DLC3 suppresses MT1-MMP-dependent matrix degradation by controlling RhoB and actin remodeling at endosomal membranes

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Abbreviations

Akt	protein kinase B
AJ	adherens junctions
GDP	guanosine diphosphat
GTP	guanosine triphosphat
APS	ammonium persulfate
BSA	bovine serum albumin
Cdc42	cell division cycle 42
DAPI	4,6-diamadin-2-phenylindol
Dia	orthologs of Drosophila melanogaster diaphanous protein
DLC (1-3)	deleted in liver cancer
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
CFP	cyan fluroescent protein
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ERC	endocytic recycling compartement
FA	focal adhesion
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GST	gluthatione-S-transferase
HCC	hepatocellular carcinoma
HRP	horseradish peroxidase
IF	immunofluorescence
mAb	monoclonal antibody
NaDoc	sodium deoxycholate
ns	not significant
NSCLC	non-small cell lung cancer
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBR	polybasic region
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
PDZ	PSD-95, ZO-1 and discs large domain
PDZL	PDZ ligand motif
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol-4,5-biphosphate
PMSF	phenylmethylsulphonyl fluoride

PVDF	polyvinylidene difluoride
Rac	Ras-related C3 botulinum toxin substrate
Ras	rat sarcoma
RhoA/B/C	Ras homology protein A/B/C
RhoGAP	GTPase-activating protein for Rho proteins
Rho proteins	Rho GTPase proteins (e.g. RhoA, Rac1 and Cdc42)
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
ROCK	Rho-associated, coiled-coil containing kinase
RPMI	medium named after the Roswell Park Memorial Institute
RT	room temperature
SAM	sterile α motif
SDS	sodium dodecyl sulfate
s.e.m.	standard error of the mean
siRNA	small interfering RNA
SNX27	sorting nexin 27
StAR	steroidogenic acute regulatory
STARD	StAR-related lipid transfer domain
START	StAR-related lipid transfer
TEMED	N,N,N,N-tetramethyl ethyldiamine
TfnR	transferrin receptor
Tris	tris-hydroxymethyl aminomethane
WB	western blot
WT	wild type
YFP	yellow fluorescent protein
EEA1	early endosome antigen 1
GFP	green fluorescent protein
LAMP1	lysosome-associated membrane protein 1
HOPS	homotypic fusion and vacuole protein sorting
Rab	Ras-related in brain
TGN	trans-golgi network
PTM	post-translational modification
CME	clathrin-mediated endocytosis
CLIC	clathrin-independent carriers
GEEC	glycophosphatidylinositol-anchored protein-enriched endosomal compartments
EE	early endosome
LE	late endosome
SNARE	soluble NSF attachment protein receptor
PM	plasma membrane
MET	mesenchymal-to-epithelial transition
HNSCC	head and neck squamous cell carcinoma
RTK	receptor tyrosine kinase
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1 matrix metalloproteinase
BM	basement membrane
TIMP-2	tissue inhibitor of metalloproteinases 2
IQGAP1	IQ Motif Containing GTPase Activating Protein 1
VAMP7	vesicle associated membrane protein 7

c-vc	crossveinless
LOH	loss of heteroygosity
NLS	nuclear localization signal
CTX	cholera toxin
PEI	Polyethylenimine
HS	horse serum
DMEM	Dulbecco's Modified Eagle Medium
TGF-β	transforming growth factor β
TIRF	total internal reflection fluorescence
JACoP	just another colocalization plugin
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein
DRF	diaphanous-related formin
Arp2/3	actin-related proteins 2/3
CXCR2	C-X-C Motif Chemokine Receptor 2
FRAP	fluorescence recovery after photobleaching

Summary

The malignant transformation of epithelial cells and the resulting increase in cell motility and invasion into foreign tissue is one of the greatest challenges in cancer therapy. Small Rho GTPases function as key regulators of cytoskeleton dynamics and are therefore involved in almost all processes driving tumorigenesis like cell proliferation, cell polarity, cell-cell adhesion or cell migration. Furthermore, Rho GTPases like RhoB are driving organelle movement and intracellular trafficking by regulating the actin polymerization of the cytoskeleton. To maintain the cell homeostasis, spatiotemporal regulation of Rho GTPases is of great importance. Nevertheless, the spatial control of Rho signalling by its regulators, namely guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), that ensure the balanced GTPase activation in space and time are still poorly understood.

The deleted in liver cancer proteins (DLC1/2/3) are a family of RhoGAP proteins acting as negative regulators of Rho GTPases and are frequently downregulated in various types of cancer. All DLC proteins contain a catalytically active GAP domain, a steroidogenic acute regulatory protein-related lipid transfer (START) domain and sterile α motif (SAM). Although the least characterized isoform DLC3 has been shown to be downregulated in several cancer cell lines and primary tumors, the subcellular localization of DLC3 and contribution to spatial Rho regulation remains highly elusive. So far, DLC3 was shown to be essential for the maintenance of cell polarity and cell-cell contacts in epithelial cells by localizing to adherens junctions. In the context of membrane trafficking, DLC3 was shown to localize to Rab8-positive membrane tubules, where it regulates receptor trafficking. Although structurally related, growing evidence suggests that DLC3 has non-redundant functions compared to the other family members, which can be explained by its distinct subcellular localization. Furthermore, the subcellular localization of DLC3 within the cell and thus, spatiotemporal control of local Rho GTPase activity, seem to be dependent on the cellular context and cell state, and may differ e.g. between epithelial and mesenchymal cells. However, the defined localization and function of DLC3 in motile, mesenchymal cells, where cell-cell adhesion is lost, are still elusive. This lack of knowledge also involves the process of epithelial-to-mesenchymal transition (EMT). Thus, the aim of this thesis was to extend the current knowledge of DLC3 localization and function in epithelial cells to transformed cells, to identify targeted Rho GTPases and investigate the impact DLC3 has on endocytic trafficking and cell invasion.

In this thesis, I provide strong evidence that DLC3 regulates the activity of the Rho GTPase RhoB at the level of early endosomes in different cell lines. The depletion of DLC3 leads to an increase in actin polymerization at endosomes and an accumulation of endosomal vesicles, resulting in the resorting of cargo and a switch in recycling routes. In the case of the model

cargo matrix metalloproteinase MT1-MMP, the depletion of DLC3 rerouted the recycling route of MT1-MMP, enhanced the surface protein levels and significantly increased the capability of transformed cells to degrade extracellular matrix. Taken together, these results unravelled a novel role for DLC3 in the spatial regulation of endosomal RhoB, affecting endocytic trafficking and recycling and highlighting the importance of DLC3 in the maintenance of cell homeostasis.

Zusammenfassung

Die bösartige Transformation von Epithelzellen und die daraus resultierende verstärkte Zellmotilität und Invasion in fremdes Gewebe ist eine der größten Herausforderungen in der Krebstherapie. Kleine Rho GTPasen fungieren als Schlüsselregulatoren der Zytoskelettdynamik und sind daher an fast allen tumorerzeugenden Prozessen wie Zellproliferation, Zellpolarität, Zellzelladhäsion oder Zellmigration beteiligt. Darüber hinaus treiben Rho GTPasen wie RhoB den intrazellulären Transport an, indem sie die Aktin-Polymerisation des Zytoskeletts regulieren. Zur Aufrechterhaltung der Zellhomöostase ist die räumliche und zeitliche Regulation von Rho GTPasen von großer Bedeutung. Dennoch ist die räumliche Kontrolle der Rho-Signalwege durch ihre Regulatoren, nämlich GEFs (*guanine nucleotide exchange factors*) und GAPs (*GTPase-activating proteins*), die eine ausgewogene GTPase-Aktivierung in Raum und Zeit sicherstellen, noch kaum erforscht.

Die DLC-Proteine *(deleted in liver cancer*) sind eine Familie von RhoGAP-Proteinen, die als negativ Regulatoren der Rho GTPasen fungieren und häufig bei verschiedenen Krebsarten herunterreguliert werden. Alle DLC3-Proteine enthalten eine katalytisch aktive GAP-Domäne, eine START (*steroidogenic acute regulatory protein-related lipid transfer*)-Domäne und eine SAM (*sterile α motif*)-Domäne. Obwohl die am wenigsten charakterisierte Isoform DLC3 in mehreren Krebszelllinien und Primärtumoren herunterreguliert ist, ist über die subzelluläre Lokalisation von DLC3 und den Beitrag zur räumlichen Rho-Regulation nach wie vor sehr wenig bekannt. Bisher hat sich gezeigt, dass DLC3 eine entscheidende Rolle in der Aufrechterhaltung der Zellpolarität und der Zell-Zell-Kontakte in Epithelzellen durch die Lokalisierung an Adhäsionsverbindungen spielt. Im Kontext des Membrantransports konnte gezeigt werden, dass DLC3 an Rab8-positive Membrantubuli lokalisiert und den Rezeptortransport reguliert. Obwohl strukturell verwandt, zeigt sich zunehmend, dass DLC3 auch nicht-redundante Funktionen besitzt, verglichen mit den anderen Familienmitgliedern, die auf seiner ausgeprägten subzellulären Lokalisation beruhen. Darüber hinaus scheint die subzelluläre Lokalisation von DLC3 innerhalb der Zelle und damit die räumlich-zeitliche

Kontrolle der lokalen Rho GTPase-Aktivität abhängig vom zellulären Kontext und Zellzustand zu sein und kann sich z.B. zwischen epithelialen und mesenchymalen Zellen unterscheiden. Die definierte Lokalisation und Funktion von DLC3 in beweglichen, mesenchymalen Zellen, bei denen die Zell-Zell-Adhäsion verloren geht, ist jedoch noch wenig erforscht. Gleiches gilt für die epithelial-mesenchymale Transition (EMT). Deshalb war es das Ziel dieser Arbeit das aktuelle Wissen über die Lokalisierung und Funktion von DLC3 in Epithelzellen auf transformierte Zellen zu übertragen, betroffene Rho GTPasen zu identifizieren und die Auswirkungen von DLC3 auf den endozytischen Transport und die Zellinvasion zu untersuchen.

In dieser Arbeit liefere ich starke Beweise, dass DLC3 die Aktivität der Rho GTPase RhoB auf der Ebene der frühen Endosomen reguliert, sowohl bei Zellen, die einer partiellen EMT unterzogen wurden, als auch in komplett transformierten Zellen. Der Verlust von DLC3 führt zu einer Zunahme der Aktinpolymerisation an Endosomen und einer Anhäufung von endosomalen Vesikeln, was zu einer Umverteilung der Transportproteine und veränderten Recyclingwegen führt. Im Falle des Modelproteins Matrix-Metalloproteinase MT1-MMP, führte der Verlust von DLC3 zu einer Umleitung der Recycling-Route von MT1-MMP, zu einem Anstieg des Oberflächenproteine und erhöhte deutlich die Fähigkeit der transformierten Zellen extrazellulären Matrix abzubauen. Zusammengenommen haben diese Ergebnisse eine neuartige Rolle von DLC3 bei der räumlichen Regulation des endosomalen RhoB aufgelöst, die den endozytischen Transport und das Recycling betrifft und die Bedeutung von DLC3 für die Aufrechterhaltung der Zellhomöostase unterstreicht.

1. Introduction

1.1 The family of Rho GTPases

Rho GTPases are small GTP/GDP binding proteins that are highly conserved from lower eukaryotes to plants and mammals (Boureux et al. 2007). Once activated, Rho GTPases are targeted to the plasma membrane (PM) by C-terminal lipid modifications (for details see section 1.1.3) where they bind different effector molecules and trigger a signalling cascade to direct cellular responses, including cell cycle progression, cell migration and invasion and cell polarity (Hall 1998) .

The Rho GTPase family is part of the Ras superfamily and consists of 22 family members which can be classified into eight subgroups (Vega and Ridley 2008). The most intensively studied and characterized members are RhoA, Rac1 and Cdc42 (Bos, Rehmann, and Wittinghofer 2009). RhoA activation is essential for actin-myosin contraction by promoting stress fiber formation. At the same time, active RhoA can mediate clustering of integrins and integrin-associated proteins forming focal adhesions (Hall 1998). Rac1, in comparison, is an important regulator of cell migration by polymerizing actin filaments in the cell periphery and forming membrane ruffles and lamellipodia, whilst generation of filopodia requires the activity of Cdc42 at the inner PM (Hall 1998).

The first Rho GTPases being discovered in 1985 were RhoA, RhoB and RhoC in humans (Madaule and Axel 1985) . These proteins are all members of the RhoA subfamily, are highly homologous and share more than 85% amino acid sequence identity (Wheeler and Ridley 2004). Although they have been discovered around the same time, RhoB and RhoC have received less attention than RhoA. The major divergence of sequence between RhoA, RhoB and RhoC is in their C-terminus, which is responsible for the correct localization of the proteins. This indicates that their localization is likely to be differentially regulated (L. Wang et al. 2003). RhoA, RhoB and RhoC are post-translationally modified by prenylation of a conserved C-terminal cysteine (Shao and Dixon 2003), anchoring the GTPases into membranes, making this modification essential for cell growth, transformation and cytoskeleton organization.

In the RhoA subfamily, RhoB takes on a special role due to specific post-translational modifications. RhoA and RhoC, for example, can only be prenylated with a 20-carbon geranylgeranyl group, localizing them to the PM, whereas RhoB can also be farnesylated by a 15-carbon farnesyl group, enabling it to localize to endomembranes (Adamson, Paterson, and Hall 1992). Thus, RhoB has an additional function not only at the PM, but also in the context of endocytic trafficking by functioning at endosomes. It is able to bind and activate effector

molecules like the Diaphanous-related formin, Dia1, and leading to the formation of the actin coat around endosomes, connecting the regulation of actin dynamics with membrane trafficking(Fernandez-Borja et al. 2005).

Therefore, the correct localization and balanced activity of Rho GTPases is crucial for the correct execution of a variety of cellular processes and as a consequence, deregulation can lead to cell transformation and disease.

1.1.2 Rho GTPases in tumor progression

With the numerous essential cellular processes regulated by Rho GTPases in mind, it is not surprising that deregulated activity of Rho GTPases can be observed in malignant cell transformation. They also contributed to the establishment of the so called “hallmarks of cancer” defined by Hanahan and Weinberg in 2000. Those six hallmarks include proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis programs (Hanahan and Weinberg 2000). Several Rho GTPases have been found to be overexpressed in various human tumors, mostly correlating with cancer progression (Erik Sahai and Marshall 2002). RhoA is upregulated in a variety of human tumour types. RhoA stimulates cell cycle progression and cytokinesis, as well as regulates cell migration (Vega and Ridley 2008). RhoB has been shown to be overexpressed in breast cancer and to correlate with the progression of the disease (G. Fritz et al. 2002) while other studies report a downregulation of RhoB expression in squamous cell carcinoma and loss of expression in lung cancer progression, suggesting a tumor suppressor role for RhoB. Thus, depending on the cancer type and progression, RhoB can fulfil either oncogenic or tumor suppressive functions (Ju and Gilkes 2018). Overexpression of RhoC has been identified in inflammatory breast cancer (Van Golen et al. 2000) and colorectal carcinoma (H. B. Wang et al. 2009), and it correlates with progression and poor prognosis in pancreatic adenocarcinoma (Suwa et al. 1998) and in melanoma (Boone et al. 2009). Overall, the frequent overexpression of Rho GTPases in cancer highlights the important role of Rho proteins in the different steps of tumorigenesis.

Furthermore, the contribution of Rho GTPases to tumor development and progression seems to be caused by alterations in gene expression or activity of Rho regulators (Orgazy, Herraizy, and Sanz-Moreno 2014). In general, Rho GTPases have been described to participate in mostly all steps of cancer initiation and progression (Figure 1).

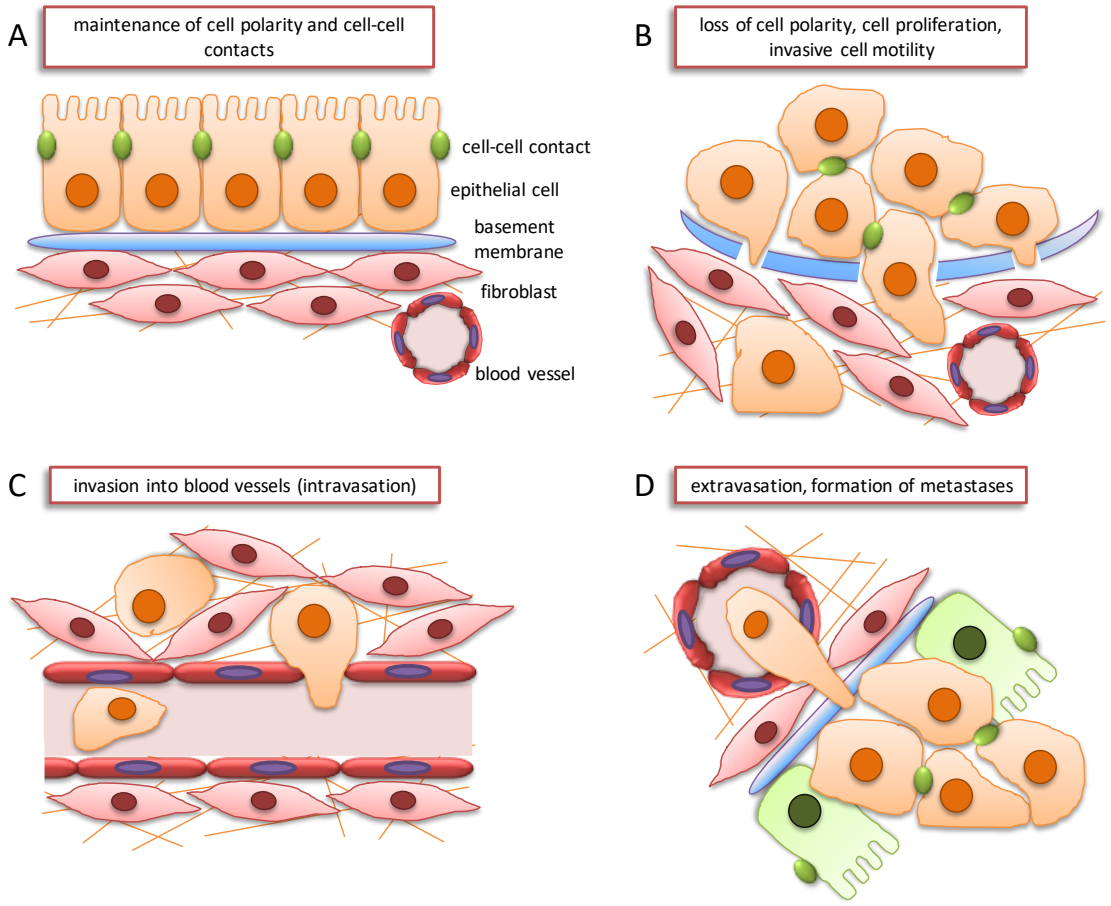


Figure 1: The role of Rho GTPases during tumor initiation and progression.
Depicted are the single steps of the tumor progression cascade. Epithelial, polarized cells (A) transform into mesenchymal, highly proliferative and invasive cells (B) that invade into adjacent tissues and blood vessels (C) and form metastasis in distant tissue (D). Modified from Noll et al., 2016

In healthy epithelial cells, Rho GTPases help to stabilize tissue integrity by establishing and maintaining apical-basal cell polarity and cell junctions. Deregulated Rho activity advocates the conversion of cells towards a more mesenchymal, motile phenotype during the so called epithelial-to-mesenchymal-transition (EMT) (Erik Sahai and Marshall 2002). Furthermore, the involvement of nearly all the important Rho GTPases in cell migration and invasion during tumor progression highlights the crucial role of this protein family. But not only cell migration and invasion are influenced by Rho GTPases, also intracellular processes like organelle movement or vesicular trafficking are targeted by Rho GTPases. Tumor cells hijack this mechanism to enhance the transport of surface proteins essential for cell migration and invasion, like the later discussed matrix metalloproteinases (Kessenbrock, Plaks, and Werb 2010).

Thus, the activity of Rho GTPases has to be tightly regulated in space and time to prevent cellular transformation.

1.1.3 Regulation of Rho GTPases

Rho GTPases are often referred to as molecular switches, because they shift between an active, GTP-bound state and an inactive, GDP-bound state and this switch is controlled by three main classes of regulators: GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs) and guanine nucleotide dissociation inhibitors (GDIs). Until today, over 70 GAPs and over 80 GEFs have been identified to regulate the 22 Rho GTPases, highlighting the importance of a precise Rho regulation (Vigil et al. 2011). GAPs are proteins that activate Rho GTPases by providing an essential catalytic group that accelerates the low intrinsic GTPase activity of the GTPases, thereby promoting the hydrolysis of GTP and resulting in an inactive GDP-bound state. In contrast, GEF proteins accelerate the exchange of Rho-bound GDP for GTP, leading to an activation of Rho GTPase signaling by enabling the binding of downstream effectors (Figure 2). The third group of regulators, the GDIs, prevent Rho GTPase activation and binding to effectors by prohibiting the GDP release or masking the C-terminal lipid anchor, thereby sequestering the Rho GTPase in the cytoplasm (Bos, Rehmann, and Wittinghofer 2009). Those C-terminal lipid anchors are part of another regulatory mechanism of Rho GTPases, the post-translational modifications (PTMs). These include lipid modifications, ubiquitination, phosphorylation and sumoylation, altering the intracellular localization of the Rho GTPases, their stability or their ability to signal to downstream effectors. Lipid modifications are performed at the C-terminal CAAX motif of the GTPases where C stands for cysteine, A is an aliphatic amino acid, and X a terminal amino acid. In detail, this motif is post-translationally prenylated on the cysteine, followed by proteolytic removal of the 3 C-terminal amino acids (AAX) and methylation of the prenylated cysteine. Depending on the prenylation, the translocation of the Rho GTPases differs. RhoB shows a predominant localization to the PM when the farnesylation is inhibited, indicating that the farnesylated and geranylgeranylated forms of RhoB show different intracellular localizations (Wherlock et al. 2004). Taken together, the variety of regulatory mechanisms for Rho GTPases by GEFs, GAPs, GDIs or PTMs ensure a highly specific control of Rho GTPase activity in space and time, enabling the Rho GTPases to sense a spectrum of external stimuli and trigger the according downstream signalling pathway.

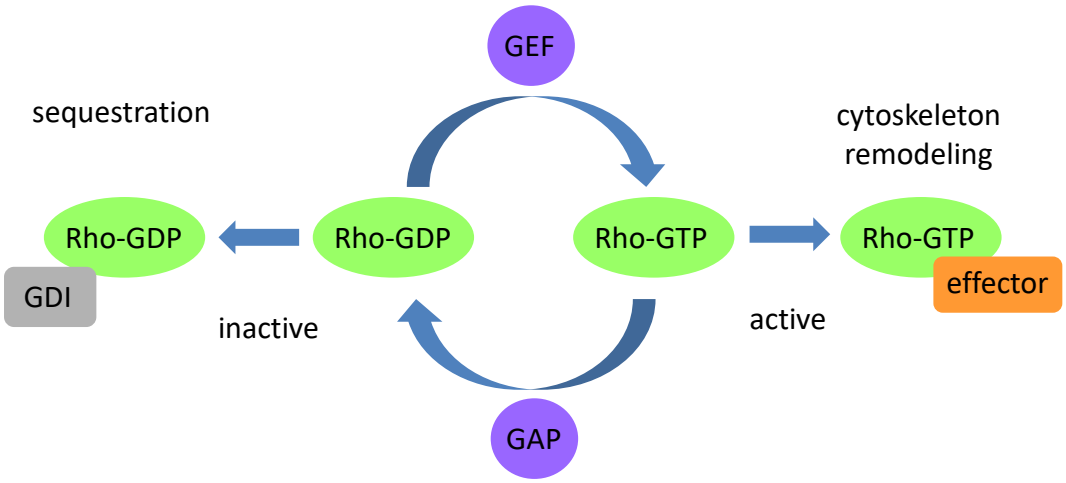


Figure 2: Regulation of Rho GTPases by GEFs and GAPs.
Schematic representation of the Rho activation cycle regulated by guanine nucleotide exchange factors (GEF), GTPase-activating proteins (GAP) and guanine nucleotide dissociation inhibitors (GDI).

The visualization and measuring of Rho GTPase activity on single cell level and connecting the activity to intracellular localization of Rho GTPase molecules is a challenging task. In this thesis, we used specific Biosensors and the Förster-Resonance-Energy-Transfer (FRET) to measure Rho GTPase activity not only for the whole cell, but also distinguish between Rho GTPase pools active at the PM and at vesicular structures. In a nutshell, the FRET-Biosensors (see 2.1.7) is comprised of a Rho-binding domain (RBD), a cyan-fluorescent protein (CFP), a yellow-fluorescent protein (YFP) and the Rho GTPase. Upon Rho GTPase activation, RBD can bind to the Rho GTPase, bringing CFP (donor) and YFP (acceptor) in close approximation. Excitation of CFP leads to an energy transfer (FRET) from donor to acceptor, which is increased when they are in close proximity. The Rho GTPase activity is proportional to the FRET/CFP ratio at a given subcellular localization.

1.2 Endocytic trafficking

Rho GTPases are involved in the regulation of endocytic trafficking (Qualmann and Mellor 2003). The endocytic network is important for the internalization, sorting and redistribution of diverse subcellular components and mediates proper shuttling and processing of elements of neighbouring cells or the extracellular milieu, enabling the cells to sense their environment, communicate with other cells or modulate their surrounding extracellular matrix (ECM). With the advancement of electron microscopy, the visualization of the specialized membrane domains revealed two morphologically and mechanistically distinct pathways: clathrin-mediated endocytosis (CME) (Roth and Porter 1964) and caveolae uptake (Kiss and Botos 2009). Since then, the selective inhibition of these two pathways led to the finding of additional pathways: The cholesterol-sensitive clathrin- and caveolae-independent pathway (Hansen, Sandvig, and Van Deurs 1991; Lamaze et al. 2001) and the pathway involving clathrin-independent carriers (CLIC) and glycophosphatidylinositol-anchored protein-enriched endosomal compartments (GEEC), called the CLIC/GEEC pathway (Kirkham et al. 2005). Once internalized through one of these different endocytic pathways, the different cargo is trafficked and sorted by a pleiomorphic series of tubulovesicular compartments, called endosomes (Scott, Vacca, and Gruenberg 2014). The function of the endosomal network is to collect all the internalized cargo, sort, and disseminate them to their final destinations (Huotari and Helenius 2011). Depending on their function, internalized surface proteins and macromolecules can have many different fates. They can be recycled back to the PM, forwarded to the lysosome for degradation, or in the case of polarized cells, sent across the cell by transcytosis, which is essential for transport across endothelia, epithelia and the blood brain barrier (Preston, Joan Abbott, and Begley 2014).

All incoming material is routed to various destinations at two major sorting stations, namely the early endosome (EE) and the late endosome (LE).

1.2.1 Early Endosome

Despite the complex and varied routes of endocytosis, all material entering the cell converges at the EEs. This pleiomorphic structure serves as a first locus after internalization for the sorting of membrane-associated molecules. To do so, EEs contain a selective subset of lipids and proteins, including Rab GTPases (Scott and Gruenberg 2011) (details see in section 1.2.2).

Single EE elements can also undergo homotypic fusion (Gruenberg and Howell 1989) and can thus form functional networks. This fusion is mediated by the Rab GTPase Rab5 and its effector protein early endosomal antigen 1 (EEA1). EEA1 has been shown to specifically associate with the cytoplasmic face of the early endosomal membrane and appears to be one of the most specific early endosomal markers known to date (Mu et al. 1995). It contains a cysteine-rich motif, called the FYVE finger, which interacts with phosphatidylinositol-3-phosphate (Gaullier et al. 1999) and has been shown to be required for endosomal targeting of EEA1 (Stenmark et al. 1996). Furthermore, EEA1 might act as a docking protein that confers targeting specificity before SNARE-dependent early endosome fusion events take place (Christoforidis et al. 1999).

A few minutes after arrival to EEs, material destined for degradation or recycling is segregated. Many receptors that need to be recycled are uncoupled from their ligands at the mildly acidic pH (pH ≈ 6.2) found in EEs (Goldstein et al. 1985). In addition to pH, endosome geometry is important for sorting of internalized cargo (Maxfield and McGraw 2004). While EE mature, they can exhibit tubulation to help facilitate cargo sorting and recycling. They form a large vacuolar portion, which is around 100-500 nm in diameter (Huotari and Helenius 2011), maximizing the volume-to-surface area ratio and enabling dissociated ligands to accumulate in the lumen of this region. At the same time, narrow tubules that extend from EE have a maximum surface area-to-volume ratio, allowing the accumulation of transmembrane receptors destined for recycling. In some cells, the released tubules accumulate in the perinuclear region before returning to the cell surface, forming the recycling endosome (Huotari and Helenius 2011). In addition to physical sorting by pH and geometry, efficient endocytic cargo recycling requires the active association of a sorting machinery (Hsu, Bai, and Li 2012). Sorting nexin family proteins (SNXs) also interact with endosomal membranes, induce tubulation and control cargo sorting (Van Weering et al. 2012). They are involved in the formation of the evolutionary conserved retromer complex. The retromer complex is a core component of the endosomal protein sorting machinery that recognises specific cargo proteins, concentrates them in distinct regions of the endosome and assures the transport of that cargo proteins to the appropriate destination (Seaman 2012). Thus, it “rescues” cargo receptors from the degradative lysosomal pathway by diverting them for recycling to the PM (Hsu, Bai, and Li 2012).

While ligands are generally transported from EE toward lysosomes, receptor molecules can be returned to the PM via a direct and rapid recycling pathway controlled by the small GTPase Rab4, or indirectly via recycling endosomes in a Rab11-dependent manner (see details in section 1.2.2). Members of the Rab family are selectively associated to each compartment of the endocytic machinery and control the function and identity of intracellular membranes (Galvez et al. 2012).

1.2.2 Regulation of endocytic trafficking by Rab GTPases

Rab GTPases are small monomeric GTPases and comprise the biggest family within the Ras superfamily of small GTPases. Rabs contribute to the functional and structural identity of the endomembrane system by recruiting sets of effector proteins to the surface of specific membrane compartments (Stenmark 2009; Zerial and Mcbride 2001) (Figure 3). Similar to Rho GTPases, Rab GTPases function as “molecular switches” and cycle between an active, GTP-bound, and an inactive, GDP-bound state. Further similarities to Rho GTPases are the regulators of Rab GTPases, the GEFs and GAPs that catalyse the exchange and hydrolysis reactions, respectively. Once activated, Rab GTPases recruit effector proteins to the endomembrane and control vesicle formation, targeting and fusion, thereby controlling the directionality of transport (Gurkan et al. 2005).

As mentioned before, the recruitment of the Rab5 effector EEA1 promotes endosomal membrane fusion, leading to the formation of EEs. During EE maturation, the cargo and receptors are sorted for either recycling or degradation. Receptors can be directly sorted back to the PM or indirectly via the recycling endosome and the slow recycling route. The fast recycling is mediated by the small GTPase Rab4. The precise role of Rab4 and the sites of Rab4 activation are still controversially discussed in the field, but the expression of a dominant-negative Rab4 mutant inhibited the fast recycling of cargo proteins (Grant and Donaldson 2009), underlining its importance in the recycling pathway. The other, slow recycling route involves recycling endosomes and is mainly regulated by the Rab GTPase Rab11 (Takahashi et al. 2012). Other studies have indicated that Rab35 is also an important regulator of fast recycling (Kouranti et al. 2006).

As the EE maturates further, it undergoes a so-called early-to-late endosome maturation, requiring a process known as “Rab-conversion”. In this process, Rab5 is being exchanged by Rab7 by the homotypic fusion and vacuole protein sorting (HOPS) complex (Rink et al. 2005). Rab7 also regulates transport from LEs to lysosomes, biogenesis of lysosomes and clustering

and fusion of LEs and lysosomes in the perinuclear region (Bucci et al. 2000; Girard et al. 2014). For the final degradation of cargo, LEs fuse with acidic lysosomes, in which internalized components are degraded due to the low pH and the presence of acidic hydrolases.

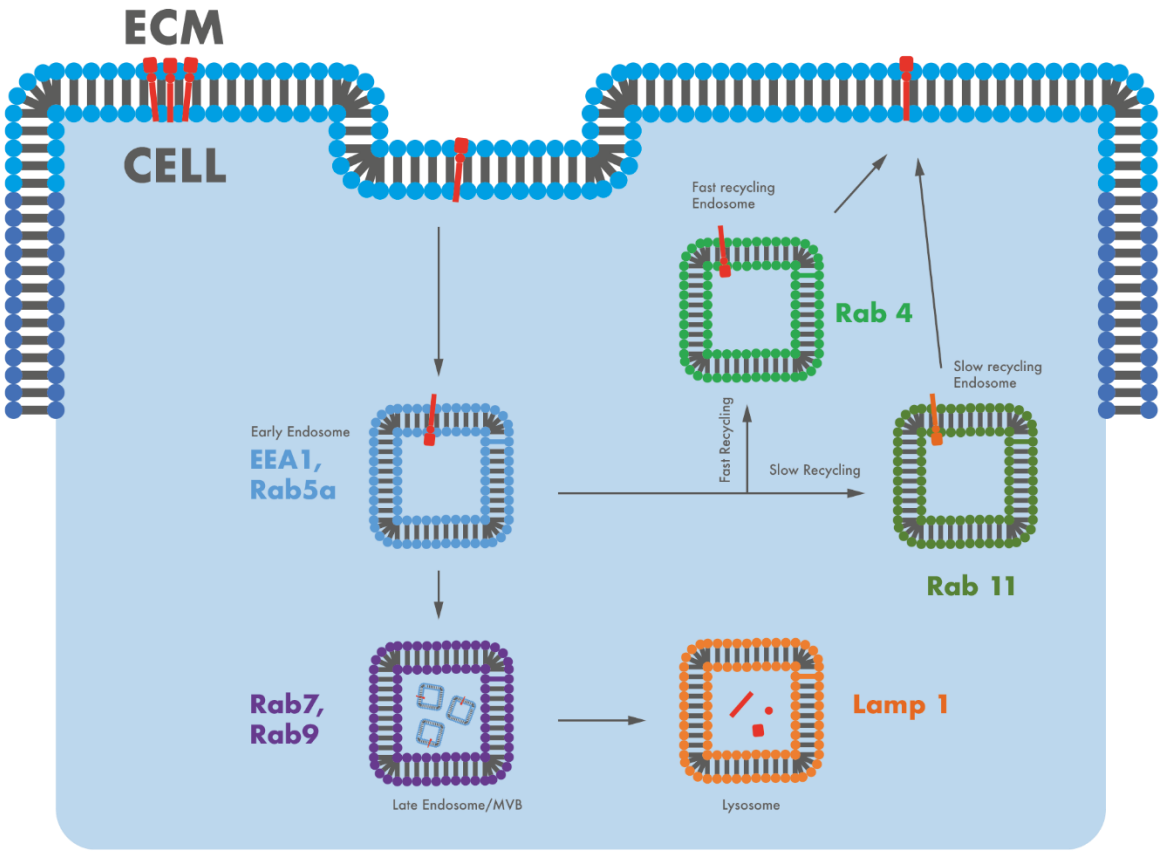


Figure 3: Regulation of endocytic trafficking by Rab GTPases.
Internalized vesicles undergo homotypic fusion to form EEs, mediated by Rab5. From here, internalized cargo can be sorted and recycled back to the PM through the Rab11-mediated slow recycling route or the Rab4-mediated fast recycling route, or trafficked through Late Endosomes (LEs) und Multivesicular bodies (MVBs) through Rab7 and Rab9 to the lysosome for degradation, where LAMP1 maintains structural integrity.

In summary, Rab GTPases are essential for the different steps in endocytic trafficking and a dysfunction or deregulation of the key players can lead to severe diseases and cancer progression. For example, Rab5a expression is associated with higher-grade and lymph node metastasis in breast cancer patients (Yang et al. 2011) and it is overexpressed in cervical cancer, where again it promotes cell motility and invasion (Liu et al. 2011).

1.2.3 Endocytic trafficking in epithelial-mesenchymal transition (EMT) and cancer progression

Cells are able to adapt to micro-environmental changes due to their intrinsic and extrinsic plasticity, altering their phenotypic and morphological characteristics. The endocytic circuit is a key factor for plasticity and is often hijacked by malignant cells to drive tumor progression and invasion. One of those processes is the EMT, which shows an upregulation of several membrane proteins (D'Souza et al. 2014). EMT is a developmental process during which epithelial, adherent cells lose their polarity and cell-cell contacts and acquire the ability to migrate. EMT as well as its reverse process, mesenchymal-epithelial transition (MET), take place during development, fibrosis and wound healing, but also during tumor progression. It contributes to nearly all of the hallmarks of cancer, thus playing a significant role in cancer progression (Hanahan and Weinberg 2000).

During EMT, an epithelial cell loses its connections to neighbouring cells, converts from apico-basal to front-back polarity and develops characteristics of migratory mesenchymal cells (Greenburg and Hay 1982).

As EMT increases cell motility, it is not surprising that it contributes to the dissemination of tumor cells. Cells within a carcinoma often express mesenchymal markers or lose epithelial markers at the invasive front of the tumor and in circulating cells (Kyung-A Hyun et al. 2016; Paterson et al. 2013), representing the early steps in the metastatic cascade. Although the increase in cell motility is the primary consequence of EMT, it is also associated with therapy resistance, stemness and immune evasion, leading to a poor clinical outcome in gastric cancer and breast cancer, among others (L. Huang, Wu, and Xu 2015). Tumor cells that are induced to undergo EMT acquire resistance to certain chemotherapy (Yin et al. 2007) and the inhibition of EMT can lead to an increased drug sensitivity (Fischer et al. 2015).

Emerging evidence suggests that EMT is not a black and white situation, with the cells being either fully epithelial or fully mesenchymal. Instead, EMT seems to be a spectrum, and tumor cells often fall somewhere in between the two states, referred to as partial EMT (Nieto et al. 2016). Partial EMT appears to create tumor cells with enhanced epithelial-mesenchymal plasticity, which is essential for metastasis, tumor recurrence and therapy resistance. A study of Puram and colleagues (Puram et al. 2017) demonstrated that partial EMT cells are invasive and highly plastic. Furthermore, in this study, the EMT signature correlated with the malignant basal head and neck squamous cell carcinoma (HNSCC) subtype and lymph node metastasis, implying that partial EMT promotes loco-regional invasion.

For a deeper understanding of the role and regulation of EMT-related processes, the cellular model system of MCF10A cells will be used in this thesis. This non-tumorigenic human mammary epithelial cell line is a widely used in vitro model for studying normal breast cell function and transformation. These epithelial cells can undergo a partial EMT when treated for several days with transforming growth factor beta (TGF- β). TGF- β is a cytokine known known to have a biphasic effect on tumor progression. Despite the function as tumor suppressor through inhibition of cell proliferation in nontransformed cells, it has been shown to act as an oncogene by inducing EMT to promote increased invasion in breast epithelial cells and cancer cells (Dumont and Arteaga 2000).

Deeper understanding about the signature and mechanisms that drive phenotypic plasticity is necessary to develop new therapeutic strategies in cancer therapy to prevent tumor progression, metastasis and resistance to therapy in human cancers. How cancer metastasizes remains an outstanding open question and involves many steps from dissemination to migration and invasion. Growing evidence indicates that membrane trafficking is frequently harnessed by tumors of epithelial origin to obtain a mesenchymal program of invasiveness (Mellman and Yarden 2013).

1.2.4 Endocytic trafficking in cell invasion

Coordinated cell migration is required for diverse physiological processes ranging from embryonic development to the induction of immune responses. Recent advances reveal that endocytic trafficking contributes to cell migration in multiple ways.

First, at the level of surface receptors. The efficiency of cell migration is strongly influenced by the number of chemokine receptors on the cell surface and the receptor’s responsiveness to chemokines. Chemokine receptor internalization and intracellular trafficking are essential for regulating cell migration. Once internalized, the chemokine receptor either proceeds to LE/lysosomes to be degraded or enters the recycling compartment to traffic back to the PM (Neel et al. 2005). The intracellular trafficking steps are regulated by Rab GTPases as depicted in chapter 1.2.2 (Stenmark 2009). But not only chemokine receptors, also receptor tyrosine kinases (RTK) like the epidermal growth factor receptor (EGFR) are influencing cell migration. Deregulated EGFR internalization or trafficking can lead to impaired signalling and severe migration defects (Jékely et al. 2005). The prolyl hydroxylase PHD3, for example, has been shown to regulate EGFR activity through the control of internalization to restrain tumor growth.

PHD3 inactivation in the hypoxic microenvironment of solid tumors provides EGFR activation and sustains proliferative signalling (Garvalov et al. 2014)

Second, at the level of focal adhesion dynamics. Cells establish adhesive contacts to the substratum called focal adhesions (FAs), which have to be dynamically turned over to allow cell migration. This again involves endocytic trafficking. Integrins, transmembrane proteins and the main component of focal adhesions, link the ECM to the intracellular actin cytoskeleton and thereby provide the necessary anchorage. It is well established that integrins continually and rapidly cycle between the PM and endosomal compartments, approximately every 30 min (Caswell and Norman 2006). Endocytosed integrins are transported to EEs where sorting into degradative or recycling pathways occurs (Bridgewater, Norman, and Caswell 2012). Furthermore, integrins are not only regulated by endocytic trafficking themselves, but also regulate in turn the endocytic transport of other migration-relevant cargos like the above-mentioned EGFR (Caswell, Vadrevu, and Norman 2009).

Third, at the level of ECM reorganization. The ECM provides on one hand the substrate for cell adhesion, but on the other hand acts as a physical barrier for invading cells (Bonnans, Chou, and Werb 2014). To achieve ECM remodelling, mesenchymal cells have developed a strategy to remodel and proteolytically degrade the ECM using matrix metalloproteinases (MMPs) (Bonnans, Chou, and Werb 2014). Delivery of MMPs, like the key player membrane type 1 (MT1)-MMP, to the PM requires for example, the master regulators of endocytosis, Rab5 and Rab4 (Frittoli et al. 2014).

Overall, endocytic trafficking takes a central stage in the process of dynamic cell migration and invasion and the deregulation of its key components can lead to inflammatory diseases and metastasis during cancer progression.

1.3 The role of the matrix metalloproteinase MT1-MMP in cell invasion

Metastasis is a complex process that starts with the dissemination of cancer cells from a primary tumor to distant tissues. As described before, EMT is one of the processes necessary for epithelial cells to migrate. Furthermore, the motile cells have to interact with and remodel the ECM. MMPs have been identified as essential enzymes engaged by tumor cells during metastasis (Egeblad and Werb 2002). There are around 25 members of the MMP family in humans, and they are multifunctional zinc-dependent endopeptidases that can degrade a variety of ECM components. Nearly all MMPs are secreted into the extracellular milieu, except six membrane-anchored (MT) MMPs, of which MT1-MMP, also called MMP-14, has been characterized extensively and seems to be a key enzyme in tumor-cell invasion. MMPs are

produced as zymogens (proenzymes) and require proteolytic cleavage of the propeptide for activation (Egeblad and Werb 2002). MT1-MMP is activated by the conversion of proMT1-MMP (~64kDa) to a catalytically active enzyme (~55kDa) by proteolytic cleavage by furin prior to its arrival at the PM (Mazzone et al. 2004). Once at the PM, MT1-MMP can directly cleave ECM components including collagen, fibronectin, gelatine and cell—surface proteins such as CD44 or integrins (Overall and Dean 2006). In addition to direct cleavage of the ECM, MT1-MMP is also required for the activation of other MMPs by cleavage of their secreted proenzymes, like proMMP-2. In this case, MT1-MMP at the cell surface forms a homo-dimer complex and together with its inhibitor, TIMP-2, it can cleave and activate proMMP-2 (Itoh et al. 2001).

During the metastatic process, tumor cells have to breach the basement membrane (BM), a 50-100 µm thick highly crosslinked meshwork that separates epithelial cells from the connective tissue (Kalluri 2003). Because the pores of the BM are too small for passive invasion, this physically tough structure represents a mechanical barrier for cancer cell migration. BM degradation is one of the first detectable signs of metastatic development and it correlates with a poor prognosis (Barsky et al. 1983). Using native mesothelial BM and BM produced in vitro, it was demonstrated that the activity of MT-MMPs, like MT1-MMP, is responsible for BM breaching by MDA-MB-231 human breast cancer adenocarcinoma cells (Hotary et al. 2006). These findings point out the essential role of MT1-MMP in proteolytic remodelling of the ECM (Hotary et al. 2006).

Most interestingly, MT1-MMP and MMP-2 were shown to accumulate at the invasive front of tumors (Hofmann et al. 2003) and are enriched at so called invadopodia, which are specialized actin-based membrane protrusions that can degrade the matrix (Linder 2007). The accumulation of MT1-MMP at invadopodia is required for focal pericellular degradation of the ECM (Nakahara et al. 1997). Recent observations also suggest that MT1-MMP itself contributes to the maturation and stabilisation of invadopodia (Clark et al. 2007; Sakurai-Yageta et al. 2008).

As a multifunctional protease, MT1-MMP is highly regulated at the levels of gene transcription, intracellular transport and proteolytic activation. Deregulation of these mechanisms can lead to several human diseases such as diabetes, vascular diseases, and cancer (Deryugina and Quigley 2006; Savinov and Strongin 2007). The proteolytic activity of MT1-MMP on the cell surface is controlled by efficient internalization via clathrin-mediated or caveolar endocytosis, where it reaches early- and late-endosomal compartments and can be sorted into lysosomes for degradation or recycled back to the cell surface (X.-Y. Li et al. 2008; Remacle, Murphy, and Roghi 2003) (Figure 4). Several studies also show that TIMP-2, the major inhibitor of MT1-MMP, can be internalized in an MT1-MMP-dependent manner and that binding of TIMP-2

enhances uptake of MT1-MMP (Maquoi et al. 2000; Remacle, Murphy, and Roghi 2003). The internalization of inactive MT1-MMP-TIMP-2 complexes seems to represent a mechanism to dissociate TIMP-2 from MT1-MMP within the endocytic pathway in order to regenerate an active protease (X.-Y. Li et al. 2008; Maquoi et al. 2000). Furthermore, a recent study showed that type-I collagen can interfere with clathrin-mediated uptake of MT1-MMP, representing a mechanism to increase the level of active protease at the cell-matrix interaction sites (Lafleur et al. 2006). A significant amount of internalized MT1-MMP is recycled back to the cell surface, additionally MT1-MMP might be mobilized from the secretory pathway, supported by the discovery of a Rab8-dependent pathway for delivery of MT1-MMP from a storage compartment to collagen contact sites (Jose J. Bravo-Cordero et al. 2007). Further studies on the identification of components involved in the delivery of MT1-MMP to the invadopodia suggest a key role for components like cortactin, IQGAP1, the exocyst complex and VAMP7 (Clark and Weaver 2008; Sakurai-Yageta et al. 2008; Steffen et al. 2008) .

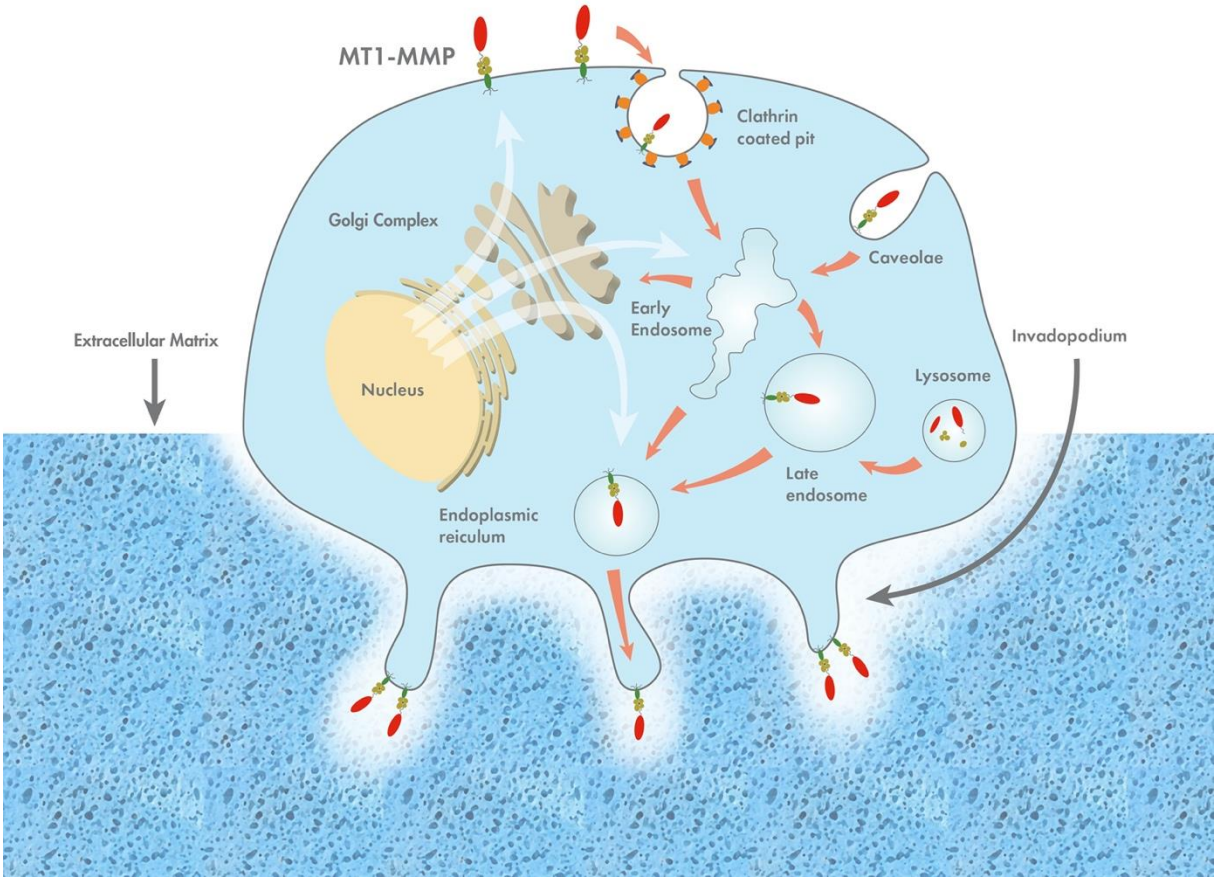


Figure 4: Intracellular trafficking of MT1-MMP.
MT1-MMP is synthesized as a proenzyme and is transported through the biosynthetic pathway to the PM (white arrows). MT1-MMP is internalized from the PM by the caveolar and clathrin-dependent endocytic routes and reaches early and late endosomes. While a fraction of the enzyme is degraded in lysosomes, most is recycled back to the PM and transported to invadopodia, which are specialized actin-based membrane protrusions of invasive cells that have the capacity to degrade the matrix.

Taken together, MT1-MMP is a key player in matrix degradation and is essential for tumor cell invasion during metastasis. The diverse mechanisms of regulation via endocytosis, recycling and lysosomal degradation highlight the importance of a functioning endocytic trafficking network.

1.4 The family of deleted in liver cancer (DLC) RhoGAP proteins

The first DLC-like protein, named RhoGAP88c, was discovered in the fruit fly *drosophila melanogaster*. Whilst several mutations in the gene were lethal at the embryonic stage, the non-lethal mutations lead to the loss of the crossvein in the fly's wing. Thus, the gene was named crossveinless-c (cv-c) (Denholm et al. 2005). During embryonic development, RhoGAP88c is required in tissues undergoing morphological changes to regulate Rho GTPase activity and thereby coordinate the organisation of the actin cytoskeleton, for example during the epithelial invagination of the fly's trachea (Brodu and Casanova 2006; Denholm et al. 2005).

In humans, the DLC protein family consists of three family members: DLC1, DLC2 and DLC3, which are quite similar in their structure, but show differences in subcellular localization (Braun and Olayioye 2015). DLC1, the first found family member in 1998, was originally identified as a potential tumor suppressor in human hepatocellular carcinoma (HCC) (Yuan et al. 1998). The expression of DLC proteins is lost in many human cancers. In some human cancers DLC1 is deleted with the same frequency as p53 (P. Kandpal 2006; Xue et al. 2008). The deregulated DLC1 levels are caused by deletion of the *DLC1* locus found on chromosome 8 (8p21.3-22) or hypermethylation of the promoter (Marian E. Durkin et al. 2007). The tumor suppressing function of DLC1 was also proven in a mouse model of liver cancer (Xue et al. 2008).

The DLC2 gene was discovered in a loss of heteroygosity (LOH) region on chromosome 13 (13q12.3), where also other tumor suppressors like BRCA2 are encoded (Ching et al. 2003). Similar to DLC1, DLC2 is highly expressed in normal tissues, especially liver, heart and brain, and a loss of expression can be observed frequently in various human cancers and tumor-derived cell lines, suggesting a role as a tumor suppressor (Ching et al. 2003; Ullmannova and Popescu 2006). This is supported by the findings that growth of MCF7 breast cancer cells can be suppressed by DLC2 overexpression (Nagaraja and Kandpal 2004).

The third protein of the DLC family, DLC3, is still the most poorly understood family member, but not less important. It is encoded on the X chromosome (Xq13) and was first found in 1996,

by isolating its cDNA from a human myeloid cell line library (Nagase et al. 1996). So far, two DLC3 isoforms are described, DLC3α and DLC3β, with DLC3β lacking the amino-terminal SAM domain (M. E. Durkin et al. 2007). Analogous to its family members, DLC3 expression is reduced in various different human cancers and overexpression of DLC3 in human breast and prostate cancer cell lines inhibited cell growth, proliferation and colony formation (M. E. Durkin et al. 2007). In addition, expression of DLC3 in HeLa cells showed an altered cell morphology due to the GAP-dependent reduction of stress fibres (Braun et al. 2015). Altogether, these results also suggest a tumor suppressor function for DLC3, like its two family members, however, this has to be finally proven in vivo.

1.4.1 Multi-domain structure of the DLC proteins

All three DLC proteins have a conserved structural organization and possess three functional domains, a SAM domain, a catalytic RhoGAP domain and the START domain (Figure 5). The SAM domain comprises around 70 amino acids at the N-terminus of DLC proteins and can also be found in many signalling proteins and transcription factors. They can form homo- or hetero-oligomers or other protein interactions and some SAM domains have been described to bind RNA, DNA and lipid molecules (H. Li et al. 2007; Qiao and Bowie 2005). Although the role of the SAM domain in DLC proteins still has to be elucidated, there is some evidence for a role in intramolecular protein regulation. The involvement of the SAM domain in the regulation of GAP activity is also speculated, as the deletion of the SAM domain yields a more active form of DLC1 (Healy et al. 2008). Thus, the SAM domain could have an auto-inhibitory effect on the GAP activity.

The RhoGAP domain encompasses around 150-200 amino acids and is the most conserved region among the three DLC proteins with about 70% sequence identity. Crucial for their catalytic GAP activity is a conserved arginine region (Scheffzek, Ahmadian, and Wittinghofer 1998). With this arginine finger, a positive charge is introduced into the catalytic site of the Rho GTPase, stabilizing the transition state during GTP hydrolysis. Furthermore, two additional conserved amino acids, a second arginine and a lysine residue, were identified to be essential for the GAP activity (Braun and Olayioye 2015; M. E. Durkin et al. 2007; G. Holeiter et al. 2012). Due to the conserved GAP domain, all DLC proteins have shown GAP activity towards RhoA and weak GAP activity towards Cdc42, at least in *in vitro* studies (Ching et al. 2003; G. Holeiter et al. 2012; Gerlinde Holeiter et al. 2008; Kawai et al. 2007). Additionally, DLC1 has been described to also control the activity of the RhoA subfamily members RhoB and RhoC *in*

vitro (Healy et al. 2008). The overexpression of DLC proteins leads to a common phenotype due to their GAP activity, including a round cell shape, elongated protrusions and the loss of actin stress fibres (Kawai et al. 2007; Wong et al. 2008).

Adjacent to the RhoGAP domain a N-terminal polybasic region (PBR) was discovered, conserved for all three DLC proteins. Although the PBR is poorly studied, in the case of DLC1 studies showed the importance of an intact PBR for binding to negatively charged phospholipids like phosphatidylinositol-4,5- bisphosphate ($PI(4,5)P_2$) and its capability to regulate Rho activity (Erlmann et al. 2009). Furthermore, an unstructured region between the SAM and RhoGAP domain has been described. It shows the least conservation between the three family members, but contains stretches like the consensus LD motif (position 469-476 in DLC1) which mediates binding to focal adhesion-associated proteins and thereby determines DLC localization (G. Li et al. 2011). In addition, potential phosphorylation sites for serine-threonine kinases in the form of serine-rich sequences have been identified in this unstructured region, enabling the regulation of DLC localization and function (Marian E. Durkin et al. 2007).

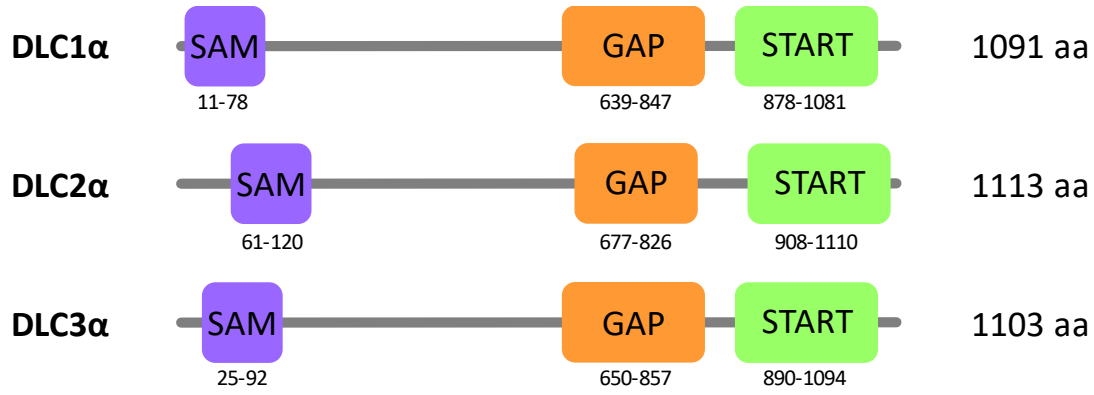


Figure 5: Multi-domain organization of DLC proteins.
Schematic representation of the three functional domains contained in DLC proteins including a sterile α-motif (SAM) domain, a catalytic RhoGAP domain and a StAR (steroidogenic acute regulatory protein)-related lipid transfer domain (START). Based on Lukasik et al., 2011.

Of most importance, being GAP proteins for Rho GTPases, the balanced expression levels of DLC proteins, the tight regulation of their activity and their intracellular localization are essential for normal cell behaviour.

1.4.2 Subcellular localization of DLC proteins

The three different DLC family members can be found at partially similar, but also distinct localizations within the cell, depending on differential protein interactions (Figure 6). This enables a spatiotemporal control of Rho activity within the cell (Braun and Olayioye 2015).

The DLC proteins were first detected at focal adhesions (FA), adhesive structures connecting integrins with the actin cytoskeleton to control stress fibre formation and cell motility. They consist of integrins, signalling molecules and adapter proteins including vinculin, paxillin and tensin. Until today this is the best studied localization of DLC proteins and all three human DLC proteins were described to associate to FA, primarily via tensin binding (Cao et al. 2012; Kawai et al. 2007; Yam et al. 2006). DLC1 has been described to bind to each member of the tensin family, whereas DLC2 and DLC3 are recruited to focal adhesions by binding to tensin1 and tensin2 (Kawai et al. 2007).

Another subcellular site where DLC1 and DLC3 reside, are the adherens junctions (AJ) (G. Holeiter et al. 2012; Tripathi, Popescu, and Zimonjic 2014). DLC1 was reported to bind to α-catenin at AJ, an interaction which seems to be crucial for AJ stability. Furthermore, DLC1 was described to inactivate RhoA and RhoC, increasing E-cadherin expression associated with stronger cell aggregation and reduced cell invasion (Tripathi, Popescu, and Zimonjic 2014).

DLC1 has also been described intensively to act at membrane protrusions and the leading edge of migrating cells. For example, after the depletion of DLC1 an enrichment of the Rho effector Dia1 at membrane protrusions of migrating breast cancer cells was observed, increasing their wound healing ability (Gerlinde Holeiter et al. 2008). Furthermore, the regulation of Rho GTPase signalling by DLC1 at the cell cortex of HCC cells was demonstrated (Wong et al. 2008).

In addition, DLC1 has been described to translocate in and out of the nucleus (Yuan et al. 2007), a mechanism that is impaired by masking the nuclear localization signal (NLS) by the adaptor protein 14-3-3 (Scholz et al. 2009). Another subcellular localization where DLC1 has been described are the caveolae, invaginations of the PM containing various receptors and signalling molecules. Caveolin-1 seems to recruit DLC1 through a targeting mechanism that depends on the START domain of DLC1 and affects its tumor suppressive properties (X. Du et al. 2012; Yam et al. 2006). Moreover, DLC2 was reported to localize to mitochondria (Ng et al. 2006).

The detailed localization of DLC3, the isoform of most importance for this thesis, is described in detail in the following chapter.

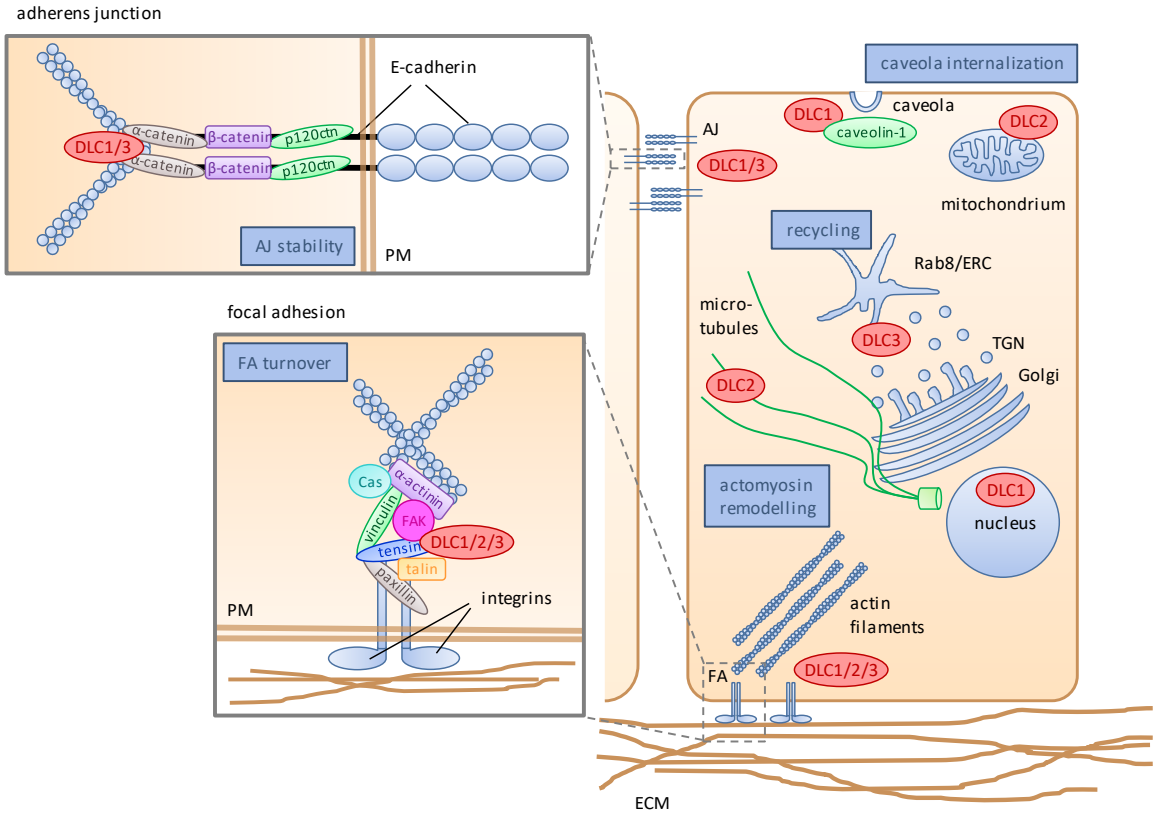


Figure 6: Subcellular localization of DLC1, DLC2, DLC3.

The different DLC isoforms and their localization at focal adhesions (FA), adherens junctions (AJ), the Rab8-positive endocytic recycling compartment (ERC), trans-Golgi network (TGN), nucleus, caveolae and mitochondria are depicted. Taken form Braun and Olayioye, 2015.

In summary, the different localization of the DLC proteins enables a tight, spatiotemporal regulation of Rho activity within the cell and the deregulation of protein interactions such as interaction with tensin and talin adaptors (G. Li et al. 2011; Qian et al. 2007), followed by mislocalization of the DLC proteins and aberrant Rho signalling can lead to tumor growth and progression. This highlights the importance to better understand the defined mechanisms responsible for correct localization and function of the DLC proteins. This is highlighted by their attributed tumor suppressive characteristics and the inhibition of tumor progression in non-small cell lung cancer (NSCLC) (Sun, Sun, and Song 2019).

1.4.3 Cellular localizations and functions of the DLC3 isoform

The investigation of the localization of DLC3 is essential for a deeper understanding of the role it might play in cancer progression. The full length DLC3α isoform has been described to localize to cell-cell adhesions and showed a strong colocalization with β-catenin and E-Cadherin at the adherens junctions of MCF7 breast cancer cells and was found at the basolateral membrane of MCF10A acini (G. Holeiter et al. 2012). Furthermore, the depletion of DLC3 led to mislocalization of β-catenin, E-Cadherin and p120-catenin at adherens junctions, followed by cell disaggregation and enhanced cell migration. The inhibition of ROCK rescued this phenotype, indicating that DLC3 is essential for the maintenance of adherens junctions by regulating Rho-ROCK signalling and the loss of DLC3 compromises epithelial integrity (G. Holeiter et al. 2012) and might contribute to carcinogenesis. First hints are emerging that DLC3 is an important regulator of Rho activity and the correct localization and function of DLC3 is essential for normal cellular behaviour.

DLC3 has also been shown to localize to focal adhesions (Kawai et al. 2007), and our group could identify it at Rab8-positive membrane tubules and the Golgi apparatus in HeLa cells (Braun et al. 2015). Depletion of DLC3 in HeLa cells lead to increased perinuclear RhoA activity and the fragmentation of the Golgi and Rab8 compartments. Moreover, the impaired internalization and endocytic trafficking of transferrin and EGFR after DLC3 depletion identified DLC3 as a novel component in the endocytic trafficking machinery and gave a first indication of the role DLC3 plays in maintaining organelle integrity and the regulation of membrane trafficking through the regulation of Rho activity (Braun et al. 2015). To identify molecular factors that specify DLC3´s subcellular localization, a mass spectrometry analysis of DLC3-interacting proteins was carried (Hendrick et al. 2016). As expected from the role of DLC3 in transferrin receptor trafficking, several proteins involved in membrane trafficking were contained in the list, including the early membrane protein Rab5. Intriguingly, also “cell junction” proteins were found to interact with DLC3, like the polarity protein Scribble. Scribble contains four PDZ domains (Humbert et al. 2008) which frequently mediate protein interactions with C-Terminal PDZ ligand (PDZL) motifs (Nourry, Grant, and Borg 2003). Most interestingly, DLC3 carries such a PDZL-motif (ETKL) which is missing in DLC1 and DLC2 and was shown to being recruited to adherens junctions (AJs) by interaction with Scribble (Hendrick et al. 2016). The maintenance of AJ integrity by Rho GTPase activity is highly delicate and requires a fine balance. Low levels of Rho activity favour downstream signalling through Dia1, leading to actin polymerization and stabilization of AJs (Carramusa et al. 2007), whereas high levels activate the Rho-associated protein kinase (ROCK), generating contractile forces that lead to AJ disruption (Erick Sahai and Marshall 2002). Thorough research of our group shed light on

the role of DLC3 in this context, providing evidence that DLC3 is recruited to AJs by Scribble (Hendricks et al, 2016), where it acts as negative regulator of junctional Rho and that the loss of DLC3 contributes to carcinogenesis by compromising epithelial integrity and promoting EMT (Holeiter et al., 2012).

Thus, the interaction partners of DLC3 and the resulting subcellular localization are an important regulatory mechanism to control Rho GTPase activity. However, here the question arises where DLC3 localizes to and fulfils a function when cell-cell contacts are lost, for example during EMT or in mesenchymal cells, which do not form stable cell-cell contacts anymore. The answer of this question is subject of the presented thesis.

1.5 Aims of the thesis

Tumor progression, especially the invasion of cancer cells from the primary tumor into adjacent tissue and the subsequent formation of metastasis, correlates strongly with a poor prognosis for the patient. Important regulators of this process are Rho GTPases with their key function in a variety of cytoskeleton-mediated cellular processes and the transduction of external stimuli into appropriate downstream signalling. Although the importance of stringent regulation of Rho GTPase is out of question, the current knowledge about the underlying molecular mechanisms regulating Rho proteins in space and time is still limited. Here, the family of DLC proteins has obtained growing attention over the last decades as their GAP activity towards Rho GTPases is known to be crucial for Rho regulation. Due to their multidomain-structure with a START and SAM domain in addition to the GAP domain, DLC proteins can be distinguished from other RhoGAPs. The additional differential subcellular localization of the three family members implies non-redundant functions depending on their localization and binding partners. The proven tumor suppressive function of DLC1 *in vivo* and the extensive downregulation in various cancer types (Marian E. Durkin et al. 2007) highlights the outstanding role of DLC proteins in oncogenic transformation. In contrary to the well-studied DLC1 protein, the DLC3 protein is still poorly characterized and regulation of its subcellular localization and the identity of molecular interaction partners remain highly elusive. Recent work in our lab revealed a first insight into DLC3 localisation, interaction partners and the regulatory mechanism at the PM or the endocytic recycling compartment (Braun et al. 2015; Hendrick et al. 2016). Nevertheless, it still remains unclear which Rho GTPases in detail are regulated by DLC3, especially concerning cells undergoing oncogenic transformation like EMT thereby acquiring a more motile phenotype, and which subcellular Rho pools are affected in these cells.

To address this issue, in this thesis I elucidate the localization and role of DLC3 in a breast epithelial model of partial EMT and highly invasive mesenchymal cells. By using FRET sensors, I will first address the question which Rho GTPases are regulated by DLC3 and whether there are noticeable differences depending on the subcellular localization of DLC3. The next aim is to investigate the effects DLC3 depletion has on these cells, especially in regards to endocytic trafficking. I will focus on MT1-MMP as a model cargo, as its key function in extracellular matrix degradation can link endocytic trafficking effects to biologically relevant cell invasion phenomena. In summary, by using a combination of biochemical and cellular assays I will reveal the defined role of DLC3 in the maintenance of cellular homeostasis in motile cells by controlling Rho GTPase activity in endocytic membrane trafficking.

2. Material and Methods

2.1 Material

2.1.1 Chemicals and reagents

Table 1: List of chemicals and reagents used in this thesis

Chemicals and Reagents	Company
Ammonium persulfate (APS)	Carl Roth GmbH & Co., Karlsruhe, Germany
Blocking reagent	Roche Biosciences, Basel, Switzerland
β-Mercaptoethanol	Sigma-Aldrich, St.Louis, USA
Complete Protease Inhibitor Cocktail (EDTA free)	Roche Biosciences, Basel, Switzerland
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH & Co., Karlsruhe, Germany
Ethanol	VWR, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth GmbH & Co., Karlsruhe, Germany
Fluoromount-G	Southern Biotech, Birmingham, USA
β-Glycerophosphate	Sigma-Aldrich, St.Louis, USA
Glycine	Carl Roth GmbH & Co., Karlsruhe, Germany
Goat serum	Invitrogen, Karlsruhe, Germany
Isopropanol	Carl Roth GmbH & Co., Karlsruhe, Germany
Magnesium chloride (MgCl2)	Carl Roth GmbH & Co., Karlsruhe, Germany
Methanol	Carl Roth GmbH & Co., Karlsruhe, Germany
NP-40 (Nonidet P40)	Sigma-Aldrich, St.Louis, USA
PageRuler - prestained protein ladder	Thermo Scientific, Waltham, USA
Paraformaldehyde (PFA)	Carl Roth GmbH & Co., Karlsruhe, Germany
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich, St.Louis, USA
N,N,N,N-Tetramethylethyldiamine (TEMED)	Carl Roth GmbH & Co., Karlsruhe, Germany

Sodium chloride (NaCl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium fluoride (NaF)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, St.Louis, USA
Tris-hydroxymethyl-aminomethane (Tris)	Carl Roth GmbH & Co., Karlsruhe, Germany
Triton-X-100	Carl Roth GmbH & Co., Karlsruhe, Germany
Tween-20	Carl Roth GmbH & Co., Karlsruhe, Germany
EZ-Link Sulfo-NHS-SS-Biotin	Thermo Scientific, Waltham, USA

2.1.2 Equipment

Table 2: List of equipment used in this thesis

Equipment	Company
Beckman Coulter Centrifuge Avanti J-30I (Rotor J20)	Beckman Coulter, Krefeld, Germany
Beckman Coulter Centrifuge J2-MC Centrifuge (Rotor J20)	Beckman Coulter, Krefeld, Germany
Casy (cell counter)	Roche, Basel, Switzerland
Eppendorf Centrifuge 5415D	Eppendorf, Hamburg, Germany
Eppendorf Centrifuge 5415R	Eppendorf, Hamburg, Germany
iBlot® Gel Transfer Device	Life technologies, Carlsbad, USA
NanoDrop® ND-1000 (Spectrophotometer)	peQLab, Erlangen, Germany
Semi-dry blotting chamber	Phase, Luebeck, Germany
Quantitative PCR Cfx96	Biorad, Munich, Germany
Tecan Infinite 200 Reader	Tecan, Crailsheim, Germany
Fusion Solo	Vilber Lourmat, Collégien, France
LSM 710 (confocal laser scanning microscope)	Zeiss, Jena, Germany
Axio Observer Spinning Disc microscope	Zeiss, Jena, Germany

2.1.3 Consumables

Table 3: List of consumables used in this thesis

Consumables	Company
Glass coverslips 18 mm x 18 mm	Carl Roth GmbH & Co., Karlsruhe, Germany
Roti®-PVDF transfermembrane	Carl Roth GmbH & Co., Karlsruhe, Germany
Whatman® cellulose blotting papers, 3 mm	Schleicher & Schuell, Dassel, Germany
35mm high glass bottom µ-Dish	ibidi GmbH, Planegg, Germany

2.1.4 Buffers and solutions

Table 4: List of buffers and solutions used in this thesis

Buffers and Solutions	Composition
Blocking Solution (IF)	5% (v/v) goat serum (Invitrogen), 0.1% (v/v) Tween-20 in PBS
Blocking Solution (WB)	0.5% (v/v) blocking solution (Roche), 0.05% (v/v) Tween-20, 0.01% (v/v) thimerosal in PBS
Blotting Buffer	200 mM glycine, 25 mM Tris base, 20% (v/v) methanol in ddH2O
Laemmli sample buffer (5x)	400 mM Tris pH 6.8, 500 mM dithiothreitol, 50% (v/v) glycerol, 10% (w/v) SDS, 0.2% (w/v) bromophenol blue in ddH2O
1% TEB lysis buffer	50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM Na3VO4, 10 mM sodium fluoride, 0.5 mM PMSF, 20 mM β-glycerophosphate and Complete protease inhibitors in ddH2O
PBS	140 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.5 mM KH2PO4 in ddH2O, pH 7.4
PBS-Tween	0.05% (v/v) Tween-20 in PBS
Permeabilization solution (IF)	0.2% (v/v) Triton X-100 in PBS
PFA	4% (v/v) PFA in PBS

RIPA lysis buffer	50 mM Tris pH 7.5, 150 mM NaCl, 10 mM NaF, 20 mM ß-glycerolphosphate, 1 mM EDTA, 1% (v/v) NP-40, 0.1% (v/v) SDS, 0.25% (v/v) NaDOC, 1 mM Na3VO4, 0.5 mM PMSF and Complete protease inhibitors in ddH2O
Separating gel solution	8% and 10% (v/v) acrylamide, 375 mM Tris pH 8.8, 0.1% (w/v) SDS, 0.1% APS, 0.06% TEMED in ddH2O
Stacking gel solution	5% (v/v) acrylamide, 130 mM Tris pH 6.8, 0.1% (w/v) SDS, 0.1% APS, 0.1% TEMED in ddH2O
SDS-PAGE running buffer	25 mM Tris pH 8.8, 192 mM glycine, 0.1% (v/v) SDS in ddH2O

2.1.5 Human cell lines

Table 5: List of cell lines used in this thesis

Cell line	Obtained from
MCF10A	Mohamed Bentires-Ahj, Basel
mCherry-MT1-MMP-MDA-MB-231	Philippe Chavrier, Paris

2.1.6 Cell culture reagents and media

Table 6: List of cell culture reagents and media used in this thesis

Cell culture reagents and media	Company
Cholera toxin (CTX)	Sigma-Aldrich, St.Louis, USA
Collagen R solution	Serva, Heidelberg, Germany
DMEM/F-12	Invitrogen, Karlsruhe, Germany
Epidermal growth factor (EGF)	R&D Systems, Minneapolis, USA
Fetal calf serum (FCS)	PAA Laboratories, Pasching, Austria
Leibovitz's L-15 medium	Invitrogen, Karlsruhe, Germany
Lipofectamin® RNAiMAX	Invitrogen, Karlsruhe, Germany
Lipofectamine® LTX with PLUS™	Invitrogen, Karlsruhe, Germany
OptiMEM®	Invitrogen, Karlsruhe, Germany
Polyethylenimine (PEI)	PolySciences Inc., Warrington, USA
Trypsin-EDTA (10x)	Invitrogen, Karlsruhe, Germany

Horse Serum (HS)	Invitrogen, Karlsruhe, Germany
Insulin	Sigma-Aldrich, St.Louis, USA
Hydrocortisone	Sigma-Aldrich, St.Louis, USA
G418	Merck, Kenilworth, USA

2.1.7 Plasmids

Table 7: List of plasmid vectors used in this thesis

Plasmids	Source
MT1-MMP-pHluorin	Kindly provided by Philippe Chavrier, Institut Curie, Paris, France
pCELF-GST-Dbl	Kindly provided by Chiara Russo, Instituto G. Gaslini, Genova, Italy
pCherry-MT1-MMP	Kindly provided by Philippe Chavrier, Institut Curie, Paris, France
pcR3.V62.Met-Flag vector (empty vector)	Kindly provided by Angelika Hausser, University of Stuttgart, Germany
pCRV62-Met-Flag-DLC3α K725E	described in Braun et al., 2015
pCRV62-Met-Flag-DLC3α WT	described in Braun et al., 2015
pEGFP-C1-DLC3α K725E	described in Holeiter et al., 2012
pEGFP-C1-DLC3α WT	described in Holeiter et al., 2012
pEGFPN1-Lifeact	Kindly provided by Michael Sixt, Martinsried, Austria
pEGFP-Rab4	Kindly provided by Hesso Farhan, University of Oslo, Norway
pmOrange2-C1-RhoB	described in Noll et al. 2019
RBD-CFP-Citrine-RhoA-His (RhoA Biosensor)	Addgene (#12150; Pertz et al. 2006)
RhoB-Venus (RhoB Biosensor)	Kindly provided by Peter Hordijk, University of Amsterdam, Netherlands
RhoC FLARE.sc.mCer, mVenus, wt (RhoC Biosensor)	Addgene (#65071)

2.1.8 Antibodies and fluorescent dyes

Table 8: List of primary Antibodies used for immunoblotting and immunofluorescence

Antibody	Species	Dilution	Company
anti-DLC3 (E-2)	mouse mAb	1:500 (WB)	Santa Cruz Biotechnology, Dallas, USA
anti-α-tubulin	mouse mAb	1:2000 (WB)	Sigma-Aldrich, St.Louis, USA
anti-EEA1	rabbit pAb	1:200 (IF)	Cell Signaling, Danvers, USA
anti-Rab7	rabbit pAb	1:100 (IF)	Cell Signaling, Danvers, USA
anti-LAMP1	mouse mAb	1:100 (IF)	DSHB, University of Iowa, USA
anti-mCherry	mouse pAb	1:200 (WB)	Abcam, Cambridge, UK

Table 9: List of secondary antibodies used for immunoblotting and immunofluorescence

Antibody	Species	Dilution	Company
Alexa Fluor® 488/546/633 anti-mouse	goat	1:500 (IF)	Invitrogen, Karlsruhe, Germany
Alexa Fluor® 488/546/633 anti-mouse	goat	1:500 (IF)	Invitrogen, Karlsruhe, Germany
Horseradish peroxidase (HRP)-anti-mouse IgG	sheep	1:1000 (WB)	GE Healthcare, Piscataway, USA

Table 10: List of fluorescent dyes used for immunofluorescence

Dye	Dilution	Company
DAPI	1:5000 (IF)	Sigma-Aldrich, St.Louis, USA

2.1.9 Kits

Table 11: List of Kits used in this thesis

Kits	Company
DC Protein Assay	Bio-Rad, Hercules, USA
HRP SuperSignal®West substrate pico	Pierce/Thermo, Rockford, USA
HRP SuperSignal®West substrate dura	Pierce/Thermo, Rockford, USA
QuantiTect Primer Assays®	Qiagen, Foster City, USA
NucleoSpin® RNA Kit	Macherey-Nagel, Hœrdt, France
Power SYBR® Green 1-Step Kit	Thermo Fisher Scientific, Waltham, USA

2.1.10 Small interfering RNA (siRNA)

All siRNAs were diluted in 1x siRNA buffer (MWG Biotech) to a concentration of 20 µM or 2 µM. As a non-targeting negative control (siCon), ONT-TARGETplus® non-targeting control SMARTpool siRNA (D-001810-10, Dharmacon) was used. Two independent DLC3-specific siRNAs and two independent Rab4-siRNAs were used. spDLC3 corresponds to siGENOME SMARTpool human STARD8 (M-010254, Dharmacon) and siDLC3 refers to Silencer®Select human STARD8 (s18826, Invitrogen). siRab4#1 (Silencer® Select s11677) and siRab4#2 (Silencer® Select s11675) were both purchased from Ambion Life Technologies.

2.2 Methods

2.2.1 Cell culture and transfection

Cell culture

MCF10A cells (provided by Mohamed Bentires-Ahj, Basel) were cultivated in DMEM/F12 medium (Invitrogen) supplemented with 5% horse serum (Invitrogen), 20 ng/ml EGF (R&D), 10 µg/ml insulin (Sigma), 0.5 µg/ml hydrocortisone (Sigma) and 100 ng/ml cholera toxin (Sigma) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. MDA-MB-231 cells stably expressing mCherry-MT1-MMP (kindly provided by Philippe Chavrier) were cultivated in Leibovitz's L-15 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 15% FCS and 50 ng/ml G418 (Calbiochem) and incubated in a humidified atmosphere of 1% CO2 at 37 °C. Cells were passaged every 3-4 days before they reached confluency using 1x Trypsin/EDTA. Every 3 months, fresh cultures were established from frozen stocks in cell culture medium and 10% DMSO stored in liquid nitrogen. Cell numbers were determined using a Casy® cell counter. For TGF-β stimulation, MCF10A cells were plated at 1×10^6 cells per 6 cm dish and 5 ng/ml TGF-β was added to the media the next day. Medium was replenished

every 2-3 days for a total duration of 7 days before using cells for RNAi or overexpression experiments.

Transient transfection with plasmid DNA

In MCF10A cells, plasmid transfections were performed with Polyethylenimine (PEI). $1x10^5$ MCF10A cells were seeded in 2ml medium in a 6-well format 24h prior transfection. For each well, a transfection mix was prepared containing 500µl OptiMEM®, 2µg DNA and 6µl PEI and incubated 5-10min at room temperature. In the case of double transfections, 1µg of each plasmid were used to obtain the same amount of total DNA. The transfection mix was added dropwise to the cells and the cells were analyzed 24h post transfection. MDA-MB-231 cells stably overexpressing cherry-MT1-MMP were transiently transfected with Lipofectamine® LTX with Plus reagent (Invitrogen) according to the manufacturer's instructions. Briefly, cells were seeded in 2ml growth medium 24h prior to transfection. A transfection mix for each well was prepared in 500µl OptiMEM® by adding 2,5µg DNA and 2,5µl Plus reagent. After 15 minutes incubation at room temperature, 9µl LTX reagent was added to the mix and incubated for another 30 minutes at room temperature before being added dropwise to the cells. 24h post transfection, the cells were subjected to further analysis.

Transient transfection with siRNA

For gene silencing, both cell lines were reverse transfected with small interfering RNAs (siRNAs) using Lipofectamine® RNAiMAX (Invitrogen). To avoid RNA degradation, only RNAse-free reaction tubes, solutions and pipette tips were used. 2x105 cells were seeded in 2 ml medium in a 6-well format. For each well, 5 nM siRNA (6 µl of a 2 µM stock solution) and 5 µl Lipofectamine® RNAiMAX were diluted in 500 µl OptiMEM® and incubated for 10-15 min at room temperature. The transfection mix was added dropwise to the freshly seeded cells which were analyzed 72 h post transfection. For immunofluorescence experiments, 1x105 cells were seeded on collagen-coated glass coverslips in 1 ml growth medium. The transfection mix was scaled down to 5 nM siRNA and 2 µl Lipofectamine® RNAiMAX in 200 µl OptiMEM®.

2.2.2 Immunoblotting

Cells were lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 0.5 mM PMSF, 0.1% SDS, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 20 mM β-glycerophosphate plus Complete protease inhibitors without EDTA (Roche) and lysates were clarified by centrifugation at 16,000 x g for 10 min. Protein concentration was determined by Bio-Rad DC protein assay. After extraction, proteins

were denaturated in 1x Laemmli sample buffer for 5 min at 100°C. Depending on the molecular weight of the analyzed proteins, the separating gel consisted of 8% or 10% polyacrylamide, the stacking gel always contained 4% polyacrylamide. For detection of MT1-MMP, lysates were run on NuPage® Novex® 4-12% Bis-Tris gels (Invitrogen) and blotted onto nitrocellulose membranes using the iBlot® device (Invitrogen). Separated proteins of the SDS polyacrylamide gels were transferred onto a polyvinylidene difluoride membrane (Roti® PVDF, Roth) by semi-dry blotting. The membrane was blocked with 0.5% blocking reagent (Roche) in PBS containing 0.1% Tween-20 and then incubated with primary antibodies (Table 8) over night at 4°C, followed by HRP-labeled secondary antibodies for ECL-based (Pierce, Rockford, IL) visualization with the Fusion Solo system (Vilber Lourmat).

2.2.3 Biotinylation assay

Cells were incubated with 1 mg/ml nonpermeable Biotin (EZ-Link Sulfo-NHS-SS-Biotin, Sigma Aldrich) for 30 min on ice under gentle agitation. Cells were then washed with PBS, quenched for 5 min and washed once more with PBS before lysis in RIPA buffer. Lysates were incubated with streptavidin beads for 2 h at 4°C to precipitate the biotinylated surface proteins, followed by 3 washes with 1% NEB buffer. Proteins were released by boiling in SDS sample buffer and analyzed by immunoblotting.

2.2.4 Immunofluorescence stainings and confocal microscopy

Cells grown on glass coverslips coated with 10 µg/ml collagen R (Serva; Heidelberg, Germany) or cross-linked gelatin, were fixed for 15 min with 4% PFA. After washes in PBS, cells were incubated for 15 min with 150 mM glycine in PBS and permeabilized for 5 min with 0.2% Triton X-100 in PBS. Blocking was performed with 5% goat serum (Invitrogen) in PBS containing 0.1% Tween-20. Fixed cells were incubated with primary antibodies diluted in blocking buffer for 2 h at RT. Following three washing steps with PBS containing 0.1% Tween-20, cells were incubated with Alexa Fluor® (488, 546, 633)-labeled secondary antibodies and phalloidin in blocking buffer for 1 h at RT. Nuclei were counterstained with DAPI and cells were washed twice with PBS containing 0.1% Tween-20. Coverslips were mounted in Fluoromount-G® (SouthernBiotech; Birmingham, AL, USA) and analyzed at RT on a confocal laser scanning microscope (LSM 710, Carl Zeiss; Oberkochen, Germany) equipped with EC Plan-Neofluar 40 × /1.30 DIC M27 and Plan-Apochromat 63x/1.40 DIC M27 (Carl Zeiss) oil immersion objectives using 488-, 561- and 633-nm excitation. Linear adjustments to brightness and contrast, as well as maximum intensity projections were made using the ZEN software (Carl Zeiss). For quantification of mean fluorescence intensities, images were acquired with the same confocal

settings and analyzed using the ImageJ software (NIMH; Bethesda, Maryland, USA). The Mander's coefficient was determined using the JACoP plugin (Bolte and Cordelières 2006).

2.2.5 TIRF microscopy

Transfected cells were replated on collagen-coated 35mm high glass bottom µ-Dish (ibidi, Cat.No: 81158). TIRF studies were performed on a Zeiss Axio Observer, equipped with a motorized TIRF illuminator (Laser TIRF 3), an EMCCD camera (Photometrics Evolve 512) and an alpha Plan-Apochromat 100x/1.46 NA Oil objective. MT1-MMP-pHluorin was visualized with a 488 nm diode laser in combination with a 525/50 nm emission filter. TIRF images were acquired at 37°C and 1 % CO_2 every 10 seconds for a time interval of 30 minutes. Image processing was done with Zen 2.3 blue software.

2.2.6 FRET analysis

Two days post siRNA transfection, control and DLC3-depleted cells were transiently transfected with the vectors encoding the Rho biosensors. The next day, cells were fixed and mounted in Mowiol® (Polysciences; Warrington, PA, USA). FRET efficiencies were determined using the acceptor photobleaching method. CFP was excited with a diode UV laser at 405 nm and emission was detected in the spectral window 454-515 nm. YFP was excited with the 514 nm laser line of an argon laser and emission was detected from 515-621 nm. Donor and acceptor images were acquired pre- and post-bleaching. Cells were bleached for YFP with the 514 nm argon laser line (90% intensity, 30 iterations, pixel dwell 50.4 µs). The FRET efficiency was calculated from the increase of the donor intensity (CFP) after acceptor bleaching using the FRET module of ZEN 2009 software (Carl Zeiss). FRET efficiency images were generated with a MATLAB script (developed by Dr. Felix Neugart, University Stuttgart, Germany) that allows background suppression and visualization of the FRET efficiency at the same time by using a two dimensional look up table (total fluorescence intensity coded by pixel brightness, FRET efficiency coded by color).

2.2.7 Quantitative PCR

RNA was isolated with the NucleoSpin® RNA Kit (Macherey-Nagel, Hœrdt, France). Q-PCR was performed with Power SYBR® Green 1-Step Kit (Thermo Fisher Scientific, USA) using a Cfx96 device (Biorad) according to the manufacturer's protocol for one-step RT-PCR. Primers used were 5′- GTCCGTGACGAGAAGTTATTACC-3′ and 5′-TGAGCGCACTTGTTTCCAAAA -3′ for Rab4 (Eurofins, Luxembourg) and Hs_PPIA_4_SG QuantiTect Primer Assay (Qiagen).

Changes in the relative expression level were determined using the $2^{-\Delta\Delta Ct}$ method (Biorad CFX manager software 3.1.). PPIA was used as a control gene for normalization.

2.2.8 Matrix degradation assays

For matrix degradation assays, coverslips were incubated with fluorescently labelled gelatine (1 mg/ml) in an inverted manner on EtOH sterilized parafilms for 30 min protected from light in a humidified chamber. Gelatine-coated coverslips were then cross-linked with 0.5% glutardialdehyde (Roth; Karlsruhe, Germany) in H_20 for 30 min, transferred to dishes, washed three times with PBS and stored at 4°C until further use. Before seeding the cells, coverslips were equilibrated with medium at 37°C for at least for 30 min. Cells were replated on gelatine coated coverslips for 3 hours (MDA-MB-231 cells stably expressing MT1-MMP) and 6 hours (MCF10A) and coverslips were processed as described in the immunofluorescence microscopy section. 40 images (40x oil magnification) per sample were acquired using identical settings (488 and DAPI channel). Quantitative image analysis of gelatine degradation was performed with CellProfiler software version 3.0.0 (Carpenter et al., 2006, Kamentsky et al., 2011). In brief, the total area of gelatine degradation per image was measured and normalized to the number of nuclei.

2.2.9 Statistical Analysis

Bar graphs show the mean ± S.E.M. Centre lines of the box blots show the medians; box limits indicate the 25th and 75th percentiles as determined by GraphPad Prism 7 software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. ‘N’ refers to the number of sample points for each box blot and ‘n’ to the number of independent experiments. Statistical significance was analyzed by one-way ANOVA followed by Tukey’s post-test (GraphPad Prism version 7; GraphPad Software Inc., La Jolla, CA). Results were considered significant when p-values were below 0.05 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; n.s. (not significant) $p > 0.05$).

3. Results

3.1 Functional and spatial validation of the RhoB biosensor

The first aim of the thesis was to investigate if DLC3 regulates the activity of the Rho GTPases RhoA, RhoB or RhoC in the utilized cell system of the MCF10A cells after induced EMT. The biosensors for RhoA and RhoC were already established in the lab, but the recently developed RhoB biosensor (Reinhard et al. 2016) needed to be validated. To show specific efficiency of the RhoB biosensor in a DLC3 dependent manner, RhoB activity was measured in HeLa cells expressing either DLC3-WT or the GAP inactive mutant DLC3-K725E. As a positive control the GEF protein Dbl was used. Only expression of DLC3-WT, but not DLC3-KE, led to a significant decrease in FRET efficiency, demonstrating that the regulation of RhoB activity by DLC3 is GAP-dependent (Figure 7A). With the FRET efficiency of the positive control being significantly higher than the control, the functionality of the RhoB biosensor was proven.

In contrast to the other Rho family members RhoA and RhoC, RhoB undergoes a unique post-translational modification, allowing it to attach not only to the PM, but also to endosomal structures. To confirm that the RhoB biosensor localized to endosomal membranes, the RhoB biosensor was expressed in TGF-β-treated MCF10A cells and detailed immunofluorescence analysis was performed. As a marker for early endosomes EEA1 was used, a protein that is important for endosomal trafficking and that binds exclusively to early endosomes. The small GTPase Rab7, a key regulatory protein for proper aggregation and fusion of late endocytic structures, served as a marker for late endosomes (Bucci et al. 2000). The RhoB biosensor was enriched at both endosomal membranes (Figure 7B), which is consistent with the original report (Reinhard et al. 2016). Thus, the RhoB biosensor was successfully validated in our hands showing mainly endosomal localization.

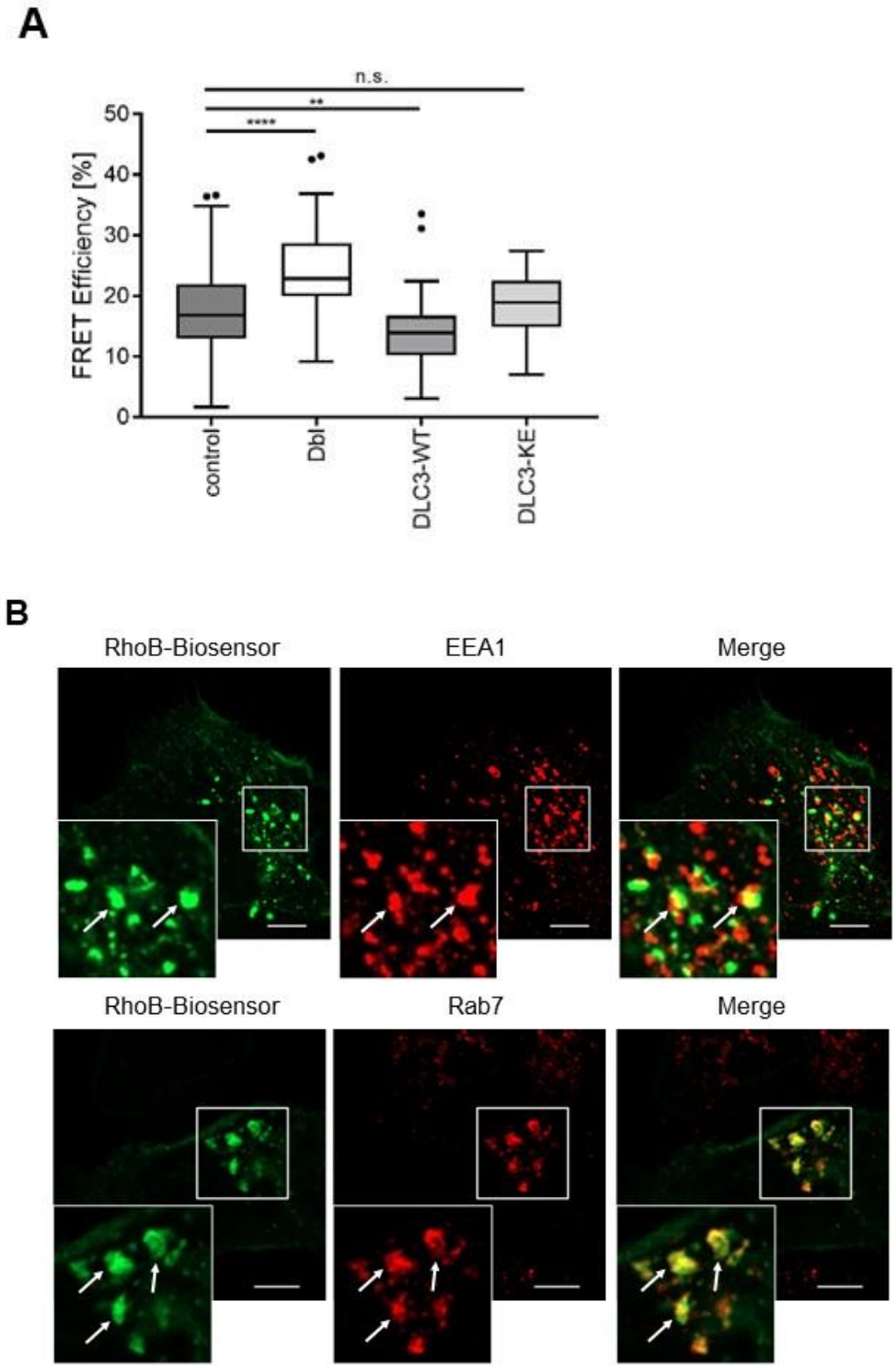


Figure 7: Functional and spatial validation of the RhoB-Biosensor.
(A) HeLa cells were seeded on collagen-coated coverslips and co-transfected with the vector encoding the RhoB-Venus biosensor and empty vector, pCELF-GST-Dbl, Flag-DLC3-WT or Flag-DLC3 K725E, respectively. 24 h later, cells were fixed and FRET efficiency was measured over the whole cell. N=61, 59, 57, 52 sample points. n=3, error bars represent mean±s.e.m.,**p<0.01, ****p<0.0001 n.s.=not significant (one-way ANOVA followed by Tukey's posttest). (B) MCF10A cells were pretreated with TGF-β for 7 days, transfected with the RhoB-Venus-Biosensor and replated on collagen-coated coverslips the next day. 24 h later, the cells were fixed and stained with EEA1- or Rab7-specific primary antibody, respectively, followed by Alexa Fluor 546-coupled secondary antibody (red). White arrows indicate colocalization events of the RhoB-Biosensor with EEA1 or Rab7. Scale bar, 20 µm.

3.1.2 DLC3 regulates global RhoB activity in TGF-β-treated MCF10 A cells

A previous study of our lab reported a regulatory role of DLC3 in adherens junctions integrity and polarized morphogenesis by locally regulating RhoA activity (Hendrick et al. 2016). To address DLC3 localization and function in a different cellular context where cell-cell contacts are lost and cells adopt a more motile phenotype, the breast epithelial cell line MCF10A was stimulated with TGF-β for 7 days to trigger a moderate, reversible EMT-like program, transforming the epithelial- like cells into a more mesenchymal phenotype. Treatment of MCF10A cells induced a moderate downregulation of the epithelial marker E-Cadherin and an upregulation of the mesenchymal markers N-Cadherin and Vimentin, an indicator of cells undergoing partial EMT (Noll et al. 2019; J. Zhang et al. 2014).

To evaluate in detail which Rho GTPases are regulated by DLC3 in TGF-β-treated MCF10A cells, we transiently transfected the cells with biosensors for RhoA, RhoB and RhoC. Using the acceptor photobleaching method, which determines the increase in donor fluorescence after completely bleaching the acceptor, FRET efficiencies of the three different biosensors and the effect of DLC3 depletion was measured. Intriguingly, only the activity of the RhoB biosensor significantly increased after DLC3 depletion, whereas the activity of the RhoA and RhoC biosensor did not show any significant changes (Figure 8A).

The knockdown efficiency of DLC3 in TGF-β-treated MCF10A cells was determined by western blotting, with α-tubulin as a loading control. The single siRNA (siDLC3) as well as the smartpool of four siRNAs targeting DLC3 (spDLC3) showed a sufficient knockdown on the protein level (Figure 8B). In sum we could show that in TGF-β-treated MCF10A cells only RhoB is regulated on a global scale.

Taken together DLC3 regulates global RhoB activity, but not that of RhoA or RhoC, in the cellular context of TGF-β-treated MCF10A cells.

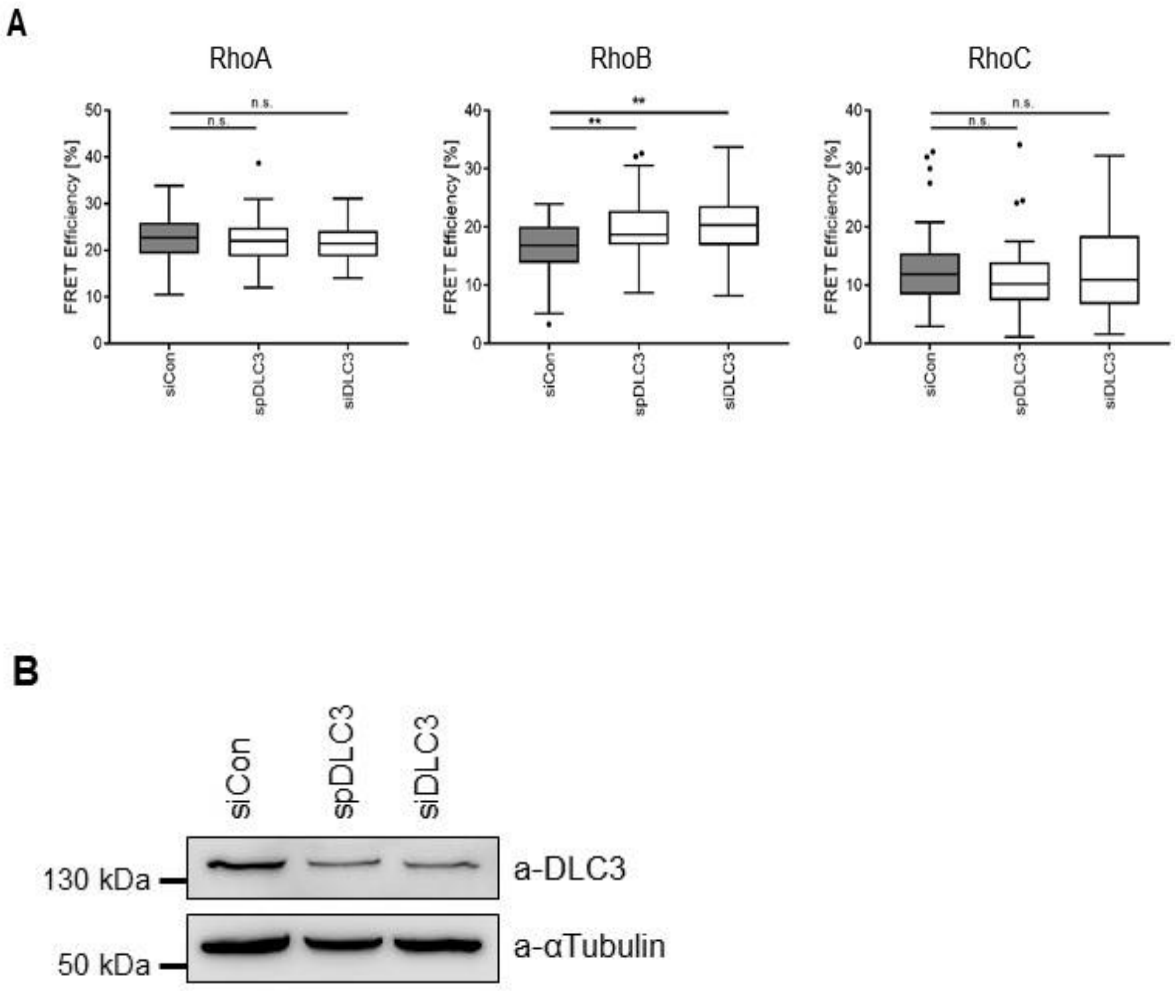
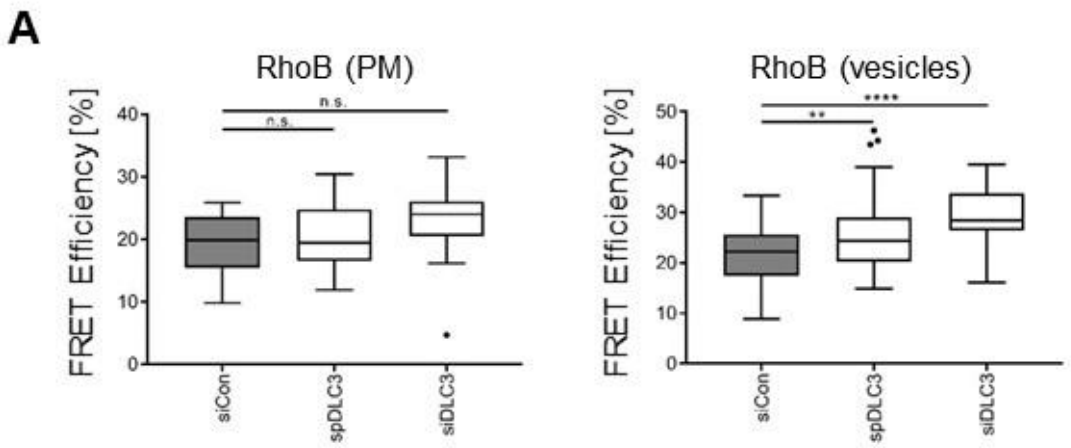


Figure 8: DLC3 regulates RhoB activity.
(A, B) MCF10A cells were pretreated with TGF-β for 7 days and then transfected with the indicated siRNAs. (A) 24 h post transfection, cells were transfected with the vector encoding the RhoA, RhoB or RhoC biosensor, respectively. 48h later, cells were fixed and FRET efficiency of the different Biosensors was measured over the whole cell. For RhoA N=49, 42, 44 sample points, for RhoB N=50, 41, 43 sample points and for RhoC N=42, 34, 46 sample points. n=3, error bars represent mean±s.e.m., **p<0.01, n.s.=not significant (one-way ANOVA followed by Tukey's posttest). (B) 72h post transfection cells were lysed and DLC3 knockdown efficiency was determined by Western blotting using the indicated antibodies. α-tubulin was used as a loading control.

3.1.3 DLC3 functions as a GAP protein for endosomal RhoB

Given the results of a correctly localized RhoB biosensor and a global effect of DLC3 on its activity, we wanted to take a closer look at the different intracellular RhoB pools which are regulated by DLC3 in TGF-β-treated MCF10A cells. As mentioned before, RhoB can localize not only to the PM, but also to endosomal membranes. Therefore, the FRET efficiencies of the intracellular RhoB pools at the PM and at vesicular structures were analyzed. Interestingly, DLC3 depletion only led to a significant increase in the FRET efficiency at the vesicular structures, but not at the PM (Figure 9A), demonstrating a local regulation of RhoB activity by DLC3 at vesicular structures. For better visualization, the FRET efficiencies of the RhoB biosensor were plotted as a pseudocolor thermal map and the areas identified as PM and vesicular structures were marked (Figure 9B).



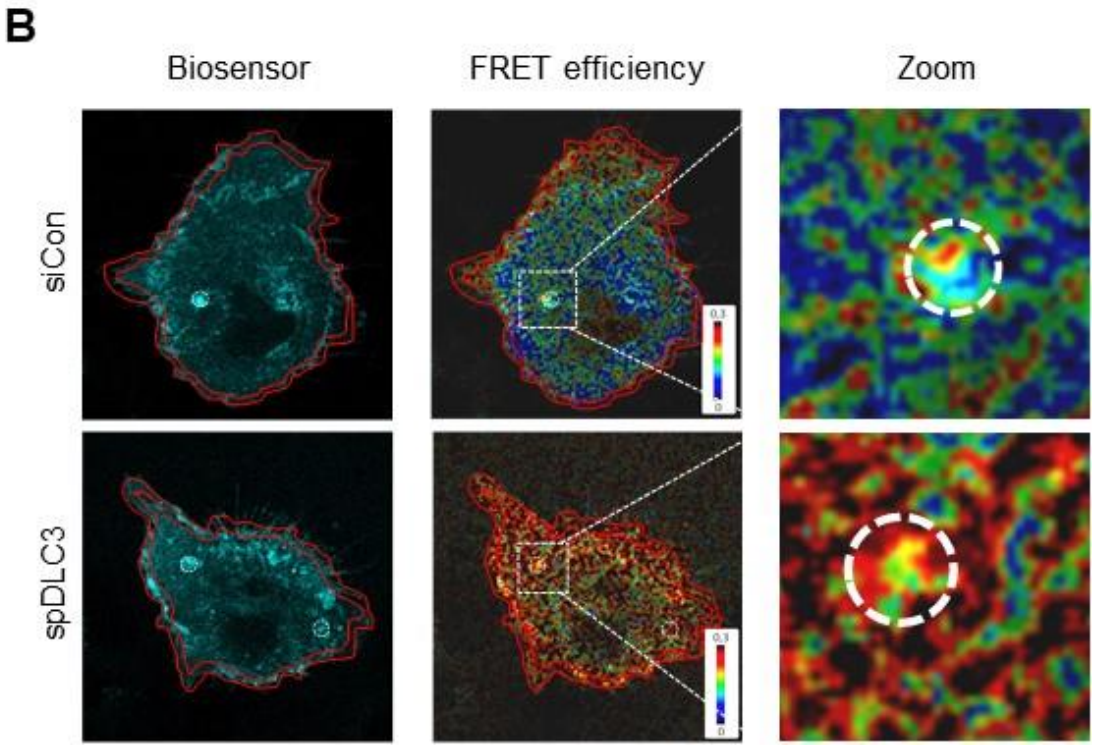


Figure 9: DLC3 functions as a GAP protein for endosomal RhoB.
(A, B) MCF10A cells were pretreated with TGF-β for 7 days and then transfected with the indicated siRNAs. 24 h post transfection, cells were transfected with the vector encoding the RhoB-Venus biosensor. 48 h later, cells were fixed and FRET efficiency for the RhoB sensor was measured at the plasma membrane (PM) and at vesicular structures. N=19, 23, 18 sample points (PM), N=47, 60, 49 sample points (vesicles) n=3, error bars represent mean±s.e.m., **p<0.01, ****p<0.0001, n.s.=not significant (one-way ANOVA followed by Tukey´s posttest). (B) Plotted are the mean FRET efficiencies which are displayed as a pseudocolor thermal map corresponding to the depicted scale, from low (blue) to high (red) RhoB activity. Marked are the areas for analysis of vesicular structures (white dotted circle) and PM (red framed area).

Form this data, it can be concluded that DLC3 regulates RhoB activity mainly at vesicular structures.

3.1.4 DLC3 colocalizes with RhoB at vesicular structures

Until now only an indirect effect of DLC3 on RhoB activity was shown, mainly at endosomal membranes, in fixed cells. In order to directly regulate RhoB at these structures, DLC3 has to interact and colocalize with RhoB. To verify this highly dynamic DLC3-RhoB interaction on endosomes, GFP-tagged DLC3-KE and orange-tagged RhoB was co-expressed in TGF-β-treated MCF10A cells and the cells were replated onto glass bottom dishes. As we were particularly interested in membrane-proximal vesicles and their dynamics, total internal

reflection fluorescence (TIRF) live cell microscopy was used. With this method, only fluorescent molecules within a few hundred nanometers above the glass surface are efficiently excited and visualized. And indeed, merging and co-trafficking of vesicles positive for GFP-DLC3-KE as well as orange-RhoB could be observed (Figure 10A). Thus, we could show the dynamic, vesicular colocalization of RhoB and DLC3 in living cells that have undergone partial EMT, supporting our hypothesis that in these cells DLC3 regulates RhoB at endosomal membranes.

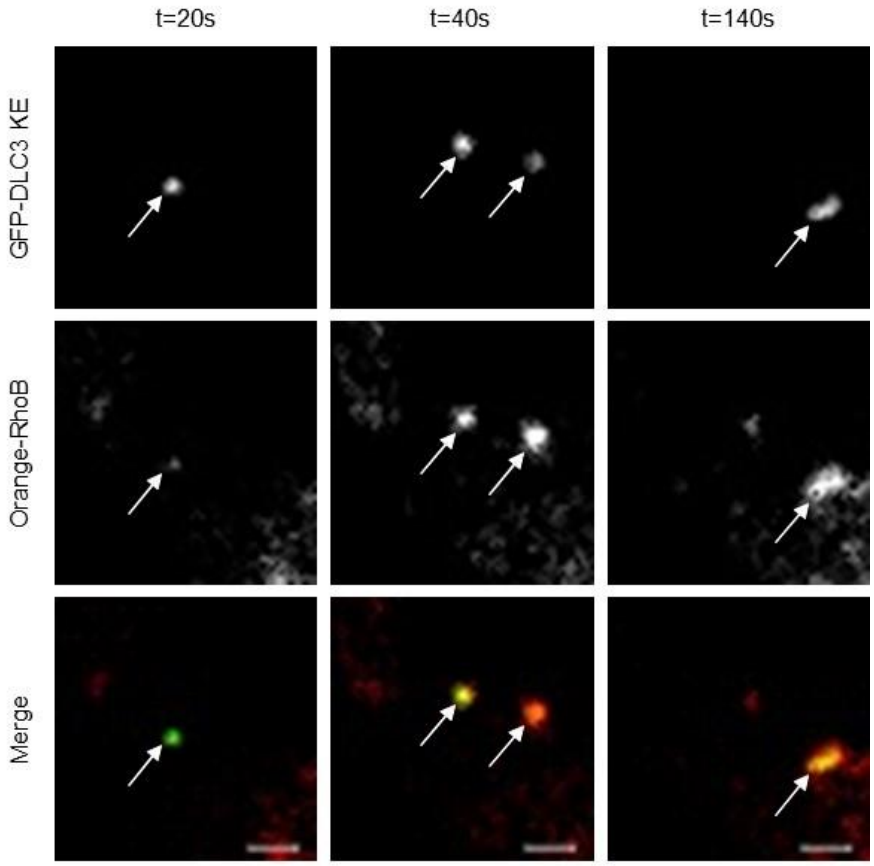
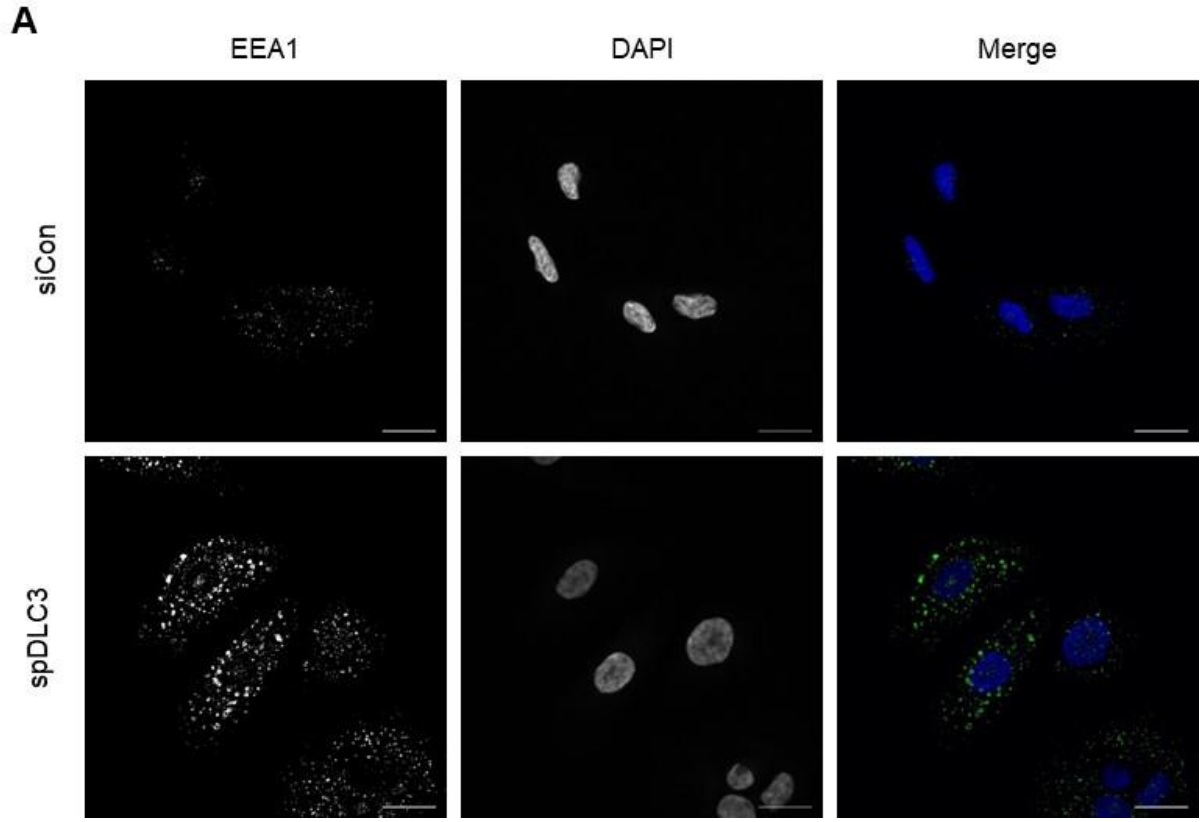


Figure 10: DLC3 colocalizes with RhoB at vesicular structures.
(A) MCF10A cells stably expressing GFP-DLC3 K725E were treated for 7 days with TGF-β, transfected with pmOrange2-C1-Endo RhoB and replated on collagen-coated glass-bottom dishes 24 h post transfection. 24 h later, living cells were imaged by TIRF microscopy. Time in seconds. Scale bar, 2 µm.

3.2 DLC3 is required for the integrity of early endosomes

Considering that active RhoB was shown to regulate endosome transport by promoting actin assembly on endosomal membranes through Dia1 (Fernandez-Borja et al. 2005), we now wanted to address in detail how DLC3 on endosomes interferes with endocytic membrane trafficking. Therefore, we depleted DLC3 in TGF-β-treated MCF10A cells, fixed the cells and stained for the early endosomal marker EEA1. Strikingly, the depletion of DLC3 led to a strong accumulation of EEA1-positive vesicles (Figure 11A). To quantify this visually detectable effect, ImageJ was used to analyze the size of the vesicles and the number of vesicles per cell. Notably, both the size of the early endosomes (Figure 11B) and the number of EEA1-postive vesicles per cell (Figure 11C) significantly increased after DLC3 depletion.



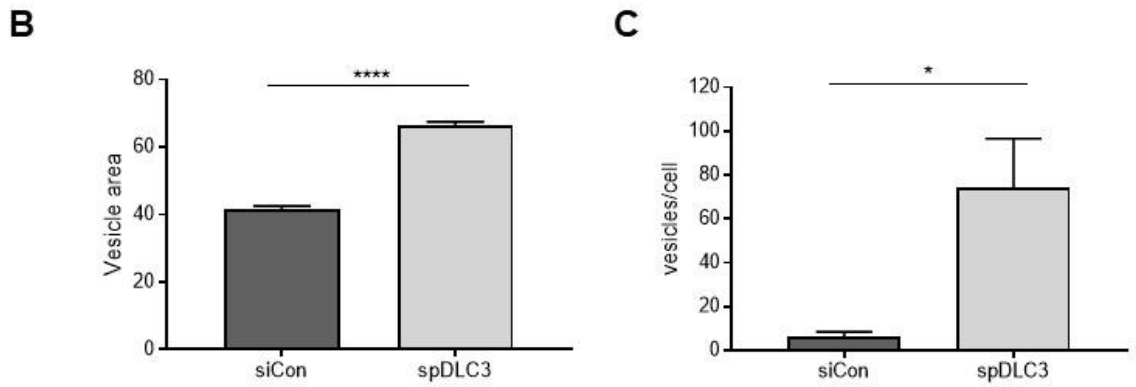


Figure 11: DLC3 depletion leads to an accumulation of early endosomes.
(A-C) MCF10A cells were pretreated with TGF-β for 7 days and then transfected with the indicated siRNAs. 48 h post transfection, cells were replated on collagen coated coverslips and fixed 24 h later. Cells were stained with EEA1-specific primary antibodies, followed by Alexa Fluor 488-coupled secondary antibodies (green). Nuclei were counterstained with DAPI (blue). Scale bar, 20µm. (B) The area of EEA1-positive vesicles per cell was quantified by ImageJ. n=3, error bars represent mean±s.e.m, ****p<0.0001 (one-way ANOVA followed by Tukey´s posttest). (C) The number of EEA1-positive vesicles per cell was quantified by ImageJ. n=3, error bars represent mean±s.e.m, *p<0.05 (one-way ANOVA followed by Tukey´s posttest).

Hence, DLC3 is required for the integrity of early endosomes and the depletion of DLC3 led to an accumulation of these endosomes.

3.2.2 DLC3 depletion has no effect on late endosomes or lysosomes

RhoB localizes not exclusively to early endosomes, but to endosomal membranes in general. This includes the vesicles further along the endocytic trafficking route. Thus, we wanted to investigate whether DLC3 depletion has a global effect on all endosomal vesicles or exclusively on early endosomes. As a marker for late endosomes the small GTPase Rab7 was used (Figure 12A). Additionally, the cells were stained for lysosomal-associated membrane protein 1 (LAMP1), which resides primarily across lysosomal membranes (Figure 12B). Intriguingly, neither Rab7- nor LAMP1-positive vesicles seemed to be affected by DLC3 depletion. Quantification of the number of Rab7-positive vesicles per cell via ImageJ showed no significant differences (Figure 12A). The same counts for LAMP1-postitive vesicles (Figure 12B).

Thus, it seems that the effect of DLC3 depletion is restricted to the level of early endosomes. Taken together, from the data so far, we assume that DLC3 maintains the integrity of the early endosomal compartment and regulates endocytic membrane transport through the control of local RhoB activity.

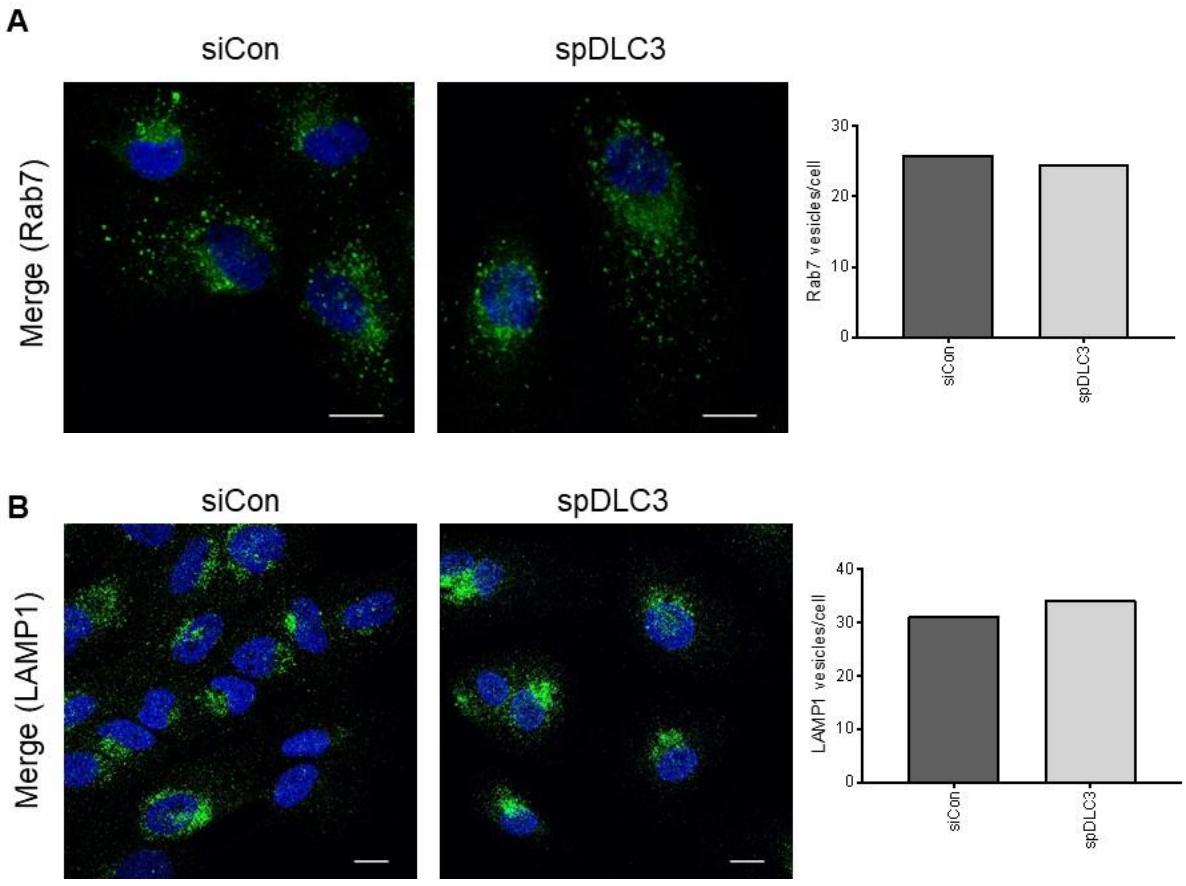


Figure 12: DLC3 depletion has no effect on late endosomal pathway.
MCF10A cells were pretreated with TGF-β for 7 days and then transfected with the indicated siRNAs. 48 h post transfection, cells were replated on collagen coated coverslips and fixed 24 h later. (A) Cells were stained with Rab7-specific primary antibodies, followed by Alexa Fluor 488-coupled secondary antibodies (green). Nuclei were counterstained with DAPI (blue). Scale bar, 20µm. The number of Rab7-positive vesicles per cell was quantified by ImageJ. n=1. (B) Cells were stained with LAMP1-specific primary antibodies, followed by Alexa Fluor 488-coupled secondary antibodies (green). Nuclei were counterstained with DAPI (blue). Scale bar, 20µm. The number of LAMP1-positive vesicles per cell was quantified by ImageJ. n=1.

3.3. DLC3 depletion leads to enhanced endosomal actin

Most of the previous experiments were performed in TGF-β-treated MCF10A cells, a suitable model system to investigate cellular processes in cells that underwent partial EMT, thereby switching from an epithelial to a more mesenchymal phenotype. Nevertheless, we wanted to validate our results in another cell line. Therefore, we switched for the following experiments to the highly invasive, triple negative breast cancer cell line MDA-MB-231, which has undergone almost full EMT, is well characterised and shows high recycling activity ((Frittoli et al. 2014) and stably expresses MT1-MMP (Sakurai-Yageta et al. 2008). This cell line has the advantage of stably expressing MT1-MMP, a matrix metalloproteinase and key regulator in

matrix degradation and invasion which could be used as a model cargo to address in detail how DLC3 on endosomes interferes with endocytic membrane trafficking and eventually the matrix degradation capacity of the cells. Our previous results in TGF-β-treated MCF10A showed that DLC3 depletion leads to an enhanced RhoB activity at endosomes and an accumulation of early endosomes, but not late endosomes or lysosomes. Experiments conducted by a bachelor student in our lab showed that this accumulation effect of early endosomes was also true for the MDA-MB-231 cells stably expressing MT1-MMP.

As a Rho GTPase, the main role of RhoB is the polymerization of actin (Fernandez-Borja et al. 2005). Thus, we were interested whether the early endosomal actin is affected by DLC3 depletion. Therefore, we transiently transfected MDA-MB-231 cells stably expressing MT1-MMP with GFP-LifeAct, a 17 amino acid small recombinant protein that stains filamentous actin (F-actin) structures (Riedl et al. 2008). The small size allows it to localize to very small actin structures without impairing any cellular processes. We then stained for EEA1 and compared the actin coat of control cells and DLC3 depleted cells, respectively. Notably, the depletion of DLC3 led to a strong increase of endosomal actin (Figure 13A). Quantifying the actin intensity at the early endosomes in relation to the global actin intensity via ImageJ confirmed a significant increase in early endosomal actin. The knockdown efficiency of DLC3 in MDA-MB-231 cells was verified by qRT-PCR (Figure 13C).

Thus, we can conclude that the enhanced RhoB activity at early endosomes due to DLC3 depletion leads to a significant increase in endosomal actin.

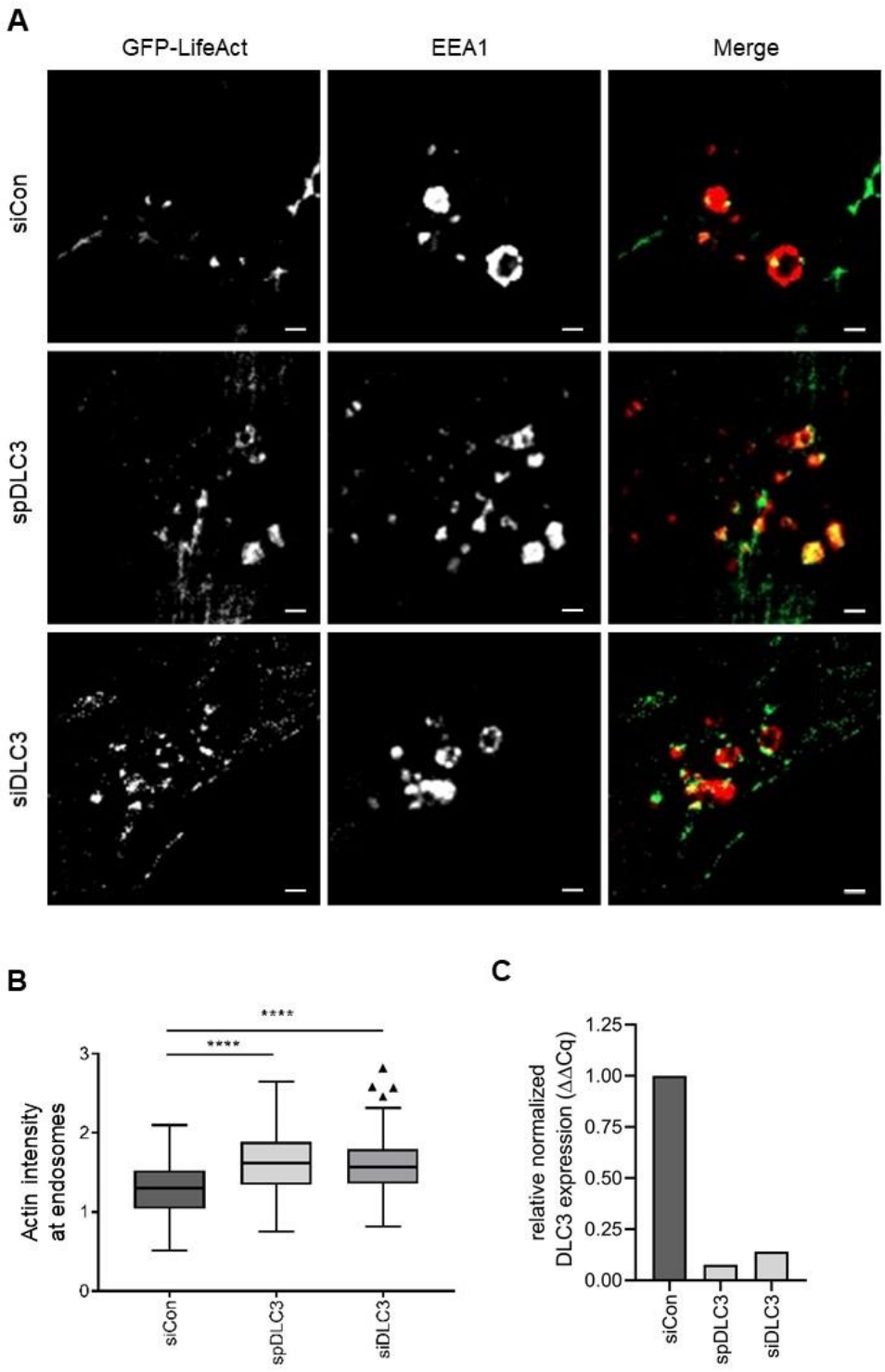
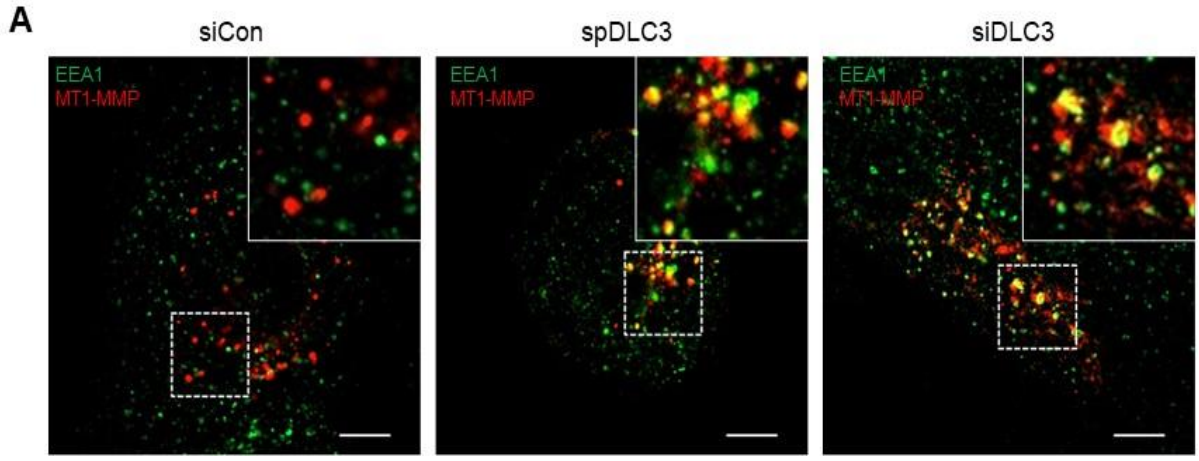


Figure 13: DLC3 depletion leads to enhanced endosomal actin.
MDA-MB-231 cells stably overexpressing cherry-MT1-MMP (red) were transfected with the indicated siRNAs. (A) 24 h post transfection, cells were transfected with pEGFPN1-Lifeact and replated on collagen-coated coverslips the next day. 24 h later, cells were fixed and stained with EEA1-specific primary antibody, followed by Alexa Fluor546-coupled secondary antibody (red). Scale bar, 2 µm. (B) The intensity of actin at endosomes was quantified using ImageJ. N=151, 374, 177 sample points, n=3, error bars represent mean±s.e.m., ****p<0.0001 (one-way ANOVA followed by Tukey´s posttest). (C) DLC3 knockdown efficiency was determined by quantitative PCR (n=1)

3.4.1 DLC3 depletion traps MT1-MMP in early endosomes

Early endosomes serve as a focal point of the endocytic pathway responsible for sorting and recycling of internalized cargo to different intracellular destinations (Jovic et al. 2010) and any malfunction of this compartment will affect trafficking or recycling of cargos such as MT1-MMP. As the loss of DLC3 impaired the early endosomal compartment we wanted to reveal whether this directly affected MT1-MMP trafficking. To do so, we depleted DLC3 in MDA-MB-231 cells stably expressing Cherry-MT1-MMP and stained for EEA1 to investigate how much MT1-MMP is trafficked through the early endosomal pathway in control and DLC3-depleted cells, respectively. Interestingly, the depletion of DLC3 seemed to increase the colocalization of MT1-MMP with EEA1-positive vesicles (Figure 14A). Quantification of the colocalization via the ImageJ plugin JACoP revealed that DLC3 depletion led to a significant increase in MT1-MMP and EEA1 colocalization (Figure 14B).



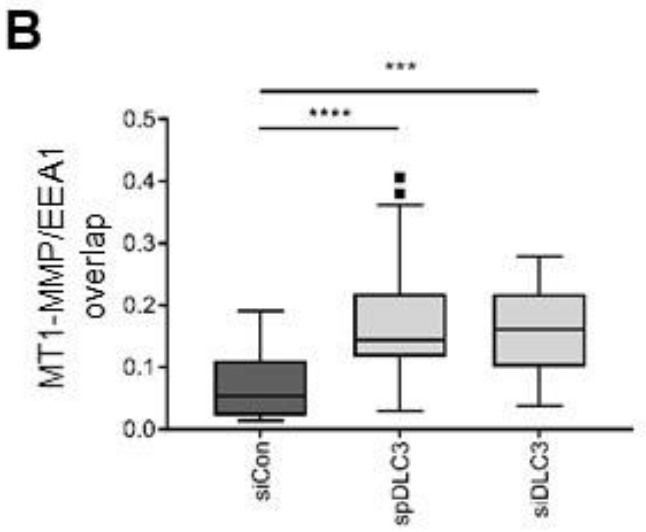


Figure 14: DLC3 depletion traps MT1-MMP in early endosomes.
(A, B) MCF10A cells were pretreated with TGF-β for 7 days, transfected with the indicated siRNAs and transfected with pCherry-MT1-MMP the next day. 24 h later, cells were fixed and stained with EEA1-specific primary antibody, followed by Alexa Fluor 488-secondary antibody (green). Shown is the merge of EEA1 and cherry-MT1-MMP. (B) The Mander‘s overlap coefficient of MT1-MMP with EEA1 was quantified using ImageJ. N= 23, 23, 24 sample points. n=3, error bars represent mean±s.e.m.,***p<0.001, ****p<0.0001 (one-way ANOVA followed by Tukey´s posttest).

Taken together, we could provide evidence that the depletion of DLC3 traps MT1-MMP in early endosomes, probably due to the enhanced endosomal actin polymerization via RhoB.

3.4.2 Late endosomal MT1-MMP localization is unaltered by DLC3 depletion

After inactivation at the PM, MT1-MMP usually becomes internalized, sorted in early endosomes and then either becomes degraded in lysosomes or is recycled back to the PM through the long-loop, Rab11-dependent recycling route. In both cases the route can pass through Rab7-positive late endosomes. In order to determine whether the accumulation of MT1-MMP is specific for the early endosomal stage or can also be observed in a late endosomal stage, we stained DLC3-depleted MDA-MB-231 cells stably expressing Cherry-MT1-MMP for the late endosomal marker Rab7. Intriguingly, no alterations in the colocalization of MT1-MMP and Rab7-positive late endosomes after DLC3 depletion could be observed (Figure 15A). The quantification of the Mander’s-Colocalization-Coefficient via ImageJ uncovered that there are no significant differences between control and DLC3-depleted cells (Figure 15B), supporting the conclusion of MT1-MMP being trapped specifically on the level of early endosomes.

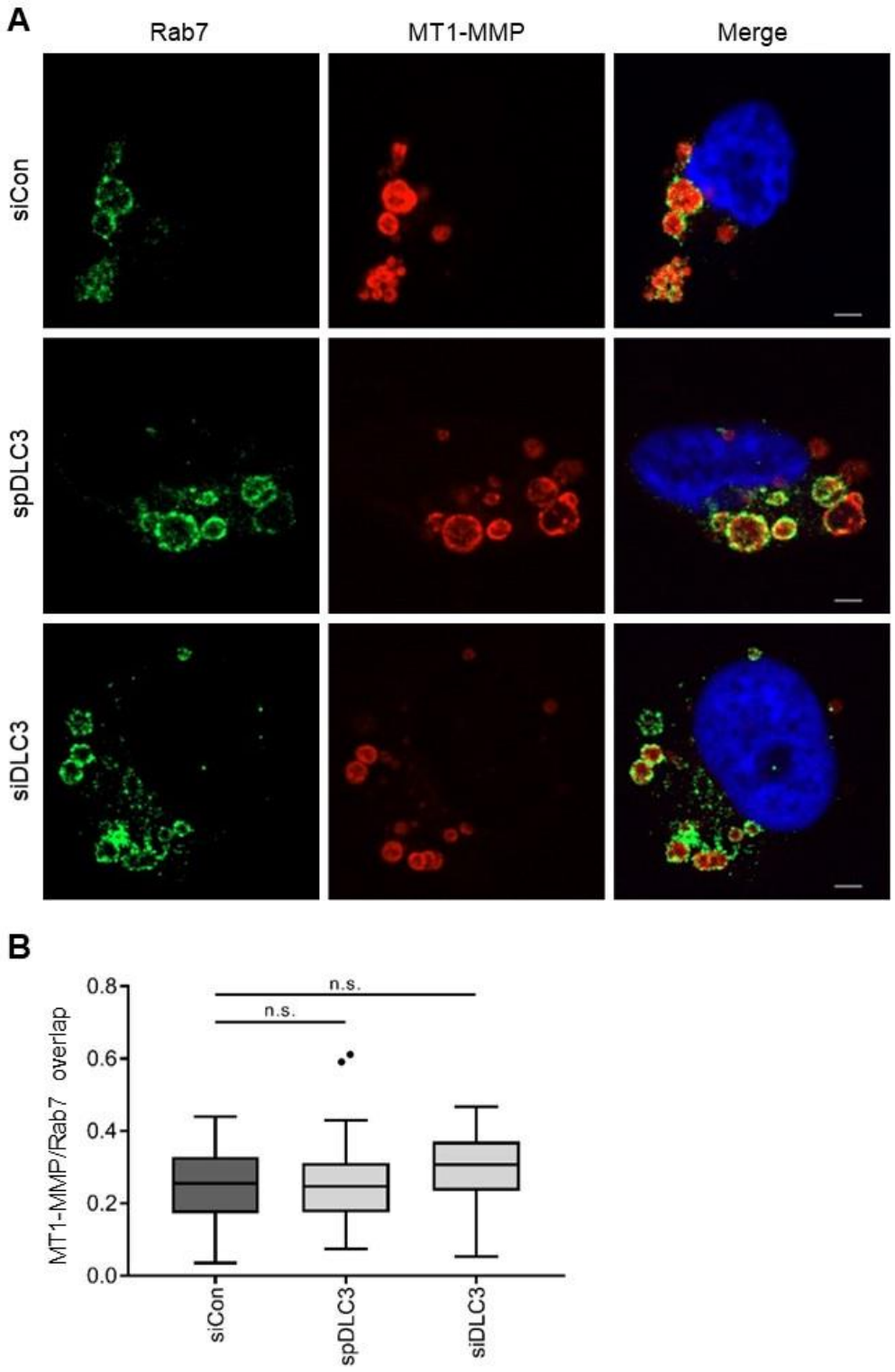
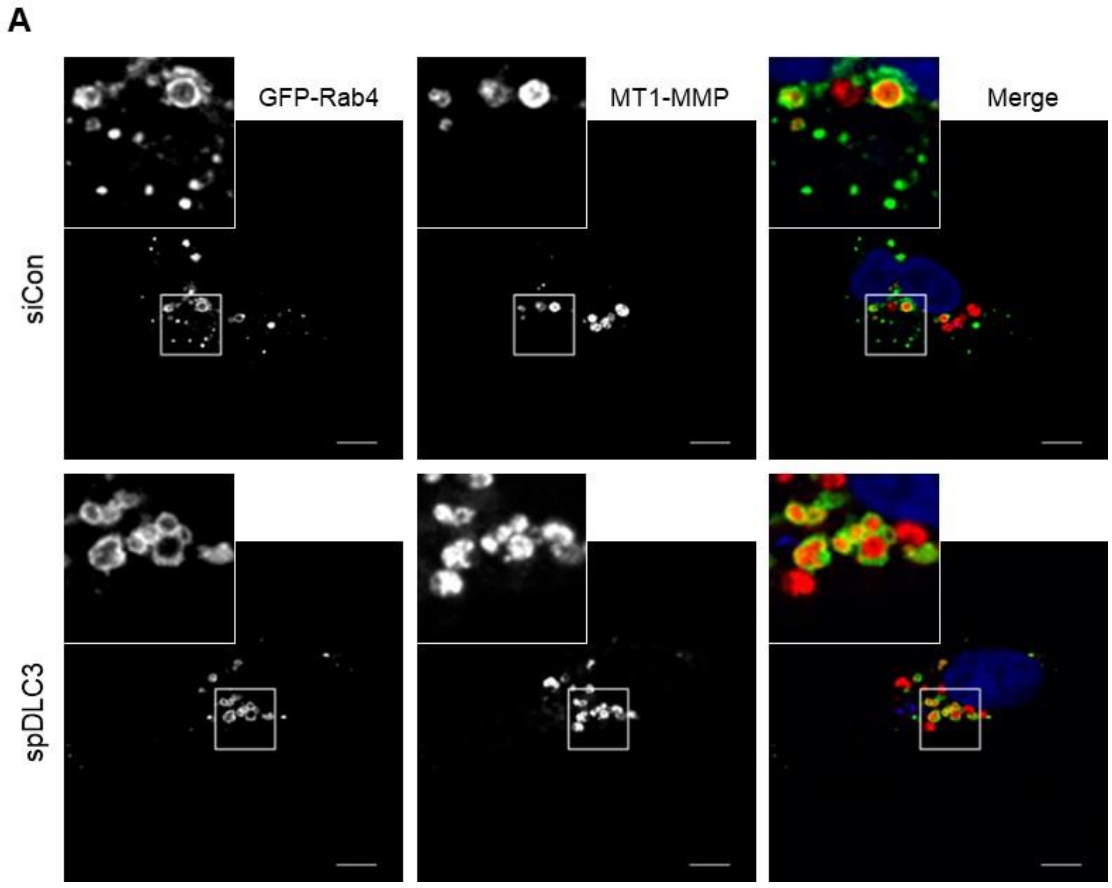


Figure 15: Late endosomal MT1-MMP localization is unaltered by DLC3 depletion.
MDA-MB-231 cells stably expressing cherry-tagged MT1-MMP were transfected with the indicated siRNAs. (A) 72 h post transfection, cells were fixed and stained with Rab7-specific primary antibody, followed by Alexa Fluor 488-secondary antibody (green). Nuclei were counterstained with DAPI (blue), scale bar 5 µm. (B) The Mander's overlap coefficient of MT1-MMP with Rab7 was quantified using ImageJ. N=29, 31, 31 sample points. n=3, error bars represents mean±s.e.m., n.s.=not significant (one-way ANOVA followed by Tukey's posttest).

3.5 DLC3 depletion increases the colocalization of MT1-MMP and Rab4-positive vesicles

As mentioned before, MT1-MMP is sorted at early endosomes to the Rab7 late endosomes, from where it is either targeted for lysosomal degradation or recycled back to the PM via the slow recycling route. But it has been described that under certain motogenic stimuli, MT1-MMP can also be directly recycled from early endosomes back to the PM via a Rab4-dependent fast recycling route (Frittoli et al. 2014; Stenmark 2009). Due to the lack of MT1-MMP accumulation at the Rab7-positive late endosomes, we hypothesized that in cells lacking DLC3 the accumulated MT1-MMP at early endosomes might be redirected to the PM via a Rab4-dependent fast-recycling route. To substantiate this hypothesis, we transiently transfected the MDA-MB-231 cells stably expressing Cherry-MT1-MMP with GFP-Rab4. And indeed, we could observe an increased colocalization of Cherry-MT1-MMP with Rab4-positive vesicles in DLC3-depleted cells compared to the control cells (Figure 16A). This is supported by the quantification of two independent experiments, with a clearly visible increase of MT1-MMP positive Rab4 vesicles (Figure 16B).



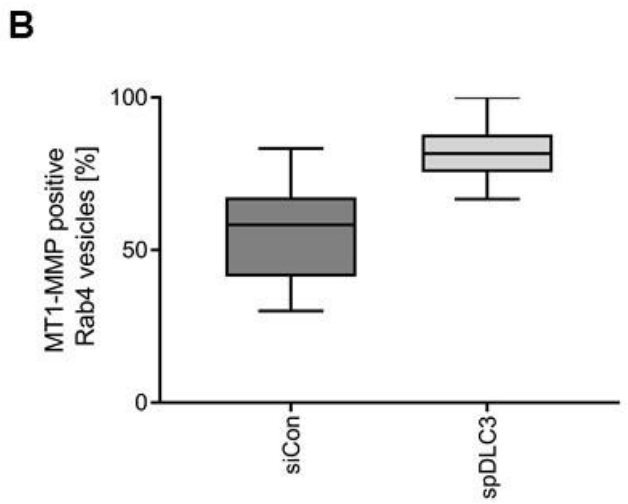


Figure 16: DLC3 depletion increases the colocalization of MT1-MMP with Rab4-positive vesicles. MDA-MB-231 cells stably overexpressing cherry-MT1-MMP were transfected with the indicated siRNAs. (A) 24 h post siRNA transfection, cells were transfected with pEGFP-Rab4 (green), replated on gelatin-coated coverslips the next day and then fixed 24 h later. Nuclei were stained with DAPI (blue). Scale bar, 20µm. (B) Quantification of cherry-MT1-MMP-positive GFP-Rab4 vesicles by ImageJ. N=18, 18 sample points, n=2, error bars represent mean±s.e.m.

Taken together, the depletion of DLC3 increases the colocalization of MT1-MMP and Rab4-positive vesicles, supporting the hypothesis of redirected MT1-MMP recycling towards the Rab4-dependent fast-recycling route.

3.5.1 DLC3 depletion enhances MT1-MMP surface levels

To investigate whether the potential Rab4-dependent rerouting of MT1-MMP affected its surface levels, we biotinylated the surface proteins of DLC3-depleted and control cells. By streptavidin pulldown, followed by SDS-Page, we could distinguish between the intracellular and the PM MT1-MMP pool. Depletion of DLC3 lead to an increase of MT1-MMP at both locations (Figure 17A), which confirms the results of previous experiments performed in our lab showing an overall increase of MT1-MMP after DLC3 depletion (data not shown). Quantifying the western blot signals, however, revealed that the increase in surface MT1-MMP was even slightly higher than the internal increase (Figure 17B).

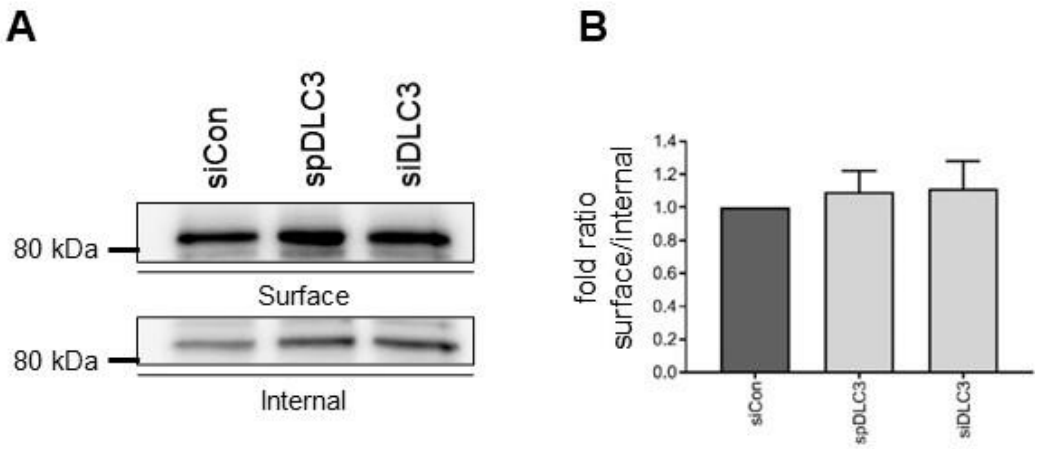


Figure 17: DLC3 depletion enhances MT1-MMP surface levels.
(A) MDA-MB-231 cells stably overexpressing cherry-MT1-MMP were transfected with the indicated siRNAs. 72 h post siRNA transfection, cells were biotinylated, lysed and biotinylated surface proteins were precipitated, followed by immunoblotting. Lysates were blotted to determine total protein levels. The blot is representative of three independent experiments. (B) The ratio of surface to internal protein was quantified using ImageJ, n=3, error bars represent mean±s.e.m.

These results provide first evidence that DLC3 depletion leads to increased surface levels of MT1-MMP.

3.5.2 DLC3 depletion enhances MT1-MMP exocytosis

To prove that the enhanced surface levels of MT1-MMP are due to an enhanced recycling of MT1-MMP and not due to impaired internalization, we used MT1-MMPpHluorin (Lizárraga et al. 2009). In this construct, MT1-MMP is fused to a fluorophore whose fluorescence is quenched in the acidic endosomal environment, but which emits green fluorescence when it is exposed to a neutral pH. Thus, the fusion of MT1-MMPpHluorin loaded vesicles with the PM can be observed in real-time TIRF live microscopy in the form of bright green bursts of fluorescence. Intriguingly, we could detect notably more fluorescent bursts in cells lacking DLC3, compared to control cells (Figure 18A). The quantification of the two independent experiments supported our observation (18B).

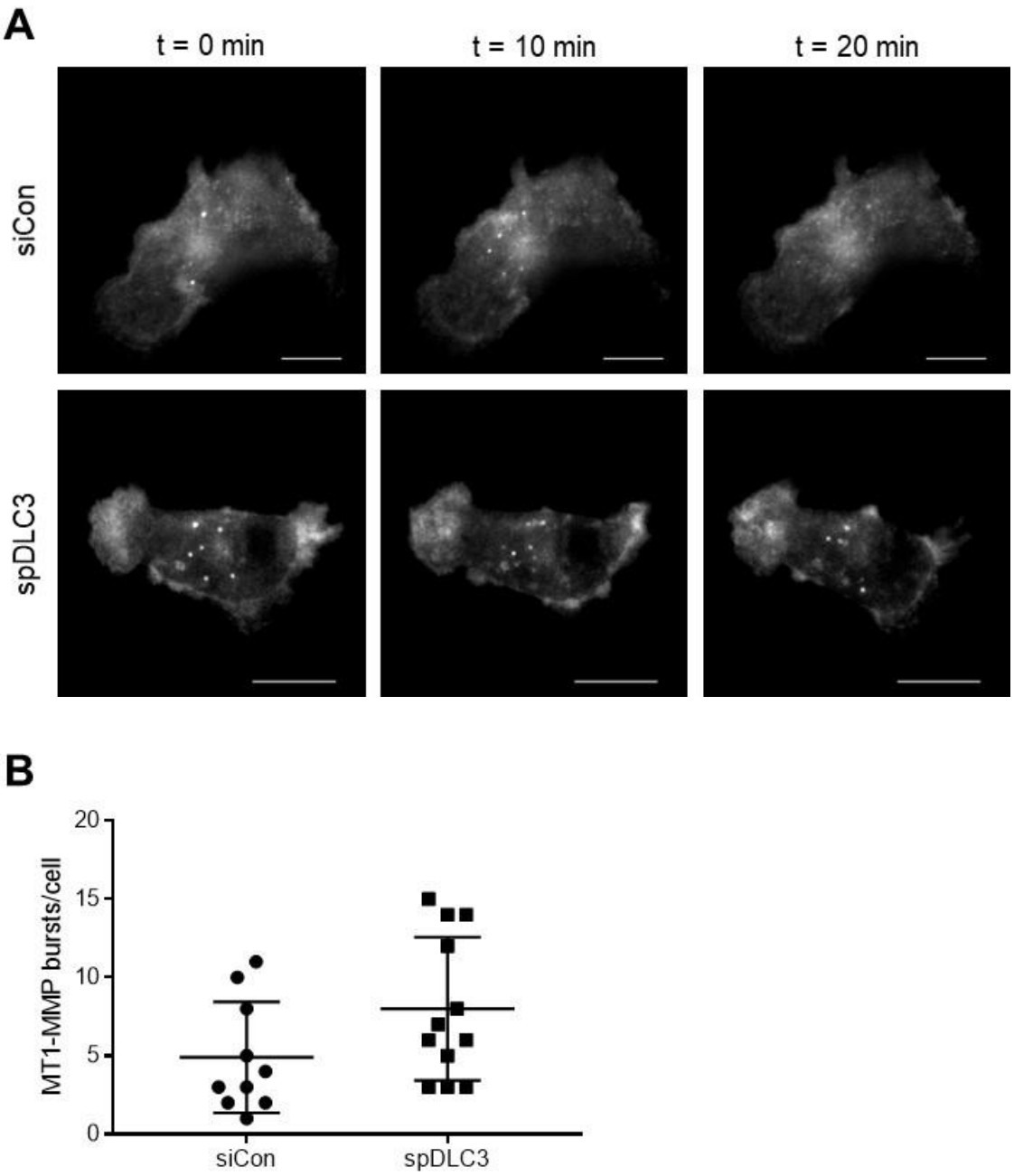


Figure 18: DLC3 knockdown enhances MT1-MMP exocytosis.
(A) MDA-MB-231 cells stably overexpressing cherry-MT1-MMP were transfected with the indicated siRNAs. 24h post transfection cells were transfected with MT1-MMP-pHluorin and replated on collagen coated TIRF dishes the next day. 24 h later, living cells were imaged by TIRF microscopy. Time in minutes, scale bar, 10µm. (B) Number of vesicle bursts at the basal membrane were quantified using ImageJ. N=10, 12 sample points, n=2, error bars represent mean±s.e.m.

Thus, the depletion of DLC3 seems to enhance the trafficking to and exposure of MT1-MMP at the PM, indicative of enhanced MT1-MMP exocytosis.

3.6 DLC3 depletion enhances matrix degradation

MT1-MMP is known to be a key player in extracellular matrix degradation. In order to evaluate whether the enhanced exocytosis and surface levels of MT1-MMP also have an effect on the capability of the cells to degrade matrix, a matrix degradation assay was performed. MDA-MB-231 cells stably expressing Cherry-MT1-MMP were plated onto fluorescently labelled, crosslinked gelatine, fixed and imaged under a confocal laser scanning microscope. The degradation area could then be identified as black spots on the otherwise green gelatine. As expected, cells lacking DLC3 degraded visibly more gelatine compared to the control cells (Figure 19A). Quantification of the degraded area of three independent experiments showed that depletion of DLC3 led to a significant increase in matrix degradation for both RNAi samples (Figure 19B).

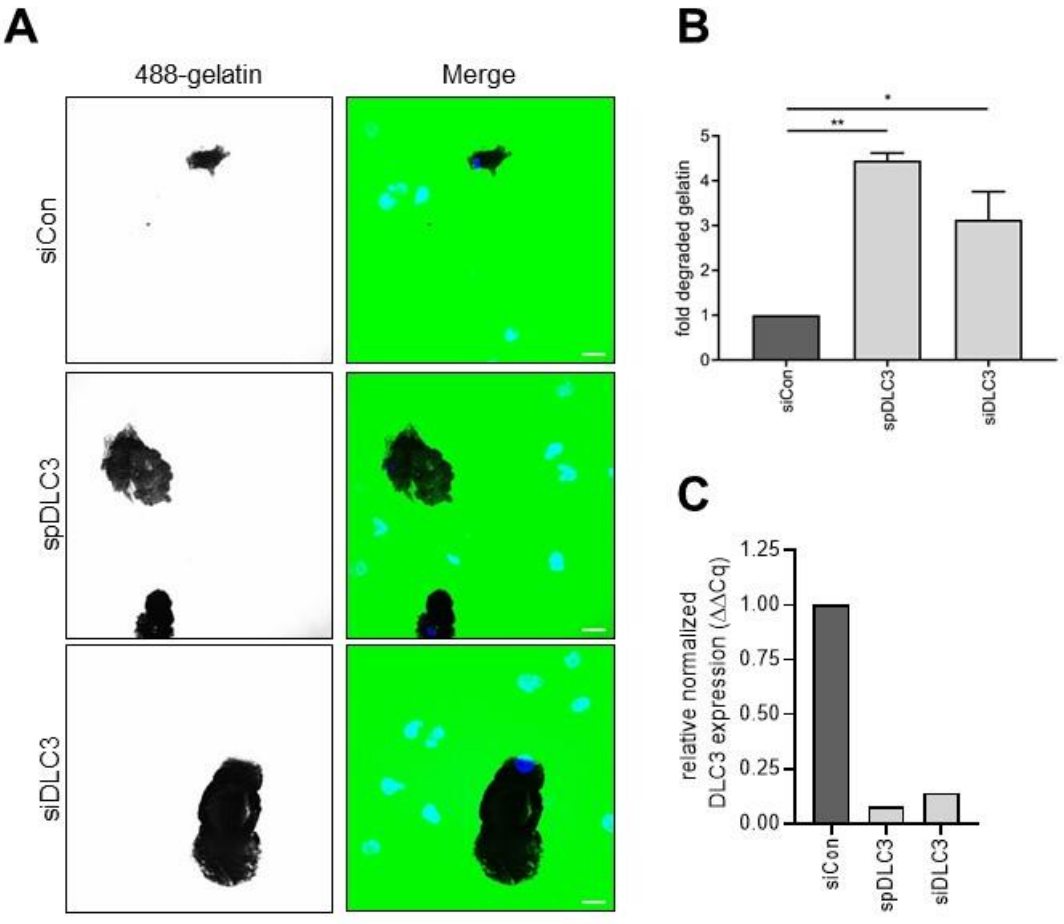


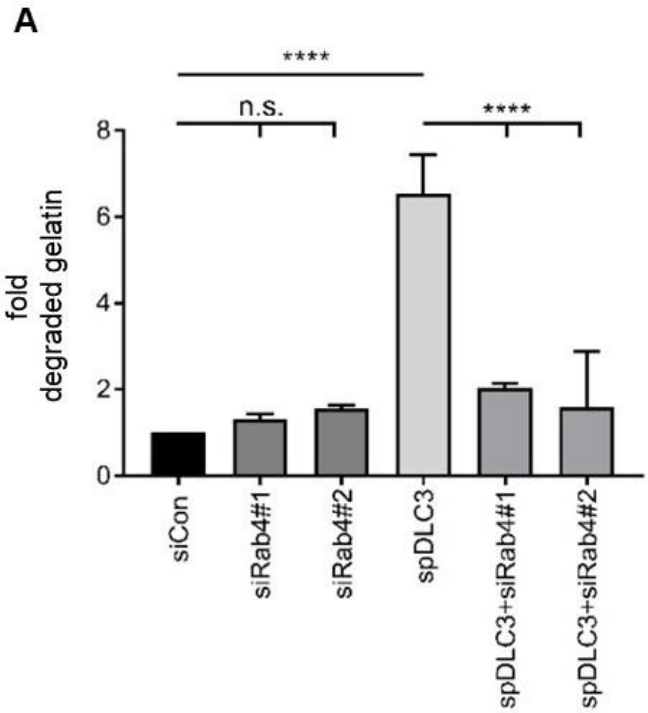
Figure 19: DLC3 knockdown enhances matrix degradation.
(A,B) MDA-MB-231 cells stably expressing Cherry-tagged MT1-MMP were transfected with the indicated siRNAs. 72 h post transfection, cells were replated onto OregonGreen488-gelatin-coated coverslips, fixed 3 h later and nuclei were stained with DAPI. (A) Scale bar, 20µm. (B) 40 images per slide were analyzed using CellProfiler Analyst and matrix degradation per cell was calculated. n=3, error bars represent mean±s.e.m., *p<0.05, **p<0.01 (one-way ANOVA followed by Tukey´s posttest).

We therefore suggest, that the depletion of DLC3 and the subsequent increase of surface MT1-MMP enhances the capacity of the cells to degrade the ECM.

3.6.1 The enhanced matrix degradation after DLC3 depletion is Rab4-dependent

In order to substantiate our theory that the increase in MT1-MMP recycling to the PM and the subsequent increase in matrix degradation after DLC3 depletion is indeed Rab4-dependent, we repeated the matrix degradation experiment, but this time co-depleted DLC3 and Rab4 in MDA-MB-231 cells stably expressing MT1-MMP. Whilst depleting Rab4 alone with two independent siRNAs had no effect on the capability of the cells to degrade the ECM, the strong increase of matrix degradation after DLC3 knockdown was fully rescued by Rab4 co-depletion, up to a level comparable to the control cells (Figure 20A). A performed qRT-PCR verified the efficient knockdown of Rab4 (Figure 20B).

Thus, our hypothesis that the increase in matrix degradation after DLC3 depletion is due to a shift of MT1-MMP recycling towards the Rab4-dependent fast-recycling route was confirmed



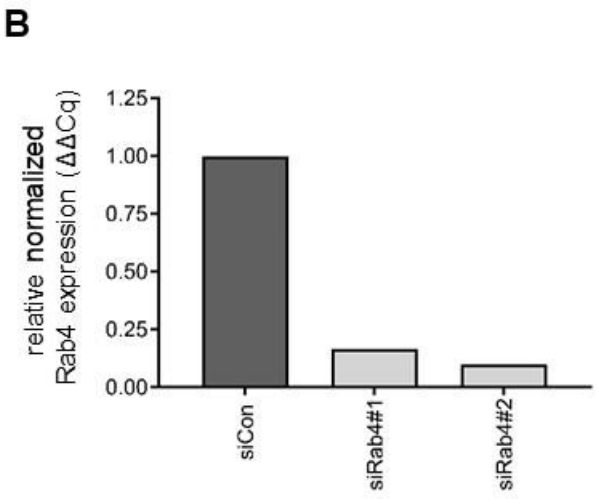


Figure 20: DLC3 depletion enhances matrix degradation via Rab4-dependent recycling of MT1-MMP. MDA-MB-231 cells stably expressing cherry-tagged MT1-MMP were transfected with the indicated siRNAs. (A) 72 h post siRNA transfection, cells were harvested and plated onto OregonGreen488-gelatin-coated coverslips and fixed 3 h later. Nuclei were stained with DAPI and 40 images per slide were analyzed using CellProfiler Analyst and matrix degradation per cell was calculated, n=3, error bars represent mean±s.e.m., ****p<0.0001, n.s. = not significant (one-way ANOVA followed by Tukey´s posttest). (B) Rab4 knockdown efficiency was determined by quantitative PCR (n=1)

3.6.2 DLC3 depletion enhances matrix degradation via Rab4-dependent recycling of MT1-MMP in MCF10A cells

To test whether the function of DLC3 is conserved across cell lines, the matrix degrading activity of TGF-β-treated MCF10A cells was investigated upon DLC3 knockdown. Again, the depletion of DLC3 led to a significant increase in matrix degradation compared to the control cells (Figure 21A). We furthermore added the broad spectrum MMP inhibitor GM6001 to prove that MMP activity was required in this assay (Figure 21A). Importantly, as seen in MDA-MB-231 cells, co-depletion of Rab4 prevented the increased matrix degrading activity of DLC3 knockdown cells (Figure 21B), demonstrating that the suppression of matrix degradation by DLC3, most likely by controlling the degree of Rab4-dependent fast recycling of MT1-MMP, is conserved in the two cell systems.

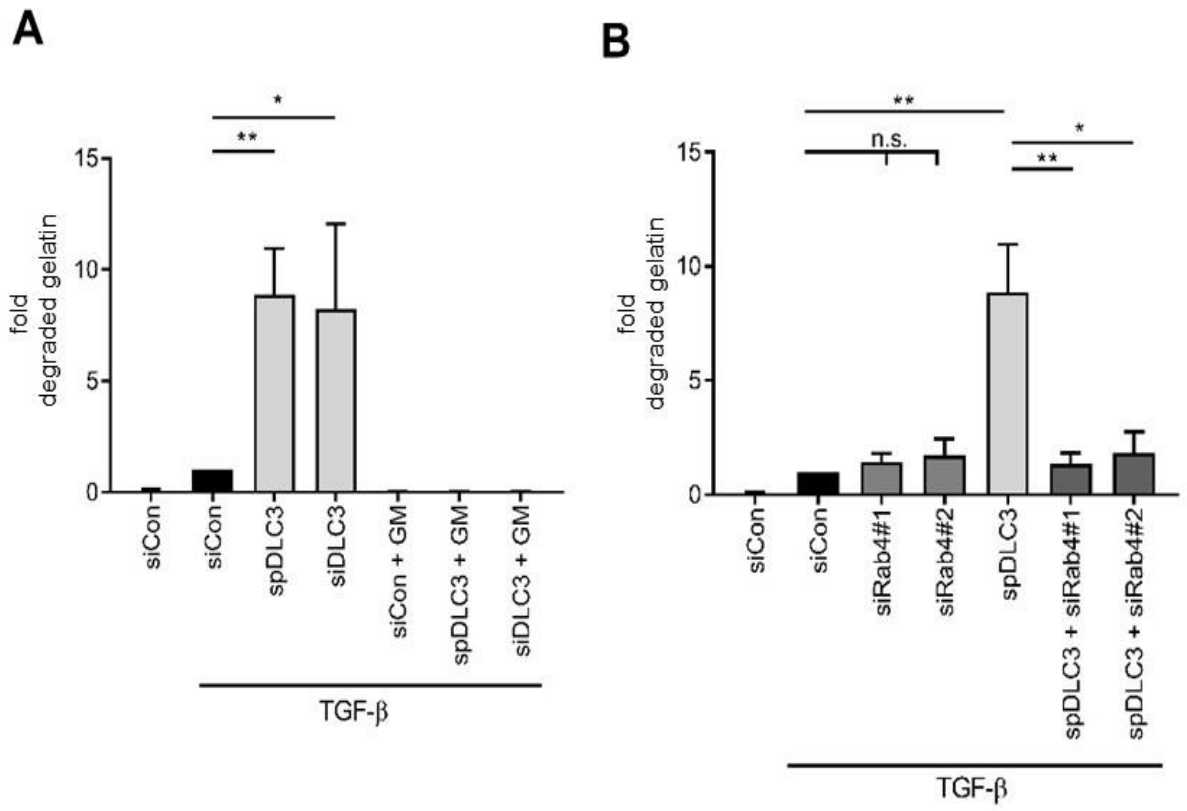


Figure 21: DLC3 depletion enhances matrix degradation via Rab4-dependent recycling of MT1-MMP. (A,B) MCF10A cells were pretreated with TGF-β for 7 days and transfected with the indicated siRNAs. 72 h post transfection cells were harvested in growth medium (-/+ TGF-β and GM6001 as indicated) and replated onto OregonGreen488-gelatin-coated coverslips and fixed 6 h later. Nuclei were stained with DAPI and 40 images per slide were analyzed using CellProfiler Analyst and matrix degradation per cell was calculated. (A) n=3, error bars represent mean±s.e.m. *p<0.05, **p<0.01 (one-way ANOVA followed by Tukey´s posttest). (B) n=3, error bars represent mean±s.e.m. *p<0.05, **p<0.01, n.s.=not significant (one-way ANOVA followed by Tukey´s posttest).

Taken together, the findings of this thesis prove that the loss of DLC3 promotes matrix degradation by affecting recycling of the model cargo MT1-MMP. In DLC3-depleted cells, the enhanced RhoB activity at early endosomes led to increased actin assembly and consequently to an accumulation of MT1-MMP at these structures. Instead of being transported to late endosomes, MT1-MMP appeared to be directly recycled back to the PM via Rab4-dependent fast recycling.

4. Discussion

4.1 Summary of Results

Rho GTPases are involved in a broad range of cellular processes important for the intact functioning of cells, and a deregulation of their activity are often the cause of tumor progression events. Especially proliferative signalling and the activation of invasion and metastasis programs correlate with the dysregulation of Rho GTPase activity. Unlike the Ras GTPases, which are frequently mutated in human cancers (Hobbs, Der, and Rossman 2016), deregulated Rho GTPase signalling is caused by either Rho GTPase overexpression or altered GEF or GAP levels. Nevertheless, despite the importance of Rho GTPase regulation, the regulators like GEFs and GAPs, their subcellular localization in space and time and the underlying signalling mechanisms are still poorly understood.

The members of the DLC family of RhoGAPs have been attributed a tumor suppressing role, with DLC1 being the main target of research. Over the last years, our group focused on DLC3, the least characterized member of the family, which is downregulated in various cancers including breast, lung and ovarian (M. E. Durkin et al. 2007). In epithelial cells (MCF10A, MCF7 and Caco2), DLC3 is recruited to the PM by the scaffold protein scribble where it is required for the maintenance of adherens junctions and polarized 3D-morphogenesis (Hendrick et al. 2016; G. Holeiter et al. 2012). DLC3 also localized to Rab8 positive endomembranes in HeLa cells and the depletion of DLC3 blocked the degradation of EGFR and increased Akt activation (Braun et al. 2015), supporting the idea of a regulatory role for DLC3 in endocytic trafficking.

Thus, the major aim of this thesis was to investigate the localization of DLC3 cells with induced trafficking and enhanced intrinsic turnover, like the utilized MCF10A cells after TGF-β treatment and in the triple negative breast cancer cell line MDA-MB-231, to identify the Rho GTPases regulated by DLC3 in this system and to reveal the role of DLC3 in endocytic trafficking and recycling. First, the regulation and targeting of different Rho GTPases by DLC3 was examined using FRET-biosensors, identifying RhoB at endosomes as the main target of DLC3 regulation. Moreover, using FRET and TIRF microscopy, convincing evidence was provided showing that DLC3 colocalizes with RhoB at early endosomes where it specifically regulates RhoB activity and actin polymerization. Furthermore, depletion of DLC3 and the resulting perturbation of the early endosomal compartment caused aberrant Rab4-dependent MT1-MMP recycling, resulting in a significant increase in the matrix degradation capacity of the cells, clearly manifesting the role DLC3 plays in the coordination of endocytic membrane trafficking and tumor progression.

4.2 DLC3 functions as a GAP protein for RhoB

Traditionally, active Rho GTPase proteins were proposed to reside and function predominantly at the PM, but active pools of multiple Rho GTPases are in part localized to endomembranes (Olayioye, Noll, and Hausser 2019; Phuyal and Farhan 2019). For example, Cdc42 was the first Rho GTPase found to localize to the Golgi apparatus (Erickson et al. 1996), and RhoB was identified as the first Rho GTPase to mainly reside at endosomes (Gampel, Parker, and Mellor 1999, Adamson, Paterson, and Hall 1992) . By now, more Rho GTPases have been identified at endosomes, like RhoD, Rac1 or Cdc42, but the recruiting mechanisms are mostly elusive. In the case of RhoB, the main factor determining its precise localization seems to be the type of prenylation. Despite sharing about 85% amino acid sequence identity with the other RhoA family members, a unique post-translational modification at the C-terminal hypervariable region allows RhoB to be prenylated differently. Whilst the farnesylated form of RhoB localized mainly to the PM, the geranylgeranylated form was detected predominantly at late endosomes (Wherlock et al. 2004). The interaction with negatively charged membranes is further regulated by a polybasic sequence in the hypervariable region of Rho GTPases (Williams 2003). The distinct Rho pools and their functions highlight the importance of the spatiotemporal regulation of these subcellular Rho signaling complexes. Nevertheless, it is still poorly understood which GEFs/GAPs for RhoGTPases are targeted to the endosomes and how they get recruited. In this work, DLC3 was found as the first GAP to colocalize with RhoB at vesicles (Figure 10) and regulate endosomal RhoB activity (Figure 7). Recruitment of RhoGTPases to endosomes facilitates spatially restricted signals that have consequences for various cellular processes. Wherlock and colleagues could show that the farnesylated pool of RhoB at the PM appeared functionally distinct from the geranylgeranylated pool at endosomes by treating cells with farnesyl-transferase inhibitor, abolishing the farnesylated pool of RhoB, resulting in an increased recycling of endocytosed EGFR (Wherlock et al. 2004). Consistent with these results, my FRET experiments showed a regulatory effect of DLC3 on RhoB activity only at the endomembranes, but not the PM, confirming the distinction of two subcellular RhoB pools (Figure 9). In further studies, it will be interesting to investigate whether RhoB is directly activated at endosomal membranes or if active RhoB is trafficked from the PM to endosomes to locally exert its function. Here, the question arises which GEFs are responsible for local RhoB activation and whether the two RhoB pools communicate with each other or exist as independent pools. In the case of Cdc42, for example, Cdc42 activated at the Golgi membrane is regulated independently of the PM pool, but can be recruited to the PM if required (Baschieri et al. 2014).

Intriguingly, neither RhoA or RhoC activity were affected by DLC3 depletion (Figure 7) in TGF-β treated MCF10A cells. This was in so far surprising as previous studies of our group showed

an effect of DLC3 depletion on RhoA activity at the Golgi in HeLa cells (Braun et al. 2015). However, in the presented work, RhoA and RhoC levels were only measured globally, so an effect of DLC3 on local RhoA (or RhoC) pools at the Golgi or invadopodia can not be ruled out. Especially at invadopodia, a potential role for DLC3 would be highly intriguing. It is known, that Rho GPTases like RhoC regulate actin polymerization at invadopodia and a deregulation of their activity could drive cell invasion and metastasis. Additional experiments focusing on smaller structures like the f-actin-rich matrix-degradation protrusions called invadopodia using super resolution microscopy might give additional information on subcellular Rho regulation by DLC3 in space and time. The RhoA family member RhoC has been described to regulate actin dynamics at invadopodia by enhancing phosphorylation of cofilin, a key regulator of actin dynamics at the leading edge of motile cells (Jose Javier Bravo-Cordero et al. 2011). A role as negative regulator of DLC3 in this context could be a further explanation of the enhanced matrix degradation after DLC3 depletion. Furthermore, the application of optogenetic tools enables the upregulation and downregulation of Rho activity with a high spatiotemporal accuracy. In the past it was already used for localization studies of GEFs (Valon et al. 2017), but also GAPs like DLC3 could be targeted to specific subcellular sites to investigate the local effects.

While the role of RhoA and RhoC in tumor progression and invasion is often explained by their capability to assemble and disassemble the actin cytoskeleton and thereby regulate cell motility, the actin remodelling capacity of RhoB is focused more on the endosomal structures it is anchored to (Fernandez-Borja et al. 2005). Due to the several post-translational modifications, RhoB is argued to be the most diverse protein of the Rho family, leading to a controversy about the role RhoB might play in tumor progression. While many argue that RhoB is an oncogene due to the poor prognosis of patients with overexpressed RhoB (Gerhard Fritz, Just, and Kaina 1999), others like the group of Kazerounian demonstrated that RhoB differentially controls Akt function in tumor versus endothelial cells, resulting in decreased proliferation of early stage cancer (Kazerounian et al. 2013). Akt signaling is essential for tumor progression as numerous genetic lesions have been discovered in Akt signaling components in human breast cancer (Stemke-hale et al. 2009). Thus, it is highly likely that RhoB functions in a contextual manner and the function may depend on the influence of the microenvironment. However, the function and regulation of RhoB with respect to endocytic trafficking and the impact on tumor progression is still poorly understood. Therefore, I focused in my studies on the function of RhoB at endosomes, its regulation by DLC3 and the effect on actin polymerization and endocytic trafficking in epithelial cells.

4.3 DLC3 regulates actin polymerization at endosomes

Over the last two decades, extensive research resulted in a reliable role for RhoB in the regulation of endosomal trafficking mainly through actin cytoskeleton remodeling. Actin nucleator proteins like Dia1 were shown to mediate actin polymerization downstream of endosomal RhoB (Fernandez-Borja et al. 2005). Thus, activated RhoB promotes the polymerization of an actin coat around the endosomes and the association to subcortical actin cables, inhibiting further endosomal transport (Fernandez-Borja et al. 2005). However, the exact regulatory mechanisms underlying RhoB activity at endosomes are still poorly understood.

In this context, it was intriguing to see how the depletion of DLC3 and the enhanced RhoB activity would influence the actin polymerization around endosomes. Using LifeAct as a tool to visualize the endosomal actin coat (Riedl et al. 2008), I could detect a strong increase in actin polymerization after DLC3 depletion (Figure 13). Actin plays multiple roles in vesicle trafficking (Smythe and Ayscough 2006). It not only facilitates membrane deformation, cytoskeleton remodeling and the formation of vesicles, but also contributes to vesicle movement and targeting within the cell (Schuh 2013). Remarkably, the depletion of DLC3 with the resulting enhanced RhoB activity at endosomes and increased actin polymerization led to an accumulation of early endosomes and an increase in size and number (Figure 11) but appeared to have no effect on late endosomal or lysosomal integrity (Figure 12). These results demonstrate a novel regulation of RhoB by DLC3 exclusively at early endosomes.

It was shown for another Rho GTPase, RhoD, that cells possessing active RhoD display reduced velocity of early endosome movement, slowing down membrane trafficking events (Murphy et al. 1996). RhoD has also been shown to localize to Rab5-positive endosomes by binding the Rab5 effector Rabankyrin-5 and regulating RTK trafficking (Nehru et al. 2013). Keeping in mind that crosstalk between Rho GTPases has already been demonstrated for RhoB and Rac1 (Marcos-Ramiro et al. 2016) or RhoB and Cdc42 (Minzhou Huang et al. 2011), it is tempting to speculate about a possible crosstalk between RhoB and RhoD in this context. However, this potential connection needs further experimental elucidation and could depend on the cellular context. To my best knowledge, this is the first time a GAP protein was demonstrated to regulate RhoB specifically at endosomes, influencing the actin coat and endosomal integrity. Nevertheless, the results will have to be substantiated and validated by follow-up experiments. So far, the effect on actin coating has only been shown via immunofluorescence microscopy. Although our group proved that actin is affected by measuring matrix degradation, comparing control cells to DLC3 depleted cells and cells treated with low doses of the actin-disrupting agents latrunculin B or cytochalasin D (Noll et al. 2019),

further experiments using high-resolution microscopy will deliver more insight into endosomal actin remodeling. Another angle to evaluate the effect of RhoB regulation at endosomes through DLC3 would be to look at the sorting and trafficking of other cargos as our group already showed that DLC3 depletion affected the trafficking of the EGFR and transferrin receptor, prolonging EGF signalling (Braun et al. 2015). It has been shown that RhoB-mediated actin polymerization at endosomes can coordinate the transport and activation of cargo like the tyrosine-protein kinase Src (Sandilands et al. 2004), but also other proteins or receptors could be affected. Identifying and analysing of affected cargos on a global scale will be the next valuable step to obtain a better understanding of DLC3 functions in motile cells.

4.4 DLC3 depletion traps MT1-MMP in early endosomes and alters recycling

Rho GTPases actively control vesicular trafficking and thereby determine associated cellular processes such as proliferation, motility and polarity among many others (Frittoli et al. 2014; Pálfy, Reményi, and Korcsmáros 2012). Endosomal RhoB was also shown to recruit the actin-regulating kinase PRK1, a Ser/Thr kinase, to endosomes, resulting in its activation and regulation of the trafficking of EGFR in a way leading to prolonged signaling and preventing its degradation (Gampel, Parker, and Mellor 1999; Mellor et al. 1998). These findings are in line with previous results from our group (Braun et al. 2015). Furthermore, RhoB was shown to function in regulating degradation and signal termination of the chemokine receptor CXCR2, suggesting a more general role for RhoB in receptor trafficking (Neel et al. 2007).

In a very recent study, the direct binding of endosomal actin was shown to be required for efficient constitutive recycling of the EGFR and MT1-MMP (MacDonald et al. 2018). The directed distribution of cargo like MT1-MMP, for example to the leading edge in migrating cells, are one of the more complex features of the recycling pathway and can drive cancer cell invasion (Steffen et al. 2008). As MT1-MMP is a key regulator of matrix degradation and cell invasion in motile cells, it was used as a model cargo to study in detail the effect of DLC3 depletion on vesicular trafficking. Of most importance, the depletion of DLC3 in MDA-MB-231 cells stably expressing Cherry-tagged MT1-MMP (Marchesin et al. 2015) not only led to an accumulation of early endosomes, it also led to enhanced MT1-MMP localization to these endosomes, suggesting that MT1-MMP was trapped in early endosomes (Figure 14).

Usually, MT1-MMP is sorted at early endosomes into late endosomes from where it is either targeted for lysosomal degradation or recycled back to the PM via the slow, Rab11-dependent recycling route. MT1-MMP can also be directly recycled from early endosomes back to the PM via a Rab4-dependent fast recycling route (Frittoli et al. 2014; Stenmark 2009). Intriguingly, the

depletion of DLC3 led to an increase in MT1-MMP-containing Rab4 vesicles (Fig. 16). A similar observation was made in previous work from our group, where the transferrin receptor as a model cargo in HeLa cells was also shown to shift towards Rab4-positive endosomes after DLC3 depletion (Braun et al. 2015). Thus, our hypothesis that the perturbation of the early endosomal compartment due to aberrant RhoB-dependent actin polymerization might redirect MT1-MMP recycling towards the Rab4-dependent fast-recycling route was further substantiated.

Intriguingly and in line with this, the perturbation of MT1-MMP recycling led to an increase in MT1-MMP surface levels (Figure 17), suggesting that the shift to the Rab4-dependent recycling route due to DLC3 depletion actually increased MT1-MMP recycling to the cell surface. To further validate this, I used MT1-MMP-pHluorin which is a useful tool to specifically address recycling and exocytosis of MT1-MMP. The notable increase in bursts of cells lacking DLC3 (Figure 18) confirmed our hypothesis that mainly the enhanced recycling of MT1-MMP and not impaired internalization was the reason for the elevated surface protein levels in these cells. Surface expression of MT1-MMP is generally low, and upregulation correlates with malignancy in cancer (Lodillinsky et al. 2016; Marchesin et al. 2015). Nevertheless, further experiments have to be conducted to prove that the elevated MT1-MMP protein levels on the surface consist of active MT1-MMP fulfilling a biological purpose. The useful tool of MT1-MMP-pHluorin can be used to investigate the duration of MT1-MMP on the cell surface and also the motility by using FRAP experiments (El Azzouzi, Wiesner, and Linder 2016). As MT1-MMP usually concentrates at invadopodial structures, analysis of the stability and motility of MT1-MMP in combination with invadopodial markers will lead to further insight on the importance of DLC3 in endocytosis and trafficking. Another approach would be a more global view on surface protein levels to identify other target proteins affected by DLC3-regulated transport. In a first, flow cytometry-based screen of approximately 300 cell surface receptors (LEGENDScreen™) of control and DLC3 depleted MDA-MB-231 cells our group showed the upregulation of target proteins involved in cell-matrix or cell-cell adhesion processes, whilst other proteins were downregulated after DLC3 depletion. Identifying proteins and pathways affected by DLC3 is essential for the deeper understanding of DLC3 mechanisms and functions.

4.5 DLC3 depletion leads to increased matrix degradation

Interestingly, the Zech group could recently prove that abrogation of actin-dependent recycling of MT1-MMP resulted in defective matrix degradation and invasion of triple-negative breast cancer cells (MacDonald et al. 2018). Thus, we were curious whether the enhanced RhoB

activity at endosomes due to DLC3 depletion and the resulting change in MT1-MMP recycling also affected the capability of the cells to degrade the extracellular matrix. MT1-MMP is activated by the conversion of proMT1-MMP (~64kDa) to a catalytically active enzyme (~55kDa) by proteolytic cleavage by furin prior to its arrival at the PM. Active MT1-MMP has been ascribed a variety of cell surface activities, with one of the most prominent being a pericellular collagenase. The collagenolytic activity of MT1-MMP appears to be essential for transmigration of tumor cells, endothelial cells, and fibroblasts through collagen matrices (Sabeh et al. 2004; W. Zhang et al. 2006). But enhanced surface levels of MT1-MMP do not necessarily correlate with increased degradative capacity of the cells. Surface MT1-MMP is only active for a very short period of time before inactivation by tissue inhibitor of metalloproteinases-2 (TIMP2) occurs (Bernardo and Fridman 2003). By performing a matrix degradation assay, I could measure the capability of the cells to degrade the extracellular matrix. Intriguingly, quantification of the degraded area relative to the fluorescence intensity of the non-degraded matrix revealed a significant increase in matrix degradation upon loss of DLC3 (Figure 19), an effect that was rescued by the simultaneous depletion of Rab4 (Figure 20). These results confirmed our hypothesis that regulation of RhoB activity by DLC3 at early endosomes not only completely changes the integrity of the early endosomal compartment due to enhanced actin remodelling, but also the sorting and recycling routes of cargos such as MT1-MMP and thus, strongly affects the matrix degrading capacity of the cells. Of note, the effect on matrix degradation was not only true for the two cell lines presented in this thesis, but also for other tumor cell lines like BT549 (Noll et al. 2019). To draw further conclusions about the role of DLC3 in cancer cell invasion, specific migration and invasion assays have to be performed. Our group already performed Transwell assays, showing that the enhanced matrix degrading capacity after DLC3 depletion correlate with an increase in migration and invasion of the cells. In summary, the regulation of RhoB via DLC3 seems to be important for endocytic trafficking which underlies important cellular processes such as extracellular matrix remodelling and invasive motility of motile cells.

4.6 Conclusion and outlook

In conclusion, the presented data in this thesis support the idea of DLC3 regulating RhoB activity at early endosomes in a GAP-dependent manner. The depletion of DLC3 leads to an increase in actin polymerization at early endosomes, impairing their integrity and affecting the sorting of cargos such as MT1-MMP. As a consequence, the depletion of DLC3 results in a

shift of MT1-MMP recycling towards the Rab4-dependent fast-recycling route and an increase in protein surface levels, ending in enhanced matrix degradation. Along these lines, with only one cargo investigated in this context our scope has to be widened by other cargos in the future. The trafficking and recycling of any receptor or surface protein is an essential part of its function, and an impairment of the transport can lead to the malfunction of the pathway or even malignant transformation of the cell. My data suggests an important role of DLC3 in the regulation of RhoB activity and endocytic trafficking, showing how essential DLC3 is for a well-balanced cell homeostasis and underscoring its possible role as a tumor suppressor. Especially in the context of CME, responsible for the uptake of receptors, antibodies and growth factors and thereby a key event in tumor progression.

The role Rho GTPases play in tumorigenesis and tumor progression has been a focus of scientists for years, and although the knowledge about the function of Rho GTPases has grown, the regulatory mechanisms and proteins involved in the highly sensitive balance of enzyme activity are still poorly understood. Due to recent advancements in Rho GTPase biosensors and microscopy techniques we gained increasing knowledge about the complexity of Rho GTPase signalling. By identifying a growing number of Rho effectors, the functions of Rho GTPases are now regarded not to be limited to cell motility, but also involve receptor trafficking, cell proliferation, differentiation and polarity (Phuyal and Farhan 2019). The use of large global approaches and screens has provided useful information about the Rho GTPase network, but is lacking the exact spatial information. In this thesis, I could demonstrate how the Rho GTPase RhoB is differentially regulated depending on its localization, and how specific the RhoGAP DLC3 protein acts. Using state of the art biosensors and microscopy techniques, I could track the localization of RhoB at endosomal membranes, identifying a highly dynamic process of trafficking. The fact that Rho GTPases themselves are trafficked adds another layer of complexity to their regulation. While RhoB is trafficked from the PM through Rab5-positive early endosomes to Rab7-positive late endosomes (Gong et al. 2018), Cdc42 was demonstrated to traffic to the PM (Osmani et al. 2010). Therefore, the importance of tools and methods allowing the specific spatiotemporal analysis of subcellular Rho pools is undeniable. One promising tool emerging over the last years is the use of optogenetics. This light-sensitive tool, where spatial and temporal control is gained through reversible signal triggering, has already been developed to control the activity of endogenous Rho GTPases (Valon et al. 2017).

In this thesis, most of the insight on the role of DLC3 was gained by transient knockdown by RNAi. One disadvantage regarding this methodology is that it is completely dependent on the half-life of the protein, which can range from a few hours to several days. Additionally, the consecutive gene knockdown over days can lead to compensatory mechanisms that may

falsify the results. Again, the use of next generation techniques could help reducing this factor, for example the auxin-inducible degradation technology. With this method, primary molecular responses can be observed due to rapid and controlled protein depletion, without the negative effects down-stream of protein deregulation (Lambrus, Moyer, and Holland 2018). Additionally, the rapid development of high-resolution microscopy techniques, especially in the field of live-cell imaging, will make it possible to elucidate the processes taking place at intracellular structures like endosomes and the proteins involved. The development of new methods, techniques and equipment is in so far of importance, as the field of Rho GTPases and their regulation is gaining growing importance in the understanding of tumor progression and metastasis.

For example, RhoB has been reported to inhibit tumor growth, cell migration and invasion (M. Huang and Prendergast 2006), but the mechanism by which it suppresses tumor progression and invasion remains elusive. The role RhoB plays in tumorigenesis seems to be context specific (Ju and Gilkes 2018). Depending on the type of prenylation, RhoB can possess either pro- or anti-proliferative activity. While the geranylgeranylated, endosome-bound form of RhoB has been connected to tumor growth inhibition, farnesylated RhoB was shown to have pro- or anti-proliferative effects (W. Du and Prendergast 1999). Also, in the context of the TGF-β pathway and EMT, the role of RhoB is discussed controversially. While appearing to suppress EMT and acting as a tumor suppressor in a recent lung cancer study (Dubois et al. 2016), the loss of RhoB has also been shown to impair TGF-β-induced migration of prostate cancer cells (Vasilaki et al. 2010). In this work, I could show that endosomal RhoB activity increases upon DLC3 depletion in TGF-β-treated MCF10A cells, leading to enhanced Rab4-dependent MT1-MMP-recycling and increased matrix degradation, an observation I also made in triple-negative MDA-MB-231 breast cancer cells. Therefore, downregulation of DLC3 seems to favour a pro-invasive phenotype. The Rab5-Rab4 trafficking route has been described in the literature to be required for MT1-MMP fast recycling and matrix degradation in HeLa cells, and the upregulation of Rab5 and Rab4 in invasive breast cancer has been linked with poor prognosis and metastasis formation (Frittoli et al. 2014). Given the data of this thesis, I propose that the anti-invasive function of DLC3 antagonizes the pro-invasive function of Rab5 and Rab4. This is substantiated by the downregulation of DLC3 observed in breast cancer (M. E. Durkin et al. 2007), suggesting a tumor suppressive role for DLC3. Nevertheless, the precise role of DLC3 in Rho regulation and eventually cell invasion and metastasis, remains to be elucidated. Future efforts should focus on identifying tools and methods to specifically modulate subcellular Rho pools to increase our knowledge of Rho regulation and help us in the understanding of tumorigenesis.

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Declaration

I hereby assure that I performed this work independently without further help or other materials than stated. Passages and ideas from other sources have been clearly indicated.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen, als die angegebenen, Quellen und Hilfsmittel benutzt habe. Wörtlich oder sinngemäß übernommenes Gedankengut habe ich als solches kenntlich gemacht.

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Signature/Unterschrift:

Date/Datum: 10.12.2020 _____