Regulation of endocytic membrane trafficking by the GTPase-activating protein Deleted in Liver Cancer 3 (DLC3)

Von der Fakultät Energie-, Verfahrens- und Biotechnik der Universität Stuttgart zur Erlangung der Würde eines Doktors der Naturwissenschaften (Dr. rer. nat.) genehmigte Abhandlung

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Table of content

List of figures .......................................................................................................................... 6
Abbreviations ........................................................................................................................ 7
Summary .................................................................................................................................. 10
Zusammenfassung .................................................................................................................... 11
1. Introduction ........................................................................................................................ 13
   1.1. Rho GTPases in cell biology ...................................................................................... 13
   1.2. Membrane trafficking ............................................................................................... 14
       1.2.1. Receptor-mediated endocytosis ......................................................................... 15
       1.2.2. Regulation of endocytosis by Rab GTPases ...................................................... 16
       1.2.3. Regulation of endocytosis by Rho GTPases ...................................................... 17
   1.3. Rho GTPase regulation by GEFs and GAPs .............................................................. 19
       1.3.1. RhoGEFs and -GAPs in membrane trafficking ................................................. 20
       1.3.2. The RhoGAP family of proteins ...................................................................... 21
   1.4. The ‘Deleted in Liver Cancer’ RhoGAP protein family .............................................. 22
       1.4.1. Multi-domain organization of DLC proteins .................................................. 23
       1.4.2. Biological functions of the DLC isoforms in time and space .......................... 25
       1.4.3. DLC proteins in focal adhesions ..................................................................... 26
       1.4.4. DLC proteins at adherens junctions ................................................................. 27
       1.4.5. DLC proteins at the plasma membrane and cell protrusions ......................... 29
       1.4.6. DLC proteins in caveola ................................................................................. 30
       1.4.7. Additional subcellular localizations of the DLC proteins ............................... 31
   1.5. Aims of the thesis ....................................................................................................... 32
2. Material and methods ......................................................................................................... 33
   2.1. Material ....................................................................................................................... 33
       2.1.1. Buffers and solutions ....................................................................................... 33
       2.1.2. Kits .................................................................................................................. 34
       2.1.3. Enzymes ........................................................................................................... 35
       2.1.4. Human cell lines ............................................................................................. 35
       2.1.5. Cell culturing .................................................................................................... 35
       2.1.6. Antibodies and fluorescent dyes ...................................................................... 36
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.7.</td>
<td>small interfering RNA (siRNAs)</td>
</tr>
<tr>
<td>2.2.</td>
<td>Methods</td>
</tr>
<tr>
<td>2.2.1.</td>
<td>Cloning strategies</td>
</tr>
<tr>
<td>2.2.2.</td>
<td>Site-directed PCR mutagenesis</td>
</tr>
<tr>
<td>2.2.3.</td>
<td>Transformation of <em>Escherichia coli</em></td>
</tr>
<tr>
<td>2.2.4.</td>
<td>Isolation of plasmid DNA</td>
</tr>
<tr>
<td>2.2.5.</td>
<td>Cell culture</td>
</tr>
<tr>
<td>2.2.6.</td>
<td>Cell transfections</td>
</tr>
<tr>
<td>2.2.7.</td>
<td>Immunoblotting</td>
</tr>
<tr>
<td>2.2.8.</td>
<td>Production of GST-fusion proteins</td>
</tr>
<tr>
<td>2.2.9.</td>
<td>Protein-lipid overlay assay</td>
</tr>
<tr>
<td>2.2.10.</td>
<td>Immunofluorescence staining</td>
</tr>
<tr>
<td>2.2.11.</td>
<td>FRET analysis</td>
</tr>
<tr>
<td>2.2.12.</td>
<td>Transferrin uptake</td>
</tr>
<tr>
<td>2.2.13.</td>
<td>EGFR trafficking and signaling assays</td>
</tr>
<tr>
<td>2.2.14.</td>
<td>Fluorescence activated cell sorting (FACS)</td>
</tr>
<tr>
<td>2.2.15.</td>
<td>Migration assay</td>
</tr>
<tr>
<td>2.2.16.</td>
<td>Aggregation assay</td>
</tr>
<tr>
<td>3.</td>
<td>Results</td>
</tr>
<tr>
<td>3.1.</td>
<td>Regulation of endocytic trafficking by DLC3</td>
</tr>
<tr>
<td>3.1.1.</td>
<td>DLC3 co-localizes with Rab8 and impacts ERC and Golgi morphology in a GAP-dependent manner</td>
</tr>
<tr>
<td>3.1.2.</td>
<td>DLC3 is required for the integrity of the Rab8 and Golgi compartments</td>
</tr>
<tr>
<td>3.1.3.</td>
<td>Depletion of p190RhoGAP does not induce perinuclear vesiculation</td>
</tr>
<tr>
<td>3.1.4.</td>
<td>DLC3 depletion enhances perinuclear RhoA activity</td>
</tr>
<tr>
<td>3.1.5.</td>
<td>The SAM domain of DLC3 associates with Golgi membranes</td>
</tr>
<tr>
<td>3.1.6.</td>
<td>A targeted DLC3 GAP domain rescues Golgi fragmentation</td>
</tr>
<tr>
<td>3.1.7.</td>
<td>DLC3 is required for transferrin trafficking to the ERC</td>
</tr>
<tr>
<td>3.1.8.</td>
<td>Simultaneous depletion of RhoA and RhoB rescues impaired Tfn trafficking</td>
</tr>
<tr>
<td>3.1.9.</td>
<td>DLC3 depletion alters EGFR trafficking</td>
</tr>
</tbody>
</table>
3.1.10. DLC3 depletion prolongs EGFR signaling .................................................64
3.1.11. DLC3 depletion causes N-cadherin accumulation at the perinuclear region ..........................................................................................................................66
3.1.12. DLC3 depletion reduces cell aggregation ..................................................67

3.2. Association of DLC3 with Rab8-positive recycling tubules .........................68

3.2.1. Amino acids 1-232 in DLC3 are necessary for its localization to the Rab8 compartment ........................................................................................................68

3.2.2. A novel polybasic region mediates binding to negatively charged lipids ........................................................................................................................................70

4. Discussion and outlook ......................................................................................72

4.1. Regulation of endocytic trafficking by DLC3 ................................................72

4.1.1. DLC3 regulates Rab8-dependent recycling in a GAP-dependent manner ...........................................................................................................................72

4.1.2. Endosomal RhoB - a possible new substrate for DLC3 ...............................74

4.1.3. DLC3-mediated Rho regulation and anterograde membrane trafficking ...............................................................................................................................75

4.1.4. DLC3-depletion promotes fast recycling of the EGFR and affects receptor signaling ........................................................................................................77

4.1.5. Cadherin transport defect upon DLC3 depletion suggests a more global role for DLC3 in membrane trafficking .........................................................77

4.2. Association of DLC3 with Rab8-positive recycling tubules .........................78

4.3. Outlook .............................................................................................................80

References ..................................................................................................................81
Publications ..................................................................................................................93
Acknowledgments .......................................................................................................96
Curriculum vitae .........................................................................................................98
Declaration ...................................................................................................................100
List of figures

Fig. 1 Pathways of receptor-mediated endocytosis. .......................................................... 16
Fig. 2 Cytoskeleton-based processes within vesicle formation, fission, transport and
fusion. .................................................................................................................................. 18
Fig. 3 Regulation of Rho GTPases. ....................................................................................... 20
Fig. 4 Domain organization of the human DLC isoforms and their homologs from rat and
fruit fly. .................................................................................................................................. 23
Fig. 5 DLC proteins and their subcellular localizations. ...................................................... 27
Fig. 6 Ectopically expressed wild type DLC3 co-localizes with Rab8 and impacts ERC and
Golgi morphology. .................................................................................................................. 48
Fig. 7 Morphological effects upon DLC3 overexpression. .................................................. 50
Fig. 8 Co-localization of GAP-inactive DLC3 with Rab8-positive tubules. ......................... 51
Fig. 9 Co-localization of GAP-inactive DLC3 with Rab6, TGN46 and RhoB. ....................... 52
Fig. 10 Active RhoA causes vesiculation of the Rab8 recycling compartment and Golgi
fragmentation. ........................................................................................................................ 53
Fig. 11 DLC3 depletion causes vesiculation of the Rab8 recycling compartment and Golgi
fragmentation. ........................................................................................................................ 54
Fig. 12 Depletion of p190RhoGAP does not induce perinuclear vesiculation ..................... 55
Fig. 13 DLC3 depletion enhances cellular RhoA activity. .................................................. 57
Fig. 14 The isolated DLC3α N-terminus localizes to Golgi membranes. ............................... 58
Fig. 15 Expression of a targeted DLC3 GAP domain rescues Golgi fragmentation in DLC3
knockdown cells. .................................................................................................................... 59
Fig. 16 DLC3 depletion impairs Tfn transport to the ERC. .................................................. 60
Fig. 17 Simultaneous depletion of RhoA and RhoB rescues the Tfn trafficking defect in
DLC3 knockdown cells. ........................................................................................................... 62
Fig. 18 DLC3 depletion alters the endocytic trafficking of EGF. ........................................ 63
Fig. 19 Internalized EGF is missorted in DLC3-depleted cells. ............................................ 64
Fig. 20 DLC3 depletion prolongs EGFR signaling. ................................................................ 65
Fig. 21 DLC3 knockdown causes N-cadherin accumulation at the Golgi complex. ............ 66
Fig. 22 DLC3 knockdown reduces cell aggregation. .............................................................. 67
Fig. 23 Schematic representation of the N-terminal DLC3α constructs. .............................. 68
Fig. 24 Mapping studies of the DLC3α N-terminus. ........................................................... 69
Fig. 25 DLC3-SAM-(81-232), but not DLC3-SAM, is recruited to Rab8 tubules. ............... 70
Fig. 26 The polybasic region between amino acid 196 and 232 mediates lipid binding and
contributes to DLC3 recruitment to Rab8 tubules. .............................................................. 71
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ</td>
<td>adherens junction</td>
</tr>
<tr>
<td>ADF</td>
<td>actin-depolymerizing factor</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>APPL1</td>
<td>a4 precursor protein like 1</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>ARAP1</td>
<td>ankyrin repeat and PH domain 1</td>
</tr>
<tr>
<td>Arf</td>
<td>ADP ribosylation factors</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Cdc42</td>
<td>cell division cycle 42</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CERT</td>
<td>ceramide transfer protein</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>CIAP</td>
<td>calf intestine alkaline phosphatase</td>
</tr>
<tr>
<td>COPI/II</td>
<td>coat protein complex I/II</td>
</tr>
<tr>
<td>CTEN</td>
<td>C-terminal tensin-like</td>
</tr>
<tr>
<td>cv-c</td>
<td>crossveinless-c</td>
</tr>
<tr>
<td>CytD</td>
<td>cytochalasin D</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidin-2-phenylindol</td>
</tr>
<tr>
<td>Dia</td>
<td>orthologs of <em>Drosophila melanogaster</em> diaphanous protein</td>
</tr>
<tr>
<td>DLC (1-3)</td>
<td>Deleted in Liver Cancer</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EEA1</td>
<td>early endosome antigen 1</td>
</tr>
<tr>
<td>EF1A1</td>
<td>eukaryotic elongation factor 1A1</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGF-555</td>
<td>Alexa Fluor 555-labeled EGF</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EHD</td>
<td>Eps15 homology domain</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ERC</td>
<td>endocytic recycling compartment</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GEF-H1</td>
<td>guanine nucleotide exchange factor H1</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GIMP</td>
<td>Gem-interacting protein</td>
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<tr>
<td>GLUT</td>
<td>glucose transporter</td>
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</table>
Abbreviations

GM130  Golgi matrix protein of 130 kDa
GST   gluthatione S-transferase
GTP   guanosine triphosphate
HCC   hepatocellular carcinoma
HOPS  homotypic fusion and vacuole protein sorting
HRP   horseradish peroxidase
IPTG  isopropyl β-D-1-thiogalactopyranoside
IQGAP IQ motif containing GTPase activating protein 1
JFC1/Slp1 synaptotagmin-like protein
LAMP1 lysosome-associated membrane protein 1
LB    Luria-Bertani
LIM   LIN-11, ISL-1 and MEC-3 proteins
LOH   loss of heterozygosity
mAb   monoclonal antibody
MAPK  mitogen-activated protein kinase
MLC   myosin light chain
NaDoc sodium deoxycholate
ns    not significant
PA    phosphatidic acid
pAb   polyclonal antibody
PAGE  polyacrylamide gel electrophoresis
PBR   polybasic region
PBS   phosphate buffered saline
PC    phosphatidylcholine
PCR   polymerase chain reaction
PE    phosphatidylethanolamine
PFA   paraformaldehyde
PG    phosphatidylglycerol
PI    phosphatidylinositol
PI3K  phosphatidylinositol 3-kinase
PLCδ1 phospholipase Cδ1
PMSF  phenylmethylsulfonyl fluoride
PRK1  protein kinase C-related kinase 1
PS    phosphatidylserine
PTB   phosphotyrosine-binding
PTEN  phosphatase and tensin homolog
PVDF  polyvinylidene difluoride
Rab   Ras-related in brain
Rac   Ras-related C3 botulinum toxin substrate
Ran   Ras-related nuclear protein
Ras   rat sarcoma
RhoA/B/C Ras homology protein A/B/C
Rho proteins Rho GTPases (e.g. RhoA, Rac and Cdc42)
RhoGAP GTPase activating protein for Rho proteins
RIPA  radioimmunoprecipitation assay buffer
RNA   ribonucleic acid
RNAl  RNA interference
ROCK  Rho-associated, coiled-coil containing kinase
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RPMI</td>
<td>medium named after the Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAM</td>
<td>sterile α motif</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Sec5</td>
<td>secretory protein 5</td>
</tr>
<tr>
<td>s.e.m.</td>
<td>standard error of the mean</td>
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<tr>
<td>SH</td>
<td>Src homology</td>
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<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>Src</td>
<td>sarcoma</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory</td>
</tr>
<tr>
<td>STARD</td>
<td>StAR-related lipid transfer domain</td>
</tr>
<tr>
<td>START</td>
<td>StAR-related lipid transfer</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N´,N´-tetramethyl ethyldiamine</td>
</tr>
<tr>
<td>Tfn</td>
<td>transferrin</td>
</tr>
<tr>
<td>Tfn-555</td>
<td>Alexa Fluor 555-labeled Tfn</td>
</tr>
<tr>
<td>TfnR</td>
<td>transferrin receptor</td>
</tr>
<tr>
<td>TGN</td>
<td>trans-Golgi network</td>
</tr>
<tr>
<td>Tris</td>
<td>tris-hydroxymethyl aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus G protein</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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Summary

Small GTPases of the Rho family are key regulators of the actin and microtubule cytoskeleton, whereby many cellular functions including cell migration, adhesion and polarity, as well as cell cycle progression are controlled. Increasing evidence suggests that Rho proteins are also critically involved in the regulation of membrane trafficking pathways within exocytosis and endocytosis. Although the molecular mechanisms are not well understood, Rho GTPases apparently have to govern and finely tune cytoskeletal remodeling, in order to support the formation, fusion and motility of transport carriers. However, the identity of their regulators, the guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) that ensure the balanced GTPase activation in space and time is largely elusive.

The ‘Deleted in Liver Cancer’ (DLC1/2/3) proteins are a structurally conserved subfamily of RhoGAP proteins that act as negative regulators of Rho GTPases. In addition to the catalytically active GAP domain, all DLC proteins contain a sterile α motif (SAM) and steroidogenic acute regulatory protein-related lipid transfer (START) domain. Expression of the best studied member, DLC1, is frequently lost in various types of human cancers and a tumor suppressive function associated with its RhoGAP activity has been established in vivo. Although DLC3 was also observed to be downregulated in several cancer cell lines and primary tumors, the cellular functions of DLC3 are still poorly characterized. So far, GAP activity for RhoA has only been demonstrated in vitro and, associated with its localization at cell-cell contacts, a Rho-regulatory role in adherens junction stability was described. Thus, the aims of this thesis were to further investigate the subcellular localization of DLC3 and to shed light on the role of DLC3 in the regulation of Rho-mediated cellular processes, in particular endocytic membrane trafficking.

This study provides convincing evidence that DLC3 is a functional, Rho-specific GAP protein in living cells and that its loss enhances perinuclear RhoA activity. DLC3 is recruited to Rab8-positive membrane tubules and required for the integrity of the Rab8 and Golgi compartments. Depletion of DLC3 impairs the transport of internalized transferrin to the endocytic recycling compartment, which is restored by the simultaneous downregulation of RhoA and RhoB. As a consequence, DLC3 loss interferes with epidermal growth factor receptor (EGFR) degradation and causes prolonged receptor signaling. Furthermore, it was found that DLC3-depleted cells show reduced surface N-cadherin levels, leading to decreased cell aggregation. Together, these findings identify DLC3 as a novel component of the endocytic trafficking machinery, wherein it maintains organelle integrity and regulates membrane transport via the control of local Rho activity.
Zusammenfassung


DLC3-depletierte Zellen verringerte N-Cadherin-Mengen auf der Zelloberfläche zeigen, was wiederum zu einer geschwächten Zellaggregation führt. Zusammengenommen identifizieren diese Ergebnisse DLC3 als eine neue Komponente innerhalb der endozytotischen Transportmaschinerie, in welcher es die Integrität der Organellen aufrecht erhält und den Membrantransport über die lokale Regulation der Rho-Aktivität kontrolliert.
1. Introduction

1.1. Rho GTPases in cell biology

Rho GTPases constitute a distinct family of small GTPases within the Ras superfamily, which was named after its founding member Ras and can be divided into five families: the Ras, Rho, Rab, Arf and Ran families, which together regulate a huge variety of cellular processes (Bos et al., 2007). Small GTPases are often called ‘molecular switches’, since they can be turned ‘on’ and ‘off’, depending on whether they are bound to guanosine triphosphate (GTP) or guanosine diphosphate (GDP), respectively. So far, 22 members of the Rho family have been identified, of which RhoA, Rac1 and Cdc42 are best characterized and most intensively studied (Bos et al., 2007). The discovery of their regulatory function in actin cytoskeleton and focal adhesion dynamics was a hallmark in understanding the role of Rho GTPases in cell migration (Ridley and Hall, 1992). In migrating cells, coordinated activation of Rho GTPases is required to form actin-dependent protrusions at the front and establish contractile filaments at the retracting tail. Moreover, microtubules have to orientate towards the direction of migration, in order to target vesicles and transport proteins to the leading edge (Jaffe and Hall, 2005). In particular, RhoA activation leads to the assembly of myosin-containing actin stress fibers at the back of the cell, whereas active Cdc42 and Rac1 induce the formation of protrusive, actin-rich lamellipodia and filopodia at the leading edge. Stabilization of microtubules and formation of mature focal adhesions, sites of cell-matrix interactions, can as well be attributed to RhoA activity.

Due to their crucial role in the regulation of the actin and microtubule cytoskeleton, Rho GTPases were found to be involved in many more cellular functions, including cell polarity, cell cycle progression, cell differentiation and membrane trafficking (Jaffe and Hall, 2005). Since all these processes are abnormal in cancer, it is not surprising that deregulation of Rho proteins contributes to cellular transformation and tumorigenesis in vivo. With the recent development of genetically encoded biosensors that allow the visualization of the Rho activation status, it became clear that Rho signaling has to be finely tuned in space and time. However, the molecular mechanisms by which the regulators, the GEFs and GAPs, coordinate Rho GTPase activity remain largely elusive. Nevertheless, the fact that aberrant Rho signaling in cancer can be caused by overexpression or deletion of GEFs and GAPs, emphasizes the importance of these regulators in maintaining balanced Rho activity (Vigil et al., 2010).
1.2. Membrane trafficking

Intracellular membrane trafficking is a fundamental process, which is essential for the maintenance of cell architecture and moreover enables cells to communicate with each other and their environment (Enns, 2001). The ability to sense and adapt to internal and external signals is crucial to establish and maintain cellular homeostasis. Mammalian cells have developed a sophisticated internal membrane system, in which proteins, lipids and other molecules, so-called cargo, can be transported from one cellular compartment to the other in membrane-covered, vesicular and tubular transport carriers. These carriers are delivered to their destination along highly ordered routes that are determined by the cargo itself, as well as by a specific signature of coat and adaptor proteins (e.g., COPI/I, clathrin, Rab proteins) (Alberts et al., 2011). Although many diverse transport routes have been identified so far, two main pathways can be broadly distinguished: first, the secretion of proteins into the direction of the plasma membrane, also known as exocytosis or anterograde trafficking, and second, the uptake of proteins at the plasma membrane, also known as endocytosis or retrograde trafficking.

Within the exocytic pathway, cargo is transported from the endoplasmic reticulum (ER), through the Golgi complex to the plasma membrane, where it either resides or is secreted into the extracellular matrix. This stepwise ER-to-Golgi transport allows the modification of newly synthesized proteins, e.g. by the addition of sugars and lipids (Alberts et al., 2011). Cargo proteins have to pass the Golgi complex cisternae by first entering at the ER-faced cis-side, then traversing the internal medial cisternae and finally leaving the Golgi at its trans-side that faces the plasma membrane. The trans-Golgi network (TGN) functions as major sorting compartment, since here each cargo is packed into the right vesicular carrier, depending on the cellular destination (Bard and Malhotra, 2006). In the last step of exocytosis, cargo-loaded vesicles fuse with the plasma membrane to either release or deliver their soluble or membrane-bound cargo, respectively.

The endocytic pathway enables cells for the uptake of nutrients, receptor-associated ligands and membrane proteins, which are taken up via a whole set of different endosomal vesicles and can get transported to lysosomes for their degradation and release into the cytosol or are recycled back, which is the case for many transmembrane receptors. It is now widely accepted that there exist a large number of specialized endocytic pathways, which are complex and partially interconnected (Maxfield and McGraw, 2004). Importantly, the massive membrane flow within endocytosis and exocytosis needs to be tightly controlled to maintain the proper composition and size of organelles, as well as to deliver essential molecules to the appropriate compartments, where they carry out specific functions and
determine the compartment identity (Maxfield and McGraw, 2004).

### 1.2.1. Receptor-mediated endocytosis

Receptor-mediated endocytosis is the most well understood endocytic process, by which cells internalize extracellular ligands and activated plasma membrane receptors. By direct interaction with adaptors, e.g. the clathrin coat adaptor complex, receptors are recruited and concentrated in clathrin-coated pits that invaginate inwards with the help of other accessory proteins and subsequently pinch off, forming a clathrin-coated vesicle (Fig. 1) (Maxfield and McGraw, 2004). Classic examples for clathrin-dependent endocytosis are the iron-bound transferrin receptor, as well as the receptors for the epidermal growth factor (EGF) and low density lipoprotein. Clathrin-independent routes have been described for several endocytic cargo molecules, but their internalization mechanisms are not well understood (e.g. the glucose transporter GLUT1 and the cell adhesion proteins β-integrin and E-cadherin) (Grant and Donaldson, 2009). Independent of the internalization mechanism, many receptors are re-utilized and recycled back to the cell surface, because their biosynthesis is expensive for the cell. And moreover, the internalized membranes and resident proteins need to be returned to the plasma membrane to maintain its composition.

Endocytosed receptors first enter the early endosomes, where most ligands are released due to the weakly acidic pH within the lumen (Fig. 1). Upon uncoupling of the ligands, the receptors are differentially sorted into the endocytic recycling pathways (Maxfield and McGraw, 2004). Receptors can be delivered to three main destinations: the plasma membrane, the endocytic recycling compartment (ERC) or late endosomes (Fig. 1). Direct recycling to the plasma membrane occurs via the incompletely understood fast-recycling pathway. Upon delivery to the ERC, most molecules return to the plasma membrane via the slow-recycling pathway, although delivery to the TGN occurs as well. To terminate signaling, receptors are routed from early endosomes to late endosomes and finally fuse with lysosomes, where they are degraded. Thus, the regulation of internalization and sorting of activated receptors critically determines the signal transduction. Dysregulation of endocytosis is therefore implicated in many pathological processes including cancer (Mellman and Yarden, 2013). For example, the activation of the EGFR triggers the downstream signaling of two major pathways, the phosphatidylinositol 3-kinase (PI3K) and the mitogen-activated protein kinase (MAPK) pathway, which are both crucial for cell survival and growth. By interfering with the ability to internalize, recycle or degrade the activated receptor, the EGFR-mediated signaling continues, leading to uncontrolled cellular signaling and neoplastic transformation (Tomas et al., 2014).
Introduction

Fig. 1 Pathways of receptor-mediated endocytosis. Receptors are recruited to clathrin-coated pits that invaginate and pinch off the plasma membrane to form a clathrin-coated vesicle (CCV). Upon internalization, Rab5 is recruited to early endocytic vesicles, where it mediates the maturation to early endosomes (EE) by promoting endosomal fusion. Internalized receptors can either recycle back to the plasma membrane via a fast Rab4-dependent mechanism or traffic back to the surface by passing the Rab8/11-containing ERC. The maturation of early to late endosomes (LE) requires the exchange of Rab5 to Rab7. Late endosomes can mediate trafficking to the TGN or fuse with lysosomes (LY) to promote cargo degradation. The dashed lines indicate unknown Rab proteins and recycling pathways, respectively.

1.2.2. Regulation of endocytosis by Rab GTPases

Rab GTPases are the master regulators of membrane trafficking and their functions in vesicle-associated processes, including vesicle budding, fusion and motility have been studied extensively (Hutagalung and Novick, 2011; Stenmark, 2009). Within the regulation of receptor-mediated endocytosis, the Rab GTPases Rab4/5/7/8/9 and Rab11 play a major role. Each of these Rab proteins defines a specific subset of endosomal vesicles and mediates its function by the recruitment and activation of effector proteins. Upon removal of the clathrin coat, the fusion of primary endocytic vesicles with sorting endosomes is regulated by Rab5 (Fig. 1). In particular, recruitment of the Rab5 effectors EEA1 (early endosome antigen 1) and phosphatidylinositol 3-kinase facilitates the endosomal membrane fusion, leading to the formation of so-called early endosomes (Stenmark, 2009). Besides, Rab5 activity is important for the movement of endosomes on microtubules (Somsel Rodman and Wandinger-Ness, 2000). Via the Rab4-dependent recycling pathway, receptors are directly sorted back from early endosomes to the plasma membrane (Fig. 1). Although the sites of
Rab4 activation are not known and contradictory results exist about the precise role of Rab4, the expression of a dominant-negative Rab4 mutant inhibited the fast recycling of cargo proteins (Grant and Donaldson, 2009). Rab4 has as well been implicated in the regulation of cargo recycling to the ERC. Nevertheless, Rab4 and Rab5 seem to act sequentially, in order to control the balance of membrane influx into and efflux out of the early endosomes, respectively (Somsel Rodman and Wandinger-Ness, 2000). Early-to-late endosome maturation requires a process known as ‘Rab conversion’, in which the exchange of Rab5 to Rab7 by the HOPS (homotypic fusion and vacuole protein sorting) complex is the key process that has to take place (Fig. 1) (Rink et al., 2005). Another Rab protein found on late endosomes is Rab9, which has been shown to regulate the transport of cargo molecules from late endosomes to the TGN (Fig. 1) (Stenmark, 2009). To promote cargo degradation, late endosomes have to fuse with acidic lysosomes, in which internalized components are degraded due to the low pH and presence of acidic hydrolases (Fig. 1). Receptors that are sorted back to the cell surface via the slow recycling pathway, have to pass the juxtanuclear located ERC, a tubulovesicular compartment that associates with the microtubule organizing center and Golgi complex and is defined by the presence of Rab11 and/or members of the Eps15 homology domain (EHD) family (Grant and Donaldson, 2009). From the ERC Rab11-positive endosomes emerge that are delivered to the plasma membrane (Fig. 1). Since perturbation with Rab11 function causes the concentration of recycled cargo at the ERC, an important role for Rab11 in the regulation of recycling from the ERC has manifested (Stenmark, 2009). Rab11 has also been found localized to the TGN, where it regulates exocytic vesicle transport (Fig. 1) (Urbe et al., 1993). More recent studies identified Rab8 as further component of the ERC, since it co-localizes with the markers Rab11 and EHD1/3 (Hattula et al., 2006; Roland et al., 2007). Moreover, it was found that Rab8 controls the delivery of transferrin from the plasma membrane to the perinuclear ERC (Hattula et al., 2006). In sum, Rab GTPases regulate different functions within the endocytic trafficking, depending on their localization and interaction with effector proteins.

1.2.3. Regulation of endocytosis by Rho GTPases

There is increasing evidence that the coordinated remodeling of actin and microtubule dynamics by Rho GTPases is important for the formation and movement of vesicular and tubular transport carriers and also assists in organelle positioning (Lanzetti, 2007). During the biogenesis of carriers, microtubules, F-actin and their associated motor proteins cooperate to shape membranes (Fig. 2). After the initial step of membrane curvature by the assembly of coat proteins, acto-myosin dynamics are needed to bend membranes and initiate the formation of a future vesicle or tubule (Fig. 2). In cooperation with myosin and
kinesin motor proteins the donor membrane is elongated along a microtubule track (Eitzen, 2003). Thereby actin polymerization leads to growing F-actin filaments, which push membranes forward and provide the needed forces (Fig. 2). To produce a single vesicle, actin filament coats or rings have to form and exert contractile forces that lead to the fission of a vesicle (Fig. 2) (Nightingale et al., 2012). To mediate the last step, the fusion of donor and acceptor membranes, highly dynamic disassembly and reassembly of actin filaments is required to mediate the movement of the transport carriers through the actin cytoskeleton and allow access to the sites of docking and fusion (Fig. 2) (Eitzen, 2003).

Fig. 2 Cytoskeleton-based processes within vesicle formation, fission, transport and fusion. (i) For membrane deformation, myosin motor proteins tether actin filaments to generate motile forces. (ii) With the help of microtubule-dependent kinesin motors tubules are extended along polymerizing actin filaments. (iii) Acto-myosin contraction around the neck of the elongated tubule causes vesicle fission. (iv) Vesicle trafficking occurs along microtubule and actin tracks. (iv) Dynamic actin remodeling is required to allow the access to docking and fusion sites.

Moreover, it is already known that actin fibers and microtubules function as tracks for short- and long-range transport, respectively, on which vesicular carriers are transported between cellular compartments (Fig. 2) (Soldati and Schliwa, 2006). Thus, the actin and microtubule cytoskeleton play important roles in the formation of carriers and their bidirectional transport between organelles (Anitei and Hoflack, 2011).

Within the Rho subfamily of Rho GTPases, there exist three highly homologous members: RhoA, RhoB and RhoC. Because of their sequence divergence in their C-terminus, the three isoforms were found to localize to different subcellular sites (Chi et al., 2013). Whereas RhoA and RhoC are mainly localized at the plasma membrane, RhoB is found at endosomes. The three isoforms exert different cellular functions, but little is known about
their role within receptor-mediated endocytosis. For example, it was found that activated RhoA inhibits clathrin-dependent internalization of the transferrin receptor at the plasma membrane (Lamaze et al., 1996). Since the actin filaments were not required for clustering transferrin receptors into coated pits, it is very likely that the right balance of actin polymerization and depolymerization is necessary for subsequent membrane deformation and vesicle budding (Lamaze et al., 1997).

In accordance with the distinct localization of RhoB to endocytic vesicles (Adamson et al., 1992; Robertson et al., 1995), a specific function in the regulation of endosomal traffic was identified. Activated RhoB induced actin assembly on endosomes, causing reduced motility by their strong association with subcortical actin fibers (Fernandez-Borja et al., 2005). The downstream effector mDia1, also known as mammalian Diaphanous-related formin, was identified to mediate the effects of RhoB on endosomal transport. Upon RhoB activation, mDia1 is recruited to endosomes, where it promotes the assembly of an actin coat on the vesicle membrane, which associates the endosome with actin fibers that run underneath the plasma membrane (Fernandez-Borja et al., 2005). Moreover, by activating its target protein kinase C-related kinase 1 (PRK1), RhoB delays the delivery of internalized EGFR to lysosomes for receptor degradation (Gampel et al., 1999). Thus, Rho GTPases are able to regulate endocytosis depending on their different localization either at the plasma membrane or within the endosomal pathway.

Although the different trafficking steps appear to be controlled by Rho signaling, only very few studies have investigated cellular Rho GTPase activation in time and space. In the context of pinocytosis, a process in which cells take up external substances by engulfing them in membranous vesicles, biosensors were used to measure spatiotemporal Rho activity. These experiments revealed that a sudden burst of RhoA activation occurred during pinosome formation and levels of active RhoA remained high during pinosome trafficking (Pertz et al., 2006). By contrast, RhoC activity was needed in the actin ring structure formed prior to vesicle closure (Zawistowski et al., 2013). Together, these data demonstrate that not only the precise activation and inactivation of Rho GTPases has to be regulated, but also the crosstalk between different Rho isoforms needs to be finely tuned.

### 1.3. Rho GTPase regulation by GEFs and GAPs

Rho proteins cycle between an active GTP-bound and an inactive GDP-bound state, a process that is controlled by three classes of regulators (Fig. 3) (Bos et al., 2007). (i) GEFs promote the exchange of bound GDP for GTP, leading to activation of the Rho GTPase and subsequent binding of downstream effectors. Activated Rho GTPases are targeted to cell
membranes by their C-terminal prenyl groups (geranyl-geranyl or farnesyl), which serve as lipid anchors. (ii) GAPs enhance the low intrinsic GTPase function of the Rho proteins by stabilizing the transition state from GTP to GDP and thereby lead to their inactivation. (iii) Guanine nucleotide dissociation inhibitors (GDIs) keep Rho GTPases in the inactive state by preventing the release of GDP or by masking the prenyl group. Thus, binding of GDI sequesters Rho GTPases in the cytoplasm and prevents interactions with effectors.

Fig. 3 Regulation of Rho GTPases. Rho proteins shuttle between an inactive, GDP-bound and an active, GTP-bound state. Activation is mediated by GEFs, which promote the exchange of GDP for GTP. GAPs promote the hydrolysis of GTP to GDP, whereas GDIs prevent the nucleotide exchange. Activated, GTP-bound Rho proteins bind to effectors triggering downstream signaling. PM = plasma membrane

Together these regulators tightly control the activation and inactivation of Rho GTPases. The fact that the number of GEFs (~80) and GAPs (~70) greatly exceeds the number of Rho GTPases (~20) can be explained by the required control of Rho activation in space and time, preventing inappropriate or prolonged signaling (Iden and Collard, 2008). Due to the large variety of regulator and effector molecules, Rho GTPases are able to integrate information of a spectrum of extracellular stimuli and subsequently trigger the appropriate downstream signaling pathways.

1.3.1. RhoGEFs and -GAPs in membrane trafficking

Although GEFs and GAPs are crucial for the control of Rho GTPases, within membrane trafficking only a few regulators are known so far. In the context of exocytosis, the Rho-specific GAP Gem-interacting protein (GIMP) and the GEF-H1 protein were recently identified to control RhoA activity (Johnson et al., 2012; Pathak et al., 2012). In particular,
Introduction

GIMP is a GAP that binds the secretory factor JFC1 and regulates actin remodeling by RhoA inactivation, leading to enhanced exocytosis (Johnson et al., 2012). The activity of GEF-H1 depends on its localization on microtubules: when associated with microtubules GEF-H1 is inactive, whereas their depolymerization leads to GEF-H1 activation (Krendel et al., 2002). Thus, disassembly of microtubules can induce GEF-H1-mediated activation of RhoA, implicating this GEF in the regulation of cellular processes that require high microtubule dynamics, e.g. during cytokinesis or migration. Moreover, GEF-H1 has been demonstrated to interact with the exocyst complex Sec5, thereby facilitating RhoA activation and regulating exocytosis (Pathak et al., 2012).

The GAP protein ARAP1 was suggested to be a possible candidate for Rho GTPase regulation at the Golgi complex and later shown to be recruited to Rab5-positive structures to regulate EGFR trafficking (Miura et al., 2002; Yoon et al., 2008). Besides a RhoGAP domain, ARAP1 possesses an ArfGAP domain, complicating mechanistic studies and leaving open the question of GTPase specificity in the context of receptor-mediated endocytosis. Similarly, overexpressed p50RhoGAP, which displays broad substrate specificity for Rho, Rac and Cdc42, was reported to co-localize with Rab5- and Rab11-positive endosomal membranes and inhibit transferrin uptake, but the underlying molecular mechanisms were not addressed (Sirokmány et al., 2006).

1.3.2. The RhoGAP family of proteins

The RhoGAP family is defined by the presence of a conserved RhoGAP domain that stimulates the intrinsic GTP hydrolysis activity of Rho GTPases, promoting their inactivation. Firstly, the formation of the GAP-GTPase complex stabilizes the position of the catalytic glutamine residue of the Rho protein, which coordinates the GTP-attacking water molecule (Bos et al., 2007). Secondly, all GAP domains contain a highly conserved arginine residue in a loop structure called the ‘arginine finger’ (Gamblin and Smerdon, 1998). The positive charge of arginine is essential for the GAP activity, since it is introduced into the catalytic site of the GTPase and stabilizes the transition state by neutralizing the negatively charged γ-phosphate of GTP (Bos et al., 2007). Some GAPs display broad specificity whereas others are specific to a single Rho GTPase. How the substrate specificity of RhoGAPs is achieved is still an open question.

Apart from the highly conserved GAP domain, the RhoGAP family members typically contain other structural motifs, including catalytic domains as well as protein-protein, protein-lipid interaction domains and many putative phosphorylation sites (Bos et al., 2007). The multi-domain organization provides a broad platform for regulatory control of RhoGAP
localization, specificity and activity, allowing optimal response to Rho-mediated signaling events. Moreover, GAPs function not only as negative regulators of Rho GTPases, but can also serve as effectors or scaffold proteins, mediating cross-talk between Rho GTPases and other signaling pathways in a GAP-independent manner. For example, the RasGAP domain of IQGAP is devoid of GAP activity, but via its other domains (e.g. calponin homology domain), IQGAP participates in multiple cellular functions, e.g. Ca\(^{2+}\)/calmodulin signaling, cellular adhesion or cytoskeletal rearrangements (White et al., 2012).

Aberrant Rho signaling in cancer is caused by altered gene expression or function of their regulators, rather than by activating mutations of the Rho GTPase itself. For example, many RhoGEFs such as Dbl and Ect2 were identified to be upregulated or mutated, leading to altered signaling and malignant transformation. Although there is less evidence for the loss of GAP-mediated Rho inactivation in cancer, it has been proven that inactivation of GAP activity alone is sufficient to cause Rho hyperactivation (Vincent and Settleman, 1999). Among the ~70 human RhoGAPs, the ‘Deleted in Liver Cancer’ (DLC) protein family attracted special attention, since inactivation of its three members (DLC1/2/3) is the most common alteration reported for Rho regulators (Vigil et al., 2010). In some cancers, loss of DLC1 is as frequently observed as for p53, a bona fide tumor suppressor (Kandpal, 2006; Xue et al., 2008).

1.4. The ‘Deleted in Liver Cancer’ RhoGAP protein family

Three genes of the human genome, DLC1, DLC2 and DLC3, encode for the structurally conserved DLC subfamily of RhoGAPs (Fig. 4). DLC1, the founding member of the family, was originally identified in 1998 as a candidate tumor suppressor homozygously deleted in human hepatocellular carcinomas (HCCs) and HCC-derived cell lines (Yuan et al., 1998). Frequent loss of heterozygosity (LOH) was confirmed for the DLC1 locus at chromosome 8 (8p21.3-22), causing the underexpression of DLC1 in a wide range of human cancers, including breast, lung, colon, ovarian, pancreatic and prostate (Durkin et al., 2007a). DLC2 was identified in a LOH region at chromosome 13 (13q12.3), which is also commonly deleted in HCC and harbors other tumor suppressors such as BRCA2 (Ching et al., 2003). Similarly to DLC1, DLC2 is significantly downregulated in a wide range of human tumors and many tumor-derived cell lines (Ching et al., 2003; Ullmannova and Popescu, 2006). The DLC3 gene is situated on the X chromosome (Xq13) and although the localization within a LOH region remains to be proven, loss of DLC3 expression was found in primary tumors from different tissues (Durkin et al., 2007b).
Orthologs of the DLC proteins have also been identified in other vertebrates and invertebrates. For example, p122RhoGAP, first isolated from rat brain as an interacting partner of phospholipase Cδ1 (PLCδ1) (Homma and Emori, 1995), was later found to be the rat ortholog of human DLC1 (Fig. 4) (Yuan et al., 1998). The genome of the fruit fly Drosophila melanogaster encodes one DLC-like protein, named RhoGAP88c (Fig. 4). Several mutations within the gene encoding RhoGAP88c cause lethality at the embryonic/larval stage because of severe defects during morphogenesis (Denholm et al., 2005), whereas mutant alleles that do not impair viability are associated with the loss of the crossvein in the fly’s wing, whereby the gene was termed crossveinless-c (cv-c). During morphogenic processes, such as invaginations, RhoGAP88c is important for the coordination of cytoskeletal rearrangements by regulating the activity of Rho1, Cdc42 and Rac in a polarized and locally restricted manner (Brodu and Casanova, 2006; Denholm et al., 2005; Sato et al., 2010; Simões et al., 2006).

Fig. 4 Domain organization of the human DLC isoforms and their homologs from rat and fruit fly. The percent identity to the corresponding domain and the overall identity to human DLC1, respectively, are shown. The figure was adapted from: Durkin et al., 2007a.

1.4.1. Multi-domain organization of DLC proteins

DLC proteins possess three functional domains, the catalytic RhoGAP domain, the sterile α motif domain (SAM) and the StAR (steroidogenic acute regulatory protein)-related lipid transfer domain (START) (Fig. 4). The RhoGAP domain (~150-200 amino acids) is the most conserved region among the three DLC proteins and harbors the catalytic arginine finger. Two additional conserved amino acids, a lysine and a second arginine residue were identified to be crucial for the GAP activity (Durkin et al., 2007b; Holeiter et al., 2012; Leung et al., 2005; Sekimata et al., 1999). Consistent with GAP domain conservation, all DLC
proteins show GAP activity towards RhoA and, at least in vitro, weak activity was also reported for Cdc42, but not for Rac (Ching et al., 2003; Healy et al., 2008; Holeiter et al., 2008; Holeiter et al., 2012; Wong et al., 2003). For DLC1 in vitro substrate specificity towards the highly related RhoA homologs, RhoB and RhoC, has also been shown (Healy et al., 2008) and recently in vivo GAP activity towards Cdc42 was demonstrated for DLC1 (Qian et al., 2012).

Active Rho is known to regulate cell morphology by the activation of its main downstream effectors ROCK (Rho-associated, coiled-coil containing kinase) and Dia, which promote actin polymerization and lead to stress fiber formation. In addition, ROCK activation triggers the phosphorylation-induced inactivation of myosin light chain (MLC) phosphatase, leading to increased acto-myosin contractility. Thus, the observed phenotype of DLC overexpression, which is characterized by a rounded cell morphology with long protrusions and the loss of actin stress fibers, is in line with a strong Rho inactivation and the inability to maintain the cellular cytoskeleton (Ching et al., 2003; Kawai et al., 2007; Sekimata et al., 1999; Wong et al., 2005).

The SAM domain makes up around 70 - 90 amino acids at the N-terminus of DLC proteins. However, isoforms lacking the SAM domain have been reported for DLC2 and DLC3 (Ching et al., 2003; Durkin et al., 2007b). SAM domains occur in many transcription factors and signaling proteins. Usually they form homo- and hetero-oligomers with other SAM domain-containing proteins or non-SAM-containing proteins, but SAM-DNA and SAM-RNA interactions have also been described (Qiao and Bowie, 2005). The function of the SAM domain in the DLC proteins is unclear, but there is evidence for a role in intramolecular protein regulation. Since, the deletion of the SAM domain yields a more active form of DLC1, it is speculated that the SAM domain is involved in auto-inhibition of the RhoGAP activity (Kim et al., 2008). The findings about the contribution of this domain to the protein localization remain elusive. Whereas the artificial deletion of the SAM domain in DLC1 did not disrupt the localization to focal adhesions (Kim et al., 2008), the naturally occurring isoforms DLC2γ and DLC3β that lack this domain are not well studied yet (Ching et al., 2003; Durkin et al., 2007b).

All three members contain a START domain (~210 amino acids) at the C-terminus. START domains are known to transfer single lipid molecules between membranes and play a role in lipid metabolism (Wirtz, 2006). Structurally, START domains form a deep lipid-binding pocket covered by a lid that protects the bound hydrophobic ligand from the external environment. Only for a few members of the 15 identified human START-containing proteins the identity of the bound lipids has been unraveled. For instance, the ceramide transfer
protein (CERT) was shown to mediate the specific exchange of ceramide from donor to acceptor membranes (Hanada et al., 2003) and the StAR protein transfers cholesterol from the outer to the inner mitochondrial membrane (Miller, 2007). However, the ligands for DLC START domains have not been identified and it remains to be determined whether they possess lipid transfer or lipid presenting activity.

The linking middle region between the RhoGAP and SAM domain lacks a secondary structure and shows the least sequence conservation among the DLC family members (Durkin et al., 2007a). This region is characterized by an open conformation and a high content of serine residues, allowing the interaction with other proteins as well as the attachment of post-translational modifications. Within DLC1 and DLC2, phosphorylation sites have been identified that activate and inhibit GAP activity, respectively, or interfere with GAP-independent functions as well as with protein localization (Ko and Yam, 2014).

1.4.2. Biological functions of the DLC isoforms in time and space

Overexpression studies in various tumor cell lines revealed a similar phenotype induced by the different DLC isoforms, including the inhibition of cell proliferation, anchorage-independent growth, migration and invasion in vitro and tumorigenicity in vivo (Ching et al., 2003; Durkin et al., 2007b; Leung et al., 2005; Ng et al., 2000; Wong et al., 2005; Yuan et al., 2003). However, upcoming evidence of DLC isoform-specific localizations, interaction partners as well as subcellular functions indicate that the family members are not redundant and might possess distinct roles depending on the developmental stage or spatiotemporal cellular context.

In particular, gene deletion studies in mice revealed that DLC1-deficient embryos die at around ten days post coitum, most likely due to placental and cardiac defects (Durkin et al., 2005; Sabbir et al., 2010), whereas DLC2 knockout mice are viable (Lin et al., 2010; Yau et al., 2009). The lethality of DLC1 knockout militates against a reverse compensation by DLC2 and DLC3 and fully overlapping functions of the DLC proteins. Hence, more tissue-specific knockouts will be necessary to identify redundant and/or unique biological functions of the DLC proteins. In a cellular context the precise localization of the DLC proteins determines the spatiotemporal Rho activation pattern. For example, by using a novel probe that allows the visualization of Rho activity during the normal development of the fruit fly, it was found that activation of Rho1, the Drosophila homolog of RhoA, was spatially restricted to the apical side of the invaginating spiracle cells (Simões et al., 2006). This asymmetric activation was achieved by the local enrichment of Rho1 and the differential distribution of
Rho regulators, with apical localization of specific GEFs and basolateral localization of RhoGAP88C.

These data highlight the mandatory need of dynamic regulation of Rho GTPase activity in time and space. Within a network of Rho proteins, GEFs, GAPs and effectors, many signaling events have to be integrated and transmitted, to ensure the proper response. Depending on the different subcellular localizations identified so far, DLC proteins are able to orchestrate site-specific Rho signaling and thereby are involved in many and varied cellular processes, some of which will be highlighted in more detail below.

1.4.3. **DLC proteins in focal adhesions**

The first subcellular localization described for DLC proteins were the focal adhesions, integrin-matrix contacts that connect the cellular cytoskeleton and extracellular matrix, thereby regulating cell motility and shape. Until now, the localization of all three DLC isoforms to focal adhesions has been reported, although the interaction with tensin adaptor proteins that couple integrins with the actin filament has been studied mainly for DLC1 (Fig. 5). In particular, DLC1 was found to interact with the SH2 (Src homology 2) and phosphotyrosine-binding (PTB) domains of tensin1, tensin2 and CTEN (C-terminal tensin-like) (Liao et al., 2007; Qian et al., 2007; Yam et al., 2006) and, via the actin-binding domain of tensin3, DLC1 also forms a complex with tensin3 (Cao et al., 2012). At least two essential binding sites have been mapped within DLC1. The amino acid stretch 440SIYDNV445 interacts with the SH2 domain of tensin1 and CTEN in a phosphorylation-independent manner, wherein Tyr442 plays an important role for the interaction, since mutation of this amino acid abolished binding (Liao et al., 2007; Qian et al., 2007). Apart from that, the amino acid residues 375-385 predominantly mediated the binding of DLC1 with the tensin2 PTB domain, but had no influence on the interaction with tensin1 and CTEN (Chan et al., 2009). Although less well studied, DLC2 and DLC3 both localize to focal adhesions by binding to tensin1 and 2, suggesting that the isoforms share similar molecular mechanisms for focal adhesion recruitment (Kawai et al., 2007; Kawai et al., 2009; Kawai et al., 2010; Qian et al., 2007).

In accordance with active RhoA inducing the formation of stress fibers and focal adhesions (Ridley and Hall, 2001), DLC1 overexpression was shown to lead to a loss of focal adhesions and a reduction of the actin stress fiber content (Kim et al., 2007; Wong et al., 2005). The morphological changes required the presence of a functional RhoGAP domain (Wong et al., 2005) and co-expression of active RhoA reversed the phenotype (Kim et al., 2008). In line with this, our laboratory could show that RNA interference (RNAi)-mediated
silencing of DLC1 enhanced stress fiber formation, focal adhesion assembly and promoted cell motility (Holeiter et al., 2008). Interestingly, mutations that exclude DLC1 from focal adhesions reduce its tumor suppressing activities, without affecting the overall ability to inactivate Rho, suggesting that the spatial Rho regulation in focal adhesions is critical to DLC function (Li et al., 2011; Liao et al., 2007; Liao et al., 2008; Qian et al., 2007). However, it should be kept in mind that nascent focal complexes undergo constant turn-over and only a subset of these mature to form stable tensin-enriched fibrillar adhesions. The spatiotemporal aspects of DLC localization to these sites and the impact of DLC proteins on focal adhesion dynamics, i.e. rates of assembly and disassembly, have not yet been precisely quantified.

Fig. 5 DLC proteins and their subcellular localizations. See main text for details.
AJ = adherens junction; EC = extracellular domain; PM = plasma membrane; FA = focal adhesion; ERC = endocytic recycling compartment; TGN = trans-Golgi network; ECM = extracellular matrix; p120ctn = p120-catenin; FAK = focal adhesion kinase; Cas = Crk-associated substrate

1.4.4. DLC proteins at adherens junctions

Cell adhesion is a dynamic process in which the cadherin-catenin complex and the attached actin cytoskeleton undergo constant remodeling (Fukata and Kaibuchi, 2001). The spatiotemporal activation of Rho GTPases is essential to coordinate the formation and maintenance of adherens junctions (AJs) in order to preserve normal epithelial function and
tissue integrity (Fukata and Kaibuchi, 2001; Nelson, 2008). However, the contribution of Rho proteins to cell adhesion has been discussed controversially. In contrast to Rac1 and Cdc42, RhoA activity was shown to be downregulated during the initial phase of cell adhesion. Actually, active Rac1 promotes the inactivation of RhoA via p190RhoGAP whereupon RhoA-GTP hydrolysis is accelerated (Noren et al., 2003; Sander et al., 1999; Wildenberg et al., 2006). Moreover, elevated levels of active RhoA were shown to negatively modulate cell adhesion due to excessive stress fiber formation and cell contractility (Sahai et al., 2001). More recent spatiotemporal analysis by fluorescence resonance energy transfer (FRET) revealed that RhoA is active during de novo cell adhesion, but activity is restricted to the periphery of contacting cell membranes (Yamada and Nelson, 2007). Recently, p114RhoGEF was identified as the first GEF that activated RhoA at epithelial junctions (Terry et al., 2011). Furthermore, the mainly Rho-specific GEF Ect2 was found to be recruited to the zonula adherens, where it maintained junctional integrity by regulating RhoA-mediated actomyosin remodeling (Ratheesh et al., 2012). Obviously AJ formation demands spatially restricted and process-specific RhoA regulation achieved by the action of specific GEFs and GAPs.

To fulfil their regulatory task, GEFs and GAPs have to be in close proximity to their Rho proteins. The association of DLC3 to AJs was first revealed by our lab, by demonstrating a strong co-localization of DLC3 with the AJ proteins E-cadherin and β-catenin in the breast cancer cell line MCF7 as well as in the breast epithelial cell line MCF10A (Fig. 5) (Holeiter et al., 2012). Associated with its subcellular localization, DLC3 depletion led to mislocalization of E-cadherin, β-catenin and p120catenin, causing reduced cell aggregation and increased cell migration. Constitutively active Rho has been reported to destabilize cell junctions through the downstream ROCK signaling pathway by generating contractile forces that led to the disruption of AJs (Sahai and Marshall, 2002). DLC3-depleted MCF7 cells contained elevated levels of phosphorylated MLC, indicative of aberrant Rho–ROCK signaling. Moreover, ROCK inhibition in these cells restored junctional localization of E-cadherin and β-catenin complexes, identifying DLC3 as regulator of Rho-ROCK signaling at AJs.

One mechanism by which DLC proteins could be recruited specifically to AJs is the binding to α-catenin (Fig. 5) (Tripathi et al., 2012). The complex formation with α-catenin enables DLC1 to associate with E-cadherin and β-catenin, promoting its localization to cell-cell junctions in a GAP-dependent manner. Because association of DLC3 with cell adhesions does not require its GAP activity (Holeiter et al., 2012), it is unlikely that the same molecular machinery is involved in recruiting DLC3 to sites of cell contact. The DLC1-α-catenin-complex reduced RhoA and RhoC activation specifically at the plasma membrane, resulting
in enhanced E-cadherin expression and stabilized AJs and thus potentiated DLC1 inhibitory effects on invasion (Tripathi et al., 2012; Tripathi et al., 2014). The disruption of AJs caused by active RhoA/C could be restored by ROCK inhibition (Tripathi et al., 2014), which is in line with the data provided by our lab. Taken together, DLC’s GAP activity and the subcellular recruitment of DLC proteins to AJs enable the spatial regulation of Rho-ROCK signaling, affecting proper expression and localization of AJ molecules and ensuring the maintenance of E-cadherin-based cell–cell contacts and epithelial integrity.

1.4.5. DLC proteins at the plasma membrane and cell protrusions

Ectopic DLC1 expression in fibroblasts was shown to reduce RhoA activity preferentially at the leading edge of cellular protrusions (Healy et al., 2008). In migrating cells, RhoA has always been thought to act at the back of the cell to induce tail retraction via activation of ROCK (Machacek et al., 2009). However, new data provide evidence for a role of Rho in the initiation of protrusion at the leading edge, most likely involving the engagement of formins such as Dia1. Rho stimulates actin polymerization through activation of its effector Dia1, which promotes addition of actin monomers to the barbed end of actin filaments and further stabilizes microtubules, specifically at the leading edge (Palazzo et al., 2001). In support of a role for DLC1 in the control of Rho signaling at the cellular front, we observed an increased accumulation of Dia1 in membrane protrusions during wound closure in MCF7 cells lacking DLC1. This was associated with enhanced directed migration, which could be reversed by the simultaneous silencing of DLC1 and Dia1 (Holeiter et al., 2008). Convincing evidence that DLC1 is also involved in the regulation of the Rho-ROCK signaling axis was provided by Xue et al., demonstrating that ROCK inhibition was able to suppress colony formation of p53−/−;Myc liver progenitor cells depleted from DLC1, indicating that some tumor suppressive functions are indeed mediated by ROCK activation (Xue et al., 2008).

Interestingly, DLC2 knockdown reduced motility and enhanced adhesion as a consequence of aberrant regulation of RhoA and Rac activity in human astrocytoma cells (Khalil et al., 2014). This phenotype could be rescued by either downregulating RhoA or overexpressing dominant active Rac1, indicating that RhoA activation upon DLC2 loss indirectly caused Rac1 inhibition. Moreover, FRET analysis revealed high and persistent RhoA activation especially in the tail of knockdown cells, suggesting that cycling between RhoA-GTP and Rho-GDP was disturbed and prevented RhoA inactivation for final tail retraction. By contrast, enhanced cell migration upon DLC2 knockdown was found in HepG2 and normal vascular endothelial cells (Leung et al., 2005; Lin et al., 2010). The consequence of DLC2 protein loss with regard to cell motility thus appears to depend on the degree of Rho
activation, facilitating cell migration under certain conditions by enhancing adhesion and contractility, but immobilizing cells when too high a level.

How the DLC proteins are recruited to defined regions at the plasma membrane, whether it be the front or back, is still an open question. The first factor involved in DLC1 recruitment to the membrane periphery was the eukaryotic elongation factor 1A1 (EF1A1), a protein involved in the organization of the actin and microtubule network (Fig. 5) (Zhong et al., 2009). EF1A1 was shown to directly interact with the SAM domain of DLC1, but not that of DLC2, and facilitated EF1A1 recruitment to the membrane periphery.

1.4.6. DLC proteins in caveola

Caveola are lipid-enriched plasma membrane invaginations that contain receptors and signaling molecules (e.g. Src-like kinases, Ras, EGFR, Rho GTPases), implicating these specialized structures in the regulation of signal transduction. Although caveola resemble in their morphology clathrin-coated pits, they follow a specialized internalization and recycling route depending on their decoration with caveolin proteins (Parton and Howes, 2010). Caveolin-1, the major component, plays an important role during the internalization of caveola and associated receptors, and is thereby able to modulate transmembrane signaling. Thus, dysregulation of caveolin-1 contributes to the pathogenesis of several diseases and oncogenic cell transformation (Schwencke et al., 2006). The first evidence of DLC protein localization in caveola was provided for the DLC1 rat homolog p122RhoGAP (Fig. 5) (Yamaga et al., 2004). The actin cytoskeleton normally restricts invaginated caveola to the cell surface, but upon ectopic p122RhoGAP expression, the internalization of caveolin-1 was induced, which could be a consequence of cytoskeletal rearrangements. Later, human DLC1 was also found to interact with caveolin-1 (Yam et al., 2006) and a short region within the START domain was found to be essential for the binding of DLC1 to caveolin-1 and its targeting to caveola (Du et al., 2012). Interestingly, a caveolin-1-deficient binding mutant of DLC1 diminished the tumor suppressive activity in several biological in vitro assays and also failed to inhibit tumor growth in vivo without compromising RhoA inactivation (Du et al., 2012), indicating that either the local Rho regulation within caveola is required or the DLC1-caveolin-1 interaction exerts GAP-independent physiological functions. Whether DLC1 and the other DLC isoforms convey their tumor suppressive function through the inactivation of a caveolar subpopulation of Rho GTPases or whether the induced Rho inactivation leads to cytoskeletal remodeling and thereby regulates the actin-dependent internalization/trafficking of caveola warrants further investigation.
1.4.7. **Additional subcellular localizations of the DLC proteins**

Finally, it should be noted that DLC1 and DLC2 were also identified at additional subcellular sites (Fig. 5). Recently it was found that DLC2 localizes to microtubules and regulates their growth by controlling the Cdc42-mDia signaling axis, thereby revealing a novel role of DLC2 in spindle positioning and mitotic fidelity (Vitiello et al., 2014). Moreover, DLC1 was shown to continuously shuttle between the cytoplasm and the nucleus (Scholz et al., 2009) and the localization of DLC2 to mitochondria was reported (Ng et al., 2006). However, no molecular functions were associated with these localizations, leaving open the question on the role of the DLC proteins at these subcellular sites.
1.5. Aims of the thesis

Rho GTPases function as molecular switches that connect changes of the external environment to intracellular signaling pathways. They are active at various subcellular sites and require fast and tight regulation to fulfill their role as transducers of extracellular stimuli. New imaging technologies visualizing the active states of Rho proteins in living cells highlight the mandatory need of spatiotemporal activation of the GTPases. Although it is known that GEFs and GAPs serve as critical signaling nodes that specify the amplitude and duration of a particular Rho signaling pathway, the underlying molecular aspects of Rho regulation in time and space remain largely elusive (Pertz, 2010).

The DLC subfamily can be distinguished from other RhoGAPs, since its members possess, in addition to the GAP domain, a SAM and a START domain. Although there are reports on GAP-independent functions, the tumor suppressive activity appears to be primarily mediated by the GAP domain. Thus, the proven tumor suppressive function of DLC1 in vivo, together with the exceptionally high frequency of reduced DLC expression in many different tumor types (Durkin et al., 2007a; Xue et al., 2008), attributes an outstanding role to the DLCs in oncogenic transformation.

Contrary to former opinions, there is increasing evidence that DLC proteins behave non-redundantly, depending on their subcellular localization and binding partners present at these sites. Since the least attention has been paid to DLC3, this member is not only poorly characterized concerning its localization, but also the regulated cellular events and molecular interactors remain elusive.

In order to further elucidate the role of DLC3, I addressed three major objectives within this study.

- First, I characterized the subcellular localization and recruitment of DLC3 in HeLa cells by immunofluorescence analysis and domain mapping studies.

- Using the RNAi technique, I investigated the impact of DLC3 loss on organelle morphology and function, focusing on intracellular membrane trafficking, in particular receptor-mediated endocytosis.

- Moreover, with the use of a RhoA biosensor, I further unraveled the spatial aspects of RhoA regulation by DLC3.
2. Material and methods

2.1. Material

2.1.1. Buffers and solutions

### Agarose gel electrophoresis

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose gel solution</td>
<td>1% (w/v) agarose, 0.6 µg/ml ethidium bromide (EtBr) in TAE</td>
</tr>
<tr>
<td>TAE</td>
<td>40 mM Tris acetate, 1 mM EDTA in ddH₂O, pH 8.0</td>
</tr>
</tbody>
</table>

### Protein extraction, SDS-PAGE and Western blotting

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking solution</td>
<td>0.5% (v/v) blocking solution (Roche Diagnostics), 0.05% (v/v) Tween-20, 0.01% (v/v) thimerosal in PBS</td>
</tr>
<tr>
<td>Blotting buffer</td>
<td>200 mM glycine, 25 mM Tris base, 20% (v/v) methanol in ddH₂O</td>
</tr>
<tr>
<td>Laemmli protein sample buffer (5x)</td>
<td>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</td>
</tr>
<tr>
<td>PBS</td>
<td>140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄ in ddH₂O, pH 7.4</td>
</tr>
<tr>
<td>PBS-Tween</td>
<td>0.05% (v/v) Tween-20 in PBS</td>
</tr>
<tr>
<td>RIPA lysis buffer</td>
<td>50 mM Tris pH 7.5, 150 mM NaCl, 10 mM NaF, 20 mM β-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, protease inhibitor cocktail (Roche Diagnostics) in ddH₂O</td>
</tr>
<tr>
<td>SDS-PAGE running buffer</td>
<td>25 mM Tris pH 8.8, 192 mM glycine, 0.1% (v/v) SDS in ddH₂O</td>
</tr>
<tr>
<td>SDS-PAGE running gel solution</td>
<td>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</td>
</tr>
<tr>
<td>SDS-PAGE stacking gel solution</td>
<td>5% (v/v) acrylamide, 130 mM Tris pH 6.8, 0.1% (w/v) SDS, 0.1% APS, 0.1% TEMED in ddH₂O</td>
</tr>
</tbody>
</table>

### Production of glutathione S-transferase (GST)-fusion proteins

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elution buffer</td>
<td>50 mM Tris, 10 mM reduced glutathione in ddH₂O, pH 8.0</td>
</tr>
</tbody>
</table>
**Material and methods**

**Protein-lipid overlay assay**

- **Blocking buffer**: 3% (w/v) fatty acid-free BSA in 0.1% (v/v) Tween-20 in PBS
- **Wash buffer**: 0.1% (v/v) Tween-20 in PBS

**Immunofluorescence**

- **Acid wash buffer**: 0.5% (v/v) CH₃COOH, 0.5 M NaCl, pH 3.0
- **Blocking solution**: 5% (v/v) goat serum (Invitrogen), 0.1% (v/v) Tween-20 in PBS
- **PBS**: 140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄ in ddH₂O, pH 7.4
- **PBS-Tween**: 0.1% (v/v) Tween-20 in PBS
- **PFA fixing solution**: 4% (v/v) PFA in PBS
- **Permeabilizing solution**: 0.2% (v/v) Triton X-100 in PBS

**Bacteria culturing and mini-prep**

- **Luria-Bertani (LB) medium (5x)**: 5% (w/v) peptone, 2.5% (w/v) yeast extract, 2.5% (w/v) NaCl in ddH₂O
  - for selection: LB medium + ampicillin or kanamycin
  - for plates: LB medium + 2% (w/v) agar
- **S1 resuspension buffer**: 50 mM Tris pH 8.0, 10 mM EDTA, 0.1 mg/ml RNaseA in ddH₂O
- **S2 lysis buffer**: 200 mM NaCl, 1% (w/v) SDS in ddH₂O
- **S3 neutralization buffer**: 2.8 M CH₃CO₂K in ddH₂O, pH 5.1

**2.1.2. Kits**

- **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
- **PureLink™ HiPure Plasmid Purification Kit**: Invitrogen, Karlsruhe, Germany
- **PureLink™ PCR Purification Kit**: Invitrogen, Karlsruhe, Germany
- **PureLink™ Quick Gel Extraction Kit**: Invitrogen, Karlsruhe, Germany
- **QuikChange site-directed PCR mutagenesis Kit**: Stratagene, La Jolla, USA
2.1.3. **Enzymes**

- Calf intestine alkaline phosphatase (CIAP) - Fermentas, St. Leon-Rot, Germany
- Klenow polymerase - Fermentas, St. Leon-Rot, Germany
- PfuUltra™ High-Fidelity DNA Polymerase AD - Stratagene, La Jolla, USA
- Restriction enzymes - Fermentas, St. Leon-Rot, Germany
- T4 DNA ligase - Fermentas, St. Leon-Rot, Germany

2.1.4. **Human cell lines**

Fresh cultures of the human cell lines were established every three months from frozen stocks stored in liquid nitrogen.

<table>
<thead>
<tr>
<th>cell line</th>
<th>obtained from</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>human cervical epithelial carcinoma cell line - ATCC, Manassas, USA</td>
</tr>
<tr>
<td>MCF7</td>
<td>human breast adenocarcinoma cell line - kindly provided by Cornelius Knabbe, Institute of Clinical Pharmacology, Stuttgart, Germany</td>
</tr>
</tbody>
</table>

2.1.5. **Cell culturing**

**Cell culture media and reagents**

- Collagen R solution - Serva, Heidelberg, Germany
- Cytochalasin D - Enzo Life Sciences, Lörrach, Germany
- EGF - R&D Systems, Minneapolis, USA
- Enzyme-free cell dissociation buffer - Invitrogen, Karlsruhe, Germany
- Fetal calf serum (FCS) - PAA Laboratories, Pasching, Austria
- RPMI 1640 - Invitrogen, Karlsruhe, Germany
- Trypsin/EDTA (10x) - Invitrogen, Karlsruhe, Germany

**Transfection reagents**

- Lipofectamine™ RNAiMAX - Invitrogen, Karlsruhe, Germany
- Oligofectamine™ - Invitrogen, Karlsruhe, Germany
- TransIT-HeLaMONSTER® - Mirus Bio, Madison, USA
- TurboFect™ - Thermo Scientific, Rockford, USA
2.1.6. Antibodies and fluorescent dyes

**Immunoblotting**

All antibodies were diluted in blocking solution; for primary antibodies 0.01% (v/v) azide was added for storage at 4°C.

**Table 2: List of primary and secondary antibodies that were used for immunoblotting**

<table>
<thead>
<tr>
<th>primary antibody</th>
<th>species</th>
<th>dilution</th>
<th>company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-AKT (pan; 40D4)</td>
<td>mAb, mouse</td>
<td>1:1000</td>
<td>Cell Signaling, Cambridge, UK</td>
</tr>
<tr>
<td>anti-pAKT (Thr308; C31E5E)</td>
<td>mAb, rabbit</td>
<td>1:1000</td>
<td>Cell Signaling, Cambridge, UK</td>
</tr>
<tr>
<td>anti-DLC3 (E-2)</td>
<td>mAb, mouse</td>
<td>1:500</td>
<td>Santa Cruz Biotechnology, Dallas, USA</td>
</tr>
<tr>
<td>anti-ERK 1/2 (3A7)</td>
<td>mAb, mouse</td>
<td>1:1000</td>
<td>Cell Signaling, Cambridge, UK</td>
</tr>
<tr>
<td>anti-pERK 1/2 (Thr202/Tyr204)</td>
<td>pAb, rabbit</td>
<td>1:1000</td>
<td>Cell Signaling, Cambridge, UK</td>
</tr>
<tr>
<td>anti-EGFR (1005) (Tyr1068; D7A5)</td>
<td>pAb, rabbit</td>
<td>1:500</td>
<td>Santa Cruz Biotechnology, Dallas, USA</td>
</tr>
<tr>
<td>anti-GST</td>
<td>pAb, goat</td>
<td>1:50000</td>
<td>GE Healthcare, Piscataway, USA</td>
</tr>
<tr>
<td>anti-N-cadherin</td>
<td>mAb, mouse</td>
<td>1:1000</td>
<td>BD Transduction Laboratories, Heidelberg, Germany</td>
</tr>
<tr>
<td>anti-RhoA (26C4)</td>
<td>mAb, mouse</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology, Dallas, USA</td>
</tr>
<tr>
<td>anti-RhoB</td>
<td>pAb, rabbit</td>
<td>1:500</td>
<td>Cell Signaling, Cambridge, UK</td>
</tr>
<tr>
<td>anti-α-tubulin</td>
<td>mAb, mouse</td>
<td>1:2000</td>
<td>Sigma-Aldrich, St. Louis, USA</td>
</tr>
<tr>
<td>anti-transferrin receptor</td>
<td>mAb, mouse</td>
<td>1:1000</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>anti-p190RhoGAP</td>
<td>mAb, mouse</td>
<td>1:1000</td>
<td>BD Transduction Laboratories, Heidelberg, Germany</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>secondary antibody</th>
<th>source</th>
<th>dilution</th>
<th>company</th>
</tr>
</thead>
<tbody>
<tr>
<td>horseradish peroxidase (HRP)-</td>
<td>sheep</td>
<td>1:10000</td>
<td>GE Healthcare, Piscataway, USA</td>
</tr>
<tr>
<td>anti-mouse IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP-anti-rabbit IgG</td>
<td>donkey</td>
<td>1:10000</td>
<td>GE Healthcare, Piscataway, USA</td>
</tr>
<tr>
<td>HRP-anti-goat IgG</td>
<td>donkey</td>
<td>1:100000</td>
<td>Santa Cruz Biotechnology, Dallas, USA</td>
</tr>
</tbody>
</table>
**Material and methods**

**Immunofluorescence**

Table 3: List of primary and secondary antibodies and fluorescent dyes that were used for immunofluorescence

<table>
<thead>
<tr>
<th>primary antibody</th>
<th>species</th>
<th>dilution</th>
<th>company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-EEA1</td>
<td>pAb, rabbit</td>
<td>1:100</td>
<td>Cell Signaling, Cambridge, UK</td>
</tr>
<tr>
<td>anti-FLAG M2 (F1804)</td>
<td>mAb, mouse</td>
<td>1:1000</td>
<td>Sigma-Aldrich, St. Louis, USA</td>
</tr>
<tr>
<td>anti-giantin (ab24586)</td>
<td>pAb, rabbit</td>
<td>1:1000</td>
<td>Abcam, Cambridge, UK</td>
</tr>
<tr>
<td>anti-GM130</td>
<td>mAb, mouse</td>
<td>1:250</td>
<td>BD Transduction Laboratories, Heidelberg, Germany</td>
</tr>
<tr>
<td>anti-LAMP1</td>
<td>mAb, mouse</td>
<td>1:100</td>
<td>Developmental Studies Hybridoma Bank, University of Iowa, USA</td>
</tr>
<tr>
<td>anti-N-cadherin</td>
<td>mAb, mouse</td>
<td>1:250</td>
<td>BD Transduction Laboratories, Heidelberg, Germany</td>
</tr>
<tr>
<td>anti-N-cadherin (8C11)</td>
<td>mAb, mouse</td>
<td>1:100</td>
<td>Santa Cruz Biotechnology, Dallas, USA</td>
</tr>
<tr>
<td>anti-p230</td>
<td>mAb, mouse</td>
<td>1:200</td>
<td>BD Transduction Laboratories, Heidelberg, Germany</td>
</tr>
<tr>
<td>anti-Rab6 (D37C7)</td>
<td>pAb, rabbit</td>
<td>1:400</td>
<td>Cell Signaling, Cambridge, UK</td>
</tr>
<tr>
<td>anti-Rab8 (D22D8)</td>
<td>mAb, rabbit</td>
<td>1:400</td>
<td>Cell Signaling, Cambridge, UK</td>
</tr>
<tr>
<td>anti-Rab7</td>
<td>pAb, rabbit</td>
<td>1:50</td>
<td>Cell Signaling, Cambridge, UK</td>
</tr>
<tr>
<td>anti-TGN46 (AHP500)</td>
<td>sheep serum</td>
<td>1:150</td>
<td>BioRad Laboratories, Hercules, USA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>secondary antibody</th>
<th>source</th>
<th>dilution</th>
<th>company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 488/546/633</td>
<td>goat</td>
<td>1:500</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>anti-mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 488/546/633</td>
<td>donkey</td>
<td>1:500</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>anti-rabbit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 546 anti-sheep</td>
<td>donkey</td>
<td>1:500</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>dyes</th>
<th>-</th>
<th>dilution</th>
<th>company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 488/546/633 phalloidin</td>
<td>-</td>
<td>1:100</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>DAPI</td>
<td>-</td>
<td>1:1000</td>
<td>Sigma-Aldrich, St. Louis, USA</td>
</tr>
<tr>
<td>Alexa Fluor® 555 EGF</td>
<td>-</td>
<td>1:1000</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
<tr>
<td>Alexa Fluor® 555 transferrin</td>
<td>-</td>
<td>1:500</td>
<td>Invitrogen, Karlsruhe, Germany</td>
</tr>
</tbody>
</table>
2.1.7. small interfering RNA (siRNAs)

All siRNAs were diluted in 1x siRNA buffer (MWG Biotech) to a final concentration of 20 µM. As a negative control (termed spControl), ON-TARGETplus® non-targeting control SMARTpool, D-001810-10, from Dharmacon was used. Two independent DLC3-specific siRNAs were used, termed spDLC3 and siDLC3. spDLC3 corresponds to the siGENOME SMARTpool, human STAR08, M-010254 from Dharmacon. siDLC3 corresponds to a single duplex with the sense sequence: 5’-UCUCUGAGGCGGAAGGAAA-3’ (Eurofins MWG Operon). sip190RhoGAP corresponds to a single duplex with the sense sequence: 5’-GGAUUGUGUGGAUGUAAG-3’ (Eurofins MWG Operon). spRhoA and spRhoB correspond to the ON-TARGETplus® SMARTpool, human RhoA, L-003860 and ON-TARGETplus® SMARTpool, human RhoB, L-008395 from Dharmacon, respectively.

2.2. Methods

2.2.1. Cloning strategies

The expression vectors pEGFP-C1–DLC3α-WT, pEGFP-C1–DLC3α-K725E, pmCherry-C1–DLC3α-K725E and pEGFP-C1–DLC3β–K645E have been described previously (Erlmann et al., 2009; Holeiter et al., 2008). pEGFP-C1–DLC3–SAM (encoding the N-terminal 80 amino acids unique to DLC3α and encompassing almost the entire SAM domain) was generated by PCR amplification using pEGFP-C1–DLC3α as a template and the following forward and reverse primers 5’-CCGGAATTCTCCTCTGCTGGACGTTTTCTG-3’ and 5’-CCGGAATTCTCACAGCCTCCTACACAGGGC-3’. The PCR product was cloned into the pEGFP-C1 vector by EcoRI restriction. pmCherry-C1–DLC1–SAM was subcloned from pEGFP-C1-DLC1-SAM (Heering et al., 2009) by BgIII/BamHI and Sall/XhoI restriction into the pmCherry-C1 vector. pEGFP-C1–DLC3–SAM-GAP was generated in two steps: First, the sequence encoding amino acids 1-80 was amplified by PCR without integration of a stop codon using pEGFP-C1–DLC3α as a template and the following forward and reverse primers 5’-CCGGAATTCTCCTCTGCTGGACGTTTTCTG-3’ and 5’-GCAGTCGACCGGCCTCCTACACAGGGC-3’. The PCR product was cloned into the pEGFP-C1 vector by EcoRI restriction. Next, the GAP domain was amplified by PCR using pEGFP-C1–DLC3α as a template and the following forward and reverse primers 5’-GCAGTCGACCGGCTCAGCTGCTGGACGTTTTCTG-3’ and 5’-GCAGTCGACCTCAGCTGCTGCTGGACGTTTTCTG-3’. This PCR product was cloned into the pEGFP-C1-DLC3-SAM vector lacking the stop codon by Sall restriction. To obtain pEGFP-
C1-RhoA G14V, the wild type RhoA expression cassette was subcloned from pcDNA3.1-HA-RhoA by BglII/BamHI and EcoRI restriction into the pEGFP-C1 vector. Next, the G14V mutation was introduced by site-directed PCR mutagenesis using the following forward primer: 5'-GTGATTGTGTTGATGATGCGCTGTGGAAAGACA-3'. pEGFP-C1–DLC3α-SAM-(81-232) was generated by PCR amplification using pEGFP-C1–DLC3α as a template and the following forward and reverse primers 5'-CCGGAATTCTCCTCTGCTGGGACGTTTCTG-3' and 5'-CCGGAATTCTCAACTATGCTTGCTGCTGGTG-3'. The PCR product was cloned into the pEGFP-C1 vector by EcoRI restriction. pEGFP-C1–DLC3α-SAM-(81-195) was generated by PCR amplification using pEGFP-C1–DLC3α-SAM-(81-195) and the following forward and reverse primers 5'-CCGGAATTCTCCTCTGCTGGGACGTTTCTG-3' and 5'-CCGGAATTCTCAACCCTCTGCGGAGCTGGGT-3'. pEGFP-C1–DLC3α-SAM-(81-195) was generated by PCR amplification using pEGFP-C1–DLC3α as a template and the following forward primers 5'-CCGGAATTCTCCTCTGCTGGGACGTTTCTG-3' and 5'-CCGGAATTCTCAACCCTCTGCGGAGCTGGGT-3'. The PCR product was cloned into the pEGFP-C1 vector by EcoRI restriction. pEGFP-C1–DLC3α-SAM-(81-165) was generated by PCR amplification using pEGFP-C1–DLC3α as a template and the following forward and reverse primers 5'-CCGGAATTCTCCTCTGCTGGGACGTTTCTG-3' and 5'-CCGGAATTCTCCTCTGCTGGGACGTTTCTG-3'. The PCR product was cloned into the pEGFP-C1 vector by EcoRI restriction. pEGFP-C1–DLC3α-SAM-(81-134) was generated by PCR amplification using pEGFP-C1–DLC3α as a template and the following forward and reverse primers 5'-CCGGAATTCTCCTCTGCTGGGACGTTTCTG-3' and 5'-CCGGAATTCTCAGGCAACAGATCAGAGGAC-3'. The PCR product was cloned into the pEGFP-C1 vector by EcoRI restriction. pEGFP-C1–DLC3α-SAM-(81-100) was generated by PCR amplification using pEGFP-C1–DLC3α as a template and the following forward and reverse primers 5'-CCGGAATTCTCCTCTGCTGGGACGTTTCTG-3' and 5'-CCGGAATTCTCAGGCAACAGATCAGAGGAC-3'. The PCR product was cloned into the pEGFP-C1 vector by EcoRI restriction. pGEX-6P3-GST-DLC3α-SAM-(81-195) and -(81-232), respectively, were subcloned from the corresponding pEGFP-C1–DLC3α vector constructs by EcoRI restriction into the pGEX-6P3 vector. To generate the GFP-DLC3α WT ΔPBR and GFP-DLC3α K725E ΔPBR vectors, respectively, the amino acid stretch 196 – 232 was excised by site-directed PCR mutagenesis using the full-length pEGFP-C1–DLC3α constructs as a template and the following forward and reverse primers 5'-CCGGCCAGGAGGTCCAGGAGGCACCTCAGAGG-3' and 5'-CCGGCCAGGAGGTCCAGGAGGCACCTCAGAGG-3'. All amplified cDNAs were verified by sequencing. Oligonucleotides were purchased from Eurofins MWG Operon. The RhoA biosensor pTriEx-RhoA FLARE.sc was purchased from Addgene (Addgene plasmid 12150; Pertz et al. 2006). pEGFP-Rab11 and pEGFP-Rab4 vectors were kindly provided by Lucas Pelkman (University of Zurich, Switzerland) and Hesso Farhan (University of Konstanz, Germany), respectively.
2.2.2. **Site-directed PCR mutagenesis**

To generate deletion mutants a site-directed PCR mutagenesis was performed. Therefore, the corresponding full-length DNA construct was used as a template. 25 ng DNA were mixed with 1.5 µl of 10 pmol/µl forward and reverse primer, 1.5 µl dNTPs, 5 µl 10x PfuUltra buffer, 1 µl PfuUltra and 38.5 µl ddH₂O, yielding a total volume of 50 µl. The amplification was done in thermal cycler (RoboCycler Gradient 96, Stratagene) using the PCR program depicted in Table 4.

**Table 4: PCR program for site-directed mutagenesis**

<table>
<thead>
<tr>
<th>step</th>
<th>temperature</th>
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<th>cycles</th>
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<tbody>
<tr>
<td>initial denaturation</td>
<td>95 °C</td>
<td>1:30</td>
<td>1</td>
</tr>
<tr>
<td>denaturation</td>
<td>95 °C</td>
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<tr>
<td>annealing</td>
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<td>1:00</td>
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<td>extension</td>
<td>68 °C</td>
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The methylated template DNA was digested by adding 20 U Dpn I for 4 h at 37°C. The resulting PCR mixture contained only the PCR-amplified DNA with deleted sequence regions, which was amplified and verified by sequencing.

2.2.3. **Transformation of *Escherichia coli***

*Escherichia coli* (DH5α, Invitrogen) were transformed with recombinant plasmids using the heat shock method. 100 µl competent bacteria were thawed on ice, 2 µl ligation mixture were added, gently mixed by tapping and further incubated for 10 min on ice. After a heat shock at 42°C for 1 min, bacteria were chilled on ice for 1-2 min and 1 ml of LB medium was added. Next, the suspension was incubated for 1 h at 37°C with vigorous shaking. After incubation, cells were spun down for 1 min at 5,000 rpm, the supernatant was discarded and 100 µl were left to resuspend the pelleted cells. Finally, the bacteria suspension was spread for selection on LB agar plates containing ampicillin/kanamycin and plates were incubated overnight at 37°C.

2.2.4. **Isolation of plasmid DNA**

To amplify recombinant plasmids, 2 ml LB medium containing ampicillin/kanamycin were inoculated with a single *E.coli* colony. The inoculated cultures were incubated overnight at
37°C with vigorous shaking. The next day, 1.5 ml of the overnight culture were pelleted by centrifugation for 2 min at 13,000 rpm. The supernatant was discarded and the cell pellet was resuspended in 150 μl buffer S1. To lyse cells, 150 μl buffer S2 were mixed with the suspension and incubated for 5 min. Next, 150 μl buffer S3 were added and the lysate was mixed immediately by inverting. After incubation on ice for 5 min, the precipitate was separated by centrifugation for 10 min at 13,000 rpm. The supernatant was transferred to a new tube and centrifuged again for 2 min at 13,000 rpm. To precipitate the DNA, 1 ml of 100% ethanol was added to the supernatant. The sample was incubated on ice for 10 min and centrifuged for 5 min at 13,000 rpm. The DNA pellet was washed with 70% ethanol and centrifuged for 2 min at 13,000 rpm. After discarding the supernatant, the pellet was air dried and resolved in 30 μl ddH₂O. To check insertion and orientation of ligated DNA fragments, the isolated plasmid DNA was analyzed by restriction enzyme digestion and subsequent agarose gel electrophoresis.

To obtain higher plasmid DNA yields, 100 μl bacterial suspension from the small-scale isolation were used to inoculate 100 ml LB medium containing ampicillin/kanamycin. Cultures were grown overnight at 37°C with vigorous shaking. Isolation and purification of plasmid DNA were performed using the PureLink™ HiPure plasmid DNA purification Kit according to the manufacturer’s instructions with the exception that the cell lysate was cleared by filtrating through filter papers (Ø 125 mm). Precipitated DNA was resuspended in 200 μl ddH₂O and the concentration of the purified DNA was determined using a spectrophotometer (NanoDrop® ND-1000, Thermo Fisher Scientific).

2.2.5. Cell culture

Cultured cell lines used in this study were maintained under sterile conditions in a humidified atmosphere of 5% CO₂ at 37°C. When cells reached 70-80% confluence, they were passaged at a ratio of 1:10 using 1x trypsin/EDTA. HeLa and MCF7 cells were grown and maintained in RPMI 1640 supplemented with 10% (v/v) FCS without antibiotics.

2.2.6. Cell transfections

Transient transfection with plasmid DNA

Cells were transiently transfected with plasmid DNA using TransIT-HeLaMONSTER® (Mirus Bio) or TurboFect™ (Thermo Scientific), respectively, according to the manufacturer’s instruction. For transient transfections using TransIT-HeLaMONSTER®, 100,000 HeLa cells were seeded on coated glass coverslips 24 h prior to transfection. For each well the
transfection mix was prepared as follows: 3 µl TransIT reagent were diluted in 100 µl Opti-MEM®, incubated for 10 min and 1 µg DNA was added. In the case of double transfections, the total DNA amount did not exceed 1 µg (0.5 µg DNA 1 + 0.5 µg DNA 2) per well. Upon addition of the DNA, the mix was incubated for 10 min, before 1 µl MONSTER reagent was added. After another incubation of 10 min, the transfection mix was added dropwise to the cells. For transient transfections using TurboFect™, 140,000 HeLa cells were seeded on coated glass coverslips 24 h prior to transfection. The next day, 4 µl TurboFect™ and 1.5 µg DNA were added to 100 µl Opti-MEM®. In the case of double transfections, the total DNA amount did not exceed 2 µg (1 µg DNA 1 + 1 µg DNA 2) per well. The transfection mix was incubated for 15 - 20 min and was then added dropwise to the cells. The next day, cells were fixed and processed for immunofluorescence staining.

**Transient transfection with siRNAs**

Cells were transiently transfected with siRNAs using Oligofectamine™ or Lipofectamine® RNAiMAX (Invitrogen), respectively, according to the manufacture’s instruction. For transient transfections using Oligofectamine™, 150,000 HeLa cells were seeded in a 6-well plate 24 h prior to transfection. First, cells were washed with medium without supplements and 800 µl serum-free medium were left on the cells. For each well, 3 µl Oligofectamine™ were diluted in 12 µl Opti-MEM® and pre-incubated for 5 min. Next, 5 µl of 20 µM siRNA stock were mixed with 180 µl Opti-MEM® and added to the Oligofectamine™ mix. After 15 - 20 min incubation, 200 µl Oligofectamine™/siRNA mixture were added drop wise to the cells. After 4 hours, 500 µl of RPMI containing 30% FCS were added to the cells, yielding a final concentration of 10% FCS. For reverse siRNA transfections using Lipofectamine® RNAiMAX, HeLa cells were resuspended to a concentration of 100,000 cells/ml and 2 ml cell suspension were seeded in a 6-well plate. Next, 3 µl of a 20 µm siRNA stock and 5 µl Lipofectamine® RNAiMAX were diluted in 500 µl Opti-MEM®. After 20 min incubation at RT, the transfection mix was added dropwise to the cells. RNAi-transfection experiments performed on coated glass coverslips were scaled down according to the surface area. All steps described above were performed with RNase-free solutions, reaction tubes and pipette tips to avoid RNA degradation.

**2.2.7. Immunoblotting**

**Protein extraction**

Cells were washed once with PBS and lysed for 10 min with ice-cold RIPA buffer [50 mM Tris (pH 7.5), 150 mM sodium chloride, 1% (v/v) NP-40, 0.25% (v/v) sodium deoxycholate,
0.1% (v/v) SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 0.5 mM PMSF and 20 mM β-glycerophosphate plus protease inhibitors. Whole cell lysates were clarified by centrifugation for 10 min at 13,000 rpm and 4°C. The supernatant was transferred to a new tube and either directly separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) or stored at -20°C until use. To determine total protein concentration, the DC Protein Assay was utilized according to the manufacturer’s instructions.

**SDS-PAGE and Western blotting**

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. Separated proteins of the SDS polyacrylamide gels were transferred onto a polyvinylidene difluoride membrane (Roti® PVDF, Roth) by semi-dry blotting. For detection of RhoA and RhoB, lysates were run on NuPage® Novex® 4-12% Bis-Tris gels (Invitrogen) and blotted onto nitrocellulose membranes using the iBlot® device (Invitrogen). Membranes were blocked for 30 min with 0.5% (v/v) blocking reagent (Roche Diagnostics) in PBS containing 0.05% (v/v) Tween-20 and 0.01% (v/v) thimerosal. Membranes were incubated with primary antibodies (Table 2) overnight at 4°C, followed by 1 h incubation with HRP-conjugated secondary antibodies (Table 2) at room temperature (RT). Proteins were visualized using an enhanced chemiluminescence detection system (Thermo Scientific). Blots were exposed to X-ray films (CEA RP New, AGFA) and developed with an automatic film processor (Curix 60 processor, AGFA). Protein bands were compared to a standard protein size marker (10-170 kDa; PageRuler™ prestained protein ladder, Fermentas).

**2.2.8. Production of GST-fusion proteins**

*Escherichia coli* (BL21) were either transformed with pGEX-6P3-GST-DLC3α-SAM-(81-195) or pGEX-6P3-GST-DLC3α-SAM-(81-232) vectors. Expression was induced with 0.5 mM IPTG for 4 h at 37°C. Bacteria were harvested and resuspended in PBS containing protease inhibitors (Roche Diagnostics). After sonification, Triton X-100 was added to a final concentration of 1% (v/v) and lysates were incubated on ice for 15 min. Next, GST-fusion proteins were purified from clarified lysate with glutathione sepharose 4B (GE Healthcare). After several washing steps, bead-bound GST-fusion proteins were eluted with elution buffer [50 mM Tris, 10 mM reduced glutathione in ddH2O, pH 8.0].
2.2.9. Protein-lipid overlay assay

The protein-lipid overlay assay was carried out according to the manufacturer's instructions. In brief, membrane lipid strips from Echelon were blocked for 1 h in 0.1% (v/v) Tween-20 in PBS containing 3% (w/v) fatty acid-free BSA. Equal volumes of eluted GST-fusion proteins (100 µl) were incubated on the membranes for 1.5 h in blocking buffer. Next, membranes were washed three times with 0.1% (v/v) Tween-20 in PBS and were subsequently incubated with anti-GST antibody for 1 h at RT. Following, three washing steps, the membranes were incubated with secondary anti-goat antibody for another hour. Following further washing steps, bound GST-tagged proteins was visualized with an enhanced chemiluminescence detection system.

2.2.10. Immunofluorescence staining

Cells grown on glass coverslips coated with 10 µg/ml collagen R (Serva) were fixed for 15 min with 4% (v/v) 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% (v/v) Triton X-100 in PBS. For staining of surface N-cadherin, fixed cells were not permeabilized, staining and washing procedures were carried out in PBS. Blocking was performed with 5% (v/v) goat serum (Invitrogen) in PBS containing 0.1% (v/v) Tween-20. Fixed cells were incubated with primary antibodies (Table 3) diluted in blocking buffer for 2 h at RT. Following three washing steps with PBS, cells were incubated with Alexa Fluor® (488, 546, 633)-labeled secondary antibodies (Table 3) in blocking buffer for 1 h at RT. In the case of F-actin staining, cells were incubated with Alexa Fluor® Phalloidin in PBS for 20 min prior to a final wash with PBS-Tween and mounting of coverslips. Nuclei were counterstained with DAPI and coverslips were mounted in Fluoromount-G® (SouthernBiotech). All samples were analyzed at RT using a confocal laser scanning microscope (LSM 710, Carl Zeiss) equipped with an Plan-Apochromat 63x/1.40 DIC M27 (Carl Zeiss) oil immersion objective. 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, USA). Fluorescence intensities along a line of interest were measured using the ZEN software. For co-localization and line-scan experiments, all images were acquired with identical dimensions in x and y direction and the same pinhole settings (1 airy unit in the red channel). The fraction of Rab8 staining overlapping with DLC3 K725E signals was analyzed using the ImageJ plugin JACoP measuring the Manders’ M2 coefficient (Bolte and Cordelières, 2006).
2.2.11. FRET analysis

For FRET measurements, cells were transfected overnight with the RhoA biosensor pTriEx-RhoA FLARE.sc and pCR.V62-Met-FLAG, pCR.V62-Met-FLAG-DLC3α-WT or pCR.V62-Met-FLAG-DLC3α-K725E, respectively. The next day, cells were fixed and immunostained with anti-Flag M2 and Alexa Fluor® 633-labeled secondary anti-mouse antibodies. In the case of siRNA transfection, control and DLC3-depleted cells were co-transfected with the RhoA biosensor two days post siRNA transfection and, the next day, cells were fixed and immunostained for the cis-/medial-Golgi marker giantin using an Alexa Fluor® 633-labeled secondary anti-mouse antibody. The staining was performed according to the immunofluorescence staining protocol described above and samples were then mounted in Mowiol® (Polysciences) mounting solution. FRET efficiencies were determined using the acceptor photobleaching method. CFP (cyan fluorescent protein) was excited with a diode UV laser at 405 nm and emission was detected in the spectral window 454-515 nm. YFP (yellow fluorescent protein) 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. Whole cells were bleached for YFP with the 514 nm argon laser line (80% intensity, 20 iterations). 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). For quantification, the mean FRET efficiency of the Golgi region was normalized to the mean FRET efficiency of the whole cell. The Golgi region was determined via a region of interest (ROI) drawn around the giantin signal (Alexa 633 was excited with the 633 nm helium-neon laser line, its emission was detected from 621-735 nm).

2.2.12. Transferrin uptake

Transferrin uptake was performed as described previously (Hattula et al., 2006). In brief, cells were washed with serum free RPMI 1640 and starved for 1 h. Cells were then incubated with 10 μg/ml Alexa Fluor® 555-labeled transferrin (Invitrogen) for 1 h. To remove surface-bound transferrin, cells were rinsed for 45 sec with acidic buffer [0.5% CH₃COOH, 0.5 M NaCl, pH 3.0], washed three times with PBS, fixed with 4% (v/v) PFA and processed for immunofluorescences staining.
2.2.13. **EGFR trafficking and signaling assays**

To analyze EGFR endocytosis, cells were incubated for the indicated time points with 200 ng/ml Alexa Fluor® 555-labeled EGF (Invitrogen), washed with PBS, fixed and processed for immunofluorescence staining. For EGFR signaling experiments, cells were starved overnight in medium supplemented with 0.5% FCS. Cells were then stimulated with either 10 ng/ml EGF (HeLa) or 50 ng/ml EGF (MCF7) for the indicated time points. Next, cells were lysed and processed for Western blotting.

2.2.14. **Fluorescence activated cell sorting (FACS)**

**Transferrin recycling**

For transferrin recycling, cells were starved in serum free medium prior to incubation with 10 μg/ml Alexa Fluor® 555-labeled transferrin (Invitrogen) for 1 h. Cells were then rinsed for 45 sec with acidic buffer [0.5% acetic acid, 0.5 M sodium chloride, pH 3.0] to remove surface-bound transferrin, followed by washes with PBS and medium containing 10% FCS. During the chase phase, cells were incubated in medium containing 10% FCS for the indicated times. Next, cells were washed with ice-cold PBS and trypsinized for 3 min at 37 °C. Finally, cells were washed with ice-cold FACS buffer [PBS + 2% FCS + 0.1% sodium azide] and analyzed with the EPICS FC500 flow cytometer (Beckman Coulter). The fluorescence intensity was measured in FL-2 channel; unlabeled cells were used for gating. Post-acquisition data analysis was performed using FlowJo software (Tree Star).

**Transferrin surface labeling**

To measure surface-bound transferrin, cells were harvested by trypsinization and washed once with ice-cold FACS buffer. After pre-chilling on ice for 10 min, cells were incubated with 10 μg/ml Alexa Fluor® 555-labeled transferrin for 45 min. Cells were then washed with ice-cold FACS buffer and processed for flow cytometry as described above.

2.2.15. **Migration assays**

For migration assays, the bottom chamber of the Transwell unit (pore size 8.0 μm; Costar; Cambridge, MA, USA) contained 0.5% FCS RPMI supplemented with or without 50 ng/ml EGF. Cells were harvested and washed once with 0.5% FCS RPMI. 40,000 cells were added to the top chamber and were allowed to migrate for 4 h. Migrated cells on the underside of the Transwell membrane were fixed with 4% (v/v) PFA and stained with 0.1% (w/v) crystal violet solution. After washing the Transwells, cells were counted in five
independent microscopic fields at a 20-fold magnification (CX2, Olympus; Tokio, Japan). Experiments were performed in duplicate.

2.2.16. Aggregation assay

Cells were washed with PBS and detached from culture dishes using enzyme-free cell dissociation buffer (Invitrogen) to preserve surface cadherins. Next, cells were resuspended to a concentration of \(4 \times 10^5\) cells/ml in medium and seeded into uncoated culture dishes. The cell suspension was rotated at 37°C and 5% CO\(_2\) for different time points. After 4 h, 6 h and overnight incubation, aggregates were photographed at a 4-fold magnification (EVOS® FL, Invitrogen)
3. Results

3.1. Regulation of endocytic trafficking by DLC3

3.1.1. DLC3 co-localizes with Rab8 and impacts ERC and Golgi morphology in a GAP-dependent manner

To obtain a better understanding of the cellular functions of DLC3, its subcellular localization was investigated in HeLa cells. For these studies, cells were transiently transfected with fluorescent protein-tagged DLC3 variants, corresponding to the full-length DLC3α isoform, because neither commercially available nor our custom-made DLC3-specific antibodies were suited for the detection of the endogenous protein by immunofluorescence. Ectopically expressed DLC3 wild type (WT) protein concentrated in the perinuclear region (Fig. 6) and, consistent with its function as a GAP protein, caused severe morphological changes and a complete loss of actin stress fibers (Fig. 7a, b, compare cells expressing GFP alone with DLC3 WT-expressing cells). However, although DLC3 WT was located in the perinuclear region, little co-localization with the cis/medial Golgi protein giantin was observed (Fig. 6).

![Fig. 6 Ectopically expressed wild type DLC3 co-localizes with Rab8 and impacts ERC and Golgi morphology.](image)

Therefore, the potential co-localization of DLC3 with the ERC was analyzed, which is located in close vicinity to the Golgi complex. The small GTPase Rab11 is the best
characterized component of the ERC, but has also been found localized to the TGN. Its main function is the regulation of the slow recycling of endocytosed cargo from the ERC to the plasma membrane (Grant and Donaldson, 2009; Stenmark, 2009). Whereas DLC3 WT only partially co-localized with GFP-tagged Rab11, a strong co-localization was observed with endogenous Rab8, a further constituent of the ERC involved in membrane recycling (Fig. 6) (Peränen, 2011). Interestingly, expression of DLC3 WT caused obvious compaction of the Rab8 compartment, which in contrast to untransfected cells displayed no tubular structures. Although to a lesser extent, a more compact morphology of the Golgi complex and Rab11-positive ERC could also be observed upon DLC3 WT expression (Fig. 6, compare transfected (*) with untransfected cells (>)).

To investigate the contribution of the GAP activity to DLC3 localization and function, a mutant containing an inactivating amino acid substitution in the GAP domain (K725E) was expressed (Holeiter et al., 2012). RhoA activity was measured in cells expressing either DLC3 WT or K725E, using the FRET-based RhoA biosensor pTriEx-RhoA (Pertz et al., 2006). This single-chain biosensor is composed of the Rho-binding domain of rhotekin and full-length RhoA, flanking the two donor (CFP) and acceptor (YFP) fluorophores, and leaving the RhoA terminus accessible for reversible membrane interactions. The FRET efficiency was measured by the acceptor photobleaching method, which determines the increase in donor fluorescence after completely bleaching the acceptor. Expression of DLC3 WT caused a significant decrease in FRET efficiency, proving the inhibitory function of DLC3 towards RhoA (Fig. 7c). In line with the inactivation of its GAP activity, DLC3 K725E expression had no impact on the FRET efficiency (Fig. 7c). Consistent with this, ectopic expression of DLC3 K725E caused no obvious changes in stress fiber formation and cells maintained a spread morphology (Fig. 7a, b), making the GAP-inactive mutant suited for more detailed localization studies of the protein. Based on the comparable RhoA activities and F-actin contents in control and DLC3 K725E-expressing cells, GAP-inactive DLC3 does not appear to act in a dominant negative manner, at least with respect to Rho signaling.

Similar to DLC3 WT, only minimal overlap with the Golgi membranes was observed in cells expressing GAP-inactive DLC3 (Fig. 8a). Interestingly, in many cells, DLC3 K725E was observed on tubular structures, which emerged from the perinuclear region and reached to the cell periphery (Fig. 8a). These tubules displayed a remarkable co-localization with Rab8-positive tubules or were decorated by orderly aligned Rab8 puncta (Fig. 8a). The formation of Rab8 tubules is dependent on actin dynamics and biogenesis of tubules can be induced by treatment with the actin-depolymerizing agent cytochalasin D (CytD) (Hattula et al., 2006). To manifest that DLC3 is associated with Rab8 tubules, DLC3 K725E-expressing cells were treated with CytD. Upon treatment the number of cells exhibiting prominent Rab8
tubulation increased significantly (Fig. 8a). This was accompanied by the distribution of DLC3 K725E to a network of tubular membranes that extensively overlapped with Rab8 (Fig. 8a-c). Quantification revealed that approximately 60% of the total cellular Rab8 signal overlapped with that of DLC3 K725E (Fig. 8d).

To examine whether DLC3 K725E associates with other tubulovesicular carriers or whether this association is restricted to Rab8-positive structures, DLC3 K725E and Rab11 were co-
expressed. Here, CytD treatment did not cause obvious redistribution of Rab11, which was found in small vesicular structures near the plasma membrane and the perinuclear region, and Rab11 did not localize to DLC3 K725E-positive tubules before and after CytD treatment (Fig. 8a).

Moreover, Rab6, which localizes to Golgi membranes (Goud et al., 1990; Liu and Storrie, 2012) and also associates with Rab8-positive post-Golgi carriers (Wakana et al., 2012), failed to co-localize with DLC3 K725E-positive tubules (Fig. 9). Although partial overlap with the trans-Golgi marker TGN46 was detected at the center of emerging tubules, TGN46 was not found on DLC3 K725E-positive tubules (Fig. 9). Real-time imaging further revealed the presence of DLC3 on vesicles moving towards and away from the plasma membrane (data not shown), a subset of which were positive for endosomal RhoB (Fig. 9). Together these data suggest that the dynamic recruitment of ectopically expressed DLC3 to the Golgi-ERC interphase occurs independently of its GAP activity, whereas the morphological and cytoskeletal changes associated with the inactivation of RhoA signaling require a functional GAP domain.

**Fig. 8 Co-localization of GAP-inactive DLC3 with Rab8-positive tubules.** (a) HeLa cells expressing GFP-tagged DLC3 K725E (green) were either stained for giantin or Rab8 (red). In the case of Rab11, cells were co-transfected with vectors encoding mCherry-DLC3 K725E (depicted in green) and GFP-Rab11 WT (depicted in red). Where indicated, cells were incubated with 0.1 µm CytD for 30 min (+ CytD). Scale bars, 10 µm. See next page for Fig. 8 (b – d).
Results

Fig. 8  (b) DLC3 K725E-expressing cells (green) were treated with CytD and stained for Rab8 (red). Scale bars, 5 µm. (c) The fluorescence intensities of both signals along the indicated line are depicted. (d) Quantification of the fraction of Rab8 overlapping with GFP-DLC3 K725E (N=20; mean ± s.e.m.).

Fig. 9  Co-localization of GAP-inactive DLC3 with Rab6, TGN46 and RhoB. HeLa cells transiently expressing GFP-DLC3 K725E (green) were stained for Rab6 and TGN46 (red), respectively. In the case of RhoB, cells were co-transfected with vectors encoding mCherry-DLC3 K725E (depicted in green) and CFP-RhoB (depicted in red). Scale bars, 10 µm.
3.1.2. DLC3 is required for the integrity of the Rab8 and Golgi compartments

Due to the localization of the ectopically expressed protein, DLC3 is a candidate GAP that regulates the morphology and/or function of the Rab8-positive ERC via the regulation of Rho activity. Indeed, overexpression of a constitutively active RhoA mutant (RhoA G14V) induced vesiculation of the tubular Rab8 compartment (Fig. 10). In addition, RhoA hyperactivation was shown to cause severe Golgi fragmentation (Zilberman et al., 2011) (Fig. 10). Considering the partial co-localization of DLC3 with TGN46 (Fig. 9) and the fact that spatial analysis of RhoA activity revealed an inactive, Golgi-localized pool of RhoA (Pertz et al., 2006), DLC3 may potentially be involved in the negative regulation of Rho signaling at this subcellular site as well.

![GFP-RhoA G14V overlay with DAPI](image)

**Fig. 10 Active RhoA causes vesiculation of the Rab8 recycling compartment and Golgi fragmentation.** HeLa cells expressing GFP-RhoA G14V (green) were either stained for Rab8 or giantin (red). Nuclei were counterstained with DAPI (blue). Note the dispersed morphology of the Rab8 and Golgi compartments in cells expressing active RhoA (*). Scale bars, 10 µm.

To assess the effects of DLC3 depletion on organelle morphology, HeLa cells were transiently transfected with DLC3-specific SMARTpool siRNAs and efficient downregulation of endogenous DLC3 expression was confirmed by immunoblotting (Fig. 11b). Endogenous Rab8 staining of control cells revealed prominent Rab8 tubules that originated from the perinuclear region (Fig. 11a). By contrast, DLC3-depleted cells showed a vesicular distribution of Rab8 in membrane protrusions and the perinuclear region. The number of cells displaying Rab8 tubules was significantly decreased by 30% and, in contrast to control cells, could not be enhanced by treatment with CytD (Fig. 11c). This could possibly be explained by a stabilized actin cytoskeleton, which was more resistant to CytD treatment (data not shown). Immunoblotting revealed that Rab8 levels were not altered in DLC3-depleted cells (Fig. 11b), indicating a change in cellular distribution rather than expression.
Fig. 11 DLC3 depletion causes vesiculation of the Rab8 recycling compartment and Golgi fragmentation. HeLa cells were transfected with control (spControl) or DLC3-specific (spDLC3) siRNAs, respectively, and analyzed three days later. (a) Cells were stained for Rab8 (green) and nuclei were counterstained with DAPI (blue). (b) Whole cell lysates were analyzed by Western blotting with anti-DLC3, anti-Rab8 and anti-tubulin antibodies. (c) Cells were left untreated or treated with 0.1 µM CytD and then stained for Rab8. The number of cells containing Rab8-positive tubules was counted and normalized to the total number of analyzed cells (n=3; one-way ANOVA followed by Tukey’s post-test; mean ± s.e.m.). (d) Cells were stained for giantin (red), F-actin was labeled with phalloidin (green) and nuclei were counterstained with DAPI (blue). (e) The degree of Golgi fragmentation was calculated for the Golgi markers giantin, GM130 and p230, respectively. Values were normalized to the mean Golgi compaction index of control cells (N≥20 cells; one-way ANOVA followed by Tukey’s post-test; mean ± s.e.m.). (a, d) Scale bars, 10 µm.
Furthermore, the compact Golgi ribbon organization was lost in DLC3-depleted cells and single Golgi fragments were distributed throughout the cell, as visualized by giantin staining (Fig. 11d). The degree of Golgi dispersion was quantified using the Golgi compaction index (Bard et al., 2003). Compared with control cells, the value of this index dropped two-fold in cells depleted from DLC3 (Fig. 11e). Fragmentation of the Golgi complex was not specific for either the cis or trans face and was also observed with the Golgi markers GM130 and p230 (Fig. 11e). Importantly, vesiculation of Rab8 recycling tubules and fragmentation of the Golgi complex were reproducible with an independent siRNA targeting DLC3 (data not shown). Moreover, DLC3 depletion induced the formation of actin stress fibers. Although actin cables in depleted cells were less thick than in control cells, they traversed the cytoplasm forming a dense actin network (Fig. 11d). Enhanced actin polymerization is in accordance with DLC3’s function as a RhoGAP protein and increased Rho activity upon DLC3 knockdown.

3.1.3. Depletion of p190RhoGAP does not induce perinuclear vesiculation

Most probably due to their short structure (Egea et al., 2013), we were unable to detect Golgi-associated actin microfilaments in both control and DLC3-depleted cells. Therefore, to rule out that vesiculation of the perinuclear region was a general consequence of RhoGAP downregulation, a p190RhoGAP knockdown was performed (Fig. 12a).

![Figure 12](image-url)

**Fig. 12 Depletion of p190RhoGAP does not induce perinuclear vesiculation.** HeLa cells were transfected with control (spControl) or siRNAs specific for p190RhoGAP (sip190RhoGAP), respectively. (a) Whole cell lysates were analyzed by Western blotting with anti-p190RhoGAP and anti-tubulin antibodies. (b) Three days later, cells were stained for Rab8 and giantin (green), respectively, and nuclei were counterstained with DAPI (blue). Scale bars, 10 µm.
Results

This widely expressed RhoGAP has proven GAP specificity towards Rho and Rac and localizes to membrane ruffles as well as to AJs (Arthur and Burridge, 2001; Settleman et al., 1992; Wildenberg et al., 2006). In contrast to DLC3 downregulation, cells depleted from p190RhoGAP showed no obvious morphological changes of the Rab8 compartment or Golgi complex (Fig. 12b). These findings thus support the hypothesis that vesiculation of the Rab8 and Golgi compartments are specific to DLC3 depletion and most likely a consequence of Rho hyperactivation.

3.1.4. DLC3 depletion enhances perinuclear RhoA activity

To investigate the spatial aspects of RhoA GTPase regulation by endogenous DLC3, the RhoA activity was measured in DLC3-depleted HeLa cells using the RhoA biosensor and FRET was analyzed over the whole cell by acceptor photobleaching. The FRET efficiency was then plotted as pseudocolor images representing high (red) and low (blue) RhoA activity (Fig. 13a). In control cells, high RhoA activity was observed near the plasma membrane (Fig. 13a) as previously reported (Pertz et al., 2006). Upon DLC3 depletion RhoA activity was enhanced by 30% within the cell (Fig. 13b), with especially high levels of active RhoA in the perinuclear region (see pseudocolor image in Fig. 13a). Considering the reports on the localization of RhoA to the Golgi complex (Camera et al., 2003; Pertz et al., 2006) and the observed Golgi fragmentation upon loss of DLC3, the RhoA activity was specifically measured at this compartment. The Golgi complex was visualized by co-staining of the cis/medial-Golgi protein giantin using far-red conjugated secondary antibodies that do not interfere with the FRET fluorophores. Quantification of RhoA activity at the Golgi complex revealed a significant increase by 50% in DLC3-depleted cells compared to control cells (Fig. 13b). It is very likely that DLC3 loss also causes RhoA hyperactivation at the ERC, however, due the dispersion of the Rab8 compartment in DLC3-depleted cells, this subcellular site cannot easily be discerned. Importantly, enhanced total and Golgi-localized RhoA activity were also observed with an independent siRNA targeting DLC3 (data not shown), confirming the specificity of this effect.
Fig. 13 DLC depletion enhances cellular RhoA activity. HeLa cells were transfected with control (spControl) or DLC3-specific (spDLC3) siRNAs, respectively. Two days post siRNA transfection, cells were co-transfected with the pTriEx-RhoA FLARE.sc biosensor and, on the next day, stained for giantin. (a) Pre- and post-bleach images of the acceptor (YFP) are depicted. Red lines denote the region of interest (ROI) where the acceptor was bleached; while lines represent the Golgi ROI. FRET efficiencies are displayed as a pseudocolor thermal map corresponding to the scale shown on the left, from low (blue) to high (red) RhoA activity. Scale bars, 5 µm. (b) Plotted are the mean FRET efficiencies within the cell and at the Golgi complex. Results of the cells from two independent experiments are depicted as mean ± s.e.m. (N≥40 cells; unpaired t-test).

3.1.5. The SAM domain of DLC3 associates with Golgi membranes

All DLC proteins possess an N-terminal SAM domain, which potentially mediates protein-protein or protein-lipid interactions. To address whether this domain was involved in DLC3 recruitment to the Golgi-ERC interphase, the isolated, GFP-tagged DLC3α N-terminus, encompassing almost the entire SAM domain (DLC3-SAM) was expressed and found to accumulate in the perinuclear region (Fig. 14). Interestingly, DLC3-SAM was not detected on Rab8-positive tubules, but extensively co-localized with TGN46 and giantin (Fig. 14).
Results

Fig. 14 The isolated DLC3α N-terminus localizes to Golgi membranes. HeLa cells transiently expressing GFP-tagged DLC3-SAM, GFP-tagged DLC3β K645E or mCherry-tagged DLC1-SAM (all depicted in green) were stained for Rab8, TGN46 or giantin (red) as indicated. Scale bars, 10 µm.

To provide additional evidence that the SAM domain is necessary for the subcellular localization of DLC3, cells were transfected with GAP-inactive DLC3β, an isoform that can be produced by alternative splicing and naturally lacks the SAM domain (Durkin et al., 2007b). Indeed, DLC3β K645E showed a cytosolic distribution without any co-localization with the Golgi complex or the Rab8 compartment (Fig. 14; data not shown). The isolated SAM domain of DLC1 was fully cytosolic (Fig. 14), indicating that the DLC3α N-terminus specifically determines localization of this particular DLC isoform.

3.1.6. A targeted DLC3 GAP domain rescues Golgi fragmentation

To prove that Golgi fragmentation was a consequence of local RhoA activation in DLC3-depleted cells, a rescue experiment was designed in which the Golgi-associated, N-terminal region was used to target DLC3 GAP activity to the Golgi complex. This was achieved by the direct fusion of the DLC3 GAP domain with DLC3-SAM. Figure 15a shows a representative image of a DLC3-depleted cell expressing Golgi-localized, GFP-tagged SAM-GAP (marked with an asterisk). In contrast to untransfected cells, DLC3-depleted cells expressing SAM-GAP regained a compact Golgi complex (Fig. 15a, compare (*) and (>)), which was confirmed by quantification of the Golgi compaction index in these cells (Fig. 15b). No rescue of the Golgi compaction index was seen for DLC3-SAM alone (Fig. 15b).
These results provide strong evidence that the RhoGAP activity of DLC3 indeed limits RhoA activation at the Golgi complex and most likely at the ERC.

![Image of a targeted DLC3 GAP domain rescues Golgi fragmentation in DLC3 knockdown cells.](image)

**Fig. 15 Expression of a targeted DLC3 GAP domain rescues Golgi fragmentation in DLC3 knockdown cells.** HeLa cells were transfected with control (spControl) or DLC3-specific (spDLC3) siRNAs, respectively. Two days post siRNA transfection, cells were transfected with vectors encoding the GFP-tagged DLC3-SAM domain fused to the DLC3 GAP domain (SAM-GAP) or the GFP-tagged N-terminus alone (SAM). Cells were fixed and stained for giantin 24 h later. (a) Representative maximum projections of DLC3-depleted cells are shown. Note the morphological difference of giantin (red) in untransfected cells (> compared to SAM-GAP-expressing (green) cells (*). Nuclei were counterstained with DAPI (blue). Scale bars, 10 µm. (b) The Golgi compaction index was calculated for at least three independent experiments. Values were normalized to untransfected control cells and are depicted as mean ± s.e.m. (n=3-6; two-way ANOVA followed by Bonferroni post-test).

3.1.7. **DLC3 is required for transferrin trafficking to the ERC**

Upon internalization, transferrin (Tfn) is delivered to EEA1-positive endosomes from where it is transported to the ERC to then recycle back to the plasma membrane via the Rab11-dependent slow recycling pathway (Grant and Donaldson, 2009). To address whether DLC3 affects ERC function, Tfn recycling was measured by pulse-chase experiments. Cells were pulsed with Alexa Fluor 555-labeled Tfn (Tfn-555) for 1 h and the fluorescence intensity of chased cells was then determined by FACS analysis. In both control and DLC3-depleted cells, the loss of internal fluorescence was similar (Fig. 16a), indicating that the rate at which Tfn recycles to the plasma membrane is unchanged upon DLC3 depletion. Previously, Rab8 was found to be important for the delivery of internalized Tfn to the ERC, but not for Tfn...
internalization or recycling from the ERC to the plasma membrane (Hattula et al., 2006). Next, the question was addressed whether DLC3 affects the subcellular distribution of internalized Tfn. After 1 h incubation, Tfn was transported to the perinuclear region in control cells (Fig. 16b). By contrast, DLC3-depleted cells showed a dispersed localization of Tfn accumulating in large vesicles that were localized in the cell periphery (Fig. 16b, c).

Fig. 16 DLC3 depletion impairs Tfn transport to the ERC. (a-e) HeLa cells were transfected with control (spControl) or DLC3-specific (spDLC3) siRNAs, respectively, and analyzed three days later. (a) Serum-starved cells were incubated with Tfn-555 for 1 h (pulse), and then chased for the indicated times. Decrease of Tfn-555 fluorescence intensity was measured by FACS and two representative experiments are depicted as mean ± s.e.m. (b, c) Serum-starved cells were incubated with Tfn-555 (red) for 1 h and stained for EEA1 (green). Representative maximum projections are shown and imaging parameters were identical. (c) The number of cells displaying Tfn-555 either at the perinuclear region or in the cell periphery was counted and normalized to the total number of analyzed cells (n=3; two-way ANOVA followed by Bonferroni post-test; mean ± s.e.m.). (d) Whole cell lysates were analyzed by Western blotting with anti-TfnR and anti-tubulin antibodies (left). FACS analysis of surface-bound Tfn; unlabeled cells (filled grey) (right). (e) Two days post siRNA transfection, cells were transfected with a vector encoding GFP-Rab4 WT (green). The next day, serum-starved cells were incubated with Tfn-555 (red) for 1 h. (b, e) Scale bars, 10 µm.
Western blot analysis revealed similar total transferrin receptor (TfnR) levels in control and DLC3-depleted cells, in addition, similar levels of surface-bound Tfn were measured by flow cytometry (Fig. 16d), excluding differences in Tfn uptake due to varying TfnR levels. Immunostaining analysis revealed that internalized Tfn in DLC3-depleted cells strongly co-localized with EEA1, whereas at this time point of uptake control cells showed only little overlap of Tfn with EEA1 (Fig. 16b). Moreover, the early endosomes in depleted cells were enlarged compared to control cells (Fig. 16b) and internalized Tfn strongly co-localized with overexpressed GFP-tagged Rab4 (Fig. 16e), a Rab GTPase that mediates fast endocytic recycling directly from early endosomes (Stenmark, 2009). Although overall Tfn recycling kinetics were similar, our findings on the impaired Tfn delivery from early endosomes to the ERC and its presence in Rab4-positive vesicles indicate that DLC3 loss leads to altered endosomal cargo routing.

3.1.8. Simultaneous depletion of RhoA and RhoB rescues impaired Tfn trafficking

Next, it was analyzed whether the defect in Tfn localization upon DLC3 depletion was Rho-dependent. Depletion of DLC3 together with RhoA only partially restored Tfn accumulation at the ERC (Fig. 17a). This is most likely due to the compensatory upregulation of RhoB upon RhoA knockdown (Fig. 17b), which was observed previously (Ho et al., 2008; Vega et al., 2011). Furthermore, it has been suggested that constitutively active RhoB prevents endosomal transport (Fernandez-Borja et al., 2005) and, indeed, downregulation of RhoB in DLC3-depleted cells also led to a partial rescue of the Tfn transport to the perinuclear region (Fig. 17a). Importantly, although the knockdown was not complete, the simultaneous suppression of RhoB expression in DLC3- and RhoA-depleted cells completely restored Tfn transport to the ERC, whereas depletion of RhoA and RhoB alone had no effect (Fig. 17a, b). DLC3 thus regulates endosomal Tfn trafficking via the regulation of RhoA and most likely also RhoB, when taking into account the co-localization of DLC3 with endosomal RhoB (Fig. 9).
Results

Fig. 17 Simultaneous depletion of RhoA and RhoB rescues the Tfn trafficking defect in DLC3 knockdown cells. HeLa cells were transfected with control siRNAs (spControl) or siRNAs specific for DLC3 (spDLC3), RhoA (spRhoA), and RhoB (spRhoB), respectively, and analyzed three days later. (a) Serum-starved cells were incubated with Tfn-555 for 1 h. The percentage of cells displaying Tfn-555 at the perinuclear region was determined and normalized to the control (n=3; one-way ANOVA followed by Tukey’s post-test, mean ± s.e.m.). (b) Whole cell lysates were analyzed by Western blotting with anti-DLC3, anti-RhoA, anti-RhoB and anti-tubulin antibodies.

3.1.9. DLC3 depletion alters EGFR trafficking

The EGFR is a receptor tyrosine kinase that is rapidly endocytosed upon EGF ligand binding and transported to lysosomes in a RhoB-dependent manner. Active RhoB was shown to delay the intracellular trafficking of the EGFR and its lysosomal degradation (Gampel et al., 1999). To analyze how EGFR trafficking may be affected by DLC3 expression, cells were incubated with Alexa Fluor 555-labeled EGF (EGF-555) for different time points. After 20 min, EGF-positive endosomes were primarily localized in the perinuclear region in control cells, whereas in DLC3-depleted cells EGF-555 accumulated in enlarged EEA1-positive endosomes in the cell periphery (Fig. 18a). Although the levels of internalized EGF were comparable between control and depleted cells, significantly more peripheral EGF-555 localization was found upon DLC3 depletion (Fig. 18b). After 40 min, EGF-555 still failed to accumulate in the perinuclear region in DLC3-depleted cells (Fig. 18a), indicating that EGF-555 was not delivered to later endosomal compartments. Indeed, after 120 min, DLC3-depleted cells showed a significant pool of remaining EGF-555, while the signal was strongly reduced in control cells (Fig. 19a, b). This remaining EGF-555 pool did not colocalize with the late endosomal/lysosomal marker LAMP1 (lysosome-associated membrane protein 1) (Fig. 19a), excluding incomplete EGF-555 degradation due to lysosomal dysfunction. To further investigate the identity of the remaining EGF pool in DLC3-depleted cells, immunostaining analysis of different Rab recycling markers was
performed. 120 min post-internalization, EGF-555 in DLC3-depleted cells neither co-localized with EEA1 nor with Rab7 (Fig. 19c), but extensively overlapped with GFP-tagged Rab4 (Fig. 19c). DLC3 depletion thus impairs EGFR lysosomal degradation and, reminiscent of the TfnR, appears to cause receptor re-routing to the Rab4-dependent recycling pathway.

**Fig. 18** DLC3 depletion alters the endocytic trafficking of EGF. (a, b) HeLa cells were transfected with control (spControl) or DLC3-specific (spDLC3) siRNAs. Three days post siRNA transfection, cells were incubated with EGF-555 (red) for 20 and 40 min, respectively, and stained for EEA1 (green). Nuclei were counterstained with DAPI (blue). Scale bars, 10 µm. (b) The number of cells displaying EGF-555 in the cell periphery after 20 min incubation was counted and normalized to the total number of analyzed cells (n=3; **p=0.0013, paired t-test, mean ± s.e.m.).
Results

3.1.10. DLC3 depletion prolongs EGFR signaling

Because the receptor trafficking route determines the signaling response, EGFR levels and activity were examined by immunoblotting cell lysates of EGF-stimulated HeLa cells. Basal EGFR levels of DLC3-depleted cells were comparable to control cells (Fig. 20a; EGFR). Immediately after EGF stimulation, strong phosphorylation of EGFR at Tyr1068 was detected in both control and depleted cells (Fig. 20a; pEGFR). After 15 min of stimulation, the level of EGFR was already decreased in control cells, while even at late time points the

![Fig. 19 Internalized EGF is missorted in DLC3-depleted cells.](image)

- HeLa cells were transfected with control (spControl) or DLC3-specific (spDLC3) siRNAs. (a, b) Three days post siRNA transfection, cells were incubated with EGF-555 (red) for 120 min, and stained for Lamp1 (green). Nuclei were counterstained with DAPI (blue). (b) Quantification of the mean fluorescence intensity of EGF-555 per cell after incubation for 120 min (n=3; **p-value=0.0066, paired t-test, mean ± s.e.m.). (c) Three days post siRNA transfection, cells were incubated with EGF-555 (red) for 120 min, and stained for EEA1 or Rab7 (green), respectively. In the case of Rab4, cells were transfected with a vector encoding GFP-Rab4 WT (green) two days post siRNA transfection. The next day, cells were incubated with EGF-555 (red) for 120 min. Nuclei were counterstained with DAPI (blue). Note the overlapping distribution of EGF-555 and GFP-Rab4 (>). (a, c) Scale bars, 10 µm.
receptor levels persisted in DLC3-depleted cells (Fig. 20a; EGFR and Fig. 20b for quantification).

**Fig. 20 DLC3 depletion prolongs EGFR signaling.** HeLa cells were transfected with control (spControl) or DLC3-specific (spDLC3) siRNAs. (a, b) Two days post siRNA transfection, cells were serum-starved overnight and, prior to lysis, stimulated with 10 ng/ml EGF for the indicated times. (a) Whole cell lysates were analyzed by Western blotting with anti-pEGFR (Tyr1068), anti-EGFR, anti-pAKT (Thr308), anti-AKT, anti-pERK (Thr202/Tyr204), anti-ERK and anti-tubulin antibodies. (b) EGFR levels from two independent experiments including the blot shown in (a) were quantified and normalized to tubulin (mean ± s.e.m.). (c) Three days post siRNA transfection, cells were seeded into the upper chamber of a Transwell unit and were allowed to migrate for 4 h under non-gradient conditions (-EGF) or towards a chemotactic EGF gradient (+EGF), respectively. The number of migrated cells was determined by counting five independent microscopic fields. Bars represent the mean of duplicate wells and are representative for two independent experiments (two-way ANOVA followed by Bonferroni post-test, mean ± s.e.m.).

EGFR activation triggers downstream signaling including the activation of the PI3K and MAPK pathways, resulting in the activation of AKT (protein kinase B) and ERK (extracellular signal-regulated kinases) kinases, respectively. Compared with control cells, the total levels of these kinases were reduced in DLC3-depleted cells for unknown reasons (Fig. 20a; AKT and ERK). Nevertheless, correlating with the delayed EGFR degradation, phosphorylation and thus activation of AKT was prolonged in DLC3-depleted cells (Fig. 20a; pAKT). In the breast cancer cell line MCF7 prolonged AKT and also ERK phosphorylation were observed, indicating that this effect is not restricted to a particular cell line (data not shown). Finally, EGF is known to be a potent stimulator of cell motility. Depletion of DLC3 significantly increased EGF-induced chemotactic cell migration by 13-fold compared to the control (Fig. 20c). However, DLC3 depletion also increased random cell motility in the absence of an EGF gradient, albeit to a lower extent, indicating that not only the enhanced EGF response, but most likely also the cytoskeletal changes contribute to the enhanced cell migratory behavior.
3.1.11. **DLC3 depletion causes N-cadherin accumulation at the perinuclear region**

Our group previously reported that in MCF7 and MCF10A breast epithelial cells DLC3 predominantly localizes to cell-cell contacts. In the absence of DLC3, the AJ proteins E-cadherin and β-catenin were mislocalized, which was associated with enhanced cell motility (Holeiter et al., 2012). Whether the observed receptor missorting is restricted to the TfnR and EGFR, or can be observed for other transmembrane proteins, was further investigated by immunofluorescence analysis of N-cadherin, an AJ protein known to be recycled from the plasma membrane (Kawauchi et al., 2010; Mary et al., 2002). Upon DLC3 depletion N-cadherin strongly co-localized with giantin (Fig. 21a) and Rab8 (data not shown). Quantification revealed a significant 3-fold increase in the number of cells showing N-cadherin accumulation at the Golgi complex in DLC3 knockdown cells (Fig. 21b).

**Fig. 21 DLC3 knockdown causes N-cadherin accumulation at the Golgi complex.** HeLa cells were transfected with control siRNA (spControl) or siRNAs specific for DLC3 (spDLC3), respectively. (a, b) Three days post siRNA transfection, cells were stained for N-cadherin (green) and giantin (red). (b) The degree of N-cadherin accumulation was quantified for three independent experiments (n=3; *p= 0.0202, unpaired t-test, mean ± s.e.m.). (c) Whole cell lysates were analyzed by Western blotting with anti-N-cadherin and anti-tubulin antibodies. (d) Staining of non-permeabilized cells with an anti-N-cadherin antibody directed against the extracellular domain. (a, d) Scale bars, 10 µm.
This was not due to different expression, since total levels of N-cadherin were comparable between control and DLC3-depleted cells (Fig. 21c). To provide further evidence that loss of DLC3 decreases N-cadherin surface levels, N-cadherin was immunolabeled in intact cells by an antibody reactive with the extracellular domain. Indeed, DLC3 knockdown cells showed a reduced staining intensity at AJs (Fig. 21d), allowing the conclusion that either the transport of N-cadherin from the Golgi complex via the ERC to the plasma membrane is inhibited, or that endocytic recycling rates of N-cadherin differ between control and DLC3-depleted cells.

### 3.1.12. DLC3 depletion reduces cell aggregation

Reduced N-cadherin levels on the plasma membrane can be associated with changes in cell adhesion. To study the functionality of cell-cell contacts, an aggregation assay was performed, in which the extent of aggregation reflects the intrinsic ability of cells to form intercellular connections. Under constant rotation, control cells rapidly aggregated to compact cell clusters, whereas cells lacking DLC3 showed retarded cell aggregation (Fig. 22). After six hours DLC3-depleted cells remained largely dispersed, with only loosely associated cells. Upon overnight incubation control cells produced spherical and compact cell aggregates with defined borders, whereas cells lacking DLC3 formed less dense and irregularly shaped clusters (Fig. 22). Thus, the loss of DLC3 interfered with the formation and compaction of cell aggregates. Taking into account that DLC3 possesses a potential tumor suppressor function, the ability of DLC3 to regulate trafficking of transmembrane proteins, e.g. EGFR and N-cadherin, thereby affecting cellular signaling and adhesion, could be one mechanism by which DLC3 loss contributes to cellular transformation.

![Fig. 22 DLC3 knockdown reduces cell aggregation](image)

*Fig. 22 DLC3 knockdown reduces cell aggregation.* HeLa cells were transfected with control siRNA (spControl) or siRNAs specific for DLC3 (spDLC3), respectively. Three days post siRNA transfection, a single cell suspension was seeded into uncoated culture dishes and cells were allowed to aggregate under rotating conditions for the indicated time points. Scale bar, 500 µm.
3.2. Association of DLC3 with Rab8-positive recycling tubules

3.2.1. Amino acids 1-232 in DLC3 are necessary for its localization to the Rab8 compartment

Although the isolated DLC3α N-terminus was recruited to the Golgi complex, only the full-length protein also associated with Rab8 tubules. Thus, we were interested in identifying the essential amino acids necessary for the protein recruitment to the tubulovesicular Rab8 compartment. For mapping studies, several DLC3 constructs were generated, in which the first 80 amino acids at the N-terminus of DLC3α (DLC3-SAM) were extended by amino acid stretches of different lengths (ranging from 20 to 152 amino acids) (Fig. 23). In addition to DLC3-SAM, five further constructs (DLC3-SAM-(81-100), -(81-134), -(81-165), -(81-195) and (81-232)) were ectopically expressed and analyzed for their co-localization with the Golgi complex. The localization of the four shortest constructs was indistinguishable from that of DLC3-SAM, which was characterized by the extensive co-localization with the Golgi marker GM130 (Fig. 24).

Interestingly, DLC3-SAM-(81-232) could be still observed at the Golgi complex, but its localization was further marked by many tubular structures that emanated from the perinuclear region and reached to the cell periphery (Fig. 24). Of note, addition of further 99 amino acids (DLC3-SAM-(81-331) did not change the tubular localization pattern (data not shown). Since these tubules strongly resembled the observed morphology of the Rab8 compartment, immunofluorescence analysis was performed to identify their nature.
Similar to Rab8, DLC3-SAM-(81-232) tubulation was enhanced by CytD treatment and showed a strong overlap with the Rab8 signal (Fig. 25). To confirm that the SAM domain is trapped at the Golgi complex, cells expressing DLC3-SAM were also treated with CytD.
Indeed, the SAM domain alone was not detected on Rab8-positive tubules (Fig. 25), indicating that the additional C-terminal 152 amino acids are necessary for the recruitment to the Rab8 compartment.

Fig. 25 DLC3-SAM-(81-232), but not DLC3-SAM, is recruited to Rab8 tubules. HeLa cells expressing GFP-tagged DLC3-SAM-(81-232) or DLC3-SAM (green), respectively, were incubated with 0.1 µm CytD for 30 min (+ CytD) and then stained for Rab8 (red). Scale bars, 10 µm.

### 3.2.2. A novel polybasic region mediates binding to negatively charged lipids

Considering that DLC3-SAM-(81-195) localized exclusively to the Golgi complex, whereas DLC3-SAM-(81-232) also co-localized extensively with Rab8 tubules, the amino acids 196-232 appear to be important for this shift in localization. Interestingly, this 37-long amino acid stretch contains several basic residues that may mediate the interaction with membranes containing negatively charged lipids (Fig. 23). Since recycling tubules are known to be enriched in phospholipids like PI(4,5)P₂ and phosphatidic acid (PA) (Giridharan et al., 2013; Jovic et al., 2009), it was then investigated whether this polybasic region (PBR) is able to bind lipids. To do so, a protein-lipid overlay assay was performed, in which recombinant GST-tagged fusion proteins were incubated on arrays spotted with different cellular lipids. DLC3-SAM-(81-232) was able to bind several lipids and showed the highest selectivity for PA and some PIPs, especially PI⁴P (Fig. 26a), whereas DLC3-SAM-(81-195), missing the PBR, did not interact with any of the spotted lipids (Fig. 26a). Of note, the negatively charged cardiolipin was excluded from further investigations, since it resides within the inner mitochondrial membrane and is known to cause false positive results. To provide further evidence that the PBR is important for the recruitment of the full-length DLC3 protein to Rab8-positive tubules, the subcellular localization of deletion mutants lacking the PBR
(DLC3 ΔPBR) was investigated. Indeed, deletion of the PBR strongly impaired the recruitment of both wild type and GAP-inactive DLC3 to Rab8 tubules, which could not be enhanced by CytD treatment (Fig. 26b). In summary, these data provide evidence that a novel PBR within the N-terminal region of DLC3 is necessary for the recruitment to Rab8-positive recycling tubules, which might be mediated by protein-lipid interactions with PA and PI4P.

**Fig. 26** The polybasic region between amino acid 196 and 232 mediates lipid binding and contributes to DLC3 recruitment to Rab8 tubules. (a) Membrane lipid strips were incubated with purified DLC3-SAM-(81-195) or -(81-232) GST-fusion proteins, and bound proteins were detected with GST-specific primary, followed by HRP-labeled secondary antibody. DAG = diacylglycerol, PA = phosphatidic acid, PS = phosphatidyserine, PE = phosphatidylethanolamine, PC = phosphatidylcholine, PG = phosphatidylglycerol, PI = phosphatidylinositol, sulfatide = 3-sulfogalactosylceramide. (b) HeLa cells expressing GFP-tagged DLC3 PBR deletion mutants (green), either WT or GAP-inactive K725E, were stained for Rab8 (red). Where indicated, cells were incubated with 0.1 µm CytD for 30 min (+ CytD). Scale bars, 10 µm.
4. Discussion and outlook

The Rho subfamily of small GTPases is implicated in different steps of endocytic membrane trafficking, but little is known about their spatiotemporal regulation by GEFs and GAPs in this particular context. The three members of the DLC family possess RhoA-specific GAP activity and were found to control various cellular processes, depending on the subcellular localization. However, their potential role in intracellular trafficking remains largely uninvestigated. Only for DLC1, the localization to caveola has been demonstrated and the hypothesis put forward that DLC1-mediated cytoskeletal rearrangements regulate the internalization of caveola, although this has not yet been formally proven. Thus, the aims of this thesis were to define the subcellular localization of DLC3 and elucidate whether this GAP protein might be involved in the regulation of Rho GTPases within the endocytic trafficking pathway.

First, the localization of DLC3 to the interface of the TGN and Rab8-positive recycling compartment was demonstrated in HeLa cells. Furthermore, it was proven that DLC3, as the first Rho-specific GAP protein, locally regulates RhoA activity in the perinuclear region. In the absence of DLC3 the structural integrity of the Golgi complex and Rab8 compartment was compromised, most likely due to the induction of aberrant cytoskeletal rearrangements. Moreover, it was found that downregulation of DLC3 inhibits proper Tfn uptake and delays EGFR degradation, causing prolonged receptor signaling. Loss of DLC3 also reduced surface N-cadherin levels, which led to impaired cell aggregation. Together, these data identify the still poorly characterized DLC3 protein as an important novel component of the endocytic recycling pathway that ensures the right balance of local Rho activation and thereby contributes to the maintenance of ERC and Golgi integrity. Concerning the mechanisms of DLC3 recruitment to different subcellular sites, the SAM domain was found to be essential for the association with the Golgi complex and a novel PBR was mapped within the DLC3 N-terminus that may function as lipid-binding motif.

4.1. Regulation of endocytic trafficking by DLC3

4.1.1. DLC3 regulates Rab8-dependent recycling in a GAP-dependent manner

The specific co-localization of DLC3 with Rab8 and the impact of DLC3 expression on the formation of Rab8-positive tubules suggest a role for DLC3 in Rab8-dependent processes. So far, Rab8 functions in membrane trafficking, cell migration, epithelial polarization,
ciliogenesis and neuronal differentiation have been described (Peränen, 2011). Since Rab8 as well as Rab11 are found at the ERC, both GTPases are implicated in the regulation of the endocytic recycling pathway (Hattula et al., 2006; Roland et al., 2007). However, previous studies revealed that Rab8 and Rab11 most likely control different recycling steps. Whereas Rab11 regulates TfN recycling from the ERC to the plasma membrane, Rab8 controls the delivery of TfN to the perinuclear ERC (Hattula et al., 2006; Roland et al., 2007; Ullrich et al., 1996). The impaired TfN delivery to the ERC in DLC3-depleted cells, in which TfN accumulated in enlarged EEA1-positive endosomes in the cell periphery, is in agreement with these differential Rab functions. Via rabin8, an effector of Rab11 and a GEF for Rab8, the two Rab proteins were found to cross-talk in the context of primary ciliogenesis and epithelial polarization (Peränen, 2011). But the mechanisms of cooperation between Rab8 and Rab11 in the endocytic recycling of TfN have not been resolved. The observed compact appearance of both, Rab8 and Rab11 in DLC3 WT-expressing cells may also suggest a role of DLC3 in Rab11-dependent recycling processes. Moreover, Rab8a/b, together with Rab10 and Rab13, compose a subfamily of closely related Rab proteins with overlapping distribution and similar functions (Stenmark, 2009). For example, Rab10 plays an important role in the regulation of GLUT4 trafficking (Chen and Lippincott-Schwartz, 2013). Since Rab8 and Rab10 have been found to co-localize on tubular structures and a cooperative role in GLUT4 trafficking as well as in basolateral cargo transport has been identified, the regulation of Rab10 localization or function by DLC3 cannot be excluded (Schuck et al., 2007; Sun et al., 2010).

Hattula et al. showed that Rab8 vesicles form at ruffling plasma membrane areas, move towards the cell center and fuse to tubular structures that are transported back to the plasma membrane (Hattula et al., 2006). During this course of vesiculation and tubulation, many endosomal fusion and fission events have to take place, which depend on dynamic actin polymerization and depolymerization. Constitutively active RhoA, however, reduced the number of cells displaying Rab8-positive tubules and redistributed Rab8 to vesicular structures in the perinuclear region. In accordance with this, DLC3 depletion caused the dispersion of Rab8 tubules, indicating a defect in the formation or maintenance of tubular structures. In fact, the vesiculation of Rab8 tubules might either arise from defects in the fusion of incoming Rab8 vesicles or from enhanced fission of outgoing tubules. Considering that the actin-depolymerizing agent CytD enhances Rab8 tubulation, apparently the right balance of actin turnover is crucial for the biogenesis of Rab8-positive transport carriers. This hypothesis is underlined by the observation that overexpression of DLC3 WT, leading to significantly reduced RhoA activity, induced a strong compaction of the Rab8 compartment lacking tubular structures. I therefore propose that the increased local Rho
activity in the absence of DLC3 causes an imbalance in actin dynamics, shown by the induction of numerous stress fibers, which triggers the loss of Rab8 recycling tubules and, as a consequence, leads to a transport block at the early endosomal stage. However, it also has to be considered that microtubules are necessary for the elongation of membrane tubules and the motility of transport carriers. Indeed, treatment with the microtubule-depolymerizing agent nocodazole inhibited Rab8 tubule formation, underlining the importance of an intact microtubule network (Hattula et al., 2006). A recent study identified a regulatory mechanism for DLC2 in microtubule stabilization, spindle positioning and junctional integrity (Vitiello et al., 2014). In particular, DLC2 co-localizes with microtubules and, in cooperation with the kinesin Kif1B, controls Cdc42 activity. Loss of DLC2 increased the activation of Cdc42, leading to microtubule stabilization via the downstream effector mDia3. Of note, DLC3-depleted cells revealed a reduced number of stabilized microtubules (data not shown), indicating that DLC3 might as well affect microtubule dynamics through the modulation of Rho GTPase activity. Whether the observed vesiculation of the Rab8 compartment is a direct consequence of enhanced actin polymerization or microtubule stabilization, or even both, needs further investigations.

4.1.2. **Endosomal RhoB - a possible new substrate for DLC3**

The endosomal Tfn transport block in DLC3-depleted cells was fully rescued by the simultaneous knockdown of RhoA and RhoB, demonstrating the involvement of Rho hyperactivation. The compensatory upregulation of RhoB in cells lacking RhoA provides an explanation why RhoA knockdown alone only partially restored Tfn transport in these cells. Considering the co-localization of DLC3 K725E with endosomal RhoB, DLC3 is likely to possess RhoB-inhibitory activity, too. Unfortunately, biochemical pull-down assays failed to reveal the potential RhoB regulation by DLC3 (data not shown). The development of a RhoB biosensor is required to address the question whether RhoB might indeed be a new in vivo substrate for DLC3 and, if so, at which subcellular site RhoB regulation occurs. Since the GAP activity of RhoB is important to mediate actin assembly and controls endosomal dynamics (Fernandez-Borja et al., 2005; Wallar et al., 2007), it would be very interesting to investigate whether the motile, endosomal pool of DLC3 is involved in the regulation of RhoB activity on vesicles. Although DLC3 activity towards RhoB remains to be proven, the observation that active RhoB causes a delay in EGFR degradation (Gampel et al., 1999) is supportive of this hypothesis, since DLC3 depletion phenocopied this effect. Another interesting question is how the selection of the different, but highly similar isoforms RhoA/B/C is made, representing a general open issue in terms of GAP-mediated Rho regulation.
At this point, it should be mentioned that although the GAP-inactive DLC3 mutant does not act in a dominant-negative manner with respect to Rho regulation and thus can be used for localization analyses, the overexpression of the inactive DLC3 still increases the levels of its domains, which might affect cellular physiology by sequestering adaptor proteins. To study the subcellular localization of DLC3, without manipulating any cellular function, the development of highly specific antibodies is required.

4.1.3. DLC3-mediated Rho regulation and anterograde membrane trafficking

Retrograde and anterograde trafficking are intimately connected to balance the membrane flux between intracellular compartments and ensure cellular homeostasis. Any defects at the level of endosomal recycling are likely to impact Golgi integrity and vice versa. Thus, it cannot be excluded that vesiculation of the Rab8 compartment influences integrity of the Golgi complex. For example, depletion of the ERC component EHD3 caused Golgi fragmentation in HeLa cells (Naslavsky et al., 2009). In line with this, I observed that overexpression of inactive Rab8a negatively affected Golgi compaction (data not shown), which might be due to changes within the actin and microtubule cytoskeleton.

The essential role of microtubules in the organization of Golgi complex morphology and positioning has been established over many years. In addition to microtubules, recent work has now identified a crucial role for the actin cytoskeleton in the maintenance of Golgi complex integrity (Egea et al., 2013; Yadav and Linstedt, 2011). Several groups have provided evidence for the association of actin with Golgi membranes and approaches using toxins found a compaction of the Golgi complex upon actin depolymerization (Dippold et al., 2009; Lázaro-Diéz et al., 2006; Valderrama et al., 2000). It is generally believed that Cdc42 is the major Rho GTPase regulating actin dynamics at the Golgi complex. A study in hippocampal neurons also observed RhoA at the Golgi complex, implying a direct role in the regulation of Golgi morphology or function through the control of local actin filament assembly (Camera et al., 2003). However, spatial analysis of subcellular RhoA activity in HeLa cells revealed that the Golgi-localized pool of RhoA is kept in an inactive, GDP-bound state (Pertz et al., 2006). Together with the observation that active RhoA caused severe Golgi fragmentation via mDia-mediated actin polymerization, myosin II driven contractility and microtubule-dependent movement (Zilberman et al., 2011), this indicates that RhoA activation at the Golgi complex needs to be tightly controlled to prevent actin hyperpolymerization and organelle disruption. The Golgi-localized GAP protein ARAP1 was suggested to be a possible candidate for Rho regulation at the Golgi complex (Miura et al.,
Besides an ArfGAP domain, ARAP1 also possesses a GAP domain with activity for RhoA and Cdc42, making it difficult to pinpoint precisely the local GTPase target. In contrast, DLC proteins are known to preferentially stimulate GTP hydrolysis of RhoA in living cells. Although we were not able to label actin filaments at the Golgi complex with standard reagents such as phalloidin, the observations that DLC3 depletion enhanced RhoA activation at the Golgi complex and led to Golgi fragmentation are in strong accordance with the above findings. Moreover, the expression of a targeted DLC3 GAP domain was sufficient to restore Golgi compaction in DLC3-depleted cells, providing clear evidence for a GAP-dependent Rho regulatory mechanism.

Nevertheless, I would like to point out that it cannot be completely excluded that DLC3 also regulates Cdc42 activity at the Golgi complex. However, overexpression of active Cdc42 does not lead to Golgi complex fragmentation, making it unlikely that the cellular effects in DLC3-depleted cells originate from Cdc42 hyperactivation. Experimental support for this assumption can be obtained by performing FRET experiments with the Cdc42 biosensor in DLC3 knockdown cells. Since DLC3 also localizes to the plasma membrane and plays a role in cell migration and Cdc42 activation occurs at the leading edge, it would be interesting to analyze active Cdc42 levels within migrating cells, too.

Despite the rather low activation status of Rho at the Golgi complex, there are hints for the involvement of Rho signaling in the regulation of TGN function. Thus far, no Golgi-associated Rho-specific GEF protein has been identified, but studies addressing the potential function of known downstream Rho effectors have been performed. Based on such experiments, myosin II and actin were found to play an important role in the fission of Rab6 vesicles at the Golgi complex and mDia1-mediated RhoA signaling was shown to promote Rab6 vesicle formation (Miserey-Lenkei et al., 2010; Zilberman et al., 2011). Furthermore, the actin-severing protein actin-depolymerizing factor (ADF)/cofilin and its upstream regulator LIM kinase 1 were reported to regulate fission and cargo sorting at the TGN (Salvarezza et al., 2009; von Blume et al., 2009). Although DLC3 depletion caused an increase in Rab6 transport carriers, we could not observe obvious changes in the delivery of the Rab6-dependent cargo protein VSV-G (vesicular stomatitis virus G protein; data not shown) to the plasma membrane. Nevertheless, it cannot be exclude that DLC3 is involved in controlling the formation of a specific subset of transport carriers from the TGN via local Rho regulation.

Another open question relates to the downstream effectors activated upon RhoA activation. Although the two best characterized effectors Dia and ROCK were both found to be involved in the regulation of vesicular trafficking and maintenance of Golgi complex structure, the
Golgi fragmentation induced by DLC3 knockdown could not be rescued by the simultaneous Dia depletion or ROCK inhibition (data not shown). It might be that the combined loss of several effectors is necessary to compensate for Rho hyperactivation or that other downstream effectors are involved, depending on the spatiotemporal context.

4.1.4. **DLC3-depletion promotes fast recycling of the EGFR and affects receptor signaling**

Although the majority of ligand-bound EGFR is targeted for degradation to attenuate signaling, alternative fates for activated EGFRs have been described. For example, recycling of the EGFR via the Rab11-positive ERC could be one means by which the cell amplifies growth signals during proliferation (Tomas et al., 2014). In addition, mutant EGFRs that evade from degradation were found to be recycled to the plasma membrane via endosomes that mainly contained EEA1, instead of Rab11, indicative of a distinct recycling route (Eden et al., 2012). Here, we found that loss of DLC3 interferes with the lysosomal degradation of the EGFR and thereby causes prolonged receptor signaling. In the absence of DLC3, the EGFR accumulated in Rab4-positive vesicles. Similarly, in DLC3-depleted cells, internalized Tfna was found to co-localize with Rab4, which is responsible for the fast recycling of cargo. This is in line with the observation that disturbed ERC integrity can lead to missorting of endocytosed Tfna (Hattula et al., 2006; Horgan et al., 2007; Naslavsky et al., 2006). In the case of the EGFR, disruption of the ERC by overexpression of the clathrin adaptor protein Eps15s caused the accumulation of the EGFR in early endosomes and potentiated receptor recycling (Chi et al., 2011). Although receptor internalization is a mechanism by which receptor signaling is terminated, it is now widely accepted that endosomes constitute active signaling platforms (Platta and Stenmark, 2011; Tomas et al., 2014), and, indeed, endosomal accumulation of EGF-stimulated EGFR in DLC3-depleted cells was associated with prolonged AKT activation. The selective activation of AKT may be mediated by the Rab5 effector protein APPL1 that regulates AKT activation from endosomes (Schenck et al., 2008).

4.1.5. **Cadherin transport defect upon DLC3 depletion suggests a more global role for DLC3 in membrane trafficking**

Our group previously reported that in MCF7 and MCF10A breast epithelial cells DLC3 predominantly localizes to cell-cell contacts. In the absence of DLC3, the AJ proteins E-cadherin and β-catenin were mislocalized, which was associated with enhanced migration and increased cell disaggregation (Holeiter et al., 2012). Interestingly, in HeLa
cells. DLC3 depletion caused an accumulation of the AJ protein N-cadherin at the Rab8 and Golgi compartments, resulting in reduced surface levels and impaired cell aggregation. In order to modulate cellular adhesion, cadherins are known to be recycled from the plasma membrane in clathrin-dependent and -independent pathways, as well as by caveolin-1 mediated uptake (Bryant and Stow, 2004). Although the regulatory mechanisms remain largely elusive, in cortical neurons, it was recently shown that Rab5 mediated the internalization of N-cadherin, which was then transported back to the plasma membrane via the Rab11-dependent slow recycling pathway (Kawauchi et al., 2010). Apart from retrograde transport, newly synthesized E-cadherin passes the Rab11-positive recycling endosome during post-Golgi transport to the plasma membrane (Lock and Stow, 2005), and Rab8 was also found to regulate E-cadherin transport to the plasma membrane (Yamamura et al., 2008). Thus, overlapping trafficking routes and shared Rab signaling pathways during the exo- and endocytosis of cadherins highlight the complicated scenario of cadherin recycling, in which single transport pathways cannot be easily distinguished. Nevertheless, it is tempting to speculate that DLC3 may possess a more global role in membrane trafficking and is able to regulate other Rab-mediated transport routes by the integration of Rho and Rab GTPase signaling. The general misregulation of endocytosed membrane receptors and adhesion-mediating transmembrane proteins might be associated with enhanced signaling and/or reduced adhesion. The finding that DLC3 depletion not only increased random cell motility, but also chemotactic cell migration towards an EGF gradient is supportive of this hypothesis. The regulatory effects of DLC3 on both, the actin and microtubule cytoskeleton are likely to act in concert in controlling cellular processes and, upon DLC3 loss, are also likely to cooperate in cell transformation.

4.2. Association of DLC3 with Rab8-positive recycling tubules

In this study it was found that the DLC3 SAM domain, as well as the short C-terminal extended variants, exclusively associated with the Golgi complex, whereas the isolated SAM domain of DLC1 did not localize to this compartment, but was fully cytosolic. This demonstrates that, although the SAM domain of DLC1 and DLC3 are conserved (Durkin et al., 2007a), they mediate interactions with different binding partners. For DLC1, the phosphatase and tensin homolog (PTEN) as well as EF1A1 were identified to specifically bind the SAM domain (Heering et al., 2009; Zhong et al., 2009). Overexpression of DLC1 SAM facilitated EF1A1 recruitment to the membrane periphery and suppressed cell migration in a GAP-independent manner (Zhong et al., 2009). Thus, it will be interesting to
identify new interaction partners of the DLC3 full-length protein and its single domains, in order to gain a deeper understanding of the recruitment and regulation of DLC3 and to subsequently investigate GAP-independent functions that might be exerted by the SAM or START domains.

Although the isolated SAM domain was strongly associated with Golgi membranes, the full-length protein co-localized with Rab8 tubules, indicating that additional domains are required for the transport of DLC3 itself from the Golgi complex to the ERC. The finding that the longest N-terminal DLC3 construct, DLC3-SAM(81-232) localized to the Golgi complex, but was also strongly recruited to Rab8-positive tubules, in contrast to DLC3-SAM(81-195), was indicative for an important targeting region within the polybasic amino acid stretch contained in DLC3-SAM(81-232). Of note, the minimal localization of the full-length DLC3 protein to Golgi membranes might be explained by an intramolecular folding mechanism that masks the PBR, reminiscent of the intramolecular negative regulation involving the SAM domain described for DLC1 (Kim et al., 2008). In fact, deletion of the PBR impaired the recruitment to Rab8-positive tubules in HeLa cells. The observation that the PBR interacted with the phospholipids PA and PI4P under in vitro conditions is intriguing, since recent data identified PA as an essential component of recycling tubules (Giridharan et al., 2013). Although PI4P was also found in the ERC, it is primarily enriched in Golgi membranes (Cullen, 2011). Despite the strong localization of DLC3-SAM(81-195) to the Golgi complex, no specific binding to PI4P was observed in the lipid overlay assay. Moreover, depletion of the PI4P-generating phosphatidylinositol 4-kinases, PI4KIIα and PI4KIIIβ, caused no displacement of ectopically expressed DLC3-SAM(81-195) from the Golgi complex (data not shown). Taken together these data suggest that the localization of the N-terminal DLC3α region to the Golgi complex is mediated by protein-protein interactions, whereas the recruitment to Rab8 tubules might require protein-lipid interactions. In future, more sophisticated approaches have to be performed to prove that the newly identified PBR within DLC3 is able to specifically bind PA and/or PI4P in a lipid bilayer. The protein binding to phospholipid-enriched membranes could, for example, be analyzed by liposome-based in vitro assays.

Since Rho GTPases are anchored to cellular membranes via their prenyl groups, signal transduction and regulation occurs at these subcellular sites. Thus, the direct interaction of lipid components with the DLC proteins might be a means for GAP protein targeting and regulation. For DLC1, we previously identified a polybasic phosphatidylinositol-4,5-bisphosphate (PIP2) binding region adjacent to the GAP domain, which is highly conserved across species and enhances the GAP activity of DLC1 in vitro (Erlmann et al., 2009). Since, phosphoinositides are known to regulate endocytic membrane trafficking by recruiting
adaptor proteins and Rho GTPases appear to be involved in the regulation of their metabolism (Croisé et al., 2014; Haucke, 2005). Rho GTPases may contribute to the recruitment and activation of their own negative regulators. Additionally, via its START domain, DLC3 may directly impact membrane composition or stimulate the activity of lipid metabolizing enzymes, thereby potentially controlling membrane trafficking via a second molecular mechanism. Although the lipid-binding pocket of the START domain theoretically enables DLC proteins to bind and transfer lipids between membranes, the putative ligands have not been described. In future, it will be necessary to perform lipid mass spectrometry to identify the bound lipids and investigate at which cellular membranes DLC3-mediated lipid shuttling might occur.

4.3. Outlook

All DLC family members share a common localization to focal adhesions, but additional, non-overlapping subcellular localizations were identified that might account for isoform-specific functions. Associated with its localization to cell-cell contacts, our lab previously established a Rho-regulatory role for DLC3 in AJ stability and cell differentiation. In this study, I provide evidence that DLC3 is recruited to the Rab8 and Golgi compartments, where it prevents RhoA hyperactivation and thereby ensures proper endocytic trafficking. Furthermore, I found that DLC3 co-localizes with RhoB on endocytic vesicles and observed the movement of DLC3 on tubular and vesicular structures in live cell experiments. Thus, the investigation of regulatory mechanisms that coordinate the dynamic association with different cellular membranes will be of special interest in the future. Considering that DLC3 might exert its Rho regulatory function at all these sites, it will be necessary to characterize protein and/or lipid interactors of DLC3 that not only recruit the protein, but also regulate its activity. Moreover, further efforts will be necessary to elucidate GAP-independent functions of DLC3 that are still poorly understood. Finally, knockout mouse models will be required to identify DLC3-specific functions in development and prove its tumor suppressive activity in vivo. Together, the increased understanding of DLC3 biology will shed light onto its normal physiological function and its potential function in the suppression of the different steps of neoplastic cell transformation.
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Publications

This work was published in part in the following publications:

2015  
Braun AC, Hendrick J, Eisler SA, Schmid S, Hausser A and Olayioye MA

• ‘The Rho-specific GAP protein DLC3 coordinates endocytic membrane trafficking.’
  
accepted for publication in *Journal of Cell Science*

Braun AC and Olayioye MA

• ‘Rho Regulation: DLC Proteins in Space and Time.’
  
invited review article for publication in *Cellular Signalling*

Other publications:

2012  

• ‘The RhoGAP protein Deleted in Liver Cancer 3 (DLC3) is essential for adherens junctions integrity.’
  
*Oncogenesis* (2012) 1, e13

Schopman NC, Braun AC and Berkhout B

• ‘Directed HIV-1 evolution of protease inhibitor resistance by second-generation short hairpin RNAs.’
  
*Antimicrob Agents Chemother* 56(1):479-86

Abstracts, posters and conference participations

2014  
Braun AC

• ‘DLC3 modulates Rho activity at the perinuclear region and regulates local membrane trafficking’
Talk at the annual internal meeting 'Molecular Mechanisms and Therapeutic Approaches in Cancer and Inflammatory Diseases' in Freudenstadt-Lauterbad, Germany

**Braun AC, Hendrick J, Eisler SA, Schmid S, Hauser A and Olayioye MA**

- ‘Membrane recycling is regulated by the DLC3 tumor suppressor protein via localized RhoA signaling.’

Abstract and poster at the EMBO Workshop 'Signaling to and from Endomembranes' in Konstanz, Germany

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2013  **Braun AC, Hendrick J, Eisler SA, Schmid S, Hauser A and Olayioye MA**

- ‘The Rho-specific GAP protein DLC3 coordinates endocytic membrane trafficking.’

Abstract and poster at the 5. Invadosom Meeting 'Cell Migration and Invasion in Physiology and Pathology' in Nijmegen, The Netherlands

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**Braun AC**

- ‘DLC3 modulates Rho activity at the Golgi complex and regulates local membrane trafficking.’

Talk at the annual internal meeting 'Research and Development in Cancer and Inflammatory Diseases' in Freudenstadt-Lauterbad, Germany

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- ‘The RhoGAP protein Deleted in Liver Cancer 3 (DLC3) is essential for adherens junctions integrity.’

Abstract and poster at the 15. Beatson International Cancer Conference 'Membrane Dynamics in Cancer' in Glasgow, Scotland
2012  Braun AC

• ‘DLC3 is required for N-cadherin trafficking by conserving microtubule integrity.’

Abstract and poster at the annual internal meeting 'Understanding molecular mechanisms and developing targeted drugs in cancer and inflammatory diseases' in Freudenstadt-Lauterbad, Germany

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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

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