffic (endocytosis/recycling) of integrins along with proper actin polymerization at the leading edge are the prerequisite for cell appropriate polarization, migration and invasion (Wilson, Allen et al. 2018). Any anomaly in these mechanisms is linked with cancer-related invasive processes. E.g., RAB25 (RAB11c) binds the β1-integrin cytoplasmic tail and promotes their recycling to the pseudopodia protracted in the direction of cell movement. Overexpression of RAB25 not only is linked with increased cell migration and invasion in in-vitro cancer model but also in clinical cases of breast, ovarian and colon cancer (Hong, Jeon et al. 2018, Wang, S., Hu et al. 2017, Dozynkiewicz, Jamieson et al. 2012). The table below summarizes the role of proteins involved in endocytosis or recycling of different integrins and their link with cancer-related processes and other diseases.

Table 1. Integrin traffic pathways and associated pathophysiological conditions in humans

Integrin receptor	Associated molecule/ trafficking step	Pathophysiological condition	Biological process and clinical significance	References
α1, α2, α5, α6, β1	Rab21/endocytosis	Prostate cancer, lung cancer	Migration, cytokinesis and generation of aneuploidy	Högnäs et al., 2012; Pellinen et al., 2006; Pellinen et al., 2008
β1	Rab5/endocytosis	Lung cancer, stomach cancer, breast cancer	Invasion, metastasis	Li et al., 1999; Yang et al., 2011; Yu et al., 1999
β1	Myo10/recycling	Breast cancer, pancreatic cancer	Invasion, metastasis, poor prognosis	Arjonen et al., 2014; Cao et al., 2014
β1, β3	Dab2/endocytosis	Epithelial ovarian cancer, prostate cancer, nasopharyngeal carcinoma, breast cancer	Invasion and metastasis	Mok et al., 1994; Tong et al., 2010; Wang et al., 2002; Xu et al., 2014
β1, β3	Numb/endocytosis	Breast cancer, non-small cell lung cancer, salivary gland carcinoma	Proliferation, poor prognosis	Maiorano et al., 2007; Pece et al., 2004; Westhoff et al., 2009
α5β1	Rab25/recycling	Ovarian cancer, breast cancer, colon cancer, intestinal neoplasia	Invasion	Cheng et al., 2010; Cheng et al., 2004; Dozynkiewicz et al., 2012; Fan et al., 2006; Goldenring and Nam, 2011; Nam et al., 2010
α5β1	RCP/recycling	Breast cancer, mutant p53 carcinomas, squamous cell carcinoma of the head and neck	Invasion and metastasis	Dai et al., 2012; Mills et al., 2009; Muller et al., 2009; Zhang et al., 2009
α5β1	CLIC3/recycling	Ovarian cancer, pancreatic cancer, breast cancer	Invasion, poor prognosis	Dozynkiewicz et al., 2012; Macpherson et al., 2014
α5β1, α6	GIPC1/endocytosis	Breast cancer, ovarian cancer, gastric cancer, pancreatic cancer	Invasion and metastasis, vascular development	Kirikoshi and Katoh, 2002; Muders et al., 2006; Rudchenko et al., 2008; Valdembri et al., 2009; Yavelsky et al., 2008
α5β1, αvβ3	PRKD1/recycling	Breast cancer, prostate cancer, gastrointestinal cancer, skin cancer	Proliferation, invasion	Eiseler et al., 2009; Jaggi et al., 2003; Ristich et al., 2006; Shabelnik et al., 2011
α3β1, α5β1	STX6 and VAMP3/recycling	Breast, colon, liver, pancreatic, prostate, bladder, skin, testicular, tongue, cervical, lung and gastric cancers	Proliferation	Riggs et al., 2012
α5β1, α2β1, αvβ3, α6β4	Rab11/recycling	Skin carcinogenesis, Barrett's dysplasia, mutant p53 carcinomas	Invasion and metastasis	Gebhardt et al., 2005; Goldenring et al., 1999; Muller et al., 2009
αvβ6	HAX1/endocytosis	Breast cancer, oral cancer, colon cancer	Neoplastic transformation, invasion, metastasis, poor prognosis	Bates et al., 2005; Ramsay et al., 2007; Trebinska et al., 2010
α9β1	Arf6/recycling	Axonal development and regeneration in peripheral nervous system	Tissue repair	Eva et al., 2012; Eva and Fawcett, 2014

Table 1: Copied from (De Franceschi, Hamidi et al. 2015) with the publisher's permission.

2.4 GGA family of trafficking adaptor proteins

Adaptor proteins like AP1/2/3/4/5 play a role in endocytic trafficking by associating the cargo proteins with the coat proteins like clathrin for vesicles selection, sorting and movement to the destined locations in the cell (Park, S. Y., Guo 2014). Golgi-localized, gamma adaptin ear-containing, ARF-binding protein (GGA) is a relatively newly discovered family of proteins identified in the year 2000 as the ARF effectors which interact with clathrin and primarily localizes to Trans Golgi network (TGN) and clathrin positive endosomes. Humans have GGA1, GGA2 and GGA3 while yeast has Gga1 and Gga2 (Boman, Zhang et al. 2000a, Dell'Angelica, Puertollano et al. 2000).

GGAs have three conserved domains. The N-terminal VHS domain, followed by GAT domain and GAE domain at the C-terminal. GAT and GAE domains are linked with a hinge region which is not conserved. The hinge is the binding site for clathrin. The VHS domain of GGAs is 140 amino acids long and consists of eight right-handed α helices. The cargo proteins which are known so far to bind GGAs through VHS domain directly contain dileucine acidic motifs (DXXLL where D- aspartic acid, L-Leucine and x-any residue), e.g., Mannose-6-phosphate receptors (M6PR) and Sortilin. Strikingly, VHS domain of other proteins, e.g., TOM1 does not have the specificity for the DXXLL motif. Also, GGAs do not recognize [DE]XXXL[LI]-type motif which is even though recognized by APs. This highlights the specificity of the VHS domain of GGAs for cargo recognition (Wang, T., Liu et al. 2010, Bonifacino 2004).

The GAT domain which is 150 amino acids long binds the small GTPases ARFs and this binding of ARFs to GGAs is vital for GGAs recruitment to TGN. The hinge region which interacts with clathrin heavy chain varies in length significantly (75-285 amino acids). The hinge also interacts with AP-1 by unknown mechanisms. The GAE domain interacts with accessory proteins which contain a DFGXϕ motif (X-any amino acid, ϕ-large hydrophobic amino acid), e.g., Rabaptin-5 and gamma-synergin (von Einem, Wahler et al. 2015, Zhu, G., Zhai et al. 2005, Hirst, Lui et al. 2000). Figure 6 illustrates the structure of GGA proteins.

To regulate traffic, GGAs need to become activated and engage with their cargo. First, ARF-GTP, through its myristoyl tail, recruits itself to the membrane of TGN. GGA then binds to this active ARF through its GAT domain. This recruitment causes structural changes in GGA, exposing the VHS domain for cargo engagement. Finally clathrin and accessory protein bind, which results in deformation in the membrane of TGN followed by CCV formation and complex budding off (Nakayama, Wakatsuki 2003).

Even though in mammals all the three GGAs are quite similar in structure, there is one crucial difference. GGA1 and GGA3 have a DXXLL motif in the hinge

region which mediates intramolecular binding with their VHS domain causing their autoinhibition. The precise mechanism of activation and inactivation are not known apart from the fact that the phosphorylation of serine 2-5 amino acids upstream of DXXLL promotes the autoinhibition (Doray, Misra et al. 2012, Cramer, Gustafsen et al. 2010). Interestingly, GGA2, but not GGA1 or GGA3, knockout in mice results in embryonically lethality (Govero, Doray et al. 2012a). On the trafficking side, GGA1 and GGA3 are involved in the recycling of transferrin and MET receptors respectively (Parachoniak, Luo et al. 2011a, Zhao, Keen 2008). The expression levels and traffic of EGFR though are regulated differentially by GGA1/3 and GGA2 (Uemura, Kametaka et al. 2018). More on trafficking by GGAs and specifically new understanding of the role of GGAs in integrin traffic will be presented in the results section.

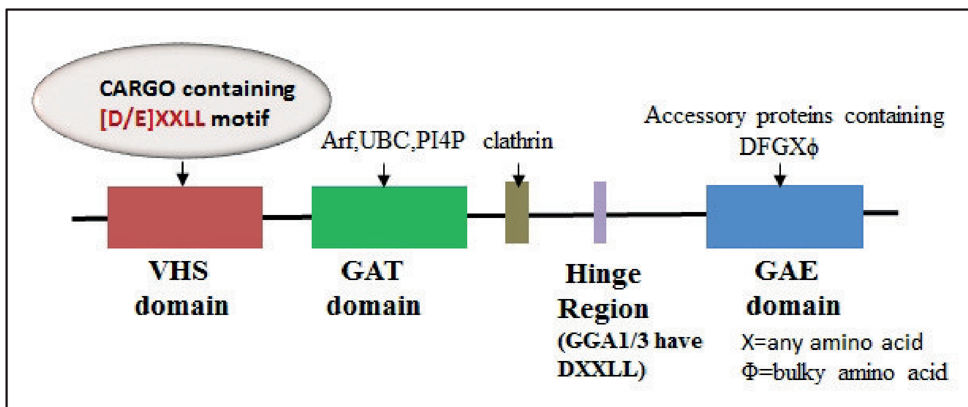

Figure 6: Structure of GGA proteins representing different domains and their interactors.

2.5 Receptor Tyrosine Kinases (RTK)

Like integrins, Receptor Tyrosine Kinases (RTKs) are also involved in cell differentiation, survival, adhesion and migration. There are about 60 known RTKs and they influence a variety of signaling pathways most importantly mitogen-activated protein kinase (MAPK) pathway, phosphoinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway and Janus kinase/ signal transducer and activator of transcription (JAK/STAT) pathway. Deregulation of these pathways especially in cancer is undisputed, and numerous attempts have been made to target various proteins involved in these pathways. After ligand binding to the extracellular domain of RTK, the receptor undergoes conformational and structural changes leading to transphosphorylation or autophosphorylation of the intracellular tyrosine binding domain (TBD). This triggers the recruitment of different down-stream adaptor proteins to the cytoplasmic tail to initiate the signal transduction (Du, Lovly 2018a, Hubbard, Miller 2007).

Majority of these adaptors contain Src homology region 2 (SH2) or phosphotyrosine binding (PTB) domain (Du, Lovly 2018a, Zwick, Bange et al. 2001). Aberrant activation of RTKs even in the absence of ligand is mostly the result of 1) activating gain-of-function mutation which causes hyper and sustained phosphorylation of the receptor. 2) Genomic amplification which results in overexpression of receptors on the plasma membrane. 3) Deregulation in the trafficking of receptors resulting in their reduced internalization and/or increased recycling hence prolonging their downstream signaling from the plasma membrane. Interestingly, this deregulation in the traffic can be either linked to reason 1) and 2) or as an outcome of an entirely different process (Fraser, Cabodevilla et al. 2017, Sorkin, Goh 2009). In the rest of this thesis, irregularities in the trafficking of two RTKs, ErbB2/HER2, an ErbB family protein, and c-MET would be discussed and shedding light on some of the new observations, mechanisms and links with cancer.

2.5.1 HER2 trafficking

ErbB/EGFR family of RTKs consists of three members: ErbB1/EGFR, HER2/ErbB2, HER3/ErbB3 and ErbB4/HER4. EGFR was one of the first receptors whose trafficking was studied and our present understanding of trafficking of all other plasma membrane receptors including integrins is based on the model of EGFR trafficking (Gorden, Carpentier et al. 1978). Details about the trafficking of ErbB2/3/4 were discovered much later. In polarized cells, the newly secreted ErbB proteins are recruited primarily to the basolateral cell surface, though a sub-pool of EGFR and HER2 in some cell types is also observed on the apical side (Balestreire, Apodaca 2007). HER2 is different from EGFR because its natural ligand is not known. The current view is that it cannot undergo efficient ligand-induced internalization hence resists its downregulation. Moreover, its internalization motif in the cytoplasmic tail is also not known (Bertelsen, Stang 2014). However, there is controversy in the literature regarding HER2 internalization, as discussed below. HER3 and HER4 whose ligands are heregulins and neuregulins when homodimerized can also undergo endocytosis which is less efficient than EGFR but more efficient than HER2. HER2 apart from being homodimerized can also undergo heterodimerization with other EGFR receptors and in this way resists their internalization as well (Giri, Ali-Seyed et al. 2005, Yarden, Sliwkowski 2001).

Owing to the constitutive open conformation of HER2, it is the favored heterodimerization partner for other EGFR members (Burgess, Cho et al. 2003, Garrett, McKern et al. 2003). Interestingly, a truncated version of HER2 known as p95HER2 which is the result of the protease activity of MMPs and ADAMs causes the ECD of full-length HER2 to shed. P95HER2 is upregulated in a subclass of HER2 positive cancer patients and its traffic is quite controversial. Some reports suggest the translocation of p95HER2 in the nucleus (Sperinde, Huang et al. 2018, Bao, Fu et al. 2010, Anido, Scaltriti et al. 2006).

One of the best-studied aspects of HER2 is its complex with a chaperon, heat shock protein 90 (Hsp90). Hsp90 interaction with HER2 is considered to be a significant factor which resists HER2 downregulation. Hsp90 together with its co-chaperon Cdc37 binds the intracellular kinase domain of HER2 (Smith, Workman 2009, Smith, de Billy et al. 2015). This interaction not only prevents the phosphorylation of HER2 but also its homodimerization hence preventing the hyperactivation and signaling of HER2 and stabilizing HER2 on the plasma membrane (Xu, Yuan et al. 2007). This mechanism prompted the pharma companies to design the inhibitors against Hsp90 and some of them are already in clinical trials (Haque, Alam et al. 2016). Geldanamycin (GA), an Hsp90 inhibitor is widely used in *in-vitro* studies. Treatment with GA or knockdown of Cdc37 causes the lysosomal-mediated downregulation of HER2 (Tikhomirov, Carpenter 2003). How this downregulation is linked with HER2 trafficking is still to be understood.

To better understand the perplexing traffic of HER2, it should be compared and contrasted with the robust EGFR trafficking (Sigismund, Avanzato et al. 2018, Fraser, Cabodevilla et al. 2017). EGFR has at least seven known ligands: EGF, transforming growth factor-a (TGFA), heparin-binding EGF-like growth factor (HBEGF), β-cellulin (BTC), amphiregulin (AREG), epiregulin (EREG), and epigen (EPGN) (Singh, Carpenter et al. 2016). The trafficking of EGFR induced by these ligands depends on their concentration and cell type, that is why some of the studies are debatable since they use very high ligand concentration (Sigismund, Algisi et al. 2013, Henriksen, Grandal et al. 2013). EGF and TGF-α induced endocytosis of EGFR is primarily clathrin-mediated with HBEGF and BTC mediated are also clathrin-independent (Henriksen, Grandal et al. 2013). Interestingly, at low EGF concentration, EGFR endocytosis is clathrin dependent due to less ubiquitination of EGFR and at high EGF concentration, the receptor endocytosis is clathrin-independent (Sigismund, Algisi et al. 2013). The fate of EGFR after endocytosis, whether it will recycle back or get degraded in the lysosome, depends on whether the ligand remains bound to the receptors and on the extent of receptor ubiquitination and activation. For example, TGF-α binding with EGFR is weak and it also induces less ubiquitination and phosphorylation to the receptor, in endosomes TGF-α dissociates with EGFR and EGFR is recycled back. On the contrary, the balanced between the recycled and degraded EGFR can be altered by using varying EGF concentration (Roepstorff, Grandal et al. 2009, Longva, Blystad et al. 2002). There are four models which help us understand why the HER2 resists its downregulation and trafficking unlike EGFR or any other receptor as shown in Figure 7 and described underneath.

A. Confinement of HER2 on the plasma membrane:

To induce ligand-dependent HER2 endocytosis, a chimeric receptor was constructed where the extracellular domain was from EGFR and was fused to the intracellular domain of HER2. This chimera, upon ligand stimulation, did not endocytose suggesting the ability of HER2 to block its endocytosis lies in its cytoplasmic tail (Sorkin, Di Fiore et al. 1993). Afterwards, it was discovered that HER2 in its c-terminal contains extra 34 amino acids between F1030 and L1075 known as the retention sequence whose function is to prevent HER2 endocytosis (Shen, Lin et al. 2008). The precise mechanism is not known though. Interestingly, on the plasma membrane, HER2 is occupied in the lipid rafts near the cell protrusions and absent from CCP, unlike other receptors. However, depletion of cholesterol did not affect the stabilization of HER2 (Hommelgaard, Lerdrup et al. 2004, Nagy, Vereb et al. 2002), suggesting that lipid raft disruption alone is not sufficient to trigger HER2 uptake. Importantly, two lipid raft-associated proteins known as Flotillin1 and Flotillin2 were found to associate with HER2-Hsp90 complex and silencing of these two lead to HER2 endocytosis and degradation (Asp, Pust et al. 2014, Pust, Klokk et al. 2013). Whether Flotillins interact with HER2 directly or associate through Hsp90 is not well known.

A. The absence of internalization sequences:

For undergoing clathrin-mediated endocytosis, the receptor should associate with the adaptor proteins which help in the formation of clathrin cage for cargo endocytosis. e.g., Grb binds pY1068/1086 in EGFR c-terminal tail. Moreover, adaptor protein AP-2 interacts directly with EGFR (Traub, Bonifacino 2013, Goh, Huang et al. 2010). HER2 though does not associate with adaptor proteins due to the absence of known internalization signal. Nonetheless, upon loss of Hsp90, HER2 becomes ubiquitinated and endocytosed. This ubiquitination might be the internalization signal necessary for endocytosis (Anania, Pham et al. 2014, Xu, Marcu et al. 2002). Other hypothesis says that the conformation itself of the cytoplasmic tail of HER2 might be preventing the buried internalization sequence to be recognized by adaptor proteins. This might be a possibility because caspase and proteosomal dependent cleavage of HER2 cytoplasmic tail downregulated HER2 expression (Lerdrup, Bruun et al. 2007, Lerdrup, Hommelgaard et al. 2006).

B. Inhibition of Clathrin-coated pits (CCP) formation:

HER2 even when activated cannot localize to CCPs. In HER2 overexpressing cells, EGFR-HER2 heterodimer upon ligand stimulation or HER2 homodimers in fact inhibits the formation of CCP suggesting HER2 overexpression does not favor CCP formation (Haslekas, Breen et al. 2005). However, in cells where HER2 expression more modest, EGFR expression and activation triggers the new CCP formation(Cortese, Howes et al. 2013).

C. Rapid Recycling:

According to some studies, it is not only the low endocytosis but also the rapid recycling of HER2 which attributes to its slow turnover (Austin, De Maziere et al. 2004, Hendriks, Opresko et al. 2003). Interestingly blocking the recycling with inhibitors did not result in accumulation of HER2 in endosomes (Longva, Pedersen et al. 2005). Also, some reports indicate that GA, which previously was used to induce the HER2 endocytosis and lysosomal degradation, does not affect endocytosis but rather inhibits receptor recycling (Cortese, Howes et al. 2013, Sorkin, Goh 2009).

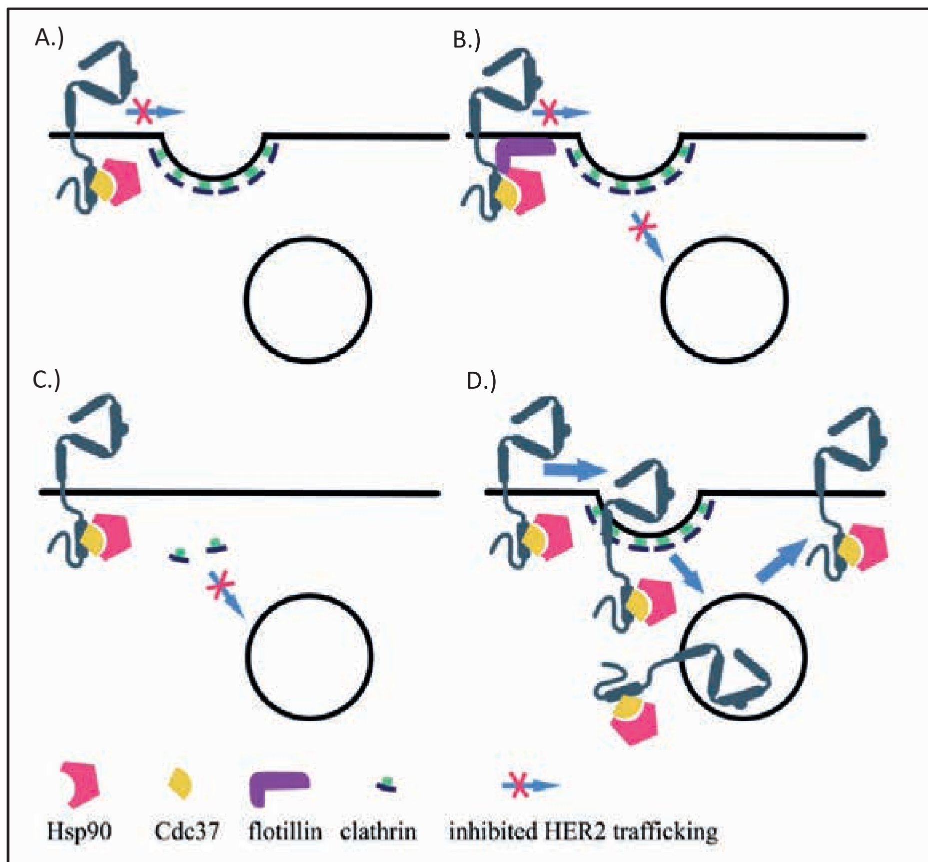

Figure 7: Four models explaining the resistance of HER2 for trafficking. A) Confinement of HER2 on the plasma membrane. B) The absence of internalization sequences. C) Inhibition of Clathrin-coated pits (CCP) formation. D) Rapid Recycling. Modified from (Bertelsen, Stang 2014).

2.5.2 Anti-HER2 antibodies in HER2 trafficking

Two murine-derived monoclonal antibodies against HER2 for treating HER2 positive cancer thus far have been approved. Trastuzumab (Herceptin) binds the

domain IV of HER2 and inhibits the activation of HER2 heterodimers (*in-vitro*) and HER2 homodimers (*in-vivo*) (Sarosiek, Morawski 2018, Junttila, Akita et al. 2009). Trastuzumab also prevents HER2 shedding (Molina, Codony-Servat et al. 2001). On the other hand, pertuzumab (Perjata) binds the domain II and inhibits the formation of HER2 heterodimers (Franklin, Carey et al. 2004, Agus, Akita et al. 2002). Either of these antibodies induces very little endocytosis of HER2, but when combined the endocytosis increases considerably. This endocytosis is not ubiquitin-mediated and the precise mechanism is still to be found (Hughes, Rodland et al. 2012, Nahta, Hung et al. 2004). Trastuzumab by itself can induce considerable endocytosis of HER2 when coated with colloidal gold or streptavidin possibly by crosslinking and clustering many HER2 receptors together (Zhu, W., Okollie et al. 2007, Maier, Xu et al. 1991).

2.5.3 MET Trafficking

MET or c-Met regulates various cellular functions such as cell migration, proliferation, survival, tissue repair, apoptosis, anchorage-independent growth and angiogenesis (Barrow-McGee, Kermorgant 2014). Unlike EGFR, which has many natural ligands, hepatocyte growth factor (HGF) is the only known ligand of MET (Park, M., Dean et al. 1987). Overexpression of HGF/MET or MET activating mutations are involved in many cancer-related processes and linked with poor prognosis (Zhuang, Jin et al. 2015, Baschnagel, Williams et al. 2014, Wang, X., Song et al. 2015).

Apart from signaling from the plasma membrane, activated MET also signals from endosomes post-endocytosis (Barrow, Joffre et al. 2014, Joffre, Barrow et al. 2011). MET also recycles back to PM or becomes lysosomally degraded similar to EGFR and integrins (Zhang, J., Babic 2016, Barrow-McGee, Kermorgant 2014, Parachoniak, Luo et al. 2011b). Hence, the balance between the MET endocytosis, recycling and degradation affects the extent of MET signaling (essentially ERK1/2, PI3K/Akt and STAT3) and MET effects on cellular homeostasis and cancer (Maroun, Rowlands 2014).

Upon HGF binding to MET, MET homodimerizes through its β-chains. This binding causes conformational changes and trans-autophosphorylation of Y1234 and Y1235 amino acids in the kinase domain of cytosolic tail and subsequential transphosphorylation of Y1349 and Y1356 amino acids in the docking site (Trusolino, Bertotti et al. 2010). Different adaptors and substrates bind to this docking side for downstream signal transduction. Grb2 associating binding protein 1(Gab1) is an essential adaptor for MET signaling. Gab1 binds MET either directly via its phosphorylated docking site or indirectly through Grb2. Gab1 and Grb2 then through their phosphorylated tyrosines can bind other regulators including kinases and phosphatases such as Crk, PI3K, Shp2, PLCgamma and activates MAPK and AKT signaling. Moreover, transcription factor STAT3, which is involved in many cancer-related processes, can be

phosphorylated and translocated to nucleus through direct binding with activated MET docking site (Trusolino, Bertotti et al. 2010, Zhang, Y. W., Wang et al. 2002, Kermorgant, Parker 2008). Figure 8 illustrates the important tyrosine in MET and proteins which interact with those residues.

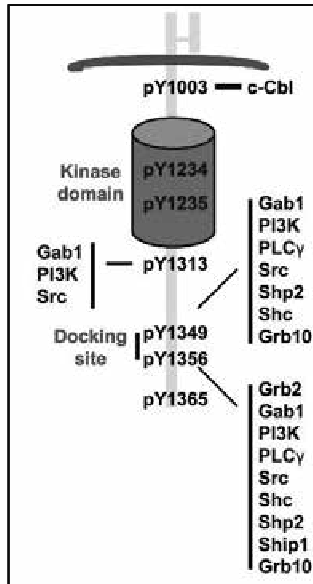

Figure 8: Multiple binding sites in MET (key tyrosines). Image from original publication (IV, 2F)

Following HGF binding, MET undergoes dynamic endocytosis which is mainly clathrin-mediated and in most instances need E3 ubiquitin ligase c-Cbl. C-Cbl associates with MET via Grb2 or functions as a scaffold between MET and endophilin for membrane curvature formation (Joffre, Barrow et al. 2011, Li, N., Lorinczi et al. 2007). MET can also undergo clathrin-independent endocytosis, but the mechanism is unclear apart from the observation that MET was observed in caveolin positive regions which is CD4410 dependent (Singleton, Salgia et al. 2007). MET accumulates in peripheral early endosomes (RAB5/EEA1 positive) and can fully activate ERK1/2 and Rac1 which get recruited to the plasma membrane for lamellipodia formation and cell migration (Joffre, Barrow et al. 2011, Abella, Parachoniak et al. 2010). Since MET endosomal signaling is relatively new, it would be interesting to see how it is distinct and relevant in different cancer subtypes.

From peripheral endosomes, MET can recycle back to the plasma membrane (RAB4 positive recycling endosomes), receive the signals for degradation or traffic to perinuclear endosomes by PKCα activation. It is in the perinuclear compartment, where MET activates STAT3. From the perinuclear compartment also, MET can recycle or is destined for proteasomal or lysosomal degradation

(Barrow-McGee, Kermorgant 2014, Kermorgant, Zicha et al. 2004). For degradation, direct binding of c-Cbl to Y1003 residue in the kinase domain of MET is essential for its ubiquitination (Carter, Urbe et al. 2004).

Adaptor GGA3, upon HGF stimulation, interacts with MET via SH3 domain of Crk and this process requires ARF6. This interaction mediates the recycling of MET through RAB4 positive endosomes. Silencing of GGA3 decreases the MET recycling and increases its lysosomal degradation (Parachoniak, Luo et al. 2011b). In some cell types with a gain-of-function p53 mutation, Rab-coupling protein (RCP) promotes the recycling of MET (Muller, Trinidad et al. 2013). Moreover, oncogenic mutation M1268T in MET induces the constitutive recycling while suppressing the MET degradation (Joffre, Barrow et al. 2011).

2.5.4 Deregulated HER2 and MET traffic in cancer

High expression and activating point mutations of HER2 and MET/HGF have been associated with many cancers and poor prognosis which in turn is the result of their altered trafficking or causes the defect in the trafficking hence affecting the stability and activity of these receptors (Du, Lovly 2018b). The above rationale hence implies that targeting the rogue trafficking in cancers dependent/addicted on HER2 or MET can produce promising results (Tang, Onitsuka et al. 2018, Garajova, Giovannetti et al. 2015a). Nearly 20-30% of all breast and ovarian cancers overexpress HER2. Its overexpression is also seen in stomach, bladder and lung cancer. One of the mechanisms by which HER2 contributes to cancer is by increasing the recycling of EGFR and decreasing its endocytosis and degradation (Lenferink, Pinkas-Kramarski et al. 1998). Activating HER2 mutations in cancers like NSCLC and gastric also messes up HER2 trafficking (Boku 2014, Krishnamurti, Silverman 2014).

Similarly, overexpression and sometimes activating point mutations in MET have been found in many cancers such as breast, ovarian, lung and liver. Mostly MET exon 14 splicing site alterations is very common with these cancers which results in impaired MET degradation as a result of the poor c-Cbl-MET interaction (Asaoka, Tada et al. 2010, Kong-Beltran, Seshagiri et al. 2006). Activating mutations M1268T and D1246N in MET kinase domain are found in many cases of renal cancer. Increased endocytosis and recycling of MET is mechanistically linked with these mutations (Joffre, Barrow et al. 2011).

3. AIMS OF THE STUDY

The object of this thesis is to gain fundamentally novel mechanistic insight into the trafficking and activity/stability of plasma membrane receptors (Integrins and RTKs) and their role in cancer thus opening avenues for new targets and markers in cancer therapy.

The specific aims of this study were:

1. To identify the new mechanisms and new regulators of adhesion receptor β1-integrin and Receptor tyrosine kinases RTK (MET and HER2) trafficking.
2. To investigate how the traffic of active and inactive integrins is differentially regulated.
3. To evaluate the functional consequences of the deregulated trafficking of β1-integrin, MET and HER2 receptors and understand the influence of this deregulation on oncogenic processes.

4. MATERIALS AND METHODS

More detailed description of the methods and reagents can be found in the original publications (I-IV)

Experimental Procedures

Method	**Used in**
BioID proximity biotinylation pulldown assay	I
Biotin-based internalization assay	I-IV
Biotin-based recycling assay	II, III, IV
Cell culture	I-IV
Cell spreading assay and focal adhesions analysis	I, II
Co-immunoprecipitation	I-IV
DCIS in-vivo xenograft models	III
DQ Red BSA assay	III
Flow cytometry	I-IV
Flow cytometry-based endocytosis assay	I, IV
Generation of lentiviral shRNA particles	III
Homology modeling	IV
Immunofluorescence, microscopy and image analysis	I-IV
Immunohistochemistry, Ki-67 and TUNEL labeling	III, IV
Inverted invasion assay	I
Live cell imaging	I-IV
Mammosphere formation assay	I
Mass spectrometry and analysis	I
Microscopy-based endocytosis assay	I, III
Migration assay	I, II, IV
Oncomine^{TM} meta-data analysis	IV
Phosphopeptide binding assay	IV
proliferation and survival assay	I, III, IV
Recombinant protein production	III, IV
RNA extraction, cDNA synthesis and qPCR	III, IV
RNAi Screening	I
siRNA transient transfection	I-IV
Stable cell-lines generation	II, III, IV
Subcellular fractionation	I, II, III
Tissue microarray construction	III,IV
Transmission electron microscopy	III
Western blot	I-IV
Yeast two-hybrid	IV
Zebrafish xenograft and metastasis assay	I

DNA Constructs

DNA construct	**Description**	**Used in**
pTagGFP2-GGA2	GGA2 wt	I
pmEGFP-GGA3	GGA3 wt	II
pmEGFP-GGA3 N194A	GGA3 with ARF uncouple mutation	II
pcDNA6.2-emGFP-RAB10	Small GTPase RAB10 wt	I
pEGFP-RAB11	Small GTPase RAB11 wt	II
pcDNA6.2-emGFP-RAB13	Small GTPase RAB13 wt	I
pEGFP-RAB4	Small GTPase RAB4 wt	II
pGEX-4T1 GST-TNS4-SH2-PTB	TNS4 with SH2 and PTB domains only	IV
pcDNA3.1- myc-BioID	To fuse protein of interest at the C-terminus of BirA(R118G)/ BioID tag	I
pcDNA3.1-myc-BioID-GGA2	GGA2 wt with myc-BioID tag	I
pmRFP-Lamp1	Lamp1 wt	IV
pLenti-SORLA-GFP	SORLA wt	III
pLenti-SORLA-GFP ECD+TM	SORLA with Extracellular and transmembrane domains only	III
pLenti-SORLA-GFP TM+CD	SORLA with transmembrane and cytoplasmic domains only	III
β1-Integrin-pEGFP	β1-Integrin wt	II
TNS3-pEGFP	TNS3 wt	IV
TNS4-pEGFP	TNS4 wt	IV
TNS4-pEGFP_R474A	TNS4 having R474A point mutation	IV

Antibodies

Antigen	**Catalog no.**	**manufacturer**	**Used In**
Active β1-integrin, clone 12G10		Developmental Studies Hybridoma Bank	I, IV
Active β1-integrin, clone 9EG7	55371	Bd Biosciences	I, IV
Alpha-Tubulin		Hybridoma Bank	I-IV
anti-mouse IRDye 680RD	926-68072	Li-Cor Biosciences	I-IV
anti-mouse IRDye 800CW	926-32212	Li-Cor Biosciences	I-IV
anti-rabbit IRDye 680RD	926-68073	Li-Cor Biosciences	I-IV
anti-rabbit IRDye 800CW	926-32213	Li-Cor Biosciences	I-IV
ARF3	610784	BD Transduction Labs	II
ARF6	SC-7971	Santa Cruz Biotechnology	II

Biotin-HRP	7075	Cell Signaling	I-IV
CD63	H5C6	Hybridoma Bank	II, III
Cleaved PARP1 (E51)	Ab32064	Abcam	III
CLTC	ab21679	Abcam	I
c-Myc tag	MMS-150R-1000	Covance	I
Cyclin D1 (72-13G)	SC-450	Santa Cruz Biotechnology	III
EEA1	3288S	Cell Signaling	I
EEA1	SC-6415	Santa Cruz Biotechnology	II, III
ErbB2 (9G6)	Ab16899	Abcam	III
GAPDH	5G4MaB6C5	Hytest	III
GFP	ab290	Abcam	I-IV
GGA1	PA5-61699	Invitrogen	I, II
GGA2	612612	BD Biosciences	I
GGA3	612310	BD Biosciences	I, II, IV
GST	MA4-004	Life Technologies	IV
HER2/ErbB2 (e2-4001 + 3B5)	MA5-14057	Thermo Scientific	III
HER2 (Trastuzumab)	CAS-180288-69-1	Roche	III
Histone H3	4499	Cell Signaling	III
IgG rabbit	sc-2027	Santa Cruz Biotechnology	II
Inactive β1-integrin, clone MAB13		Developmental Studies Hybridoma Bank	I
Integrin β1 tail (EP1041Y)	AB52971	Abcam	IV
LAMP-1	AB24170 AB25630	Abcam	II
LAMP-1 (H4A3)	SC-20011	Santa Cruz	I, II, III
LR11 (SORL1)	612633	Bd Transduction Lab	III
MET extracellular domain	MAB3582	R&D	IV
MET intracellular domain (L41G3)	3148S	Cell Signaling	IV
Na-k pump, clone a6F		Developmental Studies Hybridoma Bank	I, II
p4E-BP1 (T37/46)	2855S	Cell Signaling	III
p-AKT (S473)	9271S	Cell Signaling	III, IV
p-AKT (T308)	9275S	Cell Signaling	III
PARP1 (H-250)	SC-7150	Santa Cruz	III

Paxillin	sc-5574	Santa Cruz Biotechnology	I, II, IV
Phospho-Erk (Thr202/Tyr204)	9101S	Cell Signaling	IV
phospho-ERK p-p44/42 MAPK T202/Y204	4370S	Cell Signaling	III
Phospho-MET (Y1234/Y1235)	3077S	Cell Signaling	IV
RAB10, clone 4E2	MABN730	Millipore	I
RAB11	5589S	Cell Signaling	I, III
RAB13	NBP1-85799	Novus	I
RAB4	2167S	Cell Signaling	I
RAB5 (C8B1)	3547	Cell Signaling	I, III
RAB6	9625S	Cell Signaling	I
RAB7	9367S	Cell Signaling	I, III
SNX17	10275-1-AP	Proteintec	II
SORL1 (Histological stainings)	HPA031321	Atlas Antibodies	III
SORLA		C.M. Petersen (Aarhus University)	III
Streptavidin-Alexa 488	S11223	Invitrogen	I
Tensin-4	EPR13792	Abcam	IV
Tensin-4 (M01)	H00084951-M01	Abnova	IV
TGN46	ab50595	Abcam	I
Total 4E-BP1	9452S	Cell Signaling	III
Total AKT	9272	Cell Signaling	III, IV
Total ERK p44/p42 MAPK	9102S	Cell Signaling	III, IV
Total β1-integrin, clone N29	MAB2252	Merck-Millipore	I
Total β1-integrin, clone P5D2		Developmental Studies Hybridoma Bank	I, II
Transferrin Receptor (TfR)	ab84036	Abcam	I
TRKA	AF175	R&D	IV
Vinculin	V-9131	Sigma-Aldrich	II
VPS35	ab10099	Abcam	I, III
α1-integrin	Cbl1477	Millipore	II
α2-integrin	MAB1936	Millipore	II
α2-integrin	407286	Millipore	II
α5-integrin	328002	Biolegend	II

α5-integrin	328002	Biolegend	II
β-actin	A2228	Sigma	II, III

Peptides

Sequence	Name	Source	Used In
EMVSNESVDY^{p}RATFPED	pY1003	LifeTein	IV
GLARDMYDKEY^{p}Y^{p}SVHNKTG	pY1234-pY1235		IV
QPEYCPDPLY^{p}EVMLKCW	pY1313		IV
IFSTFIGEHY^{p}VHVNAT	pY1349		IV
VHVNATY^{p}VNVKCVA	pY1356		IV
IFSTFIGEHY^{p}VHVNATY^{p}VNVKCVA	pY1349-pY1356		IV
VNVKCVAPY^{p}PSLLSSE	pY1365		IV

Cell Lines

All the cell lines mentioned in this thesis are of human origin, except MDCK.

Cell line	Description	Used in
5637	Urinary Bladder Carcinoma	III
A549	Lung Carcinoma	IV
BT474	Breast Ductal Carcinoma	III
EBC-1	Lung Squamous Cell Carcinoma	IV
GTL-16	Gastric Carcinoma	IV
HCC1419	Breast Ductal Carcinoma	III
HCC1954	Breast Ductal Carcinoma,	III
HEK 293	Embryonic Kidney	IV
HeLa	Cervical Carcinoma	II
JIMT-1	Breast Carcinoma	III
MCF10A	Breast Fibrocystic Disease	III
MCF10A DCIS.com	Derived from xenograft originating from MCF-10AT Cells. J.F.Marshall (Barts Cancer Institute, UK)	III
MCF7	Breast Adenocarcinoma	III
MDA-MB-231	Breast Adenocarcinoma	I, II, III
MDA-MB-361	Breast Adenocarcinoma	III
MDA-MB-436	Breast Adenocarcinoma	III
MDCK	Kidney	IV
MFM-223	Breast Carcinoma	III
MKN-45	Gastric Adenocarcinoma	IV
OVCAR3	Ovarian Adenocarcinoma	IV
SKBR3	Breast Adenocarcinoma	III

siRNA and shRNA

Name or target	Description or sequence (sense)	Used in
Negative control siRNA	Qiagen, SI03650318	I-IV
GGA2#1	Qiagen, SI03190012	I, II
GGA2#2	IDT, hs.Ri.GGA2.13.1	I
RAB13#1	IDT, hs.Ri.RAB13.13.1	I
RAB13#2	IDT, hs.Ri.RAB13.13.1	I
RAB10#1	IDT, hs.Ri.RAB10.13.1	I
GGA3, D#1	CACGTTAGAGGAAGTTAACAA	II, IV
GGA3, D#2	GUGAGAUGCUGCUUCAUUA	II
ARF1	ACGUGGAAACCGUGGAGUA	II
ARF2	UAUGAACGCUGCUGAGAUC	II
ARF3	GAUGAGGGACGCCAUAAUC	II
SORLA #1	CCAUGAAUAUCACAGCUUA	III
SORLA #2	GACAGGAGCUACAAAGUAA	III
SORLA #3	CCGAAGAGCUUGACUACUU	III
SORLA #4	CCACGUGUCUGCCCAAUUA	III
SORLA 3'UTR	GGUUGGAGUGCCAAUAGAATT	III
HER2 #2	UGGAAGAGAUCACAGGUUA	III
HER2 #4	GCUCAUCGCUCACAACCAA	III
pGFP-C-shLenti shSORL1 #1	GGACTGGTCTGATGAGAAGGATTGTGGAG	III
shSORL1 – GFP #4	GTACATCTCTAGCAGTGCTGGAGCCAGGT	III
pGFP-C-shLenti Scramble (shCTRL)	Origene, TR30021	III
TNS4 3'UTR	UUCCAAAGCUGGUAUCGUA	IV
TNS4 SMARTpool		
TNS4 oligo-A	GAACGUAUGCCACCUCUUU	IV
TNS4 oligo-B	GACCUUGACUCCUACAUUG	IV
TNS4 oligo-C	GAUGUCAGCUAUAUGUUUG	IV
TNS4 oligo-D	GAGCAGGGCAUCACUCUGA	IV
β1-integrin	Qiagen, Hs_ITGB1_9 FlexiTube	IV

Reagents and chemicals

Compound	Supplier	Used in
Bafilomycin A1	Calbiochem	II, III, IV
Cell Counting kit 8	Sigma	III
Chloroquine	Biofellows	III
DAPI	Sigma-Aldrich	I-IV
DQ-BSA	ThermoScientific	III
Ebastine (in DMSO, 10mM)	Sigma	III
Fibronectin	Merck-Millipore Calbiochem	I, II, IV
Lapatinib	Selleckchem	III
Lipfectamine 2000/3000	Invitrogen	I-IV
Loratadine (in DMSO, 10mM)	Sigma	III
OptiMem	Invitrogen	I-IV
Penfluridol (in DMSO, 10mM)	Sigma	III
Phalloidin 488	Molecular Probes	II
Primaquine diphosphate	Sigma-Aldrich	I-IV
PureCol® EZ Gel Bovine Collagen Solution, Type I	Advancer Biomatrix	I, II, IV
RNAiMax	Qiagen	I-IV
Vitronectin	Sigma-Aldrich	II

5. RESULTS

5.1 GGA2 and RAB13 regulate activity-dependent β1-integrin recycling

The endocytic traffic of β1-integrins plays an essential role in cancer and cancer-related processes such as migration, invasion and focal adhesion dynamics (Campbell, Humphries 2011a). Integrins have a long half-life indicating that most of the integrins recycle back to the plasma membrane after internalization. Our laboratory has shown, using integrin activity conformation-specific monoclonal antibodies, that inactive and active integrins have very different trafficking dynamics. The inactive β1-integrins undergo rapid recycling whereas the active receptor pool is recycled to the plasma membrane more slowly (Arjonen, Alanko et al. 2012c). Apart from this, not much is known about the mechanism of activity-dependent integrin traffic or how do proteins especially adaptors regulate this specificity. Moreover, adaptor protein GGA3 diverts β1-integrin from lysosomal degradation and consequently supports the cancer-related processes (Ratcliffe, Sahgal et al. 2016) (findings from this work will be discussed in the next section). However, whether some of this traffic is integrin conformation specific is not known. To investigate other GGAs and their interactors in integrin trafficking we carried out a high throughput RNAi integrin endocytosis screen using the cell surface labeling of integrins followed by endocytosis and quenching the signal of cell-surface retained integrin on a cell spot microarray system. The screen was performed with two different siRNA for each gene in triple negative breast invasive adenocarcinoma cell line MDA-MB-231.

5.1.1 GGA2 specifically regulates the trafficking of active β1-integrin

The screen results indicated GGA2 as one of the strongest negative regulators of β1-integrin endocytosis meaning that its silencing with both siRNAs increased the β1-integrin accumulation inside the cells (I, Fig. S1A). The screen data was validated with biotin-IP based internalization assays and concurrent with the RNAi screen results; there was an increase in internalized β1-integrin signal at 10 min and 20 min after GGA2 silencing and without affecting the total β1-integrin levels (I, Fig. 1A, 1B, S1B). The other way round, upon overexpression of GGA2, the internalized β1-integrin levels reduced (I, Fig. 1C, 1D). Interestingly, unlike GGA1 which influences the trafficking of transferrin receptors, GGA2 silencing did not have any significant effect on the internalized transferrin (I, Fig. S1B, S1C).

The intracellular accumulation could be due to either increased endocytosis or reduced recycling. Considering that β1-integrin recycles to the plasma membrane with a turnover time of 10-15 minutes (I, Fig. 1A, 1B) and hence the slower accumulation of β1-integrin in the GGA2 silenced cells might be linked to recycling defect. To test this, we performed the biotin-IP based internalization

assay again but in the presence of an inhibitor of recycling, primaquine. Primaquine enhanced β1-integrin accumulation in the control cells to a level similar to the GGA2 silenced cells suggesting that it is the inhibition of β1-integrin recycling and not stimulation of endocytosis which is affected by GGA2 silencing (I, Fig. 1E, 1F).

Next, we aimed to study if GGA2 affects the trafficking of active and inactive integrins differently. We performed a FACS based internalization assay and looked at the cell surface levels of active and inactive β1-integrin. Upon β1-integrins internalization at 15 and 30 min, in GGA2 silenced samples, only the active β1-integrin surface levels reduced more compared to the control silenced cells without affecting the cell surface inactive β1-integrin uptake (I, Fig. 1G, 1H). Moreover, the micropattern based immunofluorescence analysis of β1-integrins staining revealed a significant increase in intracellular accumulation of active β1-integrins at steady state in GGA2 silenced cells (I, Fig. 1I, 1J). In addition, we also performed the imaging-based internalization assay where after 20 minutes of internalization, surface labeled active β1-integrin accumulated more in GGA2 silenced cells when compared with control silenced cells (I, Fig. 2A). To further reinforce our observation that GGA2 influences explicitly the trafficking of active β1-integrin, we performed more imaging-based internalization assays for surface labeled active and inactive β1-integrin in GFP and GFP-GGA2 expressing cells. On the contrary to the GGA2 silencing results, in GFP-GGA2 but not in GFP expressing cells, there was a decrease in the accumulation of active β1-integrin without affecting the inactive β1-integrin accumulation (I, Fig. 2C, 2D, S2A, S2B). When the cells were treated with primaquine, this difference in accumulation between the GFP and GFP-GGA2 cells was abrogated (I, Fig. 2E, 2F). These data indicate that GGA2 specifically plays a role in the recycling of active β1-integrin but not inactive β1-integrin.

5.1.2 GGA2 localizes to endosomes and plasma membrane

Intrigued by these results, we wanted to characterize the subcellular localization of GGA2. GGA2 has been mainly studied in yeast but less in the cancer cells. We either stained the cells with GGA2 or expressed GFP-GGA2 and co-stained with different endosomal markers. In line with previous publications, GGA2 colocalized with TGN46 and RAB6 (markers for the trans-Golgi network; TGN) and with clathrin heavy chain (I, Fig. S3A). In addition, we made the original observations that GGA2 colocalized with early endosome marker RAB5 and recycling markers RAB4 and RAB11. GGA2 also partially colocalized with EEA1 and a marker for retrograde transport VPS35. Interestingly, GGA2 did not show any colocalization with late endosome marker RAB7 and lysosomal marker Lamp1 (I, Fig. 3A). We also observed an overlap of GGA2 with active β1-integrin not only in endosomes but also partly on the plasma membrane (I, Fig. 3B, 3C). This observation drove us to perform total internal refraction Microscopy (TIRF) that can visualize the proteins in 200 nm range from the

plasma membrane. Using this technique, we indeed saw GGA2 colocalization with active β1-integrin on the plasma membrane (TIRF plane) (I, Fig. S3B). Furthermore, using biochemical subcellular fractionation, we observed GGA2 not only in the cytoplasmic fraction but also in plasma membrane fraction along with β1-integrin (I, Fig. S3C, S3D). Also, β1-integrin coimmunoprecipitated with GFP-GGA2 but not with GFP alone (I, Fig. 2G). This association of GGA2 with β1-integrin along with its colocalization with active β1-integrin on the plasma membrane further supports the role of GGA2 in active β1-integrin recycling from the plasma membrane.

5.1.3 GGA2 regulates cancer-related processes

GGA2 is known to be involved in receptor traffic from TGN in a clathrin-dependent manner (Boman, Zhang et al. 2000b). Our results shed new light on the active β1-integrins recycling mediated by GGA2 which indeed should affect cancer-related processes based on previous studies. Therefore, we conducted a series of functional experiments to evaluate the role of GGA2 in these processes. Focal Adhesion dynamics is an important determinant of a cell's potential for migration and invasion (Burridge 2017). GGA2 silencing though did not change the number of focal adhesions but the levels of active-β1-integrin 30 minutes post plating cells on fibronectin and collagen was less in focal adhesions of GGA2 silenced cells when compared with control silenced cells (I, Fig. 4A, 4B). Further, we also observed reduced migration speed and reduced cell invasion in the 3D collagen of GGA2 silenced cells (I, Fig. 4C, 4D). In *in-vivo* setting, we also observed reduced cell invasion in zebrafish embryos (I, Fig. 4E). Interestingly, we did not observe any change in cell proliferation in 2D, in mammospheres formation in suspension or the size of zebrafish embryos xenograft (I, Fig. S4B-D). Therefore, based on these results, it is clear that GGA2 affects cancer-related processes by altering the cell motility and focal adhesions composition.

5.1.4 Proximity biotinylation revealed RAB10 and RAB13 as new GGA2-associated proteins

GGAs through their VHS domain are known to bind dileucine acidic motif (AC-LL) in the cargo proteins they transport such as mannose-6-phosphate receptors and sortilin (Zhu, G., He et al. 2003). β1-integrin lacks this motif hence the trafficking of β1-integrin, mediated by GGA2 must be through some other mechanism. In order to find this mechanism and possibly the role of another molecule which associate β1-integrin and GGA2, we employed BioID technique (Roux, Kim et al. 2012a) to find the interacting and neighboring partners of GGA2 in its native cellular environment. This approach is based on the fusion of a promiscuous *Escherichia coli* biotin protein ligase to the targeting protein (GGA2 in this case). BioID features proximity-dependent biotinylation of proteins that are in near-neighbors of this fusion protein (I, Fig. S5A). We validated the functional integrity of our myc-BioID-GGA2 construct using

immunofluorescence (IF) and western blotting and later carried out streptavidin pull-down experiments with cell lysates having myc-BioID as a control (I, Fig. S5B, S5C).

The biotinylated proteins were identified using label-free semi-quantitative mass spectrometry. The proteins with higher peptide spectral matches (PSM) in myc-BioID-GGA2 than in myc-BioID were considered as hits. They were nine in number and included ARF6 and IGFR2 as the already known GGA2 interactors. The 'string10' based pathway enrichment analysis revealed in "recycling endosome membranes" pathway three proteins namely ARF6, RAB10 and RAB13 (I, Fig. 5A, 5B). While the role of ARF6 is linked with β1-integrin trafficking, the role of RAB10 and RAB13 is not. We validated the GGA2-RAB13 and GGA2-RAB10 association using GFP trap pulldown of GFP-GGA2, blotting for RAB13 or RAB10 and also using immunofluorescence-based colocalization studies. GGA2 and GFP-RAB10 colocalized in the cytosolic endosomes but GGA2 and RAB13/GFP-RAB13 colocalized not only in the cytosolic endosomes but also on the plasma membrane proximal endosomes (I, Fig. 5C-F, S5D).

In T-lymphocytes, active RAB13 associates with mst1 kinase and facilitates the delivery of the integrin LFA-1 (lymphocyte function-associated antigen 1) to the leading edge of lymphocytes (Nishikimi, Ishihara et al. 2014) but RAB10 and RAB13 have not been previously linked with the traffic of the ECM-binding α/β1-integrins. We evaluated the association of β1-integrin with RAB13 and RAB10 and found that endogenous β1-integrin coimmunoprecipitates both with GFP-RAB10 and GFP-RAB13 (I, Fig. 6A). Moreover, in the immunofluorescence studies, RAB13 and GFP-RAB13 colocalized with β1-integrin both in the cytosolic endosomes and on the plasma membrane. In contrast, the overlap between RAB10 and β1-integrin was not very strong comparatively and RAB10 was absent from the plasma membrane (I, Fig. 6B, 6C). These results suggest that RAB10 and RAB13 serve as the previously unknown proximal proteins of GGA2 that associate with β1-integrin as well. RAB13 particularly, not only associate with β1-integrins in the cytosolic vesicles but also on the plasma membrane.

5.1.5 RAB13 specifically regulates the trafficking of active β1-integrin

Next, we performed experiments to understand the role of RAB13 and RAB10 in β1-integrin trafficking. We first did the biotin-IP based β1-integrin endocytosis assay. RAB13 silencing but not RAB10 silencing resulted in increased β1-integrins levels similar to what we observed with GGA2 silencing (I, Fig. 7A, 7B, S6B, S6C). Next, we performed the FACS based endocytosis assay to access the role of RAB13 in mediating the internalization of active and inactive β1-integrin. Congruent with the results of GGA2 silencing, RAB13 silencing also influenced the reduction of cell surface active β1-integrin but not

inactive β1-integrin (I, Fig. 7C, 7D). Moreover, in microscopy based endocytosis assay, RAB13 silenced cells displayed more accumulation of active β1-integrin when compared with control silenced cells (I, Fig. 7E). Conversely, upon RAB13 overexpression, in GFP-RAB13 expressing cells when compared with GFP only expressing cells, there was less accumulation of active β1-integrin and no effect on inactive-β1-integrin accumulation (I, Fig. 7G, 7H, S7A, S7B). Also, the addition of primaquine in the above experimental setup led to a decrease in the difference between the internalized active β1-integrin between GFP-RAB13 and GFP expressing cells suggesting that RAB13 influences the recycling of active β1-integrin rather internalization (I, Fig. S7C, S7D). Taking these together, RAB13 phenocopies the effect of GGA2 on active β1-integrin recycling. RAB10 though associates with GGA2 and β1-integrin, it does not regulate the trafficking of β1-integrin and might be implicated in other GGA2 influenced processes.

5.2 Regulation of cell migration and β1-Integrin trafficking by the endosomal adaptor GGA3

Morag Park's lab in 2011 published the role of GGA3 in MET trafficking where they found that loss of GGA3 in HeLa cells leads to increased lysosomal degradation of activated MET and consequently decrease in ERK signaling and cancer cell migration. This mechanism was dependent on the direct interaction of GGA3 with ARF6 and GGA3 association with MET through adaptor CRK (Parachoniak, Park 2012). The following work was done in collaboration with Morag Park's lab. Here we found a surprising role of GGA3 in the recycling of β1-integrins and dependence of β1-integrins on GGA3 for the cancer cell, migration, spreading and focal adhesion dynamics.

5.2.1 GGA3 regulates cell migration and levels of a subset of integrins

As described in previous sections, GGAs are the adaptors proteins which were originally identified as small GTPase ARF effectors and bind to ARG-GTP. This interaction of GGAs and ARF prime the complex to assemble clathrin cage and mediate the traffic of receptors like M6PR from Golgi (Hirst, Lui et al. 2000). The functional role of these adaptors on integrin traffic is not well understood. We found in HeLa and MDA-MB-231 cells the reduction in their migration speeds (36% in HeLa cells and 54% in MDA-MB-231 cells) upon GGA3 loss specifically. Additionally, in MDA-MB-231 cells upon GGA2 loss, there was also a decrease in directional persistence (II, Fig. 1A-D).

Since cell migration is one of the functions regulated especially by β1-integrins, we analyzed the endogenous levels of β1-integrins in cells at basal state. GGA3 loss tends to decrease the overall β1-integrins levels along with a decrease in α2-integrin levels without affecting α5 integrins (II, Fig. 2A, 2B). Both α2 and α5-integrins dimerize with β1-integrin. α2-integrin binds collagen and α5-integrin

binds fibronectin. The processes mediated by integrins are dependent on the type of ligand present in the ECM since each heterodimer has different ligand engagement affinity. To test if GGA3 regulates the surface levels of integrin heterodimers differentially, we silenced GGA3 in HeLa cells and plated them on collagen, fibronectin or vitronectin. In all the three cases, GGA3 loss led to a decrease in surface levels of α2β1 and α5β1 without affecting the levels of αV or α1 integrins (II, Fig. 2C-E). These data suggest the differential regulation of a subset of integrins by GGA3. The mechanism of this selectivity remains to be investigated.

5.2.2 GGA3 associates with β1-integrin and regulates its trafficking

Finding this close link between GGA3 and β1-integrin and the localization of GGA3 in recycling vesicles based on previous reports, we observed in immunofluorescence, β1-integrin-GFP colocalizing with mcherry-GGA3 and co-trafficking (II, Fig. 3A). Next, we tested if GGA3 regulates β1-integrin traffic. HeLa cells were silenced for GGA3 and surface labeled with β1-integrin antibody and allowed to internalize the receptor. Specifically in GGA3 silenced cells, after 60 min of internalization, β1-integrin was mainly perinuclear and in control silenced cells peripheral (II, Fig. 3B). Also, in GGA3 silenced cells, the colocalization between β1-integrin and GFP-RAB4 decreased post internalization without affecting the degree of colocalization with GFP-RAB11 suggesting that upon GGA3 loss, β1-integrin gets internalized and this is dependent on RAB4 or the short loop recycling (II, Fig. 3C, 3D). To further study how GGA3 regulates the dynamics of β1-integrins, Biotin-IP based internalization assay was done in HeLa and MDA-MB-231 cells. Upon GGA3 loss, β1-integrin accumulated more inside the cells. When the internalization assay was performed in the presence of primaquine, an inhibitor of recycling, the difference in accumulation of β1-integrins between GGA3 and control silenced cells diminished (II, Fig. 4A-F), suggesting the role of GGA3 in β1-integrin recycling rather internalization. This result is also in line with the observation of β1-integrin reduction from RAB4 positive recycling upon GGA3 loss.

5.2.3 GGA3-ARF interaction is necessary for GGA3 dependent cancer processes

The role of ARF6 in β1-integrin recycling is well known and GGA3 being the ARF effector, we were interested in evaluating the function of GGA3-ARF interaction to maintain β1-integrin expression and β1-integrin-dependent processes. Point mutation N194A in GGA3 prevents the binding of GGA3 with ARFs. Interestingly, while re-expression of GFP-GGA3 in GGA3 silenced cells can recover the β1-integrin levels, re-expression of N194A GFP-GGA3 failed to rescues integrin levels (II, Fig. 5A, 5B). Furthermore, upon GGA3 loss, β1-integrins colocalized more with the Lamp1 positive compartment when

compared with control silenced cells suggesting the involvement of lysosomal-mediated degradation of β1-integrin. This increase in colocalization was perfectly rescued with the re-expression of GFP-GGA3 but not N194A GFP-GGA3 (II, Fig. 5E, 5F). These results hence conclude that GGA3-ARF binding is necessary to maintain β1-integrin levels and avert the lysosomal-mediated degradation of β1-integrin.

Integrin recycling is necessary for proper adhesions and cell spreading. GGA3 loss also led to a decrease in HeLa cell spreading on collagen 15 min after plating. The difference in cell spreading between control and GGA3 silenced cells reached its maximum at 120 min post cell plating. Upon expression of GFP-GGA3 but not N194A GFP-GGA3 in GGA3 silenced cells, the decrease in cell spreading phenotype was rescued (II, Fig. 6A, 6B). Since ARF1, ARF3 and ARF6 all are known to interact with GGA3, we silenced all three of them to see if they also affect spreading like GGA3, and interestingly only ARF6 silencing phenocopied GGA3 silencing of decreased cell spreading (II, Fig. 7A-C).

GGA3 loss also similarly led to a decrease in the number of vinculin-positive focal adhesions per cell and again this decrease was reverted upon expression of GFP-GGA3 but not N194A GFP-GGA3 in GGA3 silenced HeLa cells (II, Fig. 6D, 6E). Moreover, the decrease in cell migration upon GGA3 silencing was also rescued only by wild-type GFP-GGA3 (II, Fig. 8). These data indicate that GGA3 and ARF6 interaction is essential also for integrin recycling dependent processes such as cell spreading, migration and maintaining focal adhesion numbers.

5.2.4 GGA3 forms complex with SNX17 and regulates its localization

Adaptor protein SNX17 is a well-established regulator of β1-integrin traffic through RAB4 positive endosomes. SNX17 interacts directly with the β1-integrin tail and gives the identity to β1-integrin positive endosomes to recycle instead of being targeted to lysosomes (Steinberg, Heesom et al. 2012). Results from GGA3 experiments indicated the similar functionality with SNX17 and we were interested to see if somehow GGA3 loss also affects SNX17 regarding expression, localization and function. Interestingly, GGA3 loss from HeLa and MDA-MB-231 cells did not alter either SNX17 overall expression levels nor its expression in membrane fraction determined by subcellular fractionation but strikingly shifted its levels from EEA1 positive endosomes to Lamp1 positive endosomes (II, Fig. 9A-E). In other words, upon GGA3 silencing, SNX17 positive vesicles increased in size and colocalized more with Lamp1 instead of EEA1. These enlarged SNX17 endosomes were also positive for β1-integrin. In control silenced cells though the majority of SNX17 colocalized with EEA1. In addition, we also observed, in coimmunoprecipitation experiments, that GGA3, β1-integrin and SNX17 associate with each other and form a complex (II, Fig.

9F, 9G). Hence, based on these results, it is evident that upon GGA3 loss, SNX17 mislocalizes to the Lamp1 positive compartment and β1-integrins instead of getting recycled get degraded in lysosomes.

5.3 SORLA-driven endosomal trafficking regulates the oncogenic fitness of HER2 (III)

Endosomal trafficking of membrane receptors including RTKs controls the period and strength of oncogenic signaling incurred by these receptors (Watson, Arey et al. 2014). HER2 owing to the reasons explained earlier is predominantly on the plasma membrane and its traffic is poorly understood (Bertelsen, Stang 2014). In this study we unrevealed the role of Sortilin related receptor 1 (SORLA-protein name, *SORL1*-gene name), a protein previously linked with Alzheimer's disease and lipid metabolism disorders (Schmidt, Subkhangulova et al. 2017a) in HER2 trafficking in HER2 dependent breast cancer and bladder cancer cells lines. Knockdown of SORLA in these cells decreased cancer-related processes *in-vitro* and *in-vivo.* Hence, opening doors for a possible therapeutic opportunity in HER2 cancers.

5.3.1 SORLA associates with HER2 in endosomes and on the plasma membrane

We looked at the SORLA expression in a panel of breast cancer cell lines. To our surprise, SORLA levels were high only in HER2 amplified breast cancer cells lines. Also, in a bladder cancer cell line 5637 which has an activating HER2 mutation, SORLA levels were high when compared with patient-derived bladder cancer cell lines having wild-type HER2 (III, Fig. 1A, S1A). Observing this exciting trend of SORLA expression, we characterized the location of SORLA in HER2 overexpressing cancer cell lines. We found SORLA or SORLA-GFP partially colocalizing with RAB5 and EEA1 positive early endosomes, VPS35 positive retrograde traffic endosomes and RAB11 positive recycling endosomes. We did not, however, notice SORLA colocalization with RAB7 positive late endosomes or Lamp1 positive lysosomes (III, Fig. S1B). This localization indicated us the probable role of SORLA in trafficking through early endosomes, recycling endosomes but not through late endosomes or lysosomes.

We also decided to look at the localization of HER2 carefully in different cells having varying SORLA expression. Cell lines having high SORLA expression BT474, SKBR3 and HCC1419, the HER2 was mainly on the plasma membrane and this was reported by others also previously. In contrast, cell lines having intermediate or low SORLA expression MDA-MB-361, JIMT-1 and HCC1945, the HER2 was present both on the plasma membrane and in the endosomes positive for EEA1 or VPS35. These HER2 and EEA1 positive endosomes also expressed SORLA or SORLA-GFP (III, Fig. 1B, 1C, S1C, S1D). Live cell

imaging of SORLA-GFP and HER2 (trastuzumab labeled) in MDA-MB-361 cells further revealed that HER2 and SORLA colocalize and traffic together between cell membrane and endosomes (III, Fig. 1D, S1E). To further characterize the SORLA-HER2 association, we performed coimmunoprecipitation experiments (co-IP). In MDA-MB-361 and BT474 cells, endogenous SORLA and HER2 coimmunoprecipitated. Furthermore, Full-length GFP conjugated SORLA (SORLA-GFP) and extracellular domain+transmembrane domain GFP conjugated SORLA (ECD+TM) co-precipitated with HER2 but not transmembrane domain+cytosolic domain GFP conjugated SORLA (TM+CD) in MDA-MB-261 cells (III, Fig. 1E-G). When observed under the microscope, SORLA-GFP and TM+CD displayed similar endosomal localization. ECD+TM though showed dispersed cytosolic non-compartmentalized localization (III, Fig. S1F).

5.3.2 SORLA regulates HER2 expression and HER2 dependent oncogenic processes

This association of HER2 and SORLA prompted us to investigate further how SORLA regulates HER2 expression and localization. We performed FACS experiments to check cell surface levels of HER2. In BT474 cells, where HER2 is mainly on the cell membrane and SORLA expression is high, SORLA silencing led to a decrease in cell surface HER2 levels. Likewise, in MDA-MB-361 and JIMT1 cells where SORLA expression is moderate and low respectively and SORLA localized to endosomes as well, overexpression of SORLA led to an increase in surface HER2 levels (III, Fig. 2A). Total HER2 protein expression also changed proportionately (III, Fig. 2B, 2C). Noticeably, HER2 mRNA levels did not alter upon SORLA silencing indicating HER2 regulation by SORLA takes place at the post-translational stage (III, S2A, S2B).

In order to evaluate the function of SORLA in HER2 dependent cancer processes, we performed *in vitro* proliferation assays in SORLA silenced BT474 and MDA-MB-361 cells. SORLA silencing led to a decrease in cancer cell proliferation in both models. In addition, a defect in proliferation could be rescued in SORLA silenced MDA-MB-361 cells upon re-expression of GFP-SORLA (III, Fig, 2D, 2G). On an interesting note, re-expression of either of the truncated GFP-SORLA constructs did not rescue the decreased proliferation (III, Fig. 2H). On the contrary, in JIMT cells, upon SORLA-GFP expression, the proliferation increased (III, Fig. 2E). Moreover, SORLA silenced cells when xenografted in mouse, after ten weeks, formed dramatically smaller DCIS tumors when compared with those formed by control silenced cells (III, Fig. 2I). Looking at which components of cancer signaling are affected by SORLA silencing, AKT signaling reduced without affecting the ERK signaling (III, Fig. 2F, S2G). These results indicate an apparent role of SORLA in controlling the oncogenic properties of HER2.

5.3.3 SORLA controls HER2 trafficking

To find out if SORLA influences the subcellular localization and trafficking of HER2, we performed a series of experiments. In BT474 cells having HER2 expressed on the membrane, SORLA silencing led to intracellular HER2 accumulation (III, Fig. 3A, 3B). Also, when the recycling was blocked in BT474 cells with primaquine, HER2 accumulated inside the cells (III, Fig. 3C, 3D). This indicates the dynamic balance between the endocytosis and fast recycling of HER2 which can be altered by SORLA. We then evaluated the HER2 trafficking defect incurred by SORLA silencing or overexpression at different time points. Using alexa568-trastuzumab imaging and biotin-IP based trafficking assays systems, in MDA-MB-361 cells, SORLA silencing led to a marked increase in intracellular HER2 accumulation after 30 minutes of endocytosis. Intuitively, upon GFP-SORLA overexpression in JIMT-1 cells, HER2 accumulation in cells reduced after 20 minutes of endocytosis and recycling assay in these cells showed enhanced HER2 recycling when compared with GFP overexpressing cells (III, Fig. 3E-I).

5.3.4 SORLA depletion leads to dysfunctional lysosomes

Upon SORLA silencing in MDA-MB-361 cells and BT474 cells we noticed an unusual perinuclear accumulation of Lamp1, Lamp2 and CD63 positive lysosomes. These lysosomes were also enlarged in size when compared with those in control silenced cells. Moreover, in SORLA silenced cells, HER2 also accumulated in these Lamp1 positive enlarged lysosomes (III, Fig. 4A-C, S3A-D). This accumulation was perplexing because the lysosomal accumulation of receptors is linked with their enhanced degradation but upon SORLA silencing, there was an only a slight reduction in HER2 protein levels (III, fig. S3A). This prompted us to look at the functional integrity of these lysosomes in SORLA silenced cells. Indeed, under electron microscope, these enlarged lysosomes were very electron dense and resembled the lysosomes from lysosomal storage disorders such as gaucher’s disease (III, Fig. 4D). Moreover, in SORLA silenced MDA-MB-361 cells, lysosomal enzymes could not release the dequenched fluorogenic substrate from DQ-BSA and consequently producing lower fluorescence signal when compared with control silenced cells (III, Fig. 4E, S3H). These data indicate the presence of dysfunctional lysosomes in SORLA silenced HER2+ cancer cells.

5.3.5 Loss of SORLA sensitizes HER2 dependent cancer cells for CADs

Next, based on the dysfunctional lysosome in SORLA silenced cells, we tried to take advantage of this phenotype to treat HER2+ cancer cells. Cationic amphiphilic drugs (CAD) are a class of drugs which have been used to treat allergy, mental disorders and cardiac diseases (Kornhuber et al., 2010).

Surprisingly, cancer cells exhibit lysosome hypertrophy which makes the lysosomes fragile and vulnerable (Fehrenbacher et al., 2008, Fehrenbacher et al., 2004). Recently, there are reports where CADs have been used to incur lysosomal damage and hence cell death specifically in cancer cells. In MDA-MB-361 and BT474 cells, when we combined the SORLA silencing with ebastine (an anti-allergic CAD used in lung cancer), the IC50 value of ebastine to inhibit cells growth decreased drastically and we also observed increased PARP1 levels representing apoptosis, when compared with control silenced cells. This indicates increase sensitivity of SORLA silenced HER2 overexpressing cancer cells against CADs treatment (III, Fig. 4E-H).

We extended our study to HER2 dependent bladder cancer model and observed similar results as with breast cancer. In bladder cancer tissue microarray of almost 200 patients, we found a strong correlation between the levels of HER2 and SORLA. In SORLA silenced 5637 cells which have the HER2 activating mutation, cell proliferation *in-vitro* decreased compared with control silenced cells. Also, SORLA silenced 5637 cells when injected subcutaneously in nude mice, resulted in debilitated tumor growth. These tumors exhibited less intense ki-67 staining (less proliferative potential) when compared with tumors from control silenced cells (III, Fig. 5A-E).

5.4 Tensin-4-dependent MET stabilization is essential for survival and proliferation in carcinoma cells (IV)

Undesired MET activation and expression is often linked with cancer. MET interacts with several downstream effectors which initiate and sustain the MET signaling (Barrow-McGee, Kermorgant 2014). In this work we described the function of a new interactor of MET, TNS4 which controls the MET trafficking, signaling and hence MET related cancer processes.

5.4.1 Yeast two-hybrid system reveals TNS4 as new MET-associating protein

Using the kinase domain of MET as bait in the yeast two-hybrid system, we found TNS4 as one of the new MET interactors (IV, Fig. 1A). Co-immunoprecipitation experiments confirmed TNS4 and MET interaction in lung carcinoma cell line A549 and Gastric carcinoma cell line GTL-16. Moreover, TNS4 associated specifically with the active phosphorylated MET (pMET) (IV, Fig. 1B-E). In immunofluorescence studies, TNS4 and MET colocalization incremented upon MET phosphorylation by stimulating A549 cells with HGF (IV, Fig. 1F, 1G). MET binds with the adaptor and scaffold proteins which have SH2 or PTB domains. In order to better understand the TNS4-MET interaction, we generated and expressed as recombinant protein, GST tagged TNS4 having SH2 and PTB domains only. In the GST pull-down experiments, GST-TNS4-SH2-PTB but not GST pulls down MET from the cell lysates of A549 and GTL-

16 cells. In addition, this interaction was stronger when MET was phosphorylated (IV, Fig. 2C, S2A-B).

5.4.2 SH2 domain of TNS4 binds docking site of MET

The crystal structure of human TNS4 is not known. So, in order to find precisely which residues in TNS4 bind pMET, we modeled the SH2 domain of TNS4 using the known structure of the SH2 domain of Src complexed with a peptide having phosphorylated tyrosine (pY). The modeling revealed that R474 in TNS4-SH2 corresponds with already known pY binding residue R175 in Src-SH2. Hence, R474 could be the putative site in TNS4 through which it binds pMET (IV, Fig. 2D). We repeated the GST pull-down experiment with R474A point mutation and the results displayed reduced binding of GST-TNS4-SH2(R474A)-PTB with overexpressed MET in HEK293 cells when compared with that of TNS4-SH2-PTB. This indicates active MET associates with TNS4 via R474 on SH2 domain of TNS4 (IV, Fig. 2E). Next, we evaluated the TNS4 binding residues on pMET. *In-vitro* ELISA assay was done between GST-TNS4-SH2-PTB and phosphorylated biotinylated peptides corresponding to different residues of MET cytoplasmic tail. The result highlights that TNS4 interacts directly with pMET specifically through previously well-characterized pY1349-1356 in MET docking site and less well-defined site pY1313 (IV, Fig. 2F-H).

5.4.3 TNS4, MET and β1-integrins coexist in a protein complex and influence cancer signaling

TNS4, through its PTB domain, also binds β1-integrin. This interaction though unlike MET-TNS4 interaction does not depend on growth factor stimulation. Immunofluorescence studies indicated triple colocalization of TNS4-GFP, β1-integrin and MET at focal adhesion in A549 cells (IV, Fig. 3A). Further, the association among these three proteins was confirmed with co-immunoprecipitation and GST-pull down experiments (IV, Fig. 3B, 3C). Functionally, TNS4 knockdown decreased the HGF dependent A549 cells migration. Interestingly, expression of TNS4-GFP in these cells increased the cell migration but TNS4_R474A-GFP expression indeed decreases the migration of A549 cells (IV, Fig. 3E, 3F), implicating the importance of TNS4 binding with MET to initiate and sustain cancer-related processes. Interestingly in A549 cells, TNS4 silencing decreased the total and cell surface protein levels of MET and β1-integrin (IV, Fig. 4A-C, S3A, S3B). There was no downregulation on MET and β1-integrin at mRNA level suggesting that TNS4 affects MET and β1-integrin post-translationally. Depletion of MET and β1-integrin though did not change TNS4 protein levels. Moreover, TNS4 silencing also reduced the HGF dependent AKT phosphorylation suggesting its role in regulating PI3K/AKT signaling through activated MET (IV, Fig. 4D, S3C).

5.4.4 TNS4 regulates stability and trafficking of MET

Next, we evaluated if TNS4 affects MET stability. In cycloheximide chase experiments where the translation of new proteins was blocked, TNS4-GFP A549 stable cells displayed far more total MET and cell surface MET stability when compared with GFP cells (IV, Fig. 4E, S4A). This prompted us to look if the stability of MET, incurred by TNS4, is linked with the MET traffic. We observed increased MET endocytosis in TNS4 silenced A549 cells under basal conditions by biotin-IP based endocytosis assay (IV, Fig. 4H). TNS4 silencing though did not alter basal MET recycling (IV, Fig. S4B, S4C). Conversely, in TNS4-GFP A549 stable cells both the basal and HGF stimulated MET endocytosis reduced when compared with GFP A549 cells based on biotin-IP and FACS based endocytosis assay (IV, Fig. 5A, 5B). Strikingly, when compared with GGA3, which regulates the HGF dependent MET recycling (Parachoniak, Luo et al. 2011b), our data indicate that TNS4 instead regulates basal and HGF stimulated endocytosis. Also in TNS4-GFP cells, β1-integrin endocytosis was slightly lower than GFP cells (IV, Fig. 5D). This could be linked with the fact that MET and β1-integrin crosstalk and co-traffic especially after HGF stimulation and exist in a complex with TNS4. We also used the microscopy techniques to study how TNS4 influences MET trafficking. In TNS4_R474A-GFP expressing cells, after 10 min of HGF stimulation more endosomal MET was noticed and colocalizing with RFP-Lamp1 positive lysosomes when compared with TNS4-GFP expressing cells (IV, Fig. 5F). Interestingly, following 30 min of HGF stimulation, most of the lysosomal localized endosomal MET degraded (IV, Fig S5A). This suggests the protective function of TNS4 in preventing MET degradation and inefficacy of TNS4 non-binding MET mutant TNS4_R474A to keep MET stably on the plasma membrane after HGF stimulation.

5.4.5 TNS4 is necessary for MET dependent oncogenic processes

Based on the role of TNS4 in stabilizing oncogene MET, we next evaluated if there is any correlation between the expression of MET and TNS4 in patients derived tumor samples. Indeed, in the colorectal and ovarian epithelial tumors, MET and TNS4 levels correlated strongly suggesting that in MET dependent tumors, MET and TNS4 are coexpressed and TNS4 potentially regulates the MET levels and hence MET oncogenic signaling (IV, Fig. 6A, 6B). To confirm the role of TNS4 in MET related cancer processes both *in-vitro* and *in-vivo,* we performed a series of functional cancer assays in cancer cell lines. Cell proliferation in TNS4 silenced A549 cells reduced by over 50% when compared with control silenced cells. This defect in proliferation could be rescued with the expression of TNS4-SH2-PTB (IV, 7A, 7B). Similarly, in MET-amplified and dependent gastric cancer MKN-45 and GTL-16 cells, TNS4 silencing resulted in reduced proliferation (IV, Fig. 7D, S6D). Moreover, these two cell lines also displayed increased caspase3/7 activity indicative of higher apoptosis when TNS4 was silenced (IV, Fig. 7E). When control and TNS4 silenced GTL-16

cells were injected subcutaneously in nude mouse, the tumors derived from TNS4 silenced cells when compared with control silenced cells were more necrotic, stained less strongly for proliferation marker ki-67 and showed more intense TUNEL staining representative of apoptosis (IV, Fig. 7F, 7G). These results validate the crucial role of TNS4 in the MET dependent cancer-related processes *in-vitro* and *in-vivo.*

6. DISCUSSION AND CONCLUSION

6.1 Integrin traffic: significance, approaches and challenges (I, II)

Integrins, a major cell surface adhesion receptor family, play an essential role in cancer by regulating cell proliferation and migration in response to extracellular matrix (ECM) cues. Integrins are unique in their ability to act as bidirectional signaling-receptors (Hynes 2002c). Signals from other receptors or intra-cellular cues can induce receptor activation (inside-out signaling) and conversely, extracellular ligand binding triggers integrin signaling to activate the canonical integrin-dependent pathways: FAK, ERK and AKT (outside-in signaling). Most efforts to understand the molecular mechanisms involved in cancer proliferation, cell migration and cancer metastasis have overlooked the involvement of pathways regulating traffic of integrins (Akiyama 1996). Therefore, it is of paramount importance to understand integrin trafficking and to elucidate cancer progression and thereby provide new rational strategies to cure cancer.

We focused on β1-integrin to study integrin traffic. β1-integrin is the most common integrin in humans. It can heterodimerize with a variety of α subunits for binding to almost all integrins specific ECM ligands (Hynes 2002d). The most common approaches to study integrin traffic are a biotin-IP based biochemical approach or antibody-based imaging approach. The drawback of biotin-IP based approach is that it cannot distinguish between the active or inactive integrin conformations. On the other hand, using antibodies (Byron, Humphries et al. 2009) raised to recognize different exposed epitope in active or inactive integrin, can give deeper insight into activity-dependent specificity in integrin traffic. Additionally, intracellular compartments during trafficking can also be identified with antibody-based imaging approach. Moreover, studies have shown that biotin based and antibody-based integrin trafficking assays produce similar results hence the issue of receptor clustering and endocytosis does not affect the integrin trafficking results (Arjonen, Alanko et al. 2012d, Powelka, Sun et al. 2004b).

How is the trafficking (especially recycling) of active and inactive integrin governed differentially is still not well understood. In this regard, a few years ago our group made some important observations on the dynamics of active and inactive integrin trafficking and which recycling path either of the two integrin isoforms (active or inactive) prefers for recycling. We discovered that inactive integrins prefer the RAB4 dependent fast loop recycling and active integrins prefer RAB11 dependent fast loop recycling but how do the “adaptor proteins” differentially regulate this process of recycling was still unknown (Arjonen, Alanko et al. 2012a).

6.2 Overlapping and differential roles of GGA2, GGA3 and their associated proteins in β1-integrin trafficking (I, II)

GGAs family of proteins have not been studied in carcinoma cells and specifically concerning trafficking of integrins, though the role of GGA1 and GGA3 in recycling of transferrin and MET receptors respectively in cancer cells is established (Zhao, Keen 2008, Parachoniak, Luo et al. 2011c). Two of the studies presented in this thesis on GGA2 and GGA3 for the first time established their role in integrin traffic and subsequently integrin-dependent cancer-related processes. We employed high throughput RNAi screening and proximity-dependent biotinylation (BioID) to find new regulators of integrin trafficking which revealed a critical role of GGA2 and its previously unreported proximal interactor RAB13 in promoting recycling of active but not inactive β1-integrin to the plasma membrane. This function of GGA2 in influencing recycling of active β1-integrin might be linked with the cancer cell migration and invasion defects which we observed upon GGA2 depletion. In this thesis, the role of GGA2 in regulating the β1-integrin trafficking has been elucidated using both the biotin-IP based trafficking assays and antibody-based trafficking assay. Furthermore, the role of a related adaptor, GGA3, in promoting recycling of total pool of β1-integrin to the plasma membrane by influencing SNX17 subcellular localization was also demonstrated.

GGA2 is different from GGA3 because depletion of GGA2 does not reduce the total levels of β1-integrin like GGA3. Also, GGA2 and GGA3 seem to control the trafficking of β1-integrin by different mechanisms. Depletion of GGA3 leads to mislocalization of SNX17 from early endosomes to the lysosomes which result in degradation of β1-integrin. SNX17 is an important determinant of several β-integrins stability. SNX17 binds β-integrin tail on its distal NPxY motif and in this way promote their recycling (Steinberg, Heesom et al. 2012). Our results show that SNX17-GGA3-β1 integrin form a complex. On the contrary, GGA2 does not bind SNX17 (unpublished data) and that might explain why GGA2 depletion does not reduce total β1-integrin protein levels.

There are other studies also which suggest structural and functional differences between GGA2 and GGA3. GGA1 and GGA3 but not GGA2 contain an internal DXXLL motif in their hinge region. The serine 2-5 residues upstream of this motif can be phosphorylated by casein kinase-2 inducing intramolecular binding of this internal DXXLL motif with the VHS domain (Ghosh, Kornfeld 2003). Moreover, studies have demonstrated that GGA1/3 but not GGA2 recognize ubiquitin (Puertollano, Bonifacino 2004, Scott, P. M., Bilodeau et al. 2004, Shiba, Katoh et al. 2004). In a recent study, it was shown that GGA3 or GGA1 depletion increased cellular EGFR expression but GGA2 depletion drastically reduced EGFR expression (Uemura, Kametaka et al. 2018). Interestingly, loss of

GGA1 or GGA3 alone in mice is well tolerated whereas the absence of GGA2 results in embryonic or neonatal lethality (Govero, Doray et al. 2012b).
The immunofluorescence experiments revealed new subcellular locations of GGA2. Its subpopulation was found on early endosome, recycling endosomes, plasma membrane but not on late endosomes or lysosomes. The localization of GGA2 on the plasma membrane is intriguing because in that way GGA2 may associate with β1-integrin and influence the recycling. Indeed, also we observed GGA2 and β1-integrin colocalization on plasma membrane with a conventional confocal microscope, TIRF microscope and subcellular fractionation. GGA3 and β1-integrin colocalization though was observed only in endosomes and not on the plasma membrane.

The exciting function of GGA2 for recycling of active β1-integrin insisted us to explore new associating proteins of GGA2 and to study if these associating protein behave functionally same as GGA2. We focussed on new techniques to find protein-protein interaction: BioID (Roux, Kim et al. 2012b) and APEx (Lam, Martell et al. 2015). These two techniques are based on proximity biotinylation and identify proximal and interacting proteins of a target protein in the native cellular environment. Both techniques are based on different principles, BioID uses biotin-adenylate which has a half-life of minutes implying a large labeling radius and APEx uses phenoxyl radicals which have a short half-life of less than 1 ms hence smaller labeling radius. We preferred BioID in order to find not only the closely interacting but also not-directly interacting proximal hits of GGA2. We performed four BioID experiments with GGA2 and chose the hits for further investigation which were common in at least three experiments. Out of the nine hit proteins, RAB10 and RAB13 formed a separate node in interaction map and they were also enriched in "endosomal recycling" pathway. We decided to focus on these two RABs which were neither previously known to associate with GGAs nor in trafficking of collagen or fibronectin binding trafficking receptors. Moreover, these two have been implicated recently in cancer-related processes. Interestingly, in our two of the four BioID experiments we came across RABEP1, TNFAIP8 and PARP4 which were the hits in the GGA2 knocksideways knockdown (Hirst, Borner et al. 2012).

RAB13 in polarized epithelial cells is known to regulate membrane trafficking of vesicular stomatitis virus glycoprotein [VSVG], A-VSVG, and LDLR-CT27 between TGN and transferrin positive recycling endosomes (Nokes, Fields et al. 2008). In T-lymphocytes, active RAB13 associated with mst1 facilitates the delivery of the integrin LFA-1 (lymphocyte function-associated antigen 1) to the leading edge of lymphocytes (Nishikimi, Ishihara et al. 2014). RAB13 also regulates the assembly and the activity of tight junctions by endocytic recycling and transport of occludins to the plasma membrane (Morimoto, Nishimura et al. 2005, Kohler, Louvard et al. 2004). RAB13 in past few years has been linked to

cancer but the precise mechanism is still not adequately understood. e.g. in glioblastoma and carcinoma, its levels are changed (Mo, Zhang et al. 2013, Li, W., Li et al. 2014). Also, RAB13 mRNA is expressed in membrane protrusions of MDA-MB-231 breast carcinoma cells (Jakobsen, Sorensen et al. 2013). Recently, it has also been shown that RAB13 is activated by its effector DENND2B at the cell surface and this is required for cancer cell migration and invasion (Ioannou, Bell et al. 2015) and in the absence of EGF, Intersectin-small (ITSN-s) promotes the recycling of internalized EGFR by recruiting DENND2B for activation of the RAB13-dependent exocytic pathway (Ioannou, Kulasekaran et al. 2017).

Our findings show that GGA2, RAB13 and β1-integrin associate with each other and form a complex not only on the endosomes but also on the plasma membrane and this association could be the link for RAB13 related oncogenic processes described above. RAB13 depletion just like GGA2 depletion influences explicitly the recycling of active β1-integrin. The colocalization of RAB10 with GGA2 and β1-integrin was only on the endosomes and not on the plasma membrane, unlike RAB13. Also, though RAB10 co-immunoprecipitated with GGA2 and β1-integrin, RAB10 silencing did not affect β1-integrin traffic suggesting RAB10 might be connected with GGA2 and β1-integrin in regulating other cellular functions.

Our studies on GGA2 and GGA3 uncovered that while GGA2 influences specifically trafficking of active β1-integrin heterodimers, GGA3 influences the levels of subsets of certain integrin heterodimers specifically. GGA3 silencing decreases the surface levels of α2β1 and α5β1 integrins but not αV or α1 integrins. It is known that different subsets of integrins traffic through distinct pathways (De Franceschi, Arjonen et al. 2016, Morgan, Hamidi et al. 2013). Since GGA3 does not influence αV or α1 integrin levels, it might be selectively controlling the trafficking of a subset of integrin heterodimers. Our results show that GGA3 promotes the recycling of β1-integrin to the plasma membrane but which α subunits it affects during trafficking is the question for future research. Depletion of GGA3 results in a decrease in cancer cell migration, spreading and number focal adhesions. Again, these oncogenic processes regulated by GGA3 might be linked with different subsets of integrin heterodimers specifically.

In conclusion, we demonstrated the role of GGA2 and its new associating protein RAB13 in promoting the recycling of active β1-integrin but not inactive β1-integrin to the plasma membrane. Their depletion resulted in a decrease in breast carcinoma cell migration and in-vivo invasion (Figure 9A). Furthermore, we also established the role of GGA3 with intact ARF6 binding in promoting recycling of total pool of β1-integrin to the plasma membrane. Silencing of GGA3 in breast cancer and cervical cancer cells led to mislocalization of SNX17, a determinant of β1-integrin recycling to lysosomes leading to

enhanced degradation of β1-integrin and decrease in cancer cell migration, spreading and focal adhesion number (Figure 9A).

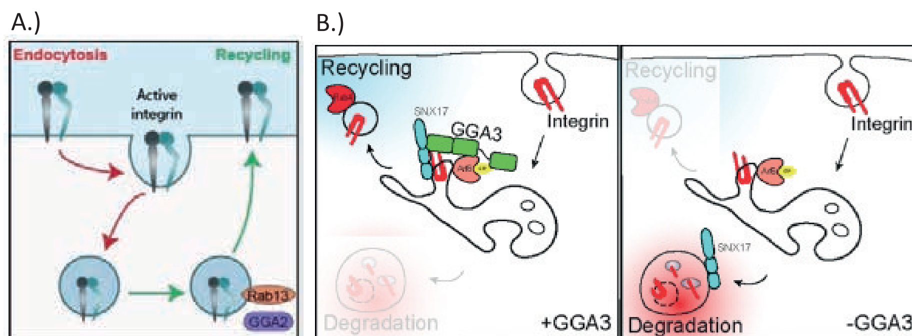

Figure 9: Schematics illustrating the role of A) GGA2 and B) GGA3 in β1-integrin trafficking. Refer text for more details. Images from original publication (I, 7I and II, Online abstract).

6.3 HER2 in cancer: prevalence, therapies and challenges (III)

After lung cancer, breast cancer in women is the second most common cause of cancer death. Metastasis arising from breast cancer contributes to a significant proportion of cancer-related deaths. Breast cancer is usually heterogeneous with mixed cell populations and different molecular signatures within the same tumor. This makes therapy selection difficult and often ineffective in the long run due to acquired mutations. Breast cancer can be classified into four subtypes based on the surface expression of hormone receptors estrogen receptor (ER) or progesterone receptor (PR) and Human epidermal growth factor receptor 2 (HER2). These are: Luminal A (ER+, PR+, HER2-); Luminal B (ER+, PR+, HER2+); HER2+ (ER-, PR-, HER2+) and Basal-like or triple negative breast cancer (ER-, PR-, HER2-) (Redig, McAllister 2013, Onitilo, Engel et al. 2009).

20-30% of all the breast cancers are HER2 positive. Moreover, a significant proportion of gastrointestinal cancers and lung cancers either have HER2 amplification or HER2 activating mutations (Bose, Kavuri et al. 2013, Lee, Soung et al. 2006). Specific therapies against HER2 dependent cancer include several approaches either as a monotherapy or a combination therapy. The widely used ones are monoclonal antibodies against HER2 (trastuzumab, pertuzumab), small molecules inhibitors against tyrosine kinase such as Lapatinib and Afatinib or inhibitors targeting downstream signaling mTOR/PI3K/Akt, hsp90 inhibitors and antibody-drug conjugate like T-DM1. These therapies many times do not work even initially as a result of de-novo drug resistance or lose their efficacy after a few months due to acquired drug resistance. Hence, new approaches to target HER2 dependent cancers should be developed (Lv, Meng et al. 2016, Tai, Mahato et al. 2010).

Endosomal trafficking of many receptor tyrosine kinases e.g. EGFR and MET critically control the strength and the duration of their signaling. There are proteins known which regulate the traffic of these RTKs. Hence there is a chance to target those in the clinic against cancer (Fraser, Cabodevilla et al. 2017, Sorkin, Goh 2009). On the contrary, the details of HER2 trafficking are poorly understood mainly because of the absence of identified ligands required to induce HER2 endocytosis and absence of a recognizable internalization motif in HER2 cytoplasmic where regulators can bind and induce endocytosis. Hence, some believe that HER2 remains stable on the plasma membrane and other believe 'rapid recycling' of HER2 which leads to almost exclusive cell-surface localization of HER2 (Bertelsen, Stang 2014). Therefore, we tried to find in detail the regulation of HER2 traffic in cancer cells and how this traffic influences cancer-related processes.

6.4 Role of SORLA in HER2 trafficking (III)

Here we identified Sortilin related receptor 1 (SORL1 or SORLA) as a novel regulator of HER2 traffic. SORLA protein previously implicated in Alzheimer's disease and obesity, was found to be a key regulator of HER2 oncogenic fitness in breast and bladder cancer cells. The results show that SORLA supports HER2 stability and signaling through supporting HER2 recycling to the plasma membrane. As contrary to the models discussed in review of literature which emphasize the reasons for poor HER2 traffic, our results indicate that HER2 undergoes potent endocytosis and recycling at least in most of the cancer cells lines we used in the study.

SORLA is a transmembrane receptor belonging to vacuolar protein sorting 10 (VPS10) receptors family and low-density lipoprotein (LDL) receptors family. SORLA has an extended extracellular domain which consists of different subdomains (VPS10P motif, β-propeller region, EGF-type repeat, complement-type repeats and Fibronectin III motif), a transmembrane domain and a short intracellular tail (Jacobsen, Madsen et al. 2001). The tail has the binding motifs for trafficking-related adaptor proteins like AP1/2, GGA1/2, SNX27 (PACS1) (Schmidt, Subkhangulova et al. 2017b). SORLA is best known for its anterograde and retrograde trafficking and processing of amyloid precursor protein (APP) through direct binding. SORLA enhances the recycling of APP and promotes its non-amyloidogenic cleavage. On the other hand, loss of SORLA leads to amyloidogenic cleavage of APP hence producing amyloid β-plaques in the brain responsible for the onset of Alzheimer's disease. Interestingly, in the presence of SORLA, amyloid β-plaques are efficiently degraded by lysosome. Downregulation of SORLA in the brain is often linked with sporadic late-onset of Alzheimer's disease (Andersen, Rudolph et al. 2016, Willnow, Andersen 2013). Other than this, SORLA also affects the lipoprotein signaling through binding of LDLR specific ligand apolipoprotein E (APOE) hence playing an important role in lipid metabolism disorders such as

atherosclerosis and obesity (Schmidt, Subkhangulova et al. 2017b). Apart from our study, to the best of our knowledge, no one has shown the mechanistic role of SORLA in cancer and cancer-related processes.

The adaptors mentioned above which bind SORLA tail also bind other receptors like integrins and other RTKs whose traffic is implicated in cancer. Keeping this in mind, we checked the expression level of SORLA in different breast cancer cell lines and noticed overexpression of SORLA specifically in HER2 amplified cell lines. The most striking observation we made was under microscope where HER2 amplified breast cancer cell lines where SORLA expression was high (BT474, SKBR3 and HCC1419), HER2 was abundantly and exclusively on plasma membrane but in the cells lines where SORLA expression was low (MDA-MB-361, HCC1954, JIMT-1), HER2 was on plasma membrane and also endosomal, colocalizing partly with EEA1, VPS35 and SORLA. These data led us to think that low SORLA expression is somehow driving HER2 in endosomes. To test this, in high SORLA expression cell lines when we silenced SORLA, some HER2 indeed reduced from the plasma membrane and appeared on endosomes. Conversely, upon expression of SORLA-GFP in cell lines where SORLA expression was less, HER2 plasma membrane level increased. Moreover, SORLA silencing led to a slight decrease in HER2 level as well. Biochemical-based trafficking assays further confirmed that SORLA promotes the recycling of HER2 to the plasma membrane. Depletion of SORLA or treating cells with an inhibitor of recycling, primaquine leads to endosomal accumulation of HER2.

This systematic immunofluorescence and biochemical study of HER2 in different cell lines instigate that HER2 indeed traffics dynamically through different endosomal compartments and manipulating this traffic of HER2 may influence HER2 dependent cancer processes. SORLA controls this trafficking process and depletion of SORLA led to decrease in HER2 oncogenic signaling, decrease in breast and bladder cancer cell proliferation, decrease in potential of these cells to form a tumor and causing dysfunctional leaky lysosomes. Lysosomal integrity is linked with proper cellular homeostasis including the metabolism of nutrient by the cell. The altered lysosomal function is a common attribute of a caner cell (Settembre, Fraldi et al. 2013). SORLA stabilizes the integrity of the lysosome probably by regulating the proteins involved in lysosomal pH and membrane integrity consequently affecting the dynamics and function of lysosomes. The precise mechanism of this regulation by SORLA remains to be understood.

Cationic amphiphilic drugs (CAD) have been long used to treat a broad spectrum of common diseases, e.g., psychiatric disorders, allergies, heart diseases and infections. Cancer cells display lysosome hypertrophy. Hypertrophy renders lysosomes fragile, increasing lysosomal membrane

permeabilization (LMP) tendency. This weakness of cancer cell lysosomes can be targeted by cationic amphiphilic drugs (CADs) that accumulate in the acidic lysosomes and induce lysosomal damage preferentially in cancer cells (Funk, Krise 2012, Petersen, Olsen et al. 2013, Ellegaard, Dehlendorff et al. 2016). Leaky lysosomes caused by SORLA silencing when combined with Cationic amphiphilic drugs (CADs) resulted in rupture of these lysosomes and eventually cell death. Hence in HER2 driven cancer specifically, combining SORLA depletion (using small molecules inhibitors or antibodies) with CADs could be a feasible therapeutic approach.

In conclusion, we identified the role of a protein called SORLA which was never linked previously with cancer in regulating the poorly understood and controversial HER2 traffic. SORLA promotes the recycling of HER2 to the plasma membrane. Silencing of SORLA led to the endosomal accumulation of HER2, reduced HER2 dependent oncogenic signaling and tumor formation along with making lysosomes dysfunctional. These SORLA depleted cancer cells having lysosomal anomaly showcased more sensitivity towards anti-cancer Cationic amphiphilic drugs (CADs) (Figure 10).

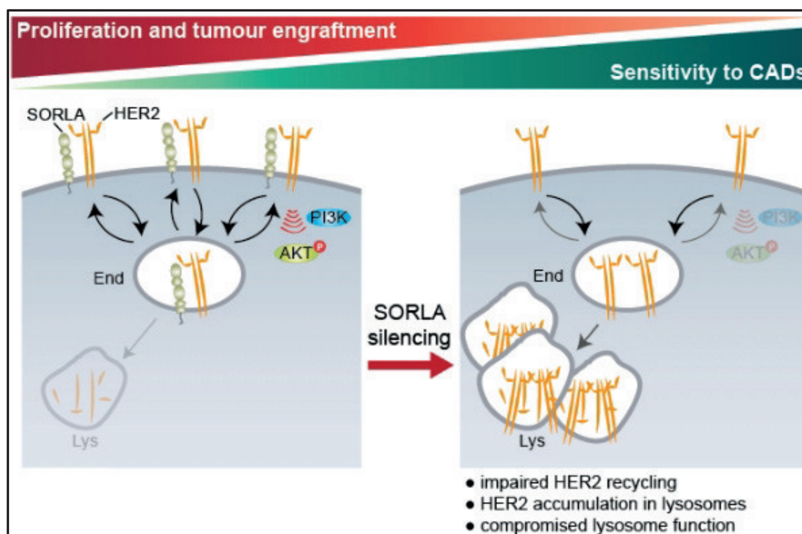

Figure 10: Schematic illustrating the function of SORLA in HER2 trafficking and in maintaining the cellular fitness of HER2-dependent cancers. Refer text for more details. Image from original publication (III, 5F).

6.5 MET oncogene: function, traffic and crosstalk (IV)

MET is involved in almost all the processes of cellular development. It is implicated in cell adhesion, motility, proliferation and survival. Oncogenic sustained MET signaling is implicated in cancer-related processes and is linked with poor prognosis and survival. MET is one of those receptors which signal

from the plasma membrane and can also signal from the endosomes after its endocytosis. MET after endocytosis can either get recycled to the plasma membrane or degraded. MET trafficking and signaling relies on activation of MET by exogenous ligand HGF and phosphorylation of cytoplasmic tails, leading to binding of adaptors and scaffold proteins consequently downstream signaling. This whole process is very tightly regulated, differs in different models and is not fully understood (Maroun, Rowlands 2014, Barrow-McGee, Kermorgant 2014)

MET can crosstalk and co-traffic with several other receptors including integrins. This crosstalk usually has an activating effect on MET activity but sometimes inhibitory as well. E.g., MET can crosstalk with different integrin heterodimers such as α5β1, α3β1 and α6β4 and positively regulate cell migration, proliferation and metastasis. Crosstalk between MET and HER2 is linked with drug resistance and increase cancer cell survival (Viticchie, Muller 2015). Co-endocytosed MET and β1-integrin are linked with sustained endosomal ERK signaling and enhanced tumorigenesis. This signaling of integrins is termed as "inside-in" signaling (Barrow-McGee, Kishi et al. 2016).

6.6 Role of TNS4 in MET trafficking (IV)

In this study, we investigated the role of putative oncogene TNS4 (Lo 2014b) in sustaining cancer-related processes by stabilizing the MET- β1-integrin complex on the plasma membrane. TNS4 does not have actin binding domain and in that way, it can uncouple the integrins from the actin cytoskeleton and enhance the cell migration (Katz, Amit et al. 2007). We established a direct interaction between TNS4 and MET. TNS4 is necessary to stabilize MET on the plasma membrane. HGF stimulation of MET strengthens this interaction and loss of TNS4 drives MET to lysosomal degradation.

Interestingly, TNS4 dependent endocytosis of MET can even take place at basal condition apart from HGF stimulation suggesting the importance of this interaction. TNS4 though is not involved in MET recycling but the previous study on MET recycling showed GGA3 in promoting the HGF dependent MET recycling to the plasma membrane (Parachoniak, Luo et al. 2011c). In line with this, we observed a reduction in MET overall protein expression upon TNS4 depletion both in basal conditions and after HGF stimulation. However, GGA3 depletion reduced MET only after HGF stimulation. Different cell models have a different level of HGF and depending on all that, endocytosis or recycling can dominate or balance each other which ultimately determines the MET signaling and effect on cellular processes.

Importantly, we have shown SH2 domain of TNS4 specifically R474 interacting with the docking site (Y1349, Y1356) and Y1313 residue on MET. Docking site of MET is well characterized and binds several other scaffold proteins like Grb2

(Barrow-McGee, Kermorgant 2014). Y1313 though is not well defined. The existence of various binding sites for TNS4 can be explained by the fact that if different other proteins occupy the busy docking site, TNS4 can still bind MET and stabilize it on the plasma membrane. This phenomenon may also play a role in drug resistance when docking site is inhibited by a peptide or a small molecule, TNS4 can still bind through a different site and sustain MET oncogenic signaling.

Notably, colon carcinoma and ovarian carcinoma tumor samples showed coexpression of MET and TNS4. Since MET overexpression is linked with the poor patient survivor (Ho-Yen, Jones et al. 2015), TNS4 expression can be a valuable marker and target for MET addicted tumors. Currently, the common problem of drug resistance for anti-EGFR and anti VEGFR therapies is linked with MET. There are different approaches to target MET, e.g. HGF inhibitors, MET kinase inhibitors, etc. but they fail at some point or other (Garajova, Giovannetti et al. 2015b). Our experiments with TNS4-SH2-R474A, showed the uncoupling of MET from TNS4 leading to MET degradation and reduced cancer cell proliferation. One promising approach in clinics could be the small molecule inhibitors binding R474 residue on TNS4 and consequently prevent MET-TNS4 binding resulting in MET endocytosis and degradation in MET dependent tumors.

In conclusion, we found putative oncogene TNS4 as the direct interactor of MET oncogene. TNS4, MET and β1-integrin together form a protein complex. TNS4 stabilizes active MET on the plasma membrane thus promoting its oncogenic signaling. TNS4 correlates with MET levels in human cancers. Silencing of TNS4 leads to enhanced MET endocytosis and lysosomal degradation resulting in a reduction of cancer-related processes in MET dependent lung, colon and ovarian cancer (Figure 11).

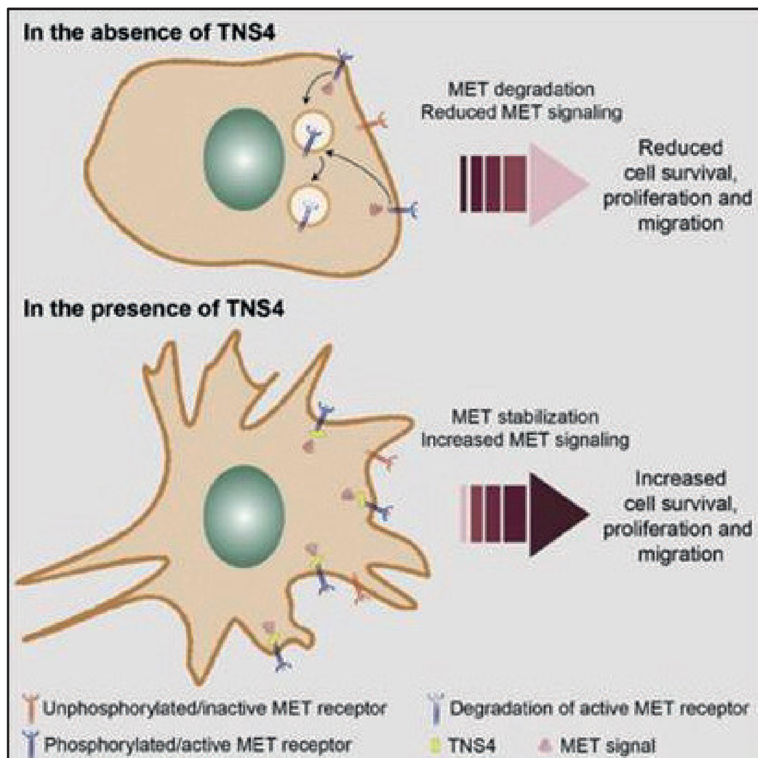

Figure 11: Schematic illustrating the function of TNS4 in MET trafficking and in maintaining the cellular fitness of MET-addicted cancers. Image from original publication (IV, Online abstract)

7. SUMMARY

In this thesis, the role of new proteins in regulating the trafficking of integrins and two RTKs, MET and HER2 in human cancer was explored and understanding how these proteins by influencing the trafficking of receptors, also affect the oncogenic processes in varied cancer models.

Trafficking of plasma membrane receptors is critical for initiating and sustaining the cellular processes such as cell proliferation and motility. Deregulation of receptor trafficking is implicated in many pathophysiological disorders including cancer. This trafficking is dynamic and spatiotemporally regulated by a variety of proteins such as small GTPases RABs. Integrins are the adhesion receptors which connect the exterior of the cell to the interior and in this way maintain the cellular contacts, signaling and consequently almost every cellular function. RTKs (HER2, MET) on the other hand are also involved in a variety of signaling and cellular processes similar to integrins but are not usually implicated in cellular adhesion. Deregulation in the traffic of integrins and RTKs eventually leads to abnormal cell adhesions, derailed signaling and progression of cancer-related processes.

We performed a high throughput RNAi screen and Proximity biotinylation (BioID) to identify new regulators of β1-integrin. β1-subunit of integrins is the most common integrin in mammals and can associate with a number of α-subunits. I found the role of GGA2 and its new associating protein RAB13 in promoting the recycling of active β1-integrin but not inactive β1-integrin. In this way, GGA2 and RAB13 influence the cancer cell migration and invasion in-vivo. Also, the role of GGA3 in regulating the recycling of the total pool of integrins was established.

I also made important discoveries about oncogenic RTK traffic in cancer. Using a yeast two-hybrid screen, we identified TNS4 as the direct binding partner of c-MET oncogene. TNS4 promotes the stability of MET and β1-integrin on the plasma membrane and hence boosts the oncogenic signaling and cancer-related processes. We also identified Sortilin related receptor 1 (SORLA) as a novel regulator of HER2 traffic in breast cancer. SORLA, a sorting protein previously implicated in Alzheimer's disease and lipid metabolism, was found to be a key regulator of HER2 oncogenic fitness in breast and bladder cancer cells. My results show that SORLA promotes HER2 stability and signaling through supporting HER2 recycling to the plasma membrane.

In summary, in this thesis, the new functions of GGA2, GGA3, MET and SORLA have been demonstrated in regulating trafficking of different plasma membranes receptors, therefore, influencing signaling and various cancer-related processes linked with these receptors in different cancel models.

8. ACKNOWLEDGEMENTS

This study was carried out at the Faculty of Medicine, Department of Cell Biology and Anatomy, University of Turku, Turku Centre for Biotechnology (TCB). I sincerely thank the previous and current directors of Turku Centre for Biotechnology, Professor Riitta Lahesmaa and Professor John Eriksson respectively, for providing such excellent research facilities and inspiring atmosphere. I express my gratitude to Professor Juha Peltonen for accepting me as a doctoral candidate in the Department of Cell Biology and Anatomy at the University of Turku. I feel honored to be part of Turku Doctoral Programme of Molecular Medicine (TuDMM), TuDMM is also acknowledged for providing continuous financial support for four years during my Ph.D. including the travel grants and also for organizing great scientific and recreational activities. Docent Kati Elima, Nina Widberg and Eeva Valve deserve special thanks for their efforts in the planning of graduate school recreation days and taking care of all the practical and bureaucratic issues so efficiently.

The most profound gratitude and appreciation I owe to my supervisor Professor Johanna Ivaska whose insightful vision and passion for science is simply incredible and will be my inspiration for the rest of my life. I have always been amazed at her way of handling things and situations and make them look simple. Without her comprehensive guidance and support, this thesis would not have been possible. There have been times when I forgot my old results, but she remembered which is extraordinary for a supervisor who is handling such a big lab. Praise and credit given by her have always been a source of motivation and confidence for me. Thank you, Johanna, for accepting me in your group and for your excellent supervision. I learned a lot from your encouraging, motivating and positive attitude.

I acknowledge the critical evaluation of my thesis by Prof. Stefan Veltel and Dr. Stéphanie Miserey-lenkei. I thank Professor Guido Serini, for accepting the invitation to be my opponent. I am thankful to my thesis committee members Docent Pieta Mattila and Docent Olli Pentikäinen for steering my thesis progress.

I express my immense gratitude to all my coauthors and collaborators in Finland and abroad. It was a pleasure working with you all: Jonna Alanko, Ilkka Paatero, Antti Arjonen, Anne Rokka, Mika Pietilä, Colin D. H. Ratcliffe, Christine A. Parachoniak, Prof. Morag Park, Emilia Peuhu, Niklas Jäntti, Assoc. Prof. Olav M.Andersen, Ghaffar Muharram, Taina Korpela, Nicola De Franceschi, Katherine Clark, David Tulasne and Prof. Olli Carpen. Earnest thanks to Petra for all those IPs and Hellyeh for helping with figures and editing.

I want to also thank all the past and present members of the Ivaska lab. We together indeed had an amazing, unforgettable time having both scientific and non-scientific discussions including the fun time outside the lab and Ph.D. dinners: Aki, Aleksi,

Anja, Camilo, Elina, Elisa, Ella-Maria, Emmi, Emmy, Guillaume, Gunilla, Habib, Hellyeh, Hussein, Ilkka, James, Jaroslav, Jenni, Jeroen, Johanna J., Johanna L., Kerstin, Maria G., Maria R., Maria T., Markku, Martina, Mika, Mitro, Nicola, Niklas, Nuria, Paulina, Petra, Reetta, Riina, Siiri. Antti, big thanks to you for helping me with different methods and techniques when I first joined the lab.

The administration and technical staff and core services at TCB deserve a special thanks for their sincere help. Thanks to Sirkku Grönroos, Sari Heikkilä, Ulla Karhunen, Susanna Pyökäri, Pasi Viljakainen, Anneli Takala, Mikael Wasberg, Petri Vahakoski, Juha Stranden, Mårten Hedman, Jouko Sandholm and Ketlin Adel. Many thanks, Markku for helping me immensely with the imaging.

Next, I want to thank all my dear friends whom I met in Finland during my master’s and Ph.D. studies. Behnoush, Shkar, Peter, Mueez, Erik, Daniel, Elena and Yaqi, you guys were great. Thanks to John and Pekka for selecting me for the BIMA program and giving me an opportunity to come to Finland where I met numerous wonderful people. Also, thanks to the past BIMA coordinators Eeva and Maritta for arranging day-to-day practical things. I want to warmly thank all my friends in TCB and outside TCB with (too many to mention all). We really had (will continue to have) quality, fun, memorable and relaxing time: Farid, Mukund, Srikar, Umar, Majid, Xi, Suvi, Roosa, Bish, Deb, Johanna T, Spiros, Mikael, Jimit and Chamu.

My deepest gratitude goes to my mum, dad and sister. Thank you for always doing/thinking best for me and encouraging me for studies and achieving highest in life. Thanks for the everlasting love, support and motivation you have been giving to me. I also thank the rest of my family, all my grandparents, cousins and relatives. Finally, thank you Emma for becoming a part of my life and for your incredible patience and understanding. Thanks for helping with the figures and making the cover for this thesis. Thank you Maikki and Ellu for all your support.

This thesis work was financially supported by Turku Doctoral Programme of Molecular Medicine (TuDMM), The Maud Kuistila Memorial Foundation, Ida Montinin Foundation, Instrumentarium Science Foundation, Finnish Cancer Foundation and University of Turku Foundation.

Turku, Finland - October 2018

Pranshu Sahgal

9. REFERENCES

ABELLA, J.V., PARACHONIAK, C.A., SANGWAN, V. and PARK, M., 2010. Dorsal ruffle microdomains potentiate Met receptor tyrosine kinase signaling and down-regulation. *The Journal of biological chemistry,* **285**(32), pp. 24956-24967.

AGUS, D.B., AKITA, R.W., FOX, W.D., LEWIS, G.D., HIGGINS, B., PISACANE, P.I., LOFGREN, J.A., TINDELL, C., EVANS, D.P., MAIESE, K., SCHER, H.I. and SLIWKOWSKI, M.X., 2002. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer cell,* **2**(2), pp. 127-137.

AKIYAMA, S.K., 1996. Integrins in cell adhesion and signaling. *Human cell,* **9**(3), pp. 181-186.

AKIYAMA, S.K., YAMADA, S.S. and YAMADA, K.M., 1989. Analysis of the role of glycosylation of the human fibronectin receptor. *The Journal of biological chemistry,* **264**(30), pp. 18011-18018.

ALANKO, J., MAI, A., JACQUEMET, G., SCHAUER, K., KAUKONEN, R., SAARI, M., GOUD, B. and IVASKA, J., 2015. Integrin endosomal signalling suppresses anoikis. *Nature cell biology,* **17**(11), pp. 1412-1421.

ALBERTS, B., 2008. *Molecular biology of the cell.* 5th edn. New York : Garland,.

ANANIA, V.G., PHAM, V.C., HUANG, X., MASSELOT, A., LILL, J.R. and KIRKPATRICK, D.S., 2014. Peptide level immunoaffinity enrichment enhances ubiquitination site identification on individual proteins. *Molecular & cellular proteomics : MCP,* **13**(1), pp. 145-156.

ANDERSEN, O.M., RUDOLPH, I.M. and WILLNOW, T.E., 2016. Risk factor SORL1: from genetic association to functional validation in Alzheimer's disease. *Acta Neuropathologica,* **132**(5), pp. 653-665.

ANIDO, J., SCALTRITI, M., BECH SERRA, J.J., SANTIAGO JOSEFAT, B., TODO, F.R., BASELGA, J. and ARRIBAS, J., 2006. Biosynthesis of tumorigenic HER2 C-terminal fragments by alternative initiation of translation. *The EMBO journal,* **25**(13), pp. 3234-3244.

ARJONEN, A., ALANKO, J., VELTEL, S. and IVASKA, J., 2012a. Distinct recycling of active and inactive beta1 integrins. *Traffic (Copenhagen, Denmark),* **13**(4), pp. 610-625.

ARJONEN, A., ALANKO, J., VELTEL, S. and IVASKA, J., 2012b. Distinct recycling of active and inactive beta1 integrins. *Traffic (Copenhagen, Denmark),* **13**(4), pp. 610-625.

ARJONEN, A., ALANKO, J., VELTEL, S. and IVASKA, J., 2012c. Distinct recycling of active and inactive beta1 integrins. *Traffic (Copenhagen, Denmark),* **13**(4), pp. 610-625.

ARJONEN, A., ALANKO, J., VELTEL, S. and IVASKA, J., 2012d. Distinct recycling of active and inactive beta1 integrins. *Traffic (Copenhagen, Denmark),* **13**(4), pp. 610-625.

ASAOKA, Y., TADA, M., IKENOUE, T., SETO, M., IMAI, M., MIYABAYASHI, K., YAMAMOTO, K., YAMAMOTO, S., KUDO, Y., MOHRI, D., ISOMURA, Y., IJICHI, H., TATEISHI, K., KANAI, F., OGAWA, S., OMATA, M. and KOIKE, K., 2010. Gastric cancer cell line Hs746T harbors a splice site mutation of c-Met causing juxtamembrane domain deletion. *Biochemical and biophysical research communications,* **394**(4), pp. 1042-1046.

ASP, N., PUST, S. and SANDVIG, K., 2014. Flotillin depletion affects ErbB protein levels in different human breast cancer cells. *Biochimica et biophysica acta,* **1843**(9), pp. 1987-1996.

AUSTIN, C.D., DE MAZIERE, A.M., PISACANE, P.I., VAN DIJK, S.M., EIGENBROT, C., SLIWKOWSKI, M.X., KLUMPERMAN, J. and SCHELLER, R.H., 2004. Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. *Molecular biology of the cell,* **15**(12), pp. 5268-5282.

BALESTREIRE, E.M. and APODACA, G., 2007. Apical epidermal growth factor receptor signaling: regulation of stretch-dependent exocytosis in bladder umbrella cells. *Molecular biology of the cell,* **18**(4), pp. 1312-1323.

BAO, W., FU, H.J., JIA, L.T., ZHANG, Y., LI, W., JIN, B.Q., YAO, L.B., CHEN, S.Y. and YANG, A.G., 2010. HER2-mediated upregulation of MMP-1 is involved in gastric cancer cell invasion. *Archives of Biochemistry and Biophysics,* **499**(1-2), pp. 49-55.

BARROW, R., JOFFRE, C., MENARD, L. and KERMORGANT, S., 2014. Measuring the role for Met endosomal signaling in tumorigenesis. *Methods in enzymology,* **535**, pp. 121-140.

BARROW-MCGEE, R. and KERMORGANT, S., 2014. Met endosomal signalling: in the right place, at

the right time. *The international journal of biochemistry & cell biology,* **49**, pp. 69-74.

BARROW-MCGEE, R., KISHI, N., JOFFRE, C., MENARD, L., HERVIEU, A., BAKHOUCHE, B.A., NOVAL, A.J., MAI, A., GUZMAN, C., ROBBEZ-MASSON, L., ITURRIOZ, X., HULIT, J., BRENNAN, C.H., HART, I.R., PARKER, P.J., IVASKA, J. and KERMORGANT, S., 2016. Beta 1-integrin-c-Met cooperation reveals an inside-in survival signalling on autophagy-related endomembranes. *Nature communications,* **7**, pp. 11942.

BASCHNAGEL, A.M., WILLIAMS, L., HANNA, A., CHEN, P.Y., KRAUSS, D.J., PRUETZ, B.L., AKERVALL, J. and WILSON, G.D., 2014. c-Met expression is a marker of poor prognosis in patients with locally advanced head and neck squamous cell carcinoma treated with chemoradiation. *International journal of radiation oncology, biology, physics,* **88**(3), pp. 701-707.

BAYS, J.L. and DEMALI, K.A., 2017. Vinculin in cell-cell and cell-matrix adhesions. *Cellular and molecular life sciences : CMLS,* **74**(16), pp. 2999-3009.

BELLIS, S.L., 2004. Variant glycosylation: an underappreciated regulatory mechanism for beta1 integrins. *Biochimica et biophysica acta,* **1663**(1-2), pp. 52-60.

BERTELSEN, V. and STANG, E., 2014. The Mysterious Ways of ErbB2/HER2 Trafficking. *Membranes,* **4**(3), pp. 424-446.

BLANGY, A., 2017. Tensins are versatile regulators of Rho GTPase signalling and cell adhesion. *Biology of the cell,* **109**(3), pp. 115-126.

BLAZEK, A.D., PALEO, B.J. and WEISLEDER, N., 2015. Plasma Membrane Repair: A Central Process for Maintaining Cellular Homeostasis. *Physiology (Bethesda, Md.),* **30**(6), pp. 438-448.

BOKU, N., 2014. HER2-positive gastric cancer. *Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association,* **17**(1), pp. 1-12.

BOMAN, A.L., ZHANG, C., ZHU, X. and KAHN, R.A., 2000a. A family of ADP-ribosylation factor effectors that can alter membrane transport through the trans-Golgi. *Molecular biology of the cell,* **11**(4), pp. 1241-1255.

BOMAN, A.L., ZHANG, C., ZHU, X. and KAHN, R.A., 2000b. A family of ADP-ribosylation factor effectors that can alter membrane transport through the trans-Golgi. *Molecular biology of the cell,* **11**(4), pp. 1241-1255.

BONCOMPAIN, G. and PEREZ, F., 2013. The many routes of Golgi-dependent trafficking. *Histochemistry and cell biology,* **140**(3), pp. 251-260.

BONIFACINO, J.S., 2004. The GGA proteins: adaptors on the move. *Nature reviews.Molecular cell biology,* **5**(1), pp. 23-32.

BOSE, R., KAVURI, S.M., SEARLEMAN, A.C., SHEN, W., SHEN, D., KOBOLDT, D.C., MONSEY, J., GOEL, N., ARONSON, A.B., LI, S., MA, C.X., DING, L., MARDIS, E.R. and ELLIS, M.J., 2013. Activating HER2 mutations in HER2 gene amplification negative breast cancer. *Cancer discovery,* **3**(2), pp. 224-237.

BOTTCHER, R.T., STREMMEL, C., MEVES, A., MEYER, H., WIDMAIER, M., TSENG, H.Y. and FASSLER, R., 2012. Sorting nexin 17 prevents lysosomal degradation of beta1 integrins by binding to the beta1-integrin tail. *Nature cell biology,* **14**(6), pp. 584-592.

BOUVARD, D., POUWELS, J., DE FRANCESCHI, N. and IVASKA, J., 2013. Integrin inactivators: balancing cellular functions in vitro and in vivo. *Nature reviews.Molecular cell biology,* **14**(7), pp. 430-442.

BRETSCHER, M.S., 1989. Endocytosis and recycling of the fibronectin receptor in CHO cells. *The EMBO journal,* **8**(5), pp. 1341-1348.

BRIDGEWATER, R.E., NORMAN, J.C. and CASWELL, P.T., 2012. Integrin trafficking at a glance. *Journal of cell science,* **125**(Pt 16), pp. 3695-3701.

BURGESS, A.W., CHO, H.S., EIGENBROT, C., FERGUSON, K.M., GARRETT, T.P., LEAHY, D.J., LEMMON, M.A., SLIWKOWSKI, M.X., WARD, C.W. and YOKOYAMA, S., 2003. An open-and-shut case? Recent insights into the activation of EGF/ErbB receptors. *Molecular cell,* **12**(3), pp. 541-552.

BURRIDGE, K., 2017. Focal adhesions: a personal perspective on a half century of progress. *The FEBS journal,* **284**(20), pp. 3355-3361.

BYRON, A., HUMPHRIES, J.D., ASKARI, J.A., CRAIG, S.E., MOULD, A.P. and HUMPHRIES, M.J., 2009. Anti-integrin monoclonal antibodies. *Journal of cell science,* **122**(Pt 22), pp. 4009-4011.

CALDERWOOD, D.A., CAMPBELL, I.D. and CRITCHLEY, D.R., 2013. Talins and kindlins: partners in integrin-mediated adhesion. *Nature reviews.Molecular cell biology,* **14**(8), pp. 503-517.

CALDERWOOD, D.A., FUJIOKA, Y., DE PEREDA, J.M., GARCIA-ALVAREZ, B., NAKAMOTO, T., MARGOLIS, B., MCGLADE, C.J., LIDDINGTON, R.C. and GINSBERG, M.H., 2003. Integrin beta cytoplasmic domain interactions with phosphotyrosine-binding domains: a structural prototype for diversity in integrin signaling. *Proceedings of the National Academy of Sciences of the United States of America,* **100**(5), pp. 2272-2277.

CAMPBELL, I.D. and HUMPHRIES, M.J., 2011a. Integrin structure, activation, and interactions. *Cold Spring Harbor perspectives in biology,* **3**(3), pp. 10.1101/cshperspect.a004994.

CAMPBELL, I.D. and HUMPHRIES, M.J., 2011b. Integrin structure, activation, and interactions. *Cold Spring Harbor perspectives in biology,* **3**(3), pp. 10.1101/cshperspect.a004994.

CARTER, S., URBE, S. and CLAGUE, M.J., 2004. The met receptor degradation pathway: requirement for Lys48-linked polyubiquitin independent of proteasome activity. *The Journal of biological chemistry,* **279**(51), pp. 52835-52839.

CASWELL, P.T. and NORMAN, J.C., 2006. Integrin trafficking and the control of cell migration. *Traffic (Copenhagen, Denmark),* **7**(1), pp. 14-21.

CASWELL, P.T., VADREVU, S. and NORMAN, J.C., 2009. Integrins: masters and slaves of endocytic transport. *Nature reviews.Molecular cell biology,* **10**(12), pp. 843-853.

CHADDA, R., HOWES, M.T., PLOWMAN, S.J., HANCOCK, J.F., PARTON, R.G. and MAYOR, S., 2007. Cholesterol-sensitive Cdc42 activation regulates actin polymerization for endocytosis via the GEEC pathway. *Traffic (Copenhagen, Denmark),* **8**(6), pp. 702-717.

CHAN, L.K., CHIU, Y.T., SZE, K.M. and NG, I.O., 2015. Tensin4 is up-regulated by EGF-induced ERK1/2 activity and promotes cell proliferation and migration in hepatocellular carcinoma. *Oncotarget,* **6**(25), pp. 20964-20976.

CHAO, W.T. and KUNZ, J., 2009. Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. *FEBS letters,* **583**(8), pp. 1337-1343.

CHAUDHARY, N., GOMEZ, G.A., HOWES, M.T., LO, H.P., MCMAHON, K.A., RAE, J.A., SCHIEBER, N.L., HILL, M.M., GAUS, K., YAP, A.S. and PARTON, R.G., 2014. Endocytic crosstalk: cavins, caveolins, and caveolae regulate clathrin-independent endocytosis. *PLoS biology,* **12**(4), pp. e1001832.

CHEN, C.S., ALONSO, J.L., OSTUNI, E., WHITESIDES, G.M. and INGBER, D.E., 2003. Cell shape provides global control of focal adhesion assembly. *Biochemical and biophysical research communications,* **307**(2), pp. 355-361.

CHI, R.J., HARRISON, M.S. and BURD, C.G., 2015. Biogenesis of endosome-derived transport carriers. *Cellular and molecular life sciences : CMLS,* **72**(18), pp. 3441-3455.

CHIA, P.Z. and GLEESON, P.A., 2011. The regulation of endosome-to-Golgi retrograde transport by tethers and scaffolds. *Traffic (Copenhagen, Denmark),* **12**(8), pp. 939-947.

CORTESE, K., HOWES, M.T., LUNDMARK, R., TAGLIATTI, E., BAGNATO, P., PETRELLI, A., BONO, M., MCMAHON, H.T., PARTON, R.G. and TACCHETTI, C., 2013. The HSP90 inhibitor geldanamycin perturbs endosomal structure and drives recycling ErbB2 and transferrin to modified MVBs/lysosomal compartments. *Molecular biology of the cell,* **24**(2), pp. 129-144.

CRAMER, J.F., GUSTAFSEN, C., BEHRENS, M.A., OLIVEIRA, C.L., PEDERSEN, J.S., MADSEN, P., PETERSEN, C.M. and THIRUP, S.S., 2010. GGA autoinhibition revisited. *Traffic (Copenhagen, Denmark),* **11**(2), pp. 259-273.

DAY, K.J., STAEHELIN, L.A. and GLICK, B.S., 2013. A three-stage model of Golgi structure and function. *Histochemistry and cell biology,* **140**(3), pp. 239-249.

DE FRANCESCHI, N., ARJONEN, A., ELKHATIB, N., DENESSIOUK, K., WROBEL, A.G., WILSON, T.A., POUWELS, J., MONTAGNAC, G., OWEN, D.J. and IVASKA, J., 2016. Selective integrin endocytosis is driven by interactions between the integrin alpha-chain and AP2. *Nature structural & molecular biology,* **23**(2), pp. 172-179.

DE FRANCESCHI, N., HAMIDI, H., ALANKO, J., SAHGAL, P. and IVASKA, J., 2015. Integrin traffic - the update. *Journal of cell science,* **128**(5), pp. 839-852.

DE LEON, N. and VALDIVIESO, M.H., 2016. The long life of an endocytic patch that misses AP-2. *Current genetics,* **62**(4), pp. 765-770.

DELL'ANGELICA, E.C., PUERTOLLANO, R., MULLINS, C., AGUILAR, R.C., VARGAS, J.D., HARTNELL, L.M. and BONIFACINO, J.S., 2000. GGAs: a family of ADP ribosylation factor-binding proteins related

to adaptors and associated with the Golgi complex. *The Journal of cell biology,* **149**(1), pp. 81-94.

DORAY, B., GHOSH, P., GRIFFITH, J., GEUZE, H.J. and KORNFELD, S., 2002. Cooperation of GGAs and AP-1 in packaging MPRs at the trans-Golgi network. *Science (New York, N.Y.),* **297**(5587), pp. 1700-1703.

DORAY, B., MISRA, S., QIAN, Y., BRETT, T.J. and KORNFELD, S., 2012. Do GGA adaptors bind internal DXXLL motifs? *Traffic (Copenhagen, Denmark),* **13**(10), pp. 1315-1325.

DOZYNKIEWICZ, M.A., JAMIESON, N.B., MACPHERSON, I., GRINDLAY, J., VAN DEN BERGHE, P.V., VON THUN, A., MORTON, J.P., GOURLEY, C., TIMPSON, P., NIXON, C., MCKAY, C.J., CARTER, R., STRACHAN, D., ANDERSON, K., SANSOM, O.J., CASWELL, P.T. and NORMAN, J.C., 2012. Rab25 and CLIC3 collaborate to promote integrin recycling from late endosomes/lysosomes and drive cancer progression. *Developmental cell,* **22**(1), pp. 131-145.

DU, Z. and LOVLY, C.M., 2018a. Mechanisms of receptor tyrosine kinase activation in cancer. *Molecular cancer,* **17**(1), pp. 58-018-0782-4.

DU, Z. and LOVLY, C.M., 2018b. Mechanisms of receptor tyrosine kinase activation in cancer. *Molecular cancer,* **17**(1), pp. 58-018-0782-4.

ECHARRI, A. and DEL POZO, M.A., 2006. Caveolae internalization regulates integrin-dependent signaling pathways. *Cell cycle (Georgetown, Tex.),* **5**(19), pp. 2179-2182.

ELLEGAARD, A.M., DEHLENDORFF, C., VIND, A.C., ANAND, A., CEDERKVIST, L., PETERSEN, N.H.T., NYLANDSTED, J., STENVANG, J., MELLEMGAARD, A., OSTERLIND, K., FRIIS, S. and JAATTELA, M., 2016. Repurposing Cationic Amphiphilic Antihistamines for Cancer Treatment. *EBioMedicine,* **9**, pp. 130-139.

FRANKLIN, M.C., CAREY, K.D., VAJDOS, F.F., LEAHY, D.J., DE VOS, A.M. and SLIWKOWSKI, M.X., 2004. Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer cell,* **5**(4), pp. 317-328.

FRASER, J., CABODEVILLA, A.G., SIMPSON, J. and GAMMOH, N., 2017. Interplay of autophagy, receptor tyrosine kinase signalling and endocytic trafficking. *Essays in biochemistry,* **61**(6), pp. 597-607.

FUNK, R.S. and KRISE, J.P., 2012. Cationic amphiphilic drugs cause a marked expansion of apparent lysosomal volume: implications for an intracellular distribution-based drug interaction. *Molecular pharmaceutics,* **9**(5), pp. 1384-1395.

GARAJOVA, I., GIOVANNETTI, E., BIASCO, G. and PETERS, G.J., 2015a. c-Met as a Target for Personalized Therapy. *Translational oncogenomics,* **7**(Suppl 1), pp. 13-31.

GARAJOVA, I., GIOVANNETTI, E., BIASCO, G. and PETERS, G.J., 2015b. c-Met as a Target for Personalized Therapy. *Translational oncogenomics,* **7**(Suppl 1), pp. 13-31.

GARRETT, T.P., MCKERN, N.M., LOU, M., ELLEMAN, T.C., ADAMS, T.E., LOVRECZ, G.O., KOFLER, M., JORISSEN, R.N., NICE, E.C., BURGESS, A.W. and WARD, C.W., 2003. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Molecular cell,* **11**(2), pp. 495-505.

GEORGIADOU, M., LILJA, J., JACQUEMET, G., GUZMAN, C., RAFAEVA, M., ALIBERT, C., YAN, Y., SAHGAL, P., LERCHE, M., MANNEVILLE, J.B., MAKELA, T.P. and IVASKA, J., 2017. AMPK negatively regulates tensin-dependent integrin activity. *The Journal of cell biology,* **216**(4), pp. 1107-1121.

GHOSH, P. and KORNFELD, S., 2003. Phosphorylation-induced conformational changes regulate GGAs 1 and 3 function at the trans-Golgi network. *The Journal of biological chemistry,* **278**(16), pp. 14543-14549.

GIRI, D.K., ALI-SEYED, M., LI, L.Y., LEE, D.F., LING, P., BARTHOLOMEUSZ, G., WANG, S.C. and HUNG, M.C., 2005. Endosomal transport of ErbB-2: mechanism for nuclear entry of the cell surface receptor. *Molecular and cellular biology,* **25**(24), pp. 11005-11018.

GOH, L.K., HUANG, F., KIM, W., GYGI, S. and SORKIN, A., 2010. Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. *The Journal of cell biology,* **189**(5), pp. 871-883.

GOLDENRING, J.R., 2015. Recycling endosomes. *Current opinion in cell biology,* **35**, pp. 117-122.

GORDEN, P., CARPENTIER, J.L., COHEN, S. and ORCI, L., 1978. Epidermal growth factor: morphological demonstration of binding, internalization, and lysosomal association in

human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America,* **75**(10), pp. 5025-5029.

GOUGH, R.E. and GOULT, B.T., 2018. The tale of two talins - two isoforms to fine-tune integrin signalling. *FEBS letters,* **592**(12), pp. 2108-2125.

GOVERO, J., DORAY, B., BAI, H. and KORNFELD, S., 2012a. Analysis of Gga null mice demonstrates a non-redundant role for mammalian GGA2 during development. *PloS one,* **7**(1), pp. e30184.

GOVERO, J., DORAY, B., BAI, H. and KORNFELD, S., 2012b. Analysis of Gga null mice demonstrates a non-redundant role for mammalian GGA2 during development. *PloS one,* **7**(1), pp. e30184.

GRANT, B.D. and DONALDSON, J.G., 2009. Pathways and mechanisms of endocytic recycling. *Nature reviews.Molecular cell biology,* **10**(9), pp. 597-608.

GU, Z., NOSS, E.H., HSU, V.W. and BRENNER, M.B., 2011. Integrins traffic rapidly via circular dorsal ruffles and macropinocytosis during stimulated cell migration. *The Journal of cell biology,* **193**(1), pp. 61-70.

HAMIDI, H. and IVASKA, J., 2018. Every step of the way: integrins in cancer progression and metastasis. *Nature reviews.Cancer,* .

HANAHAN, D. and WEINBERG, R.A., 2011. Hallmarks of cancer: the next generation. *Cell,* **144**(5), pp. 646-674.

HAQUE, A., ALAM, Q., ALAM, M.Z., AZHAR, E.I., SAIT, K.H., ANFINAN, N., MUSHTAQ, G., KAMAL, M.A. and RASOOL, M., 2016. Current Understanding of HSP90 as a Novel Therapeutic Target: An Emerging Approach for the Treatment of Cancer. *Current pharmaceutical design,* **22**(20), pp. 2947-2959.

HASLEKAS, C., BREEN, K., PEDERSEN, K.W., JOHANNESSEN, L.E., STANG, E. and MADSHUS, I.H., 2005. The inhibitory effect of ErbB2 on epidermal growth factor-induced formation of clathrin-coated pits correlates with retention of epidermal growth factor receptor-ErbB2 oligomeric complexes at the plasma membrane. *Molecular biology of the cell,* **16**(12), pp. 5832-5842.

HEGDE, S. and RAGHAVAN, S., 2013. A skin-depth analysis of integrins: role of the integrin network in health and disease. *Cell communication & adhesion,* **20**(6), pp. 155-169.

HENDRIKS, B.S., OPRESKO, L.K., WILEY, H.S. and LAUFFENBURGER, D., 2003. Coregulation of epidermal growth factor receptor/human epidermal growth factor receptor 2 (HER2) levels and locations: quantitative analysis of HER2 overexpression effects. *Cancer research,* **63**(5), pp. 1130-1137.

HENRIKSEN, L., GRANDAL, M.V., KNUDSEN, S.L., VAN DEURS, B. and GROVDAL, L.M., 2013. Internalization mechanisms of the epidermal growth factor receptor after activation with different ligands. *PloS one,* **8**(3), pp. e58148.

HIRST, J., BORNER, G.H., ANTROBUS, R., PEDEN, A.A., HODSON, N.A., SAHLENDER, D.A. and ROBINSON, M.S., 2012. Distinct and overlapping roles for AP-1 and GGAs revealed by the "knocksideways" system. *Current biology : CB,* **22**(18), pp. 1711-1716.

HIRST, J., LUI, W.W., BRIGHT, N.A., TOTTY, N., SEAMAN, M.N. and ROBINSON, M.S., 2000. A family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome. *The Journal of cell biology,* **149**(1), pp. 67-80.

HOMMELGAARD, A.M., LERDRUP, M. and VAN DEURS, B., 2004. Association with membrane protrusions makes ErbB2 an internalization-resistant receptor. *Molecular biology of the cell,* **15**(4), pp. 1557-1567.

HONG, K.S., JEON, E.Y., CHUNG, S.S., KIM, K.H. and LEE, R.A., 2018. Epidermal growth factor-mediated Rab25 pathway regulates integrin beta1 trafficking in colon cancer. *Cancer cell international,* **18**, pp. 32-018-0526-y. eCollection 2018.

HOWES, M.T., KIRKHAM, M., RICHES, J., CORTESE, K., WALSER, P.J., SIMPSON, F., HILL, M.M., JONES, A., LUNDMARK, R., LINDSAY, M.R., HERNANDEZ-DEVIEZ, D.J., HADZIC, G., MCCLUSKEY, A., BASHIR, R., LIU, L., PILCH, P., MCMAHON, H., ROBINSON, P.J., HANCOCK, J.F., MAYOR, S. and PARTON, R.G., 2010. Clathrin-independent carriers form a high capacity endocytic sorting system at the leading edge of migrating cells. *The Journal of cell biology,* **190**(4), pp. 675-691.

HO-YEN, C.M., JONES, J.L. and KERMORGANT, S., 2015. The clinical and functional significance of c-Met in breast cancer: a review. *Breast cancer research : BCR,* **17**, pp. 52-015-0547-6.

HUBBARD, S.R. and MILLER, W.T., 2007. Receptor tyrosine kinases: mechanisms of activation and signaling. *Current opinion in cell biology,* **19**(2), pp. 117-123.

HUGHES, J.B., RODLAND, M.S., HASMANN, M., MADSHUS, I.H. and STANG, E., 2012. Pertuzumab Increases 17-AAG-Induced Degradation of ErbB2, and This Effect Is Further Increased by Combining Pertuzumab with Trastuzumab. *Pharmaceuticals (Basel, Switzerland),* **5**(7), pp. 674-689.

HYNES, R.O., 2002a. Integrins: bidirectional, allosteric signaling machines. *Cell,* **110**(6), pp. 673-687.

HYNES, R.O., 2002b. Integrins: bidirectional, allosteric signaling machines. *Cell,* **110**(6), pp. 673-687.

HYNES, R.O., 2002c. Integrins: bidirectional, allosteric signaling machines. *Cell,* **110**(6), pp. 673-687.

HYNES, R.O., 2002d. Integrins: bidirectional, allosteric signaling machines. *Cell,* **110**(6), pp. 673-687.

IOANNOU, M.S., BELL, E.S., GIRARD, M., CHAINEAU, M., HAMLIN, J.N., DAUBARAS, M., MONAST, A., PARK, M., HODGSON, L. and MCPHERSON, P.S., 2015. DENND2B activates Rab13 at the leading edge of migrating cells and promotes metastatic behavior. *The Journal of cell biology,* **208**(5), pp. 629-648.

IOANNOU, M.S., KULASEKARAN, G., FOTOUHI, M., MOREIN, J.J., HAN, C., TSE, S., NOSSOVA, N., HAN, T., MANNARD, E. and MCPHERSON, P.S., 2017. Intersectin-s interaction with DENND2B facilitates recycling of epidermal growth factor receptor. *EMBO reports,* **18**(12), pp. 2119-2130.

IVASKA, J., VUORILUOTO, K., HUOVINEN, T., IZAWA, I., INAGAKI, M. and PARKER, P.J., 2005. PKCepsilon-mediated phosphorylation of vimentin controls integrin recycling and motility. *The EMBO journal,* **24**(22), pp. 3834-3845.

JACOBSEN, L., MADSEN, P., JACOBSEN, C., NIELSEN, M.S., GLIEMANN, J. and PETERSEN, C.M., 2001. Activation and functional characterization of the mosaic receptor SorLA/LR11. *The Journal of biological chemistry,* **276**(25), pp. 22788-22796.

JAKOBSEN, K.R., SORENSEN, E., BRONDUM, K.K., DAUGAARD, T.F., THOMSEN, R. and NIELSEN, A.L., 2013. Direct RNA sequencing mediated identification of mRNA localized in protrusions of human MDA-MB-231 metastatic breast cancer cells. *Journal of molecular signaling,* **8**(1), pp. 9-2187-8-9.

JIN, J.K., TIEN, P.C., CHENG, C.J., SONG, J.H., HUANG, C., LIN, S.H. and GALLICK, G.E., 2015. Talin1 phosphorylation activates beta1 integrins: a novel mechanism to promote prostate cancer bone metastasis. *Oncogene,* **34**(14), pp. 1811-1821.

JOFFRE, C., BARROW, R., MENARD, L., CALLEJA, V., HART, I.R. and KERMORGANT, S., 2011. A direct role for Met endocytosis in tumorigenesis. *Nature cell biology,* **13**(7), pp. 827-837.

JUNTTILA, T.T., AKITA, R.W., PARSONS, K., FIELDS, C., LEWIS PHILLIPS, G.D., FRIEDMAN, L.S., SAMPATH, D. and SLIWKOWSKI, M.X., 2009. Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941. *Cancer cell,* **15**(5), pp. 429-440.

KATZ, M., AMIT, I., CITRI, A., SHAY, T., CARVALHO, S., LAVI, S., MILANEZI, F., LYASS, L., AMARIGLIO, N., JACOB-HIRSCH, J., BEN-CHETRIT, N., TARCIC, G., LINDZEN, M., AVRAHAM, R., LIAO, Y.C., TRUSK, P., LYASS, A., RECHAVI, G., SPECTOR, N.L., LO, S.H., SCHMITT, F., BACUS, S.S. and YARDEN, Y., 2007. A reciprocal tensin-3-cten switch mediates EGF-driven mammary cell migration. *Nature cell biology,* **9**(8), pp. 961-969.

KERMORGANT, S. and PARKER, P.J., 2008. Receptor trafficking controls weak signal delivery: a strategy used by c-Met for STAT3 nuclear accumulation. *The Journal of cell biology,* **182**(5), pp. 855-863.

KERMORGANT, S., ZICHA, D. and PARKER, P.J., 2004. PKC controls HGF-dependent c-Met traffic, signalling and cell migration. *The EMBO journal,* **23**(19), pp. 3721-3734.

KJOS, I., VESTRE, K., GUADAGNO, N.A., BORG DISTEFANO, M. and PROGIDA, C., 2018. Rab and Arf proteins at the crossroad between membrane transport and cytoskeleton dynamics. *Biochimica et biophysica acta,* **1865**(10), pp. 1397-1409.

KOHLER, K., LOUVARD, D. and ZAHRAOUI, A., 2004. Rab13 regulates PKA signaling during tight junction assembly. *The Journal of cell biology,* **165**(2), pp. 175-180.

KONG-BELTRAN, M., SESHAGIRI, S., ZHA, J., ZHU, W., BHAWE, K., MENDOZA, N., HOLCOMB, T., PUJARA, K., STINSON, J., FU, L., SEVERIN, C., RANGELL, L., SCHWALL, R., AMLER, L., WICKRAMASINGHE, D. and YAUCH, R., 2006. Somatic mutations lead to an oncogenic

deletion of met in lung cancer. *Cancer research,* **66**(1), pp. 283-289.

KRISHNAMURTI, U. and SILVERMAN, J.F., 2014. HER2 in breast cancer: a review and update. *Advances in Anatomic Pathology,* **21**(2), pp. 100-107.

LAM, S.S., MARTELL, J.D., KAMER, K.J., DEERINCK, T.J., ELLISMAN, M.H., MOOTHA, V.K. and TING, A.Y., 2015. Directed evolution of APEX2 for electron microscopy and proximity labeling. *Nature methods,* **12**(1), pp. 51-54.

LAMPE, M., VASSILOPOULOS, S. and MERRIFIELD, C., 2016. Clathrin coated pits, plaques and adhesion. *Journal of structural biology,* **196**(1), pp. 48-56.

LANGEMEYER, L., FROHLICH, F. and UNGERMANN, C., 2018. Rab GTPase Function in Endosome and Lysosome Biogenesis. *Trends in cell biology,* .

LEE, J.W., SOUNG, Y.H., SEO, S.H., KIM, S.Y., PARK, C.H., WANG, Y.P., PARK, K., NAM, S.W., PARK, W.S., KIM, S.H., LEE, J.Y., YOO, N.J. and LEE, S.H., 2006. Somatic mutations of ERBB2 kinase domain in gastric, colorectal, and breast carcinomas. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **12**(1), pp. 57-61.

LEMMON, M.A. and SCHLESSINGER, J., 2010. Cell signaling by receptor tyrosine kinases. *Cell,* **141**(7), pp. 1117-1134.

LENFERINK, A.E., PINKAS-KRAMARSKI, R., VAN DE POLL, M.L., VAN VUGT, M.J., KLAPPER, L.N., TZAHAR, E., WATERMAN, H., SELA, M., VAN ZOELEN, E.J. and YARDEN, Y., 1998. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. *The EMBO journal,* **17**(12), pp. 3385-3397.

LERDRUP, M., BRUUN, S., GRANDAL, M.V., ROEPSTORFF, K., KRISTENSEN, M.M., HOMMELGAARD, A.M. and VAN DEURS, B., 2007. Endocytic down-regulation of ErbB2 is stimulated by cleavage of its C-terminus. *Molecular biology of the cell,* **18**(9), pp. 3656-3666.

LERDRUP, M., HOMMELGAARD, A.M., GRANDAL, M. and VAN DEURS, B., 2006. Geldanamycin stimulates internalization of ErbB2 in a proteasome-dependent way. *Journal of cell science,* **119**(Pt 1), pp. 85-95.

LI, N., LORINCZI, M., IRETON, K. and ELFERINK, L.A., 2007. Specific Grb2-mediated interactions regulate clathrin-dependent endocytosis of the cMet-tyrosine kinase. *The Journal of biological chemistry,* **282**(23), pp. 16764-16775.

LI, W., LI, K., ZHAO, L. and ZOU, H., 2014. Bioinformatics analysis reveals disturbance mechanism of MAPK signaling pathway and cell cycle in Glioblastoma multiforme. *Gene,* **547**(2), pp. 346-350.

LILJA, J., ZACHARCHENKO, T., GEORGIADOU, M., JACQUEMET, G., DE FRANCESCHI, N., PEUHU, E., HAMIDI, H., POUWELS, J., MARTENS, V., NIA, F.H., BEIFUSS, M., BOECKERS, T., KREIENKAMP, H.J., BARSUKOV, I.L. and IVASKA, J., 2017. SHANK proteins limit integrin activation by directly interacting with Rap1 and R-Ras. *Nature cell biology,* **19**(4), pp. 292-305.

LIM, J.P. and GLEESON, P.A., 2011. Macropinocytosis: an endocytic pathway for internalising large gulps. *Immunology and cell biology,* **89**(8), pp. 836-843.

LO, S.H., 2014a. C-terminal tensin-like (CTEN): a promising biomarker and target for cancer. *The international journal of biochemistry & cell biology,* **51**, pp. 150-154.

LO, S.H., 2014b. C-terminal tensin-like (CTEN): a promising biomarker and target for cancer. *The international journal of biochemistry & cell biology,* **51**, pp. 150-154.

LONGVA, K.E., BLYSTAD, F.D., STANG, E., LARSEN, A.M., JOHANNESSEN, L.E. and MADSHUS, I.H., 2002. Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. *The Journal of cell biology,* **156**(5), pp. 843-854.

LONGVA, K.E., PEDERSEN, N.M., HASLEKAS, C., STANG, E. and MADSHUS, I.H., 2005. Herceptin-induced inhibition of ErbB2 signaling involves reduced phosphorylation of Akt but not endocytic down-regulation of ErbB2. *International journal of cancer,* **116**(3), pp. 359-367.

LUO, B.H., CARMAN, C.V. and SPRINGER, T.A., 2007. Structural basis of integrin regulation and signaling. *Annual Review of Immunology,* **25**, pp. 619-647.

LUO, B.H. and SPRINGER, T.A., 2006. Integrin structures and conformational signaling. *Current opinion in cell biology,* **18**(5), pp. 579-586.

LV, Q., MENG, Z., YU, Y., JIANG, F., GUAN, D., LIANG, C., ZHOU, J., LU, A. and ZHANG, G., 2016.

Molecular Mechanisms and Translational Therapies for Human Epidermal Receptor 2 Positive Breast Cancer. *International journal of molecular sciences,* **17**(12), pp. 10.3390/ijms17122095.

MAI, A., VELTEL, S., PELLINEN, T., PADZIK, A., COFFEY, E., MARJOMAKI, V. and IVASKA, J., 2011. Competitive binding of Rab21 and p120RasGAP to integrins regulates receptor traffic and migration. *The Journal of cell biology,* **194**(2), pp. 291-306.

MAIB, H., SMYTHE, E. and AYSCOUGH, K., 2017. Forty years on: clathrin-coated pits continue to fascinate. *Molecular biology of the cell,* **28**(7), pp. 843-847.

MAIER, L.A., XU, F.J., HESTER, S., BOYER, C.M., MCKENZIE, S., BRUSKIN, A.M., ARGON, Y. and BAST, R.C.,Jr, 1991. Requirements for the internalization of a murine monoclonal antibody directed against the HER-2/neu gene product c-erbB-2. *Cancer research,* **51**(19), pp. 5361-5369.

MARGADANT, C., MONSUUR, H.N., NORMAN, J.C. and SONNENBERG, A., 2011. Mechanisms of integrin activation and trafficking. *Current opinion in cell biology,* **23**(5), pp. 607-614.

MARITZEN, T., SCHACHTNER, H. and LEGLER, D.F., 2015. On the move: endocytic trafficking in cell migration. *Cellular and molecular life sciences : CMLS,* **72**(11), pp. 2119-2134.

MAROUN, C.R. and ROWLANDS, T., 2014. The Met receptor tyrosine kinase: a key player in oncogenesis and drug resistance. *Pharmacology & therapeutics,* **142**(3), pp. 316-338.

MAYOR, S. and PAGANO, R.E., 2007a. Pathways of clathrin-independent endocytosis. *Nature reviews.Molecular cell biology,* **8**(8), pp. 603-612.

MAYOR, S. and PAGANO, R.E., 2007b. Pathways of clathrin-independent endocytosis. *Nature reviews.Molecular cell biology,* **8**(8), pp. 603-612.

MAZIVEYI, M. and ALAHARI, S.K., 2017. Cell matrix adhesions in cancer: The proteins that form the glue. *Oncotarget,* **8**(29), pp. 48471-48487.

MCNALLY, K.E., FAULKNER, R., STEINBERG, F., GALLON, M., GHAI, R., PIM, D., LANGTON, P., PEARSON, N., DANSON, C.M., NAGELE, H., MORRIS, L.L., SINGLA, A., OVERLEE, B.L., HEESOM, K.J., SESSIONS, R., BANKS, L., COLLINS, B.M., BERGER, I., BILLADEAU, D.D., BURSTEIN, E. and CULLEN, P.J., 2017. Retriever is a multiprotein complex for retromer-independent endosomal cargo recycling. *Nature cell biology,* **19**(10), pp. 1214-1225.

MELNIKOV, S. and SAGI-EISENBERG, R., 2009. Down-regulating protein kinase C alpha: functional cooperation between the proteasome and the endocytic system. *Cellular signalling,* **21**(11), pp. 1607-1619.

MO, W., ZHANG, J., LI, X., MENG, D., GAO, Y., YANG, S., WAN, X., ZHOU, C., GUO, F., HUANG, Y., AMENTE, S., AVVEDIMENTO, E.V., XIE, Y. and LI, Y., 2013. Identification of novel AR-targeted microRNAs mediating androgen signalling through critical pathways to regulate cell viability in prostate cancer. *PloS one,* **8**(2), pp. e56592.

MOLINA, M.A., CODONY-SERVAT, J., ALBANELL, J., ROJO, F., ARRIBAS, J. and BASELGA, J., 2001. Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer research,* **61**(12), pp. 4744-4749.

MORGAN, M.R., HAMIDI, H., BASS, M.D., WARWOOD, S., BALLESTREM, C. and HUMPHRIES, M.J., 2013. Syndecan-4 phosphorylation is a control point for integrin recycling. *Developmental cell,* **24**(5), pp. 472-485.

MORIMOTO, S., NISHIMURA, N., TERAI, T., MANABE, S., YAMAMOTO, Y., SHINAHARA, W., MIYAKE, H., TASHIRO, S., SHIMADA, M. and SASAKI, T., 2005. Rab13 mediates the continuous endocytic recycling of occludin to the cell surface. *The Journal of biological chemistry,* **280**(3), pp. 2220-2228.

MULLER, P.A., TRINIDAD, A.G., TIMPSON, P., MORTON, J.P., ZANIVAN, S., VAN DEN BERGHE, P.V., NIXON, C., KARIM, S.A., CASWELL, P.T., NOLL, J.E., COFFILL, C.R., LANE, D.P., SANSOM, O.J., NEILSEN, P.M., NORMAN, J.C. and VOUSDEN, K.H., 2013. Mutant p53 enhances MET trafficking and signalling to drive cell scattering and invasion. *Oncogene,* **32**(10), pp. 1252-1265.

NADER, G.P., EZRATTY, E.J. and GUNDERSEN, G.G., 2016. FAK, talin and PIPKIgamma regulate endocytosed integrin activation to polarize focal adhesion assembly. *Nature cell biology,* **18**(5), pp. 491-503.

NAGY, P., VEREB, G., SEBESTYEN, Z., HORVATH, G., LOCKETT, S.J., DAMJANOVICH, S., PARK, J.W., JOVIN, T.M. and SZOLLOSI, J., 2002. Lipid rafts and the local density of ErbB proteins influence the biological role of homo- and heteroassociations of ErbB2. *Journal of cell science,* **115**(Pt 22), pp. 4251-4262.

NAHTA, R., HUNG, M.C. and ESTEVA, F.J., 2004. The HER-2-targeting antibodies trastuzumab and pertuzumab synergistically inhibit the survival of breast cancer cells. *Cancer research,* **64**(7), pp. 2343-2346.

NAKAYAMA, K. and WAKATSUKI, S., 2003. The structure and function of GGAs, the traffic controllers at the TGN sorting crossroads. *Cell structure and function,* **28**(5), pp. 431-442.

NG, E.L., GAN, B.Q., NG, F. and TANG, B.L., 2012. Rab GTPases regulating receptor trafficking at the late endosome-lysosome membranes. *Cell biochemistry and function,* **30**(6), pp. 515-523.

NISHIKIMI, A., ISHIHARA, S., OZAWA, M., ETOH, K., FUKUDA, M., KINASHI, T. and KATAGIRI, K., 2014. Rab13 acts downstream of the kinase Mst1 to deliver the integrin LFA-1 to the cell surface for lymphocyte trafficking. *Science signaling,* **7**(336), pp. ra72.

NOKES, R.L., FIELDS, I.C., COLLINS, R.N. and FOLSCH, H., 2008. Rab13 regulates membrane trafficking between TGN and recycling endosomes in polarized epithelial cells. *The Journal of cell biology,* **182**(5), pp. 845-853.

ONITILO, A.A., ENGEL, J.M., GREENLEE, R.T. and MUKESH, B.N., 2009. Breast cancer subtypes based on ER/PR and Her2 expression: comparison of clinicopathologic features and survival. *Clinical medicine & research,* **7**(1-2), pp. 4-13.

OSBORNE, D.G., PHILLIPS-KRAWCZAK, C.A. and BILLADEAU, D.D., 2015. Monitoring receptor trafficking following retromer and WASH deregulation. *Methods in cell biology,* **130**, pp. 199-213.

PARACHONIAK, C.A., LUO, Y., ABELLA, J.V., KEEN, J.H. and PARK, M., 2011a. GGA3 functions as a switch to promote Met receptor recycling, essential for sustained ERK and cell migration. *Developmental cell,* **20**(6), pp. 751-763.

PARACHONIAK, C.A., LUO, Y., ABELLA, J.V., KEEN, J.H. and PARK, M., 2011b. GGA3 functions as a switch to promote Met receptor recycling, essential for sustained ERK and cell migration. *Developmental cell,* **20**(6), pp. 751-763.

PARACHONIAK, C.A., LUO, Y., ABELLA, J.V., KEEN, J.H. and PARK, M., 2011c. GGA3 functions as a switch to promote Met receptor recycling, essential for sustained ERK and cell migration. *Developmental cell,* **20**(6), pp. 751-763.

PARACHONIAK, C.A. and PARK, M., 2012. Dynamics of receptor trafficking in tumorigenicity. *Trends in cell biology,* **22**(5), pp. 231-240.

PARK, M., DEAN, M., KAUL, K., BRAUN, M.J., GONDA, M.A. and VANDE WOUDE, G., 1987. Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. *Proceedings of the National Academy of Sciences of the United States of America,* **84**(18), pp. 6379-6383.

PARK, S.Y. and GUO, X., 2014. Adaptor protein complexes and intracellular transport. *Bioscience reports,* **34**(4), pp. 10.1042/BSR20140069.

PAUL, N.R., JACQUEMET, G. and CASWELL, P.T., 2015. Endocytic Trafficking of Integrins in Cell Migration. *Current biology : CB,* **25**(22), pp. R1092-105.

PELLINEN, T., TUOMI, S., ARJONEN, A., WOLF, M., EDGREN, H., MEYER, H., GROSSE, R., KITZING, T., RANTALA, J.K., KALLIONIEMI, O., FASSLER, R., KALLIO, M. and IVASKA, J., 2008. Integrin trafficking regulated by Rab21 is necessary for cytokinesis. *Developmental cell,* **15**(3), pp. 371-385.

PETERSEN, N.H., OLSEN, O.D., GROTH-PEDERSEN, L., ELLEGAARD, A.M., BILGIN, M., REDMER, S., OSTENFELD, M.S., ULANET, D., DOVMARK, T.H., LONBORG, A., VINDELOV, S.D., HANAHAN, D., ARENZ, C., EJSING, C.S., KIRKEGAARD, T., ROHDE, M., NYLANDSTED, J. and JAATTELA, M., 2013. Transformation-associated changes in sphingolipid metabolism sensitize cells to lysosomal cell death induced by inhibitors of acid sphingomyelinase. *Cancer cell,* **24**(3), pp. 379-393.

POTELLE, S., KLEIN, A. and FOULQUIER, F., 2015. Golgi post-translational modifications and associated diseases. *Journal of inherited metabolic disease,* **38**(4), pp. 741-751.

POWELKA, A.M., SUN, J., LI, J., GAO, M., SHAW, L.M., SONNENBERG, A. and HSU, V.W., 2004a. Stimulation-dependent recycling of integrin

beta1 regulated by ARF6 and Rab11. *Traffic (Copenhagen, Denmark),* **5**(1), pp. 20-36.

POWELKA, A.M., SUN, J., LI, J., GAO, M., SHAW, L.M., SONNENBERG, A. and HSU, V.W., 2004b. Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. *Traffic (Copenhagen, Denmark),* **5**(1), pp. 20-36.

PUERTOLLANO, R. and BONIFACINO, J.S., 2004. Interactions of GGA3 with the ubiquitin sorting machinery. *Nature cell biology,* **6**(3), pp. 244-251.

PUST, S., KLOKK, T.I., MUSA, N., JENSTAD, M., RISBERG, B., ERIKSTEIN, B., TCATCHOFF, L., LIESTOL, K., DANIELSEN, H.E., VAN DEURS, B. and SANDVIG, K., 2013. Flotillins as regulators of ErbB2 levels in breast cancer. *Oncogene,* **32**(29), pp. 3443-3451.

RANTALA, J.K., POUWELS, J., PELLINEN, T., VELTEL, S., LAASOLA, P., MATTILA, E., POTTER, C.S., DUFFY, T., SUNDBERG, J.P., KALLIONIEMI, O., ASKARI, J.A., HUMPHRIES, M.J., PARSONS, M., SALMI, M. and IVASKA, J., 2011. SHARPIN is an endogenous inhibitor of beta1-integrin activation. *Nature cell biology,* **13**(11), pp. 1315-1324.

RATCLIFFE, C.D., SAHGAL, P., PARACHONIAK, C.A., IVASKA, J. and PARK, M., 2016. Regulation of Cell Migration and beta1 Integrin Trafficking by the Endosomal Adaptor GGA3. *Traffic (Copenhagen, Denmark),* **17**(6), pp. 670-688.

REDIG, A.J. and MCALLISTER, S.S., 2013. Breast cancer as a systemic disease: a view of metastasis. *Journal of internal medicine,* **274**(2), pp. 113-126.

RINK, J., GHIGO, E., KALAIDZIDIS, Y. and ZERIAL, M., 2005. Rab conversion as a mechanism of progression from early to late endosomes. *Cell,* **122**(5), pp. 735-749.

ROBERTS, M., BARRY, S., WOODS, A., VAN DER SLUIJS, P. and NORMAN, J., 2001. PDGF-regulated rab4-dependent recycling of alphavbeta3 integrin from early endosomes is necessary for cell adhesion and spreading. *Current biology : CB,* **11**(18), pp. 1392-1402.

ROEPSTORFF, K., GRANDAL, M.V., HENRIKSEN, L., KNUDSEN, S.L., LERDRUP, M., GROVDAL, L., WILLUMSEN, B.M. and VAN DEURS, B., 2009. Differential effects of EGFR ligands on endocytic sorting of the receptor. *Traffic (Copenhagen, Denmark),* **10**(8), pp. 1115-1127.

ROUX, K.J., KIM, D.I., RAIDA, M. and BURKE, B., 2012a. A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *The Journal of cell biology,* **196**(6), pp. 801-810.

ROUX, K.J., KIM, D.I., RAIDA, M. and BURKE, B., 2012b. A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *The Journal of cell biology,* **196**(6), pp. 801-810.

SAKASHITA, K., MIMORI, K., TANAKA, F., KAMOHARA, Y., INOUE, H., SAWADA, T., HIRAKAWA, K. and MORI, M., 2008. Prognostic relevance of Tensin4 expression in human gastric cancer. *Annals of surgical oncology,* **15**(9), pp. 2606-2613.

SANDRI, C., CACCAVARI, F., VALDEMBRI, D., CAMILLO, C., VELTEL, S., SANTAMBROGIO, M., LANZETTI, L., BUSSOLINO, F., IVASKA, J. and SERINI, G., 2012. The R-Ras/RIN2/Rab5 complex controls endothelial cell adhesion and morphogenesis via active integrin endocytosis and Rac signaling. *Cell research,* **22**(10), pp. 1479-1501.

SAROSIEK, T. and MORAWSKI, P., 2018. Trastuzumab and its biosimilars. *Polski merkuriusz lekarski : organ Polskiego Towarzystwa Lekarskiego,* **44**(263), pp. 253-257.

SATHE, M., MUTHUKRISHNAN, G., RAE, J., DISANZA, A., THATTAI, M., SCITA, G., PARTON, R.G. and MAYOR, S., 2018. Small GTPases and BAR domain proteins regulate branched actin polymerisation for clathrin and dynamin-independent endocytosis. *Nature communications,* **9**(1), pp. 1835-018-03955-w.

SCHMIDT, V., SUBKHANGULOVA, A. and WILLNOW, T.E., 2017a. Sorting receptor SORLA: cellular mechanisms and implications for disease. *Cellular and molecular life sciences : CMLS,* **74**(8), pp. 1475-1483.

SCHMIDT, V., SUBKHANGULOVA, A. and WILLNOW, T.E., 2017b. Sorting receptor SORLA: cellular mechanisms and implications for disease. *Cellular and molecular life sciences : CMLS,* **74**(8), pp. 1475-1483.

SCOTT, C.C., VACCA, F. and GRUENBERG, J., 2014. Endosome maturation, transport and functions. *Seminars in cell & developmental biology,* **31**, pp. 2-10.

SCOTT, P.M., BILODEAU, P.S., ZHDANKINA, O., WINISTORFER, S.C., HAUGLUND, M.J., ALLAMAN, M.M., KEARNEY, W.R., ROBERTSON,

A.D., BOMAN, A.L. and PIPER, R.C., 2004. GGA proteins bind ubiquitin to facilitate sorting at the trans-Golgi network. *Nature cell biology,* **6**(3), pp. 252-259.

SETTEMBRE, C., FRALDI, A., MEDINA, D.L. and BALLABIO, A., 2013. Signals from the lysosome: a control centre for cellular clearance and energy metabolism. *Nature reviews.Molecular cell biology,* **14**(5), pp. 283-296.

SHATTIL, S.J., KIM, C. and GINSBERG, M.H., 2010. The final steps of integrin activation: the end game. *Nature reviews.Molecular cell biology,* **11**(4), pp. 288-300.

SHEN, F., LIN, Q., CHILDRESS, C. and YANG, W., 2008. Identification of the domain in ErbB2 that restricts ligand-induced degradation. *Cellular signalling,* **20**(4), pp. 779-786.

SHI, F. and SOTTILE, J., 2008. Caveolin-1-dependent beta1 integrin endocytosis is a critical regulator of fibronectin turnover. *Journal of cell science,* **121**(Pt 14), pp. 2360-2371.

SHIBA, Y., KATOH, Y., SHIBA, T., YOSHINO, K., TAKATSU, H., KOBAYASHI, H., SHIN, H.W., WAKATSUKI, S. and NAKAYAMA, K., 2004. GAT (GGA and Tom1) domain responsible for ubiquitin binding and ubiquitination. *The Journal of biological chemistry,* **279**(8), pp. 7105-7111.

SIGISMUND, S., ALGISI, V., NAPPO, G., CONTE, A., PASCOLUTTI, R., CUOMO, A., BONALDI, T., ARGENZIO, E., VERHOEF, L.G., MASPERO, E., BIANCHI, F., CAPUANI, F., CILIBERTO, A., POLO, S. and DI FIORE, P.P., 2013. Threshold-controlled ubiquitination of the EGFR directs receptor fate. *The EMBO journal,* **32**(15), pp. 2140-2157.

SIGISMUND, S., AVANZATO, D. and LANZETTI, L., 2018. Emerging functions of the EGFR in cancer. *Molecular oncology,* **12**(1), pp. 3-20.

SIMONSEN, A., WURMSER, A.E., EMR, S.D. and STENMARK, H., 2001. The role of phosphoinositides in membrane transport. *Current opinion in cell biology,* **13**(4), pp. 485-492.

SINGH, B., CARPENTER, G. and COFFEY, R.J., 2016. EGF receptor ligands: recent advances. *F1000Research,* **5**, pp. 10.12688/f1000research.9025.1. eCollection 2016.

SINGLETON, P.A., SALGIA, R., MORENO-VINASCO, L., MOITRA, J., SAMMANI, S., MIRZAPOIAZOVA, T. and GARCIA, J.G., 2007. CD44 regulates hepatocyte growth factor-mediated vascular integrity. Role of c-Met, Tiam1/Rac1, dynamin 2, and cortactin. *The Journal of biological chemistry,* **282**(42), pp. 30643-30657.

SMITH, J.R., DE BILLY, E., HOBBS, S., POWERS, M., PRODROMOU, C., PEARL, L., CLARKE, P.A. and WORKMAN, P., 2015. Restricting direct interaction of CDC37 with HSP90 does not compromise chaperoning of client proteins. *Oncogene,* **34**(1), pp. 15-26.

SMITH, J.R. and WORKMAN, P., 2009. Targeting CDC37: an alternative, kinase-directed strategy for disruption of oncogenic chaperoning. *Cell cycle (Georgetown, Tex.),* **8**(3), pp. 362-372.

SORKIN, A., DI FIORE, P.P. and CARPENTER, G., 1993. The carboxyl terminus of epidermal growth factor receptor/erbB-2 chimerae is internalization impaired. *Oncogene,* **8**(11), pp. 3021-3028.

SORKIN, A. and GOH, L.K., 2009. Endocytosis and intracellular trafficking of ErbBs. *Experimental cell research,* **315**(4), pp. 683-696.

SPERINDE, J., HUANG, W., VEHTARI, A., CHENNA, A., KELLOKUMPU-LEHTINEN, P.L., WINSLOW, J., BONO, P., LIE, Y.S., PETROPOULOS, C.J., WEIDLER, J. and JOENSUU, H., 2018. p95HER2 Methionine 611 Carboxy-Terminal Fragment Is Predictive of Trastuzumab Adjuvant Treatment Benefit in the FinHer Trial. *Clinical cancer research : an official journal of the American Association for Cancer Research,* **24**(13), pp. 3046-3052.

STEINBERG, F., HEESOM, K.J., BASS, M.D. and CULLEN, P.J., 2012. SNX17 protects integrins from degradation by sorting between lysosomal and recycling pathways. *The Journal of cell biology,* **197**(2), pp. 219-230.

STENMARK, H., 2009. Rab GTPases as coordinators of vesicle traffic. *Nature reviews.Molecular cell biology,* **10**(8), pp. 513-525.

TAI, W., MAHATO, R. and CHENG, K., 2010. The role of HER2 in cancer therapy and targeted drug delivery. *Journal of controlled release : official journal of the Controlled Release Society,* **146**(3), pp. 264-275.

TANG, Q., ONITSUKA, M., TABATA, A., TOMOYASU, T. and NAGAMUNE, H., 2018. Construction of Anti-HER2 Recombinants as Targeting Modules for a Drug-delivery System Against HER2-

positive Cells. *Anticancer Research,* **38**(7), pp. 4319-4325.

TIKHOMIROV, O. and CARPENTER, G., 2003. Identification of ErbB-2 kinase domain motifs required for geldanamycin-induced degradation. *Cancer research,* **63**(1), pp. 39-43.

TRAUB, L.M. and BONIFACINO, J.S., 2013. Cargo recognition in clathrin-mediated endocytosis. *Cold Spring Harbor perspectives in biology,* **5**(11), pp. a016790.

TRUSOLINO, L., BERTOTTI, A. and COMOGLIO, P.M., 2010. MET signalling: principles and functions in development, organ regeneration and cancer. *Nature reviews.Molecular cell biology,* **11**(12), pp. 834-848.

TZENG, H.T. and WANG, Y.C., 2016. Rab-mediated vesicle trafficking in cancer. *Journal of Biomedical Science,* **23**(1), pp. 70-016-0287-7.

UEMURA, T., KAMETAKA, S. and WAGURI, S., 2018. GGA2 interacts with EGFR cytoplasmic domain to stabilize the receptor expression and promote cell growth. *Scientific reports,* **8**(1), pp. 1368-018-19542-4.

VALDEMBRI, D. and SERINI, G., 2012. Regulation of adhesion site dynamics by integrin traffic. *Current opinion in cell biology,* **24**(5), pp. 582-591.

VAN MEER, G., VOELKER, D.R. and FEIGENSON, G.W., 2008. Membrane lipids: where they are and how they behave. *Nature reviews.Molecular cell biology,* **9**(2), pp. 112-124.

VETTER, I.R. and WITTINGHOFER, A., 2001. The guanine nucleotide-binding switch in three dimensions. *Science (New York, N.Y.),* **294**(5545), pp. 1299-1304.

VITICCHIE, G. and MULLER, P.A.J., 2015. c-Met and Other Cell Surface Molecules: Interaction, Activation and Functional Consequences. *Biomedicines,* **3**(1), pp. 46-70.

VON EINEM, B., WAHLER, A., SCHIPS, T., SERRANO-POZO, A., PROEPPER, C., BOECKERS, T.M., RUECK, A., WIRTH, T., HYMAN, B.T., DANZER, K.M., THAL, D.R. and VON ARNIM, C.A., 2015. The Golgi-Localized gamma-Ear-Containing ARF-Binding (GGA) Proteins Alter Amyloid-beta Precursor Protein (APP) Processing through Interaction of Their GAE Domain with the Beta-Site APP Cleaving Enzyme 1 (BACE1). *PloS one,* **10**(6), pp. e0129047.

WANG, S., HU, C., WU, F. and HE, S., 2017. Rab25 GTPase: Functional roles in cancer. *Oncotarget,* **8**(38), pp. 64591-64599.

WANG, T., LIU, N.S., SEET, L.F. and HONG, W., 2010. The emerging role of VHS domain-containing Tom1, Tom1L1 and Tom1L2 in membrane trafficking. *Traffic (Copenhagen, Denmark),* **11**(9), pp. 1119-1128.

WANG, X., SONG, N., ZHANG, Y., CAI, Y., LIU, Y., QU, X., LI, Z., LI, D., HOU, K., KANG, J. and HU, X., 2015. Coexpression of c-Met and Notch-1 correlates with poor prognosis in resected non-small-cell lung cancer. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine,* **36**(9), pp. 7053-7059.

WANG, Y., ARJONEN, A., POUWELS, J., TA, H., PAUSCH, P., BANGE, G., ENGEL, U., PAN, X., FACKLER, O.T., IVASKA, J. and GROSSE, R., 2015. Formin-like 2 Promotes beta1-Integrin Trafficking and Invasive Motility Downstream of PKCalpha. *Developmental cell,* **34**(4), pp. 475-483.

WATSON, J., AREY, B.J. and ALT, A., 2014. *Chapter 5 - Biasing Receptor Tyrosine Kinase Signaling Pathways.* San Diego: Academic Press.

WILEY, H.S. and BURKE, P.M., 2001. Regulation of receptor tyrosine kinase signaling by endocytic trafficking. *Traffic (Copenhagen, Denmark),* **2**(1), pp. 12-18.

WILLNOW, T.E. and ANDERSEN, O.M., 2013. Sorting receptor SORLA--a trafficking path to avoid Alzheimer disease. *Journal of cell science,* **126**(Pt 13), pp. 2751-2760.

WILSON, B.J., ALLEN, J.L. and CASWELL, P.T., 2018. Vesicle trafficking pathways that direct cell migration in 3D and in vivo. *Traffic (Copenhagen, Denmark),* .

WINOGRAD-KATZ, S.E., FASSLER, R., GEIGER, B. and LEGATE, K.R., 2014a. The integrin adhesome: from genes and proteins to human disease. *Nature reviews.Molecular cell biology,* **15**(4), pp. 273-288.

WINOGRAD-KATZ, S.E., FASSLER, R., GEIGER, B. and LEGATE, K.R., 2014b. The integrin adhesome: from genes and proteins to human disease. *Nature reviews.Molecular cell biology,* **15**(4), pp. 273-288.

WOLFENSON, H., BERSHADSKY, A., HENIS, Y.I. and GEIGER, B., 2011. Actomyosin-generated tension controls the molecular kinetics of focal

adhesions. *Journal of cell science,* **124**(Pt 9), pp. 1425-1432.

WOODS, A.J., WHITE, D.P., CASWELL, P.T. and NORMAN, J.C., 2004. PKD1/PKCmu promotes alphavbeta3 integrin recycling and delivery to nascent focal adhesions. *The EMBO journal,* **23**(13), pp. 2531-2543.

XIONG, J., BALCIOGLU, H.E. and DANEN, E.H., 2013. Integrin signaling in control of tumor growth and progression. *The international journal of biochemistry & cell biology,* **45**(5), pp. 1012-1015.

XU, W., MARCU, M., YUAN, X., MIMNAUGH, E., PATTERSON, C. and NECKERS, L., 2002. Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proceedings of the National Academy of Sciences of the United States of America,* **99**(20), pp. 12847-12852.

XU, W., YUAN, X., BEEBE, K., XIANG, Z. and NECKERS, L., 2007. Loss of Hsp90 association up-regulates Src-dependent ErbB2 activity. *Molecular and cellular biology,* **27**(1), pp. 220-228.

YARDEN, Y. and SLIWKOWSKI, M.X., 2001. Untangling the ErbB signalling network. *Nature reviews.Molecular cell biology,* **2**(2), pp. 127-137.

YE, F., SNIDER, A.K. and GINSBERG, M.H., 2014. Talin and kindlin: the one-two punch in integrin activation. *Frontiers of medicine,* **8**(1), pp. 6-16.

YEAGLE, P.L., 2016. *Chapter 15 - Membrane Receptors.* Boston: Academic Press.

ZAIDEL-BAR, R., ITZKOVITZ, S., MA'AYAN, A., IYENGAR, R. and GEIGER, B., 2007. Functional atlas of the integrin adhesome. *Nature cell biology,* **9**(8), pp. 858-867.

ZHANG, J. and BABIC, A., 2016. Regulation of the MET oncogene: molecular mechanisms. *Carcinogenesis,* **37**(4), pp. 345-355.

ZHANG, Y.W., WANG, L.M., JOVE, R. and VANDE WOUDE, G.F., 2002. Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. *Oncogene,* **21**(2), pp. 217-226.

ZHAO, Y. and KEEN, J.H., 2008. Gyrating clathrin: highly dynamic clathrin structures involved in rapid receptor recycling. *Traffic (Copenhagen, Denmark),* **9**(12), pp. 2253-2264.

ZHEN, Y. and STENMARK, H., 2015. Cellular functions of Rab GTPases at a glance. *Journal of cell science,* **128**(17), pp. 3171-3176.

ZHU, G., HE, X., ZHAI, P., TERZYAN, S., TANG, J. and ZHANG, X.C., 2003. Crystal structure of GGA2 VHS domain and its implication in plasticity in the ligand binding pocket. *FEBS letters,* **537**(1-3), pp. 171-176.

ZHU, G., ZHAI, P., WAKEHAM, N., HE, X. and ZHANG, X.C., 2005. Analysis of the interaction between GGA1 GAT domain and Rabaptin-5. *Methods in enzymology,* **403**, pp. 583-592.

ZHU, J., ZHU, J. and SPRINGER, T.A., 2013. Complete integrin headpiece opening in eight steps. *The Journal of cell biology,* **201**(7), pp. 1053-1068.

ZHU, L., YANG, J., BROMBERGER, T., HOLLY, A., LU, F., LIU, H., SUN, K., KLAPPROTH, S., HIRBAWI, J., BYZOVA, T.V., PLOW, E.F., MOSER, M. and QIN, J., 2017. Structure of Rap1b bound to talin reveals a pathway for triggering integrin activation. *Nature communications,* **8**(1), pp. 1744-017-01822-8.

ZHU, W., OKOLLIE, B. and ARTEMOV, D., 2007. Controlled internalization of Her-2/ neu receptors by cross-linking for targeted delivery. *Cancer biology & therapy,* **6**(12), pp. 1960-1966.

ZHUANG, X.P., JIN, W.W., TENG, X.D., YUAN, Z.Z., LIN, Q.Q. and XU, S.T., 2015. c-Met and RON expression levels in endometrial adenocarcinoma tissue and their relationship with prognosis. *European journal of gynaecological oncology,* **36**(3), pp. 255-259.

ZWICK, E., BANGE, J. and ULLRICH, A., 2001. Receptor tyrosine kinase signalling as a target for cancer intervention strategies. *Endocrine-related cancer,* **8**(3), pp. 161-173.

Annales Universitatis Turkuensis

Turun yliopisto
University of Turku

Grano oy, Turku , Finland 2018

ISBN 978-951-29-7472-6 (PRINT)
ISBN 978-951-29-7473-3 (PDF)
ISSN 0355-9483 (Print) ISSN 2343-3213 (Online)