Regulatory roles of MICAL-L1 and EHD1 in cell signaling and mitosis

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Regulatory roles of MICAL-L1 and EHD1 in cell signaling and mitosis

By

James Reinecke

A DISSERTATION

Presented to the Faculty of The Graduate College in the University of Nebraska
In partial Fulfillment of the Requirements for the degree of Doctor of Philosophy.

Department of Biochemistry and Molecular Biology
Under the Supervision of Professor Steve Caplan

University of Nebraska Medical Center
Omaha, Nebraska

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Regulatory roles of MICAL-L1 and EHD1 in cell signaling and mitosis

James Reinecke Ph.D.

Advisor: Steven Caplan Ph.D

Membrane trafficking is a basic cell biological process that controls the distribution of proteins and lipids. Our lab is particularly interested in delineating the cellular functions as well as the molecular mechanisms that regulate the C-terminal Eps15 Homology Domain (EHD) protein family of adenosine-5'-triphosphatases in mammalian cells. EHD1-4 are ubiquitously expressed in mammalian tissues and serve partially overlapping but also distinct functions in regulating membrane shaping and fission along the endocytic pathway. Specifically, EHD1 is recruited to tubular recycling endosomes (TREs) by Molecule Interacting with CasL Like-1 (MICAL-L1) and facilitates TRE fission and release of cargo from the perinuclear endocytic recycling compartment (ERC). Recent studies from our lab have shown that the interaction between EHD1 and MICAL-L1 is crucial for TRE biogenesis, dynamics, and the efficient recycling of transferrin, major histocompatibility complex (MHC) class I, and Integrin receptors. However, the roles of EHD1 and MICAL-L1 in cellular processes are less clear. Herein, I describe novel roles for EHD1 and MICAL-L1 in modulating cell signaling through regulating the localization of the non-receptor tyrosine kinase c-Src. In addition, I demonstrate rather unexpected roles for MICAL-L1 and EHD1 in modulating the microtubule cytoskeleton during mitosis.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>1</td>
</tr>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Table of contents</td>
<td>3</td>
</tr>
<tr>
<td>Table of figures</td>
<td>8</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>10</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>13</td>
</tr>
<tr>
<td>Chapter I</td>
<td>14</td>
</tr>
<tr>
<td>1. Endocytic trafficking</td>
<td>15</td>
</tr>
<tr>
<td>1.1. Historical overview</td>
<td>15</td>
</tr>
<tr>
<td>1.2. Fundamental concepts of membrane trafficking</td>
<td>15</td>
</tr>
<tr>
<td>1.3. Introduction to nucleotide binding proteins and the importance of nucleotide cycling in membrane trafficking</td>
<td>15</td>
</tr>
<tr>
<td>2. Organization of the endocytic pathway</td>
<td>18</td>
</tr>
<tr>
<td>2.1. Internalization pathways</td>
<td>18</td>
</tr>
<tr>
<td>2.2. The sorting endosome</td>
<td>19</td>
</tr>
<tr>
<td>2.3. Degradative pathway from the sorting endosome</td>
<td>20</td>
</tr>
<tr>
<td>2.4. Recycling pathways from the sorting endosome</td>
<td>22</td>
</tr>
<tr>
<td>3. C-terminal EH domain-containing proteins (EHD)</td>
<td>23</td>
</tr>
<tr>
<td>3.1. Evolution and general characteristics</td>
<td>23</td>
</tr>
<tr>
<td>3.2. Structure</td>
<td>25</td>
</tr>
<tr>
<td>3.3. EH domain and protein interactions</td>
<td>27</td>
</tr>
<tr>
<td>3.4. MICAL-L1, EHD and TREs</td>
<td>28</td>
</tr>
</tbody>
</table>
4. Conclusions and transition 30

Chapter II

5. Introduction 39

5.1. Src family of non-receptor tyrosine kinases (SFK): evolution, structure, function 39

5.2. Regulation of SFK by protein-protein interactions and post-translational modifications 41

5.3. Spatio-temporal regulation of SFK by membrane trafficking 44

6. Materials and Methods 47

7. Results 53

7.1. MICAL-L1 associates with Src in mammalian cells and is required for Src activation and localization to the cell periphery 53

7.2. MICAL-L1 co-localizes with Src and is required for Src recruitment to circular dorsal ruffles in human foreskin fibroblasts following PDGF stimulation 56

7.3. MICAL-L1 is required for PDGF-induced focal adhesion turnover 58

7.4. MICAL-L1 regulates cell spreading 61

7.5. MICAL-L1 depletion affects directional cell migration 63

7.6. EHD1 is required for Src transport and activation and acts as a molecular ‘pinchase’ on MICAL-L1 tubules to release Src from the ERC in response to EGF 64

8. Discussion and figures 66

Chapter III 97
9. Introduction to “Novel functions for the endocytic regulatory proteins MICAL-L1 and EHD1 in mitosis” 98

9.1. The cell cycle 98

9.2. Stages of cytokinesis, central spindle assembly, and the three M’s of cytokinesis 99

9.3. Endocytic recycling proteins and cytokinesis 101

10. Materials and Methods 104

11. Results 108

11.1. MICAL-L1 or EHD1-depletion in HeLa cells impairs normal cell cycle 108

11.2. MICAL-L1 and EHD1 are required for cytokinesis and transport of recycling endosomes to the intercellular bridge (ICB) 110

11.3. Recruitment of MICAL-L1 to the ICB is independent of EHD1, Rab11 and Rab35 111

11.4. EHD1- but not MICAL-L1-, Rab11-, or Rab35 depletion affects central spindle formation 113

11.5. EHD1 and Rab35 regulate mitotic spindle orientation while MICAL-L1 controls spindle length 115

11.6. Effect of MICAL-L1-, EHD1-, and Rab35-depletion on kinetochores, inter-kinetochore tension and kinetochore fibers 116

12. Discussion and figures 118

Chapter IV 144

13. Summary 145
14. Future directions: Life beyond TREs

14.1. EHDs as regulators of microtubule nucleation and release from the centrosome

14.2. EHD1 and mitochondrial dynamics

15. References
Table of Figures

Chapter 1

Figure 1.1. Schematic drawing of molecular events that occur during endocytic trafficking.
Figure 1.2 In vivo GTP nucleotide cycle for hypothetical GTPase Protein A
Figure 1.3 Schematic of clathrin-dependent endocytosis and the endocytic pathway
Figure 1.4 Mammalian EHD proteins
Table 1.1 EHDs and select binding partners

Chapter 2

Figure 2.1 Src structure and regulation
Figure 2.2 Partial co-localization between MICAL-L1 and Src in mammalian cells
Figure 2.3 MICAL-L1-depletion in HeLa cells impairs EGF-induced Src activation and translocation out of the ERC.
Figure 2.4 MICAL-L1-depletion in HeLa cells does not affect EGF binding capacity, EGFR internalization or EGFR activation
Figure 2.5 MICAL-L1 co-localizes with Src and focal adhesion proteins along circular dorsal ruffles (CDR) in human foreskin fibroblasts (BJ).
Figure 2.6 MICAL-L1 regulates Src recruitment to CDR.
Figure 2.7 MICAL-L1-depletion affects CDR closure.
Figure 2.8 MICAL-L1-depleted BJ cells show altered distribution of focal adhesion proteins at steady-state.
Figure 2.9 MICAL-L1 regulates PDGF-induced focal adhesion turnover
Figure 2.10 MICAL-L1-depletion impedes focal adhesion turnover and leads to increased focal adhesion size
Figure 2.11 MICAL-L1 is required for proper cell spreading on fibronectin and optimal integrin-induced Src activation
Figure 2.12 MICAL-L1 is required for cell migration.
Figure 2.13 EHD1 is required for EGF-induced Src translocation and activation in HeLa cells
Figure 2.14 EHD1 is required for EGF-induced MICAL-L1 tubule vesiculation
Figure 2.15 Schematic model depicting the proposed roles of MICAL-L1 and EHD1 in mediating Src translocation from the ERC to the plasma membrane in response to growth factor or integrin stimulation as well as potential recycling or degradation pathways.

Chapter 3

Figure 3.1 Introduction to cytokinesis
Figure 3.2 Depletion of MICAL-L1 or EHD1 in HeLa cells causes cell cycle defects
Figure 3.3 Specificity of MICAL-L1- and EHD1-siRNA knockdown phenotypes
Figure 3.4 Depletion of the EHD family member, EHD2, has no gross effect on cell cycle
Figure 3.5 MICAL-L1- or EHD1-depletion leads to cytokinesis failure
Figure 3.6 MICAL-L1 and EHD1 are required for delivery of transferrin-containing recycling endosomes to the intercellular bridge (ICB)
Figure 3.7 Recycling regulatory protein requirements for their recruitment to the ICB
Figure 3.8 Depletion of EHD1 but not MICAL-L1, Rab11 or Rab35 affects central spindle formation
Figure 3.9 Recruitment of Aurora B or PLK1 is not affected by depletion of MICAL-L1, EHD1, Rab11 or Rab35
Figure 3.10 Role of recycling proteins in regulating mitotic spindle orientation and length
Figure 3.11 Role of recycling proteins in regulating inter-kinetochore tension
Figure 3.12 MICAL-L1-depletion enhances kinetochore fiber length and leads to abnormal kinetochore-microtubule interactions while EHD1-depletion decreases kinetochore fiber length
Figure 3.13 MICAL-L1-depletion enhances kinetochore fiber cold-stability
Figure 3.14 Summary of MICAL-L1 and EHD1 functions during mitosis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>ACA</td>
<td>Anti-centromere antibody</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance statistical test</td>
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<tr>
<td>AP</td>
<td>Adaptor protein</td>
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<td>APC/C</td>
<td>Anaphase promoting complex/cyclosome</td>
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<td>ADP</td>
<td>Adenosine-5’-diphosphate</td>
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<td>Arf</td>
<td>ADP-ribosylation factor</td>
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<td>Autosomal recessive hypercholesterolemia</td>
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<td>Actin-related proteins 2/3</td>
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<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
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<td>BCA</td>
<td>Bicinchoninic acid assay-protein measurement</td>
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<tr>
<td>BJ</td>
<td>Primary human foreskin fibroblast cell line</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C-terminus</td>
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<td>Clathrin-dependent endocytosis</td>
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<td>CDK1</td>
<td>Cyclin-dependent kinase 1</td>
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<td>CDR</td>
<td>Circular dorsal ruffles</td>
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<td>CEP55</td>
<td>Centrosomal protein of 55 kiloDaltons</td>
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<td>CH</td>
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<td>Charged multivesicular body protein</td>
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<td>CSK</td>
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<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole, dihydrochloride</td>
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<td>Dulbecco’s modified Eagle medium</td>
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<td>Ethylene glycol tetraacetic acid</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>Enhanced green fluorescent protein</td>
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<td>Epidermal growth factor receptor</td>
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</tr>
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<tr>
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<td>Eps15</td>
<td>Epidermal growth factor substrate 15</td>
</tr>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERC</td>
<td>Endocytic recycling compartment</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
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<tr>
<td>ESCRT</td>
<td>Endosome sorting complex required for transport</td>
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<td>F-BAR</td>
<td>Bin/amphiphysin/Rvs domain</td>
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<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FIP</td>
<td>Rab11 Family of interacting proteins</td>
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<td>FYVE</td>
<td>Fab1, YOTB, Vac1, and EEA1domain</td>
</tr>
<tr>
<td>GA</td>
<td>Golgi apparatus</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GDF</td>
<td>GDI displacement factor</td>
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<td>GDI</td>
<td>GDP dissociation inhibitor</td>
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<td>GDP</td>
<td>Guanosine-5’-diphosphate</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin tag</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HOPS</td>
<td>Homotypic fusion and vacuole protein sorting complex</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Hrs</td>
<td>Hepatocyte growth factor-regulated tyrosine kinase substrate</td>
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<tr>
<td>ICB</td>
<td>Intercellular bridge</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
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<tr>
<td>kDa</td>
<td>kiloDalton</td>
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<td>KD</td>
<td>Knockdown-referring to gene depletion with siRNA</td>
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<tr>
<td>LE</td>
<td>Late endosome</td>
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<tr>
<td>LIM</td>
<td>Lin11, Isl-1 and Mec-3 domain</td>
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<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
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<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MG132</td>
<td>Proteasome inhibitor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MICAL</td>
<td>Molecule interacting with Cas Ligand</td>
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<td>MICAL-L1</td>
<td>Molecular interacting with Cas Ligand Like-1</td>
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<td>Minute</td>
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<td>MKLP1</td>
<td>Mitotic kinesin-like protein 1</td>
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<tr>
<td>mM</td>
<td>Millimolar/ mmoles/liter</td>
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<tr>
<td>MTOC</td>
<td>Microtubule organization center</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>MVB</td>
<td>Multi-vesicular bodies</td>
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<td>N-terminus</td>
<td>Referring to the NH₂ end of a poly-peptide</td>
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<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NPF</td>
<td>Asparagine-proline-phenylalanine motif</td>
</tr>
<tr>
<td>OCRL1</td>
<td>Lowe oculocerebrorenal syndrome protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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</table>
PD  Parkinson's disease
PDGF  Platelet-derived growth factor
PH  Plekstrin homology
PHEM  PIPES, HEPES, EGTA, MgCl
PI3K  Phosphatidylinositol-4,5-bisphosphate-3 kinase
PI3P  Phosphatidylinositol 3-phosphate
PI3P  PIPES, HEPES, EGTA, MgCl
PLK1  Polo-like kinase 1
PM  Plasma membrane
PRC1  Protein Required for Cytokinesis 1
PxxP  Proline-rich motif
pY  phosphotyrosine
RhoA  Ras homolog gene family, member A
RIPA  Radioimmunoprecipitation assay buffer
RME-1  Receptor-mediated endocytosis protein 1
RPM  Revolutions per minute
RPTP  Receptor protein tyrosine phosphatase
RSV  Rous sarcoma virus
RTK  Receptor tyrosine kinase
SCAMP  Secretory carrier-associated membrane protein
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec  Seconds
SFK  Src family of kinases
SH2  Src homology 2
SH3  Src homology 3
SH4  Src homology 4 (kinase domain)
SIM  Structured illumination microscopy
siRNA  Short-interfering ribonucleic acid
SNAP  α-soluble N-ethylmaleimide-sensitive factor attachment protein
SNARE  Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
STAT  Signal transducer and activator of transcription
SYF  Src, Yes, Fyn
Tf  Transferrin
TfR  Transferrin receptor
TRE  Tubular recycling endosome
TBST  Tris-buffered saline with Tween20
Tris  Tris(hydroxymethyl)aminomethane
TSG101  Tumor susceptibility gene 101
µM  micromolar-concentration
µm  micrometers-distance
Acknowledgements

“Failures, repeated failures, are finger posts on the road to achievement. One fails forward towards success.” - C.S. Lewis

This rather pithy quote from C.S. Lewis accurately describes the process of being trained as a biomedical researcher and I imagine it will continue to be true throughout the rest of my scientific career. Fortunately, I have been well supported both professionally and personally throughout my Ph.D training.

Professionally, I am indebted to my mentor Dr. Steve Caplan. Steve has been instrumental in molding me as a biomedical researcher. He has provided me with guidance, patience and insight but has also given me the independence to develop projects that are not directly related to the main focus of the lab. It is my hope that his unreserved confidence and willingness to support my hypotheses will be rewarded in the future when I run a lab of my own, which I view as a testament to his mentorship. I am also indebted to Dr. Naava Naslavsky for her insight, especially with regards to overcoming experimental pitfalls. I also would like to thank Dawn Katafiasz, who was a co-author on both of my manuscripts, for her hard work and ingenuity. It would have been a lot harder to complete each manuscript without her valued input. I am also grateful to all the Caplan lab members, both past and present, for creating a fun and energetic working environment.

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Chapter 1

INTRODUCTION

Parts of this chapter were derived from

1. **Endocytic trafficking**

1.1. **Historical overview**

The evolution from prokaryotic to eukaryotic cells generated organelles, which are compartmentalized, membrane-bound structures that carry out distinct functions and are required for cellular homeostasis and survival. The advances in light and electron microscopy have allowed scientists to visualize these distinct compartments and dissect their functions. George Palade was awarded the 1974 Nobel Prize in physiology or medicine for his electron microscopy-based work describing the inter-connection of the Golgi apparatus (GA), endoplasmic reticulum (ER) and plasma membrane (PM) via membrane vesicles and tubules. Recently, the Nobel Prize committee awarded the 2013 Noble Prize in physiology or medicine to James Rothman, Randy Schekman, and Thomas Sudhof for their combined efforts in discovering the fundamental mechanisms that regulate communication between organelles.

1.2 **Fundamental concepts of membrane trafficking**

Organelles are both spatially and molecularly distinct. Each organelle contains different lipid compositions, composed of combinations of phospholipids, cholesterol, sugar-lipids and sphingolipids (van Meer and de Kroon 2011). The varied lipid compositions between organelles contribute to their identity. Indeed, conserved domains such as the Pleckstrin Homology (PH) and Fab1, YOTB, Vac11, EEA1 (FYVE) domain target proteins to organelles enriched in distinct phosphoinositol lipid species (Lemmon 2008).
A schematic representation of the three steps of intracellular organelle trafficking is depicted in Figure 1.1. Coat and adaptors proteins localized on the donor organelle promote membrane invagination, leading to the formation of a membrane bud. A vesicle is then generated through the process of fission, which is mediated by molecular membrane scissors or membrane ‘pinchases’. The vesicle travels along cytoskeletal tracks such as microtubules or actin filaments to the target/acceptor organelle where proteins localized on both the vesicle and the target organelle coordinate the process of fusion. Fusion induces the mixing of donor organelle membranes, cargo and proteins with the acceptor organelle.

1.3. Principles of nucleotide binding cycles, membrane recruitment and protein-protein interactions in regulating endocytic trafficking

The membrane trafficking steps described above are highly regulated in cells. A common molecular theme found throughout the endocytic pathway that modulates lipid fission and fusion is the presence of proteins that bind to and hydrolyze nucleotides such as guanosine triphosphate (GTP) and adenosine triphosphate (ATP). Nucleotide binding allows for recruitment of endocytic proteins to membranes and also promotes allosteric changes required for protein-protein interactions, which regulate membrane fusion, fission, and transport. The Rab family of small Ras-like GTPases are the most numerous and best understood GTPases in the endocytic pathway and serve as convenient markers of different endocytic organelles ((Stenmark 2009); see below). However, given that there are greater than 60 Rabs expressed in
mammalian cells and an even greater number of proteins that regulate their nucleotide cycles, I will use a hypothetical protein, Protein A, to describe the regulatory proteins that promote nucleotide binding and nucleotide hydrolysis (Barr and Lambright 2010). Upon describing nucleotide cycles, I will then describe some of the Rab proteins, their localizations to endocytic organelles, and the proteins that they recruit to these organelles.

Figure 1.2 shows the in vivo GTP cycle for the hypothetical GTPase Protein A. The localization and the rate of GTP hydrolysis for Protein A is exquisitely regulated by a number of factors. First, GDP-bound Protein A can be sequestered in the cytosol by a GDP dissociation inhibitor (GDI). The Protein A GDI acts to both inhibit the dissociation of GDP as well as to block the association of Protein A with membranes. The action of GDI is opposed by the action of a GDI displacement factor (GDF). Once Protein A has been released from its GDI by the action of its GDF, it is now free to bind membranes but remains in the inactive or GDP-bound form until a guanine nucleotide exchange factor (GEF) facilitates the exchange of GDP for GTP. The active GTP-bound Protein A recruits effector proteins. A GTPase activating protein (GAP) initiates Protein A GTPase activity, leading to the hydrolysis of GTP to GDP, the dissociation of Protein A effectors, and the return of Protein A to its inactive GDP-bound state. The GDP-GTP cycle must be reset in order for the endocytic pathway to function continuously.

2. Organization of the endocytic pathway and important regulatory proteins
I refer the reader to Figure 1.3 for a schematic representation of the endocytic pathway as well as the localization of regulatory proteins that are described below.

2.1 Internalization pathways

The majority of the lipids and proteins localized to the PM are in a state of constant flux. The PM serves as both a mechanical barrier as well as the primary mode of communication between the extracellular and intracellular environments. Transmembrane receptors localized at the PM respond to cues from the extracellular environment. Internalization of transmembrane receptors represents one mechanism utilized by eukaryotic cells to fine-tune the output of signaling pathways. In mammalian cells, there are two routes of internalization. Clathrin-dependent endocytosis (CDE) is the best-understood route of internalization (McMahon and Boucrot 2011). Coated pits on the plasma membrane were first visualized by electron microscopy of mosquito oocytes internalizing yolk protein (Roth and Porter 1964). The authors noted that the internalized yolk protein appeared to be encased inside a proteinaceous basket that differs in morphology from the amorphous pits formed during fluid uptake (pinocytosis). Clathrin, the major constituent of these proteinaceous pits, was identified from isolated membrane vesicles (Pearse 1976). Clathrin is composed of three heavy chains and three light chains that form triskelia, which self-polymerize into hexagonal and pentagonal shapes (Crowther and Pearse 1981). Interestingly, while clathrin is a self-polymerizing protein, its assembly is tightly regulated in cells. For instance, clathrin does not bind membranes
directly. The adaptor protein (AP) family, most notably AP-2, recognize tyrosine
based motifs on transmembrane receptors and subsequently recruit clathrin to
the site of internalization (Ohno, Stewart et al. 1995). Implied in the specificity
of AP-2 interactions with a portion of plasma membrane receptors is the notion
that CDE is cargo-specific. Thus, receptors that do not have an AP-2 binding
motif are internalized via clathrin-independent endocytosis (CIE). Relative to
CDE, the process and regulation of CIE is poorly understood (Mayor and
Pagano 2007). In general, CIE requires the GTPase dynamin 2 (as does CDE),
cholesterol and the actin cytoskeleton. While CIE was previously thought to be
the minor and ‘accessory’ endocytic pathway to CDE, it is now clear that a large
number of receptors are internalized through CIE and recent data supports the
notion that cell biologists have only begun to appreciate the number of intricate
regulatory mechanisms involved in CIE (Eyster, Higginson et al. 2009). Despite
the complexity of internalization pathways, the majority of CDE and CIE cargo
are transported to a common structure termed the early or sorting endosome
(EE/SE).

2.2 The Sorting Endosome
Akin to a Fed-Ex receiving station, it is the job of the SE to receive, package
and ship cargo from the plasma membrane to the correct cellular destination
((Jovic, Sharma et al. 2010)Figure 1.3-SE). Though many proteins are
required for SE biogenesis and function, Rab5 is central to defining the SE as
well as regulating its function, dynamics and subsequent transport from the SE
(Woodman 2000). GTP-bound Rab5 recruits a number of effectors, including
the phosphatidyl inositol-4-5 bisphosphate-3-kinase (PI3K; (Christoforidis, Miaczynska et al. 1999)). The recruitment of PI3K is important to establishing the early identity of the SE, as it promotes the generation of phosphoinositol 3-phosphate (PI3P). PI3P serves as a molecular platform required for the recruitment of proteins containing a FYVE domain (Stenmark, Aasland et al. 2002). Examples of FYVE domain proteins include Early Endosomal Autoantigen-1 EEA1, rabankyrin-5, rabenoysn-5. It is interesting to note that all of these FYVE domain proteins bind to Rab5 as well as PI3P (Grosshans, Ortiz et al. 2006), indicating a level of overlap in the mechanisms that mediate the recruitment of these proteins to the SE. The Rab5-dependent recruitment of a diverse number effector proteins promotes the transformation or maturation of the SE from a small vesicular organelle to a relatively bulky organelle composed of a vacuolar component and a tubular component (Huotari and Helenius 2011). In general, cargos that are sorted into the vacuolar component are targeted to the late endosomal (LE)/lysosomal pathway for degradation while cargos sorted to the tubular component are recycled directly to the plasma membrane or transported into the ERC prior to being brought back to the plasma membrane.

2.3 The late endosome/lysosome pathway

Below, I will briefly summarize the degradative pathway mediated by the LE/lysosome. The Epidermal Growth Factor Receptor (EGFR) will serve as an example cargo to describe how receptors are internalized from the plasma membrane, transported from the SE to the late endosome and degraded in the
lysosome (Goh and Sorkin 2013). As alluded to above, the endocytic pathway can fine-tune the output of signaling pathways. The degradation of EGFR is one such example of how the endocytic pathway can attenuate cell-signaling events. Indeed, preventing EGFR degradation by disrupting its trafficking to the lysosome can lead to amplified EGFR signaling, increased cell growth and cancer (Bache, Slagsvold et al. 2004). The targeting of EGFR to the degradative pathway is kinase-dependent. Active EGFR recruits ubiquitin E3 ligases that ubiquitinate several lysines present on the EGFR c-terminal cytoplasmic tail (Sigismund, Algisi et al. 2013). Ubiquitinated lysines recruit the Endosomal Sorting Complex Required for Transport (ESCRT), which is a family of proteins that act sequentially to promote the budding or ‘internalization’ of cargo such as EGFR into vesicles localized within the vacuolar component of the SE (Williams and Urbe 2007). These vesicles, termed multivesicular bodies (MVB), shield EGFR from signaling proteins localized within the cytoplasm and thus attenuate EGFR signaling.

While Rab5 is crucial for the function of the SE, the dissociation of Rab5 from the SE and the acquisition of Rab7 are necessary events required for the maturation of a SE to a LE. The Rab5-Rab7 transition is mediated by the SAND-1/Mon1 complex (Rink, Ghigo et al. 2005; Poteryaev, Datta et al. 2010). The SAND-1/Mon1 promotes the dissociation of the Rab5 GEF RABX-5 (which decreases active Rab5), the activation of Rab7 and the recruitment of the LE/lysosome fusion machinery known as the Homotypic fusion and Protein Sorting (HOPS) complex. Rab7 is to the LE as Rab5 is to the SE and as such,
is required for the molecular events that ultimately lead to LE fusion with the lysosome. LE/lysosome fusion causes the degradation of cargo via proteases localized within the lysosomal lumen.

2.4 Recycling pathways from the SE

Cargos that are destined for recycling back to the plasma membrane do so via two routes. The first is the direct or ‘fast’ recycling pathway and is regulated by Rab35 and Rab4 (Grant and Donaldson 2009). I will not expand on this pathway further but will instead focus on the ‘slow’ recycling pathway as it is the most germane pathway to my work.

Cargos localized to the tubular component of the SE that are recycled via the slow recycling pathway are transported in a microtubule- and dynein-dependent manner from the cell periphery to the peri-nuclear or peri-centriolar localized ERC (Traer, Rutherford et al. 2007). Once thought to be a contiguous membrane bound organelle (Yamashiro, Tycko et al. 1984; Hopkins, Gibson et al. 1994), unpublished super-resolution imaging from our lab demonstrates that the ERC is actually a concentrated accumulation of distinct tubulo-vesicular structures. Rab11 was one of the first proteins described to regulate the function of the ERC (Ren, Xu et al. 1998). Rab11, in association with effector proteins such as the Rab11 Family of Interacting Proteins (FIP) coordinate traffic into and out of the ERC (Hales, Vaerman et al. 2002; Horgan, Oleksy et al. 2007; Schonteich, Wilson et al. 2008). Interestingly, while microtubules are required for transport into the ERC, Rab11-FIP2 recruits the atypical actin motor myosin Vb to ERC endosomes for anterograde transport out of the ERC (Hales,
Indeed, ectopic expression of a motorless myosin Vb leads to the accumulation of cargo proteins in the ERC (Lapierre, Kumar et al. 2001). In addition to promoting transport, the rigid structures of the actin and microtubule cytoskeletons support the membrane tubules that emanate out of (or into) the ERC.

ERC tubules, alternatively referred to as tubular recycling endosomes (TRE), vary in length depending on the cell type. The cervical cancer cell line HeLa has prominent TREs, thus making HeLa cells an ideal model system to study TRE function and biogenesis. TREs are hypothesized to transport cargo into and out of the ERC. The formation and dynamics of TREs requires the coordinated actions of many types of proteins. Central to the generation, stability and fission of TREs is the Eps15 Homology Domain containing protein family (EHD) (Naslavsky and Caplan 2011).

3.C-terminal Eps15 homology domain protein family

3.1 Evolution and general characteristics

The mammalian EHD family is composed of four proteins (EHD1-4) that are capable of both homo- and hetero-oligomerization (Pohl, Smith et al. 2000; Grant and Caplan 2008). A single EHD gene is found in the genomes of lower eukaryotes such as C. elegans and D. melanogaster; interestingly, while C. elegans has only one EHD gene, it expresses several different isoforms via alternative splicing (Grant, Zhang et al. 2001; Olswang-Kutz, Gertel et al. 2009). The EHDs are highly conserved at the amino acid level even across species.
(Figure 1.3A). For instance, the entire amino acid sequence of the C. elegans EHD family member, RME-1, is 67% identical to the human EHD1 sequence. Mammalian EHD1 and EHD3 display greater than 80% sequence identity (Grant and Caplan 2008). From worms to humans, the EHDs share a highly conserved domain architecture with a N-terminal G-domain, a central helical region and a C-terminal EH domain. (Figure 1.3B). Interest in the structure and function of the EHD family increased dramatically after EHD1 and RME-1 were found to be required for endocytic recycling in human cells and C. elegans (Grant, Zhang et al. 2001; Caplan, Naslavsky et al. 2002).

Subsequent studies by our lab and several others have begun to elucidate how the EHDs coordinate membrane trafficking events. Despite the high level of amino acid identity, the EHDs carry out distinct functions. EHD1 is required for the transport of endocytic cargo from the ERC to the PM while EHD3 is required for transport from the SE into the ERC (Caplan, Naslavsky et al. 2002; Naslavsky, Boehm et al. 2004; Naslavsky, Rahajeng et al. 2006). EHD4 on the other hand, localizes to the SE, is involved in regulation of Rab5 activation, and supports trafficking of cargo to the lysosome (Sharma, Naslavsky et al. 2008). EHD2, the most disparate EHD family member and the only EHD incapable of hetero-oligomerizing with other family members, localizes to the plasma membrane and regulates the dynamics of caveolins (Hansen, Howard et al. 2011; Moren, Shah et al. 2012; Stoeber, Stoeck et al. 2012). Currently, the molecular and atomic mechanisms that are responsible for the unique functions of each EHD family are unclear. Prior to describing how the EHDs
facilitate the endocytic trafficking steps described above, I will first describe the structure of the EHD family.

### 3.2 Structure of EHDs

The crystallization of murine EHD2 provided novel insight into the mechanisms that regulate EHD structure and function (Daumke, Lundmark et al. 2007). The authors answered several important questions regarding EHD biology. First, they demonstrated that EHD2 possessed intrinsic membrane bending activity, as purified EHD2 tubulated negatively charged liposomes *in vitro*. Initial molecular characterization of the EHD1/RME-1 N-terminus demonstrated that EHD1/RME-1 shared considerable homology with the G-domain of dynamin 2, a large membrane bending GTPase (Heymann and Hinshaw 2009). The homology between the EHD1 N-terminus and the dynamin G-domain suggested that EHD1 might bind to GTP (Grant, Zhang et al. 2001; Caplan, Naslavsky et al. 2002). Surprisingly, RME-1 binds to ATP rather than GTP and ATP binding is required for the localization and function of RME-1 on endocytic membranes *in vivo* (Lee, Zhao et al. 2005). Daumke et al. confirmed that ATP binding is required for mammalian EHD2 membrane tubulation *in vivo* but also found, counterintuitively, that EHD2 can tubulate membranes in an ATP-independent manner *in vitro* (Daumke, Lundmark et al. 2007). Secondly, wild-type EHD2 had a low intrinsic ATP hydrolysis rate that was increased eightfold by lipid binding. Third, the authors provided the first crystal structure of an EHD protein. The ribbon representation created from the crystal structure of an EHD2 dimer bound to a non-hydrolyzable ATP analog is shown (with
permission) in Figure 1.3C. EHD2 dimerizes via a hydrophobic region found in the G-domain, which includes a conserved (among the 4 EHD family members) tryptophan at amino acid position 283. The importance of W283 to the dimerization of EHD2 was highlighted by the fact that mutation of this amino acid rendered EHD2 insoluble. It is important to point out that the putative membrane binding surface, while composed of several alpha helices in the central helical region (amino acids 285-400), also contains two N-terminal alpha helices (amino acids 18-55). Lastly, the EH domain, containing two calcium binding EF hands, is connected to the central helical region via a 40-residue amino acid linker region. The dimer structure suggested that dimerization occurs between opposing G-domains, creating a highly curved scissor-like structure (Figure 1.3 C; arrow). The pocket of the EHD2 scissor is composed of several alpha helices of the central helical domain. This putative membrane-binding interface contains several lysines that could support binding to negatively charged lipids (like the ones used in their initial liposome assays). Indeed, mutation of lysines 327-329 impaired EHD2 lipid binding. In total, Daumke et al provided several key answers to questions about the structure/function relationship of EHD2 (Daumke, Lundmark et al. 2007). Many important questions remained, most notably, regarding the mechanism that controls the recruitment of EHDs to membrane tubules in vivo. Subsequent studies discovered that the EH domain is crucial for the interaction of EHDs with other proteins in vivo and mediates the recruitment of several of the EHD family members to endocytic tubules.
3.3 Importance of the EH domain to EHD function *in vivo*

The EH domain is a common modular domain in the endocytic pathway (Confalonieri and Di Fiore 2002). Structural insight into the EH domain was first provided by Nuclear Magnetic Resonance (NMR) spectroscopy analysis of the second EH domain of Epidermal growth factor Substrate 15 (Eps15) (de Beer, Carter et al. 1998). Several experimental approaches elucidated that the Eps15 EH domain specifically bound to proteins containing the tri-peptide motif asparagine-proline-phenylalanine (NPF; (Salcini, Confalonieri et al. 1997; de Beer, Carter et al. 1998; Paoluzi, Castagnoli et al. 1998; de Beer, Hoofnagle et al. 2000)). Curiously, despite the structural conservation between N-terminal EH and C-terminal EH proteins, they share few common interacting proteins. The molecular mechanism underlying the high level of specificity was explained by comparing the electrostatic charge of N-terminal EH domains and C-terminal EH domains. The crystal structure of EHD2 and the NMR solution structure of the EH domain of EHD1 was critical for this comparison (Daumke, Lundmark et al. 2007; Kieken, Jovic et al. 2007). The NPF binding region on N-terminal EH proteins such as Eps15 have an overall neutral or slightly negative charge whereas the NPF binding region of the EHD proteins is highly positive. Consequently, NPF proteins flanked by neutral or positive amino acids preferentially bind to N-terminal EH proteins, while EHD proteins preferentially bind to NPF motifs flanked by several acidic residues (NPF-Ac) (Grant and Caplan 2008; Kieken, Jovic et al. 2009; Henry, Corrigan et al. 2010; Kieken, Sharma et al. 2010). Table 1.1 provides a list of select EHD binding proteins.
and their functions. For this body of work, I will focus on the interaction between MICAL-L1 and EHD1 and their important functions on TRE in human cells.

### 3.4 MICAL-L1, EHDs and TREs

While the localization of EHD1 to TRE was initially elucidated by Caplan et al in 2002 (Caplan, Naslavsky et al. 2002), it was unknown whether EHD1 was required for the biosynthesis of these membrane tubules or alternatively, if EHD1 was a resident protein on TRE that regulated their function in endocytic recycling. The answer to this important question became clear when Sharma et al uncovered an interaction between EHD1 and MICAL-L1 (Sharma, Giridharan et al. 2009). MICAL-L1 belongs to a family of conserved N-terminal flavin mono-oxygenase domain (FAD) proteins that are involved in the regulation of actin cytoskeleton morphology through oxidation of actin (Terman, Mao et al. 2002; Giridharan, Rohn et al. 2012; Lee, Peterfi et al. 2013; Giridharan and Caplan 2014). While MICAL-L1 shares several domains in common with family members MICAL1-3 such as a Calponin Homology (CH) domain, a Lin-11, Isl-1 and MEC-3 (LIM) domain and coiled-coil domains, MICAL-L1 lacks the N-terminal FAD domain but contains several NPF-Ac. The finding that MICAL-L1 bound to EHD1 (and EHD3) suggested that MICAL-L1 might be involved in endocytic recycling. Indeed, siRNA-mediated depletion of MICAL-L1 lead to the accumulation of several cargos such as transferrin, MHC Class I and integrin receptors in the ERC and significantly slowed their recycling back to the plasma membrane. Additionally, MICAL-L1 localized to TREs in an EHD1-independent
manner and MICAL-L1 was required for EHD1 localization to TREs, suggesting that MICAL-L1 may be the master regulator of TRE biogenesis. Subsequent studies showed that MICAL-L1 acts as a membrane hub that recruits EHD1 as well as other TRE resident proteins Rab8, Rab35, and the GTPase Arf6 (Giridharan, Cai et al. 2012; Rahajeng, Giridharan et al. 2012; Kobayashi and Fukuda 2013; Kobayashi, Etoh et al. 2014).

Further evidence that MICAL-L1 is required for TRE biogenesis came from a recent study in our lab that found that EHD1, EHD3 and MICAL-L1 binds to and recruits the membrane bending, F-BAR domain containing protein Syndapin2 to TREs (Giridharan, Cai et al. 2013). Interestingly, the C. elegans syndapin ortholog binds to RME-1 and mammalian syndapin2 binds to EHD2 (Braun, Pinyol et al. 2005; Hansen, Howard et al. 2011). The fact that syndapin2 was found on TREs was rather surprising given that the majority of available data suggested that syndapin2 is involved in internalization at the plasma membrane (Qualmann and Kelly 2000; Da Costa, Sou et al. 2003; Hansen, Howard et al. 2011; Senju, Itoh et al. 2011). However, syndapin2 is indeed a crucial TRE protein. Depletion of syndapin2 led to MICAL-L1 localization on vesicular structures, suggesting impaired TRE biogenesis. In addition to uncovering a novel TRE protein, our lab found that TREs are enriched in phosphatidic acid and that phosphatidic acid is required for TRE biogenesis. Both the MICAL-L1 coiled-coil domains as well as the syndapin2 F-BAR domain bound to phosphatidic acid in vitro. Importantly, inhibiting several phosphatidic acid biosynthetic pathways in vivo disrupted the localization of
MICAL-L1 and syndapin2 to TREs and impaired endocytic recycling, supporting a role for phosphatidic acid in TRE biogenesis as well as the importance of MICAL-L1 in recruiting membrane-shaping proteins such as syndapin2 to TREs.

The above study demonstrates the complexity of protein networks on TREs, as EHD1 and EHD3 both localize to TREs and can directly bind to MICAL-L1 and syndapin2. It is known that EHDs can bend and possibly promote fission or fusion of membranes \textit{in vitro} (Daumke, Lundmark et al. 2007); however, little is known regarding the ability of EHDs to shape TREs \textit{in vivo}. To address this, our lab recently developed a novel semi-permeabilized cell system assay, in which cytosolic proteins are extracted but MICAL-L1-positive TREs remain intact (Cai, Giridharan et al. 2013). Following cytosolic extraction, purified GST-EHD fusion proteins were added to the permeabilized cells in the presence of ATP and the morphology of TREs was quantified by immunofluorescence microscopy. In this system, purified EHD1 promoted the vesiculation or fission of TREs while purified EHD3 promoted tubulation or fusion of TREs. While the atomic differences that separate the functions of EHD1 and EHD3 are unclear, this study provided clear insight into the distinct functions of each protein on TRE \textit{in vivo}.

4. Conclusions, transition and figures

As the TRE molecular landscape comes into focus, it becomes increasingly important to determine the function of TREs and TRE regulatory proteins in cellular processes. EHD1 regulates cell adhesion and migration by
controlling the plasma membrane levels of adhesion receptors such as \( \beta_1 \)-integrins (Jovic, Naslavsky et al. 2007). \( \beta_1 \)-integrins are implicated in cell adhesion to extracellular matrices containing fibronectin (Albelda and Buck 1990), and EHD1-depletion impaired cell adhesion. Counter-intuitively, EHD1-depletion also stabilized focal adhesions. Focal adhesions are large molecular scaffolds built from interactions between \( \beta_1 \)-integrins and the actin cytoskeleton and their continuous turnover is required for cell migration (Petit and Thiery 2000). Thus, in addition to mediating beta-1 integrin recycling, EHD1 may be required for transporting cellular machinery to focal adhesions that are required for focal adhesion turnover. The non-receptor tyrosine kinase c-Src is an ideal candidate given that c-Src: is required for focal adhesion turnover; has been shown to localize to recycling endosomes; and kinase activity requires transport from the perinuclear area to focal adhesions (Rohrschneider 1979; Kaplan, Swedlow et al. 1992; Kaplan, Bibbins et al. 1994; Xing, Chen et al. 1994; Fincham and Frame 1998; Klinghoffer, Sachsenmaier et al. 1999; Sandilands, Cans et al. 2004; Sandilands and Frame 2008). In Chapter 2, we demonstrate that c-Src localizes to MICAL-L1-positive TREs and that both MICAL-L1 and EHD1 are required for c-Src transport and activation at the plasma membrane. Functionally, we show that MICAL-L1 is required for Src-dependent activities such as focal adhesion turnover and growth factor induced and extracellular matrix-induced actin cytoskeletal rearrangements in human fibroblasts. Lastly, we introduce a paradigm shift in TRE biology. Previous live imaging studies have suggested that TREs are static structures (Sharma,
Giridharan et al. 2009). We find that TRE vesiculation is induced by growth factor treatment, indicating that TREs are dynamic and respond to extracellular stimulation.

In Chapter 3, we show that MICAL-L1 and EHD1 play key roles during mitosis. Recycling endosomes have long been known to mediate completion of cytokinesis, the final phase of the cell cycle, and recent studies have also highlighted that recycling endosomes can control earlier mitotic events such as mitotic spindle stability and spindle orientation (Montagnac, Echard et al. 2008; Schiel and Prekeris 2011; Royle 2013; Hehnly and Doxsey 2014). We find that EHD1 and MICAL-L1 are required upstream of previously characterized recycling proteins such as Rab11/FIP3 and Rab35 during mitosis. Thus, findings from Chapter 3 introduce MICAL-L1 and EHD1 as novel and important regulators of mitotic progression.
Figure 1.1. Schematic drawing of molecular events that occur during endocytic trafficking.
Figure 1.2 *In vivo* GTP nucleotide cycle for hypothetical GTPase Protein A
Figure 1.3 Schematic of clathrin-dependent endocytosis and the endocytic pathway. Rab proteins are denoted in **green** while their effector proteins are denoted in **red**. EHD proteins are shown in **blue** while their binding partners are shown in **purple**.
Figure 1.4 Mammalian EHD proteins. A) Comparison of the amino acid sequence identity shared between EHD family members adapted with permission from (Grant and Caplan 2008). B) Linear domain architecture of EHD proteins. C) EHD2 crystal structure adopted with permission from (Daumke, Lundmark et al. 2007). Arrow denotes lipid binding region created by helical domain alpha-helices.
### Table 1.1 EHDs and select binding partners

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<th>EHD protein</th>
<th>Interacting Protein</th>
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<td>MICAL-L1</td>
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<td>Syndapin2</td>
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<td>EHD2</td>
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<td>EHD3</td>
<td>Rab11-FIP2</td>
<td>*EE-ERC transport</td>
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<td></td>
<td>Rabenosyn-5</td>
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<td>EHD4</td>
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Chapter 2

Regulation of Src trafficking and activation by the endocytic regulatory proteins MICAL-L1 and EHD1

Parts of this chapter were derived from

5. Introduction

Cell adhesion and migration are fundamental processes that are required for organ development as well as pathologic conditions such as wound healing, atherosclerosis and cancer (Friedl and Gilmour 2009). The non-receptor tyrosine kinase c-Src (Src), the founding member of the Src Family Kinases (SFK), plays key roles in regulating cell adhesion and migration (Fincham and Frame 1998), and deregulation of Src kinase activity in cancer is correlated with metastasis and poor survival (Wheeler, Iida et al. 2009).

5.1 Src family of non-receptor tyrosine kinases (SFK): evolution, structure, function

The discovery of the Rous Sarcoma Virus (RSV) by Peyton Rous in the early 20th century marked the beginning of a new era in cancer biology (Rous 1911) that went unappreciated until the latter half of the 20th century. Rous found that a filterable agent, such as RSV, could cause soft tissue tumors known as sarcomas in birds. Skepticism prevailed regarding Rous' findings and they were largely ignored until the revelation that the RSV transformative agent, v-Src, was a truncated form of a cellular protein kinase called c-Src (Src) (Czernilofsky, Levinson et al. 1980). Src, one of the first proto-oncogenes, is the founding member of the SFK, a family of 8 closely related non-receptor tyrosine kinases (Src, Fyn, Yes, Lyn, Hck, Blk, Fgr, Lck). The SFKs are highly conserved among metazoans and a Src ortholog is expressed in unicellular choanoflagellates (Segawa, Suga et al. 2006; Li, Young et al. 2008; Miller 2012; Schultheiss, Suga et al. 2012). The unicellular Src ortholog has provided novel
insight into the evolution of Src biochemical regulation in multicellular organisms (see below). In mammals, Src, Yes and Fyn (SYF) are ubiquitously expressed while the other family members display more restricted expression profiles. SFKs phosphorylate proteins such as focal adhesion kinase (FAK), p190RhoGAP, and signal transducer and activator of transcription (STAT3) to promote cell migration and proliferation. Given that SFKs are overactive in a number of cancers, understanding Src regulatory mechanisms is a high priority for researchers (Wheeler, Iida et al. 2009).

SFK share a highly conserved protein domain architecture (Fig. 2.1 A, reviewed (Boggon and Eck 2004)). The N-terminus of all SFKs is myristoylated, while some family members, such as Yes and Fyn, are also palmitoylated. N-terminal lipid modification is required for SFK membrane association and kinase activity in cells (Patwardhan and Resh 2010). The unique domain is the only domain that is not highly conserved between SFK members. The SH3 domain binds to PxxP proline-rich motifs (where x stands for any amino acid) while the SH2 domain binds to phosphotyrosine (pY) residues that are flanked by acidic residues such as glutamate and followed by isoleucine (pY-E-E-I). A type-II polyproline helix lies between the SH2 and kinase domains. The kinase domain, or SH1 domain, consists of two lobes (N and C). Between the N and C lobes is a helix containing a critical tyrosine residue (Y416 in chicken or Y419 in humans (Smart, Oppermann et al. 1981)) that must be trans-phosphorylated by an adjacent Src molecule to enable Src kinase activity.
The C-terminus of SFK contains a regulatory tyrosine (Y527 in chicken and Y530 in humans) that is phosphorylated by a regulatory kinase known as C-terminal Src kinase (CSK) (Nada, Okada et al. 1991). In keeping with the historical chicken Src numbering system used, we will abide by this numerical designation both for human Src and other Src family members such as Hck. Mutation of Y527 to phenylalanine or deletion of this residue (as is the case with v-Src) results in constitutively active Src (Nada, Yagi et al. 1993). Figure 2.1B summarizes critical amino acids found within each SFK domain. The domain architecture and phosphorylation of SFK is central to the regulation of their kinase activity.

CSK-mediated down-regulation of Src kinase activity appears to have evolved with metazoans (Segawa, Suga et al. 2006; Li, Young et al. 2008; Miller 2012; Schultheiss, Suga et al. 2012). In an eloquent biochemical study, Segawa et al. compared and characterized the regulation of Src orthologs expressed in the unicellular choanoflagellate Monosiga ovata and the multicellular primitive sponge Ephydatia fluviatilis (Segawa, Suga et al. 2006). Interestingly, CSK phosphorylation was evident in M. ovata and in E. fluviatilis; however, M. ovata Src was still active following CSK phosphorylation. In fact, ectopic expression of wild-type M. ovata Src in mammalian cells induced cellular transformation irrespective of CSK expression, indicating that exquisite Src regulation in multicellular organisms is crucial for tissue homeostasis.

5.2 Regulation of SFK by protein-protein interactions and post-translational modifications
In the following section, I will describe the cascade of events that occurs immediately after CSK-dependent phosphorylation of Y527. Phosphorylated Y527 (pY527) promotes an intramolecular interaction with the SH2 domain (Fig. 2.1C, 'off') (Xu, Doshi et al. 1999). SH2-pY527 binding causes the SH3 domain to bind to the polyproline helix SH2-kinase linker region. As a result, the SH3 domain pushes against the backside of the N-lobe of the kinase domain, which closes the cleft between the N and C lobes. The closing of the cleft between the N and C lobes effectively buries Y416 and prevents the binding of ATP and other substrates. Collectively, the sequential steps of the Src intramolecular interaction are known as the 'latch' (SH2-pY527), 'clamp' (SH3-linker) and 'switch' (kinase domain conformations) (Harrison 2003). The intramolecular interactions between the Src modular domains are substantially weaker than Src intermolecular interactions, allowing for rapid induction of Src activation under certain conditions. For example, the SH2 domain preferentially binds to pY-E-E-I thus making the pY527-Q-P-G a lower affinity substrate. Similarly, the Src SH3 domain binds with higher affinity to PxxP proline-rich motifs than it does to the kinase-linker region, which contains a type II polyproline helix rather than a PxxP motif. Therefore, there are several mechanisms that alleviate the Src intramolecular interaction and restore full kinase activity: 1) dephosphorylation of pY527; 2) pY-E-E-I substrate binding to the SH2 domain; or 3) PxxP substrate binding to the SH3 domain (Fig. 2.1C, 'on').

A plethora of Src interacting proteins become available following receptor tyrosine kinase (RTK) activation or engagement of integrin receptors with the
extracellular matrix. The auto-phosphorylation of several C-terminal tyrosine residues on RTKs following ligand binding provides high-affinity ligands for the Src SH2 domain (Alonso, Koegl et al. 1995). Similarly, activation of integrin receptors induces autophosphorylation of FAK at Y397 and Src SH2-pY397 binding induces Src activation (Xing, Chen et al. 1994). Lastly, Src kinase activity can occur through pY527 de-phosphorylation by phosphatases such as receptor protein tyrosine phosphatase-alpha (RPTP-α) (Su, Muranjan et al. 1999).

Hck SH3 binding to the HIV-1 protein Nef PQVP proline-rich motif best demonstrates the activation of SFK via SH3 interaction (termed SH3 displacement) (Moarefi, LaFevre-Bernt et al. 1997). Hck-Nef binding (Kd= 250 nM) represents one of the strongest known SH3 binding affinities and is required for HIV replication in peripheral mononuclear cells in vitro (Lee, Leung et al. 1995; Saksela, Cheng et al. 1995). Site-directed mutagenesis of Hck has also been instrumental in elucidating the interplay between the three Src regulatory mechanisms. Mutation of Y527-Q-P-G to Y-E-E-I greatly reduces Hck activation by locking its C-terminus to the SH2 domain. However, Nef binding to the Hck SH3 domain is able to induce Hck catalytic activity even in the presence of the high-affinity SH2-pY527 interaction (Lerner and Smithgall 2002), suggesting that: 1) SH3 domain displacement from the linker region is sufficient to induce Hck activity and signaling; and 2) differential activation of SFK activity by SH2-pY527 or SH3-PxxP interactions may specify downstream function. While SFK conformational change represents one mode of
modulating Src kinase activity, Src intracellular localization also dictates its downstream signaling events.

5.3 Spatio-temporal regulation of SFK by membrane trafficking

Regulation of Src intracellular localization is an additional mechanism utilized to intricately regulate Src-dependent signaling cascades (Sandilands and Frame 2008). v-Src localizes to the PM as well as internal cytoplasmic compartments (Rohrschneider 1979; Courtneidge, Levinson et al. 1980). Temperature-sensitive mutants of v-Src that are inactive at 41°C and active at 35°C demonstrated that inactive v-Src localizes to the perinuclear region, and upon activation, translocates to peripheral membranes and focal adhesions (Welham and Wyke 1988) to promote focal adhesion turnover and migration (Fincham and Frame 1998).

The initial insights gained from v-Src were also proven to be true for c-Src. Src localizes to perinuclear endosomes whereas active Src localizes to focal adhesions (Kaplan, Swedlow et al. 1992; Kaplan, Bibbins et al. 1994). The creation of triple-null fibroblasts lacking Src, Yes, and Fyn provided an ideal model to understand the endocytic machinery that regulates each SFK (Klinghoffer, Sachsenmaier et al. 1999). Expression of exogenous wild-type Src in Src-null mouse embryonic fibroblasts provided clear evidence that Src translocates to the cell periphery in an actin-dependent manner within endocytic vesicles containing the small GTPase RhoB. Disruption of the actin cytoskeleton by cytochalasin D or genetic ablation of RhoB prevented Src translocation and activation (Sandilands, Cans et al. 2004). Data from other
groups suggest that Src trafficking occurs through late endosomes (Kasahara, Nakayama et al. 2007), regulated by the ESCRT complex (Tu, Ortega-Cava et al. 2010). However, the effect of ESCRT-depletion on Src activity may be indirect, as the ESCRT complex regulates the trafficking and localization of integrins in human fibroblasts (Lobert and Stenmark 2012). Regardless, the endocytic regulatory proteins that are involved in Src localization remain largely uncharacterized.

Despite the high level of homology between SFKs, distinct endocytic pathways regulate Fyn and Yes. This phenomenon is largely driven through differences in N-terminal lipid modifications. All SFK are myristoylated at the N-terminus, specifically, a glycine at position 2. However, SFK such as Fyn, Lyn and Yes are also mono- or di-palmitoylated at nearby cysteine residues. As noted above, wild-type Src-GFP preferentially localized to RhoB-containing vesicles when expressed in SYF-/- fibroblasts, although a small subset of Src-GFP also localized to RhoD-containing endosomes (Sandilands, Cans et al. 2004). In contrast, Fyn-GFP expressed in SYF-/- cells localized primarily to RhoD vesicles (Sandilands, Brunton et al. 2007).

To test if differential lipid modifications specify RhoB vs RhoD localization, Sandilands et al. constructed a Src-GFP chimera that is palmitoylated and inversely, created non-palmitoylated Fyn-GFP mutants (Sandilands, Brunton et al. 2007). Indeed, palmitoylated Src localized to RhoD-containing early endosomes while non-palmitoylated Fyn behaved more like wild-type Src and localized to RhoB vesicles. These localization studies were
confirmed using a combination of RhoB-/ MEFs and siRNA-mediated RhoD-depletion in SYF cells. Wild-type Src-GFP was sequestered in the perinuclear region in RhoB-/ cells. However, the palmitoylated Src chimera and wild-type Fyn-GFP were able to translocate from the perinuclear region to the plasma membrane (Sandilands, Brunton et al. 2007). Conversely, wild-type Src and non-palmitoylated Fyn translocated to the plasma membrane after RhoD depletion in SYF-/-, but RhoD-depletion resulted in the perinuclear retention of the palmitoylated Src chimera and wild-type Fyn (Sandilands, Brunton et al. 2007). Sato et al. similarly found that differential lipid modifications of SFK dictate subcellular localization (Sato, Obata et al. 2009). The modes of transport for non-palmitoylated Src and palmitoylated SFK such as Fyn and Yes likely specify their distinct downstream functions. This is supported by the fact that while Src, Yes and Fyn have some overlapping functions (i.e., the loss of all three results in mouse embryonic lethality (Klinghoffer, Sachsenmaier et al. 1999)), the loss of Src alone causes bone thickening (osteopetrosis) indicative of abnormal osteoclast functioning while loss of Fyn or Yes alone results in impaired thymocyte signaling and immunoglobulin receptor trafficking respectively (Soriano, Montgomery et al. 1991; Gauen, Zhu et al. 1994; Luton, Verges et al. 1999).

Work by our lab and others have shown that EHD1 and MICAL-L1 are required for the exit of transmembrane receptors from the perinuclear ERC (Caplan, Naslavsky et al. 2002; Sharma, Giridharan et al. 2009). The potential role of MICAL-L1 and EHD1 in regulating the intracellular trafficking and
function of non-receptor tyrosine kinases such as Src has not been established. We hypothesized that MICAL-L1 and EHD1 may be required for Src transport to the cell periphery and thus may be regulators of Src-dependent functions. We demonstrate herein that Src localizes to MICAL-L1-positive endocytic compartments. Furthermore, depletion of MICAL-L1 and EHD1 impairs Src activation and localization to the periphery following growth factor treatment. Functionally, we show that the loss of MICAL-L1 impairs focal adhesion turnover, cell spreading and cell migration. We now demonstrate that inactive Src is concentrated in the perinuclear ERC, and that upon growth factor treatment, EHD1 is recruited to MICAL-L1-positive structures and promotes the release of Src from the ERC allowing Src to travel to the PM, thereby affecting key downstream cellular processes.

6. Methods

Reagents and Antibodies

Recombinant human PDGF-BB, EGF and EGF-Rhodamine were purchased from Invitrogen. Fibronectin was purchased from Sigma. The following antibodies were used: EHD1 (Caplan, Naslavsky et al. 2002); vinculin (Sigma); GM130 (BD Biosciences); Src (36D10); phospho-Src (tyrosine 416; D49G4); FAK, phospho-FAK (tyrosine 925); EGFR, phospho-FAK (tyrosine 1068); (Cell Signaling Technologies); phospho-FAK (tyrosine 397); phospho-paxillin (tyrosine 118); (Invitrogen); actin, MICAL-L1; (Novus); phospho-Src (tyrosine 416; immunofluorescence); (Millipore); human transferrin receptor (Zymed).

Cell Culture
The cervical cancer cell line HeLa cells (ATCC-CCL2) and SYF mouse embryonic fibroblasts (ATCC-CRL2459; (Klinghoffer, Sachsenmaier et al. 1999)) were grown in DMEM (high glucose) containing 10% fetal bovine serum, penicillin/streptomycin (Invitrogen) and 2 mM glutamine. Normal human foreskin fibroblasts (BJ; ATCC-2522) were grown in EMEM containing 10% fetal bovine serum, penicillin/streptomycin, 2 mM glutamine and non-essential amino acids.

**Plasmids, siRNA Transfections and Rescue**

Human c-Src-GFP was created similarly to that described previously (Sandilands, Cans et al. 2004). Briefly, human Src (Invitrogen- IOH12563) was PCR-amplified using forward primer 5’ ccgctcgagatgggtagcaacaagagcaagcc 3’ and reverse primer 5’ ccccaagctttgatctgtgaggttctccccggctgg 3’. The resulting PCR product, which contains (from 5’ to 3’): 5’ Xho1 restriction site, c-Src ORF, G-S-G-S linker and a 3’ HindIII site were ligated into pre-digested EGFP-N1 (Clontech). Plasmid was verified by sequencing. HA-MICAL-L1 was previously described (Sharma, Giridharan et al. 2009). Plasmids were transfected into SYF cells using FuGene HD (Roche).

Pooled and individual oligonucleotides targeting human MICAL-L1, custom EHD1 siRNA (Sharma, Giridharan et al. 2009) and non-targeting control siRNA were obtained from Dharmacon. HeLa cells were transfected with Oligofectamine (Invitrogen) with 150 nM siRNA while BJ cells were transfected using Lipofectamine RNAiMAX (Invitrogen) with 50 nM siRNA. Efficiency of protein knockdown was measured 48-72 h post-transfection by immunoblot or immunofluorescence for each experiment. For rescue experiments, 48 h post-
siRNA transfection, 8 X 10⁵ BJ cells were electroporated using Amaxa Nucleofector II according to the manufacturer’s protocol with a siRNA-resistant GFP-MICAL-L1 construct.

*Growth factor stimulation and phenotype analysis*

Cells grown on glass coverslips (HeLa) or 10 µg/ml fibronectin-coated coverslips (BJ) were serum-starved overnight (18 h) in Opti-MEM media. Cells were then stimulated with EGF (50 ng/ml) or PDGF (15 ng/ml) for the times indicated in the text prior to immunoblotting or immunofluorescence.

Src ERC fluorescence was measured using ImageJ. Cells were stimulated with EGF, fixed and stained with Src and TfR antibodies (as described in text). Images were imported into ImageJ and a region of interest was created in individual cells by manually tracing around perinuclear TfR (to mark ERC). Src fluorescence was measured using ‘measure’ function. All cells (control and KD cells in individual experiments) were fixed, stained and imaged on the same day and with identical confocal settings.

Src recruitment to PDGF-induced CDR (15 CDR/condition/experiment, three independent experiments total) was quantified from Src and actin fluorescence values measured by profile line-scan analysis in Pascal LSM5 Image Examiner. Data was expressed as a ratio of Src fluorescence to actin fluorescence.

MICAL-L1 tubule area was calculated as described previously (Cai, Giridharan et al. 2013).

*Immunoblotting*
Cell lysates were prepared by washing cells two times in ice-cold PBS. Cells were then scraped off the plate with a rubber policeman into ice-cold RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 1.8 mg/ml iodoacetamide, 1 mM orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM PMSF). Lysates were then clarified by centrifugation at 13,000 RPM at 4°C. Protein levels were quantified using the BCA assay (Biorad). For immunoblotting, 20-30 µg (HeLa cells) or 10-15 µg (BJ cells) of protein lysate/sample was separated by 8% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in TBST (TBS +0.1% Tween). BSA (3%) was used for blotting with phospho-protein antibodies or TBST+5% Dry milk and then incubated overnight in primary antibodies diluted in either TBST-BSA (for phospho-proteins) or TBST-Milk. Membranes were washed with TBST and then incubated with HRP-conjugated goat anti-mouse (Jackson Research Laboratories) or donkey anti-rabbit (GE healthcare) secondary antibody for 1 h at room temperature.

**Cell Spreading**

At 72 h post-siRNA transfection, BJ cells were detached from plates with 0.05% trypsin/EDTA. Trypsin was inactivated by addition of complete growth medium. Cells were pelleted and washed twice in serum-free medium and then incubated in suspension at 37°C for 30 min. Cells were then plated onto 10 µg/ml fibronectin-coated coverslips for 90 min. For immunoblots, cells in suspension were plated onto fibronectin-coated tissue culture dishes and
harvested at the time points indicated in text. Cell area was measured using Pascal LSM image examiner by manually tracing borders around cells. Focal adhesions were quantified as described below.

_Focal adhesion quantification_

Focal adhesion number and size was measured in ImageJ. Images from vinculin-stained samples were imported into ImageJ. Cropped images of single cells were thresholded. The total number of focal adhesions per cell was quantified using ‘measure particles’ function with parameters set to measure particles in the range of 1-30 µm². Focal adhesion size distribution was analyzed by categorizing focal adhesion area into three categories: 1-5 µm², 6-10 µm², and 11-30 µm².

_Scratch wound assay_

At 48 h post-siRNA transfection, BJ cells were trypsinized and plated onto 10 µg/ml fibronectin-coated cover-slips at high density in low-serum (2%) medium overnight. A single scratch was made using a P200 pipet tip. Cell debris was washed away with low-serum media and cells were then incubated in low-serum medium for the times indicated in text prior to fixation.

_Flow Cytometry_

At 48 h post-siRNA transfection, control and MICAL-L1 KD cells were trypsinized. Trypsin was inactivated by addition of complete serum. Cells were pelleted and washed twice with serum-free medium. Cells were incubated in suspension in serum-free medium for 1 h at 37°C. Cells were then pelleted at 4°C and resuspended in ice-cold serum-free medium containing 1 µg/ml EGF-
Rhodamine and incubated at 4°C with gentle rotation. Cells were pelleted, washed three times with cold PBS and fixed with 4% paraformaldehyde. At least 10,000 cells were analyzed for surface bound EGF-Rhodamine by flow cytometry analysis (BD Biosciences).

**Immunofluorescence**

Cells were treated as indicated in the text and then fixed in 3.7% paraformaldehyde in PBS (HeLa) or PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 7.0) for 15 minutes at room temperature. Cells were rinsed three times in PBS and permeabilized in 0.1% TritonX-100/PBS for 3 min (HeLa) or 10 min (BJ). Cells were then blocked for 1 h at room temperature in PBS containing 10% normal goat serum, 1% BSA and 0.1 M glycine. The cells were incubated with primary antibody in PBS/1% BSA, and where indicated, phalloidin-AlexaFluor 488 or Phalloidin-Rhodamine (Invitrogen), for 1 h at room temperature, washed 3X in PBS and then incubated with appropriate fluorochrome-conjugated secondary antibodies (Molecular Probes) plus DAPI for 1 h at room temperature. Cells were washed 3X in PBS and mounted in Fluoromount G.

Single plane confocal images were collected using a Zeiss LSM5 Pascal laser confocal microscope with a Plan-Apochromat 63X/1.4 oil objective except for scratch wound images which were imaged using Plan-Neofluor 10x/0.3 objective. For quantification, collected images were imported into ImageJ or LSM Pascal Image Examiner as described above. Images presented in figures were imported into Adobe Photoshop CS, where they were re-sized and
formatted to 300 dpi resolution with minimal image manipulation (whole-image adjustment of brightness was done using ‘levels’ function).

Statistics

Data from ImageJ was imported into Microsoft Excel. Mean and standard error of the mean were calculated from data obtained from three independent experiments. Statistical significance was calculated by one-way ANOVA and Tukey test (when comparing more than two samples) using vassarstats program (www.vassarstats.net).

7. Results

7.1 MICAL-L1 associates with Src in mammalian cells and is required for Src activation and localization to the cell periphery.

MICAL-L1 displays a highly unique localization in HeLa cells, decorating long tubular endosomes that emanate from the ERC (Sharma, Giridharan et al. 2009) and Fig. 2.2B). Under steady-state conditions, we observed that endogenous Src partially co-localized with MICAL-L1 along tubular membranes that radiate from the ERC (Figure 2.2 A and B; arrows in inset). Given that Src family members such as Fyn and Yes may also be expressed in HeLa cells, we next transiently expressed Src-GFP and HA-MICAL-L1 fusion proteins in Src/Yes/Fyn (SYF) null mouse embryonic fibroblasts to test if MICAL-L1 specifically associates with Src in mammalian cells. Src-GFP is concentrated along membrane ruffles and tubulo-vesicular structures underneath the PM (Fig. 2.2D; arrows). Interestingly, HA-MICAL-L1 was also found associated with these structures (Fig. 2.2E; arrows) and partially co-localized with Src-GFP (Fig.
suggesting that MICAL-L1 specifically associates with Src and not other Src family members such as Yes and Fyn. Although Src and MICAL-L1 could be weakly observed in a complex (Fig. 2.2G), it is likely that the insolubility of membrane-associated Src renders this interaction minimal (Fra, Williamson et al. 1994). Moreover, the failure to detect an interaction by yeast two-hybrid experiments suggests that the interactions are indirect (data not shown). Our immunofluorescence data, coupled with a weak biochemical interaction, supports the notion of a transient MICAL-L1-Src interaction, consistent with previous Src trafficking papers (Sandilands, Cans et al. 2004; Tu, Ortega-Cava et al. 2010).

Given that Src localized to MICAL-L1-decorated endosomal structures, we hypothesized that MICAL-L1 may regulate Src activation and transport. Src autophosphorylation on tyrosine 419 (in humans) is required for kinase activity (Feder and Bishop 1990; Reuter, Findik et al. 1990). Accordingly, Y419 phosphorylation is a surrogate marker for Src activation. In control siRNA-treated HeLa cells, EGF stimulation caused a time-dependent increase in Src activation (Fig. 2.3A, lanes 1-3) that was attenuated in MICAL-L1-depleted cells (Fig. 2.3A, lanes 4-6). This indicates that MICAL-L1 is required for EGF-induced Src activation. In agreement with these findings, MICAL-L1 decreased the amount of active Src localized to paxillin-positive focal adhesions (Fig. 2.3, compare B to C; see line-scan analysis).

To address the localization of Src in MICAL-L1-depleted cells, we used an antibody that recognizes both the phosphorylated and non-phosphorylated
forms of Src. Src localization was then assessed by immunofluorescence microscopy. In serum-starved cells, the majority of Src localized to the perinuclear region in control (Fig. 2.3D) and MICAL-L1-depleted cells (Fig. 2.3F). EGF stimulation caused Src transport from the perinuclear region to PM in control-siRNA cells (Fig. 2E). However, in MICAL-L1-depleted cells, the majority of Src remained in the perinuclear region upon EGF-stimulation (Fig. 2G; arrows), indicating that MICAL-L1 is required for Src transport from the interior of the cell to the PM.

Given that it is unresolved whether Src localizes to the ERC (Sandilands, Cans et al. 2004) or late endosomes (Kasahara, Nakayama et al. 2007; Tu, Ortega-Cava et al. 2010), we addressed the nature of the perinuclear region in which Src is retained upon MICAL-L1-depletion. We stimulated cells with EGF and immunostained for Src and transferrin receptor (TfR), a well-established marker of the ERC that is absent in late endosomes. Compared to EGF-stimulated control cells, which display modest overlap between Src and TfR (Fig. 2.3H), MICAL-L1-depletion resulted in substantial accumulation and overlap of both Src and TfR in the ERC (Fig. 2.3I; yellow arrows). Quantification of Src fluorescence in the TfR-positive ERC indicated that it was significantly higher in MICAL-L1 KD cells (Fig. 2.3J).

While we hypothesized that MICAL-L1 knockdown (KD) directly impacts Src localization and activation, it was necessary to rule out the possibility that MICAL-L1 might indirectly impair EGF-induced Src activation by affecting EGFR surface levels or endocytosis. Using flow cytometry with fluorescently labeled
EGF, we found that MICAL-L1-depleted cells displayed a small increase in EGF binding capacity compared to control cells (Fig. 2.4 A, B), indicating that lack of EGFR on the plasma membrane is not the underlying cause of reduced Src activation upon MICAL-L1 KD. Next, cells were pulsed with unlabeled EGF for 15 minutes and immunostained with antibodies against EGFR to evaluate if MICAL-L1 KD affects EGFR internalization. MICAL-L1 KD cells displayed no overt EGFR trafficking defects (Fig. 2.4 C-F). Lastly, MICAL-L1-depletion did not affect EGFR phosphorylation upon stimulation with EGF (Fig. 2.4 G; compare levels of EGFR-pY1068). Of note, Src does regulate EGFR trafficking (Ware, Tice et al. 1997; Wilde, Beattie et al. 1999; Donepudi and Resh 2008). Thus, MICAL-L1 KD may have subtle effects on Src-dependent EGFR trafficking. Given that MICAL-L1 KD has minimal effects on EGF binding and EGFR activation, we concluded that decreased Src activation in MICAL-L1 KD cells is not due to indirect effects.

7.2 MICAL-L1 co-localizes with Src and is required for its recruitment to circular dorsal ruffles in human foreskin fibroblasts following PDGF stimulation

We next tested if MICAL-L1 co-localizes with Src in other cells such as normal human fibroblasts and whether the regulation of Src by MICAL-L1 is specific to EGF or constitutes a general regulatory mechanism. In Src null mouse embryonic fibroblasts, Src translocates from the ERC to the PM ruffles upon platelet-derived growth factor (PDGF) stimulation (Sandilands, Cans et al. 2004). However, the localization of endogenous Src in PDGF-stimulated
human fibroblasts has not been characterized. In human foreskin fibroblasts (BJ cells), Src localized to circular dorsal ruffles (CDR; compare Fig. 2.5 A to D, see arrow) following PDGF stimulation. Similarly, PDGF stimulation resulted in MICAL-L1 recruitment to CDR, where it co-localized with Src on tubulovesicular structures (Fig. 2.5 E and F; arrow, see insets), suggesting that the MICAL-L1/Src co-localization is physiologically relevant in normal human cells. MICAL-L1 also co-localized with other focal adhesion regulatory proteins such as phosphorylated FSK (Figure 2.5 G-I; arrow and arrowhead) and phosphorylated paxillin (Figure 2.5 J-L; arrows).

We next asked if Src localization to CDR in BJ cells is MICAL-L1-independent. Previous studies have demonstrated that Src is required for CDR formation and subsequent macropinocytosis (Mettlen, Platek et al. 2006; Veracini, Franco et al. 2006). Control and MICAL-L1 KD BJ cells were stimulated with PDGF for 10 minutes and stained with Src and phalloidin (to mark actin). In control cells, Src localized to actin-rich positive CDR (Fig. 2.6 A). In contrast, MICAL-L1 KD resulted in a significant reduction of Src recruitment to CDR (Fig. 2.6 B; quantified in Fig. 2.6 C). The efficiency of MICAL-L1-depletion was confirmed using immunoblot (Fig. 2.6 D). To assess if MICAL-L1 KD affected CDR maturation into macropinocytic vesicles, we quantified the number of cells containing enlarged Rab5 and rabankyrin-5 vesicles, two well-established markers of macropinosomes (Schnatwinkel, Christoforidis et al. 2004; Mettlen, Platek et al. 2006). Compared to control cells, in which 46% of cells contained macropinosomes after 20 minutes of
PDGF stimulation (Fig. 2.6 E-G; yellow arrows), only 10% of MICAL-L1 KD cells had macropinosomes (Fig. 2.6 H-J; quantified in K). Given that Src activation at the periphery has been shown to be required for CDR formation in mouse fibroblasts (Azimifar, Bottcher et al. 2012), it is interesting that CDR form in MICAL-L1 KD cells at similar rates to control cells. However, we found that MICAL-L1 KD CDR displayed altered morphology by live-cell imaging (Fig. 2.7 A-J). Control and MICAL-L1 KD cells were stimulated with PDGF for 4 minutes (Time 0:00) and then imaged every 2 minutes for approximately 1 hour. In control cells, CDRs were present in many cells at Time 0 (Fig. 2.7 A, yellow arrows). The CDRs progressively constricted and closed in most cells by 24 minutes (Fig. 2.7 B-D; yellow arrowheads). After 62 minutes (Fig. 2.7 E; Time 58:00), most cells had begun to migrate as demonstrated by an elongated cellular morphology and presence of a single lamellipodia. In contrast, while MICAL-L1 KD cells also formed CDRs (Fig. 2.7F; Time 0:15; white arrows), the CDRs were larger and appeared to collapse without first constricting (Fig. 2.7 G-I). The failure of constriction resulted in the formation of large, oval-shaped cells with broad lamellipodia (Fig. 2.7I; Time 20:15). Strikingly, these large MICAL-L1 KD cells failed to migrate in response to PDGF (Fig. 2.7 J; Time 58:15). The combination of our fixed confocal microscopy analysis and live-cell imaging strongly supports a dual role for MICAL-L1 in regulating Src recruitment to CDRs and the efficient closure of CDR into macropinosomes. Furthermore, MICAL-L1 is required for PDGF-induced migration.

7.3 MICAL-L1 is required for PDGF-induced focal adhesion turnover
The observation that MICAL-L1 KD cells failed to migrate in response to PDGF stimulation suggested to us that focal adhesion function might be compromised in MICAL-L1 KD cells given that integrins and other focal-adhesion proteins (such as paxillin and FAK, Fig. 2.5) rapidly redistribute to CDRs upon PDGF stimulation and that this redistribution is linked to focal-adhesion turnover and to migration (Gu, Noss et al. 2011; Hoon, Wong et al. 2012). We first addressed whether MICAL-L1 KD in human fibroblasts affected focal adhesions at steady-state. MICAL-L1-depleted BJ cells displayed larger, more prominent focal adhesions compared to control cells (Fig. 2.8, compare E to D for vinculin and G to F for paxillin). Furthermore, active Src levels were decreased at focal adhesions in MICAL-L1-depleted cells (Fig. 2.8, compare C to B), suggesting that focal adhesion dynamics may be impaired due to decreased Src recruitment to focal adhesions. Because Src kinase activity is required for focal-adhesion turnover (Fincham and Frame 1998), we hypothesized that loss of MICAL-L1 may impair PDGF-induced focal-adhesion turnover in BJ cells.

Following PDGF stimulation, Src and the focal adhesion protein vinculin co-localized along CDR in control cells (Fig. 2.9 A-C; arrows), in agreement with our findings and those from other labs that focal adhesion proteins redistribute to CDRs. However, MICAL-L1-depletion caused considerable Src retention in the perinuclear region (Fig. 2.9 E; arrowheads) and impaired vinculin redistribution to CDRs (Fig. 2.9 D). The data from Fig. 2.7 and 2.8 ruled out the possibility that the failure of vinculin to localize to CDR is due to loss of CDR formation in MICAL-L1 KD cells. Interestingly, MICAL-L1-depleted cells
appeared to display an increase in number and size of vinculin-positive focal adhesions relative to control cells.

To test if MICAL-L1-depletion impairs focal-adhesion turnover, which would explain the increased number of focal adhesions, we quantified the number of focal adhesions in PDGF-stimulated cells. As shown in Figure 2.9 G-I and quantified in Figure 5M, control-siRNA-treated cells displayed a significant time-dependent decrease in the number of vinculin-containing focal adhesions (serum-starved=24.6 ± 4.1; 15 min PDGF=14.5 ± 3.1). Under non-stimulated conditions, MICAL-L1-depletion caused a significant increase in focal adhesions/cell (serum-starved=56.2 ± 8.7) relative to control (Fig. 2.9 J and M). Moreover, stimulation of MICAL-L1-depleted cells did not lead to focal adhesion disassembly (PDGF=55.1 ± 8.8). Reintroduction of MICAL-L1 into MICAL-L1-depleted cells partially rescued focal adhesion number (SS=24.3 ± 3.3, PDGF=28.4 ± 0.71; quantified in Figure 2.9 M. Furthermore, reintroduction of MICAL-L1 to MICAL-L1 KD cells partially rescued PDGF-induced cellular elongation, a phenotypic marker we used to assess cell migration in fixed cells (data not shown).

To further address the impact of MICAL-L1-depletion on increased focal adhesion stability, we quantified focal adhesion size, as this is linked to impaired turnover (Webb, Donais et al. 2004). Focal adhesion size in BJ cells was empirically categorized into three pools: small (1-5 µm²), medium (6-10 µm²) and large (11-30 µm²). Figure 2.10 A-D is a representative example of fields of cells that have been quantified. PDGF stimulation of control BJ cells
(Fig. 2.10 C) caused the disassembly of larger focal adhesions that are present under serum-starved conditions (Fig. 2.10 A). The statistics of focal adhesion size distribution are depicted in Figure 2.10 E. The majority of focal adhesions in control cells are between 1-5 µm² (serum starved=93.5%, PDGF 97.5%). In contrast, MICAL-L1-depletion led to a decrease in the small focal adhesion population (88.1%) that does not change significantly following PDGF stimulation (89.8%).

While there are many Src substrates present at focal adhesions, FAK phosphorylation at position Y925 is crucial for focal-adhesion turnover (Deramaudt, Dujardin et al. 2011). If Src activity is decreased at focal adhesions in MICAL-L1-depleted cells, we hypothesized FAK-Y925 phosphorylation would be impaired. BJ cells were stimulated with PDGF and immunoblots were used to measure FAK-Y925 phosphorylation. In control cells, there was a sharp increase in Y925 phosphorylation following 5 and 15 min. PDGF stimulation (Fig. 2.10 F Lane 1-4). This rise in phosphorylation coincided with increased levels of active Src. However, MICAL-L1-depleted cells displayed impaired PDGF-induced activation of Src (Lane 5-8). Consequently, no increase in FAK-Y925 phosphorylation was observed. These data reinforce the notion that MICAL-L1 regulates the transport of active Src to focal adhesions, thus controlling focal adhesion turnover.

**7.4 MICAL-L1 regulates cell spreading**

Adhesion molecules such as integrin receptors mediate cell attachment to the extracellular matrix. Upon attachment, cell spreading requires the continual
formation and disassembly of focal adhesions and Src is crucial for this process (Huveneers and Danen 2009). To test if MICAL-L1 is required for cell spreading in BJ cells, control siRNA-treated or MICAL-L1-siRNA-treated cells were plated onto fibronectin-coated coverslips for 90 min. Control cells spread and polarized, displaying long actin stress fibers with vinculin-positive focal adhesions localized to the end of actin stress fibers at the cell periphery (Fig. 2.11 A).

In contrast, MICAL-L1-depleted cells failed to polarize on fibronectin-coated coverslips (Fig. 2.11 B-D). We observed several abnormal phenotypes in these cells characterized by: large round cells with prominent actin cables distributed throughout the cell (Fig. 2.11 B; arrows), polygonal cells with disorganized actin stress fibers (Fig. 2.11 C), or cells with a single, broad lamellipodium containing actin arcs (Fig. 2.11 D; arrowhead). MICAL-L1-depleted cells were also larger (3537 mm$^2 \pm 754$) compared to control cells (2098 mm$^2 \pm 455$) and contained more focal adhesions per cell (MICAL-L1-siRNA = 49.2 ± 10.7, compared to control = 21.6 ± 6.6; see Fig. 2.11 E and F).

Having established that MICAL-L1 is required for BJ cell spreading, we tested if integrin-induced Src activation was affected by MICAL-L1 depletion. When control treated BJ cells were plated onto fibronectin, Src activation increased compared to cells held in suspension (Fig. 2.11 G, lanes 1-4). MICAL-L1 KD caused a modest decrease in Src activation upon fibronectin plating (Lanes 5-8; arrow) and delayed the phosphorylation of FAK-Y925 (Fig. 2.11 G, lanes 5-8) compared to control cells. Reduced Src activation and Src-
dependent FAK phosphorylation is consistent with the impaired focal adhesion turnover we observed during cell spreading (Fig. 2.11 B-D).

7.5 MICAL-L1-depletion affects directional cell migration

Focal-adhesion turnover and spreading are both necessary for cell migration. We employed scratch wound assays to test if MICAL-L1 is required for fibroblast migration. Control BJ cells migrated into the wound within 24 h (Fig. 2.12 A-C). In comparison, MICAL-L1-depleted cells were impaired in wound closure (Fig. 2.12 D-F). Interestingly, Src and MICAL-L1 co-localize along the leading edge (Fig. 2.12 G and H; arrowhead) and along tubules at the cell front (Fig. 2.12 G and H; inset and arrow) of migrating cells. Since Src is required for cell polarization to the wound (Timpson, Jones et al. 2001; Magdalena, Millard et al. 2003), we assessed polarity in migrating fibroblasts using the orientation of the Golgi apparatus and the actin cytoskeleton as well established markers of polarization. While the majority of control cells displayed Golgi oriented to the wound and stress fibers that were perpendicular to the wound (Fig. 2.12 J; 68%), MICAL-L1-depleted cells were partially impaired in their ability to polarize to the wound (Fig. 2.12 K; 40%).

Cell migration requires formation and disassembly of focal adhesions at the cell front and focal adhesion disassembly at the cell rear (Wozniak, Modzelewksa et al. 2004). In control cells, there were increased numbers of focal adhesions as well as increased intensity of vinculin staining at the cell front compared to the dimmer, less frequent staining at the cell rear (Fig. 2.12 L). In comparison, MICAL-L1-depleted cells displayed an overall increase in
the number of focal adhesions (in agreement with our previous data) but failed to polarize in the direction of the wound (Fig. 2.12 M). Taken together, these results demonstrate the requirement of MICAL-L1 for polarized cell migration in BJ cells.

7.6 EHD1 is required for Src transport and activation and acts as a molecular pinchase on MICAL-L1 tubules to release Src from the ERC in response to EGF

Having established that MICAL-L1 co-localizes with Src and regulates its trafficking and activation in response to a variety of stimuli, we sought to understand the mechanism by which MICAL-L1 regulates Src. In the absence of a direct interaction between MICAL-L1 and Src, we postulated that MICAL-L1 regulates Src release from the ERC and its subsequent activation by recruiting vesiculating proteins such as EHD1. This hypothesis is further strengthened by the fact that we have previously shown that EHD1 also regulated focal adhesion turnover and cell migration in fibroblasts (Jovic, Naslavsky et al. 2007); however, the mechanism by which this occurred was unclear. We hypothesized that impaired Src recruitment to focal adhesions could explain these deficits. Indeed, EHD1 KD in HeLa cells led to a significant retention of Src in the TfR-positive ERC (Fig. 2.13 A-F; see arrows; quantified in G). In addition, EHD1 KD impaired EGF-induced Src activation (Fig. 2.13 H; compare control lanes 1-3 to EHD1 KD lanes 4-6).

We have recently shown that MICAL-L1 is a regulator of recycling tubule biogenesis in HeLa cells (Giridharan, Cai et al. 2013). Interestingly, MICAL-L1
may also facilitate the cleavage or vesiculation of recycling tubules by recruiting molecular pinchases such as EHD1 (Cai, Giridharan et al. 2013). Whether or not MICAL-L1 and EHD1 constitutively bind to one another in cells, or if EHD1 is recruited transiently to MICAL-L1 positive TREs by certain stimuli is unknown. Under steady-state conditions, EHD1 concentrated in the perinuclear region (Fig. 2.14 A) but was also found on a subset of MICAL-L1 tubules. MICAL-L1 also concentrated in the perinuclear region; however, MICAL-L1 tubules emanating from the ERC were also visible (Fig. 2.14 B) with many being EHD1-negative. Serum starvation resulted in the displacement of EHD1 from the ERC (Fig. 2.14 D) and a modest increase in MICAL-L1 tubulation and spreading of the tubular endosomes out of the ERC (Fig. 2.14 E). Surprisingly, EGF stimulation resulted in shortening of MICAL-L1 tubules that were displaced from the ERC to the cell periphery (Fig. 2.14 H; see arrows). In addition, EGF concentrated EHD1 to MICAL-L1 tubular structures (Fig. 2.14 G; arrows), suggesting that EGF may either enhance the recruitment of EHD1 to tubules or possibly stimulate EHD1 ATPase activity, which would promote the vesiculation of MICAL-L1-containing tubular recycling endosomes.

EGF-induced morphological changes to MICAL-L1 recycling tubules was unexpected and suggested that the release of Src from the ERC in response to EGF might be mediated by the recruitment of EHD1. Therefore, we tested if EGF-induced vesiculation of MICAL-L1 tubules was dependent on EHD1. Similar to our previous experiments, EGF stimulation led to shortening, peripheral scattering, and reduction of MICAL-L1 tubules compared to serum
starved in control cells (Compare Fig. 2.14 K to J; quantified in N). In contrast, EHD1 KD resulted in a modest increase of MICAL-L1 tubular area under serum-starved conditions (Fig. 2.14 L) that did not decrease significantly with EGF stimulation (Fig. 2.14 M; quantified in N). In summary, we conclude that MICAL-L1 and EHD1 regulate Src trafficking and activation. MICAL-L1 recruits EHD1 to perinuclear ERC endosomes allowing for the release of Src from ERC and transportation to the PM, where Src functions in mediating actin cytoskeletal rearrangement (CDR), focal adhesion disassembly, and cell migration (Fig. 2.15; see model).

8. Discussion and figures

Cell adhesion and migration require the correct spatiotemporal activation of many kinases, phosphatases, and GTPases, with Src kinase being central to the entire process (Huveneers and Danen 2009). Insufficient Src activation paralyzes the cell and prevents turnover of focal adhesions (Fincham and Frame 1998), while excessive Src activation can promote cancer cell spreading, invasion and metastasis (Wheeler, Iida et al. 2009). Accordingly, exquisite regulation of Src activity is paramount to normal cell behavior.

We found that Src localizes to MICAL-L1-positive tubular recycling endosomes in HeLa cells (Fig. 2.2) and that MICAL-L1 and its binding partner EHD1 are required for stimulation-induced Src transport and activation (Fig. 2.3 and 2.13). We also interrogated the localization of Src and MICAL-L1 in human fibroblasts (Fig. 2.5). We were surprised to find that in addition to small, presumably lipid-rich tubular structures (not shown), MICAL-L1 localized to
PDGF stimulated, actin-rich CDR where it co-localized with Src. While the cell-type differences in localization of MICAL-L1 are of interest, the fact that MICAL-L1 co-localized with Src in human fibroblasts and was crucial for Src recruitment and activation was consistent with our data in HeLa cells; thus we conclude that regulation of Src localization and activity by MICAL-L1 is general and not specific to one cell type.

Lastly, we found that MICAL-L1 recycling tubules (in HeLa cells) are highly dynamic. Specifically, EGF stimulation induced a rapid shortening of MICAL-L1 tubules (Fig. 2.14). The molecular mechanisms regulating this phenomenon remain enigmatic; however, our data clearly implicate EHD1 in mediating EGF-induced MICAL-L1 vesiculation. While EHD1 and other EHD family members are phosphorylated in response to serum (Fichtman, Ravid et al. 2008), it is unclear what role phosphorylation or other potential post-translational modifications have on EHD ATPase activity or endocytic membrane recruitment.

While our data provide new information regarding the regulation of Src transport out of the ERC (Fig.2.15 ERC-PM pathway), our data also highlight an important unknown in the Src endocytosis field, namely, what is the fate of Src following activation? We predict that Src, much like other endocytic cargo, may be either degraded or recycled subsequent to its activation. Below I will discuss certain scenarios that suggest Src may be recycled while in other cellular contexts, Src may be degraded.
One peculiar phenomenon that we observed in MICAL-L1- and EHD1-depleted cells were relatively elevated levels of active Src under serum-starved conditions compared to serum-starved control cells and MICAL-L1- or EHD1 KD-stimulated cells. This suggests loss of MICAL-L1 or EHD1 may trap a relatively larger pool of Src at the PM under serum-starved conditions (compared to control cells). Accordingly, it is possible that MICAL-L1 and EHD1 also play a role in the PM-ERC pathway (Fig. 2.15), as MICAL-L1 also regulates the transport of some receptors into the perinuclear ERC by its interaction with the Collapsin Response Mediator Protein-2 (CRMP2) and dynein, which modulate microtubule-based transport (Rahajeng, Giridharan et al. 2010). Interestingly, the localization of Src to the ERC is microtubule-dependent (Fincham, Brunton et al. 2000). Testing the role of MICAL-L1 or EHD1 in Src transport into the ERC would be experimentally challenging given that the predominant phenotype of MICAL-L1- or EHD1-depleted cells is the retention of Src in the ERC. An attractive alternative experimental approach may be to assess the effect of EHD3-depletion on Src localization and activation since the dominant role of EHD3 is to mediate the trafficking of recycling cargo into the ERC (Naslavsky, Rahajeng et al. 2006). I hypothesize that EHD3-depletion would result in the retention of Src in the periphery and perhaps promote prolonged Src-dependent signaling pathways.

In addition to recycling endosomes, there is also evidence that late endosomes and lysosomes regulate Src transport, which promotes the idea that Src may be degraded. In HeLa cells, over-expressed c-Src-GFP rapidly
shuttles back and forth from the plasma membrane to perinuclear regions that contain the lysosomal hydrolase cathepsin D (Kasahara, Nakayama et al. 2007). Over-expression of Src in HeLa cells induces macropinosome formation (Kasahara, Nakayama et al. 2007). Given that Src localizes to macropinosomes that eventually fuse with lysosomes, it is not surprising that over-expressed Src localizes with the lysosomal compartment. Furthermore, the ESCRT complex component Tsg101 regulates v-Src trafficking from the plasma membrane to lysosomes (Tu, Ortega-Cava et al. 2010). Using conditional Tsg101 knockout mouse embryonic fibroblasts, Tu et al. showed that early loss of Tsg101 (1-3 days) induces higher levels of active v-Src compared to control cells. On the other hand, chronic Tsg101 deletion caused a decrease in active v-Src to levels below those of control cells, but also increased Src protein stability.

In human fibroblasts, transient depletion of several ESCRT components resulted in the retention of endogenous active c-Src in enlarged early endosomes containing β1 integrins (Lobert and Stenmark 2012). Functionally, the ESCRT complex is best characterized for its role in the transport of endocytic cargo to multivesicular bodies; examples of such cargo include ubiquitinated RTK and β1 integrins (Lobert, Brech et al. 2010; Babst and Odorizzi 2013). Interestingly, Src ubiquitination is required for its degradation, and both Src ubiquitination and degradation are dependent on Src activation (Hakak and Martin 1999; Harris, Shoji et al. 1999). Lastly, constitutively active Src phosphorylates the ESCRT component Hrs and localizes with Hrs to
enlarged early endosome structures suggesting that Src may act directly on the ESCRT complex to mediate its own downstream trafficking (Bache, Raiborg et al. 2002). Indeed, loss of ESCRT function results in sequestration of Src in enlarged endosomes. The signaling consequences of this phenomenon remain to be explored, although, as Tu et al. show, the chronic sequestration of active v-Src in enlarged endosomes may impair its function in mediating cell migration (Tu, Ortega-Cava et al. 2010). Accordingly, we hypothesize that active Src may be ubiquitinated and transported from the plasma membrane to endosomes (or macropinosomes) and to the late endosomal-lysosomal compartment for degradation in an ESCRT-dependent manner.

Data from constitutively active Src mutants, as is the case with v-Src, suggests that Src ubiquitination is highly correlated with kinase activity and C-terminal phosphorylation and that ubiquitination mediates ESCRT-dependent lysosome trafficking and degradation. Several lines of evidence to support this notion: 1) v-Src is highly ubiquitinated compared to c-Src, although this ubiquitination is dependent on v-Src kinase activity; 2) ubiquitinated v-Src is degraded in a proteasome-dependent manner; 3) while chronic TSG-101 depletion impairs v-Src activity, it also increases v-Src protein stability (Hakak and Martin 1999; Harris, Shoji et al. 1999; Tu, Ortega-Cava et al. 2010). This suggests that the fate of ubiquitinated v-Src may be analogous to that of ubiquitinated RTK, such as EGFR.

c-Src stability is significantly decreased in CSK-/- cell lines (Harris, Shoji et al. 1999). This raises the idea that the fate of c-Src may be dictated by its
conformational state. For example, v-Src is constitutively active and therefore constitutively ‘open’. However, phosphorylation of c-Src by CSK promotes an intramolecular interaction between its c-terminal tail with its SH2 domain; accordingly, depletion of CSK likely renders c-Src mostly in the ‘open’ conformation.

These observations support the notion that CSK expression leads to Src C-terminal phosphorylation and recycling to the ERC (Fig. 2.15; ‘PM to ERC’ pathway). On the other hand, we predict that v-Src (or c-Src in CSK null cells) undergoes ubiquitination-dependent degradation via the ESCRT complex (Fig. 2.15; ‘PM to lysosome pathway). This is further supported by the fact that in v-Src-expressing fibroblasts, expression of the E3 ligase Cbl-c inhibits cellular transformation (Kim, Tezuka et al. 2004). Given that EHD4 is required for transport from the early endosome to the late endosome (Sharma, Naslavsky et al. 2008), I predict that EHD4 is required for this putative Src degradation pathway.

Alternatively, new lines of evidence suggest that the late-endosome/lysosome acts as a signaling platform, much like that of the early endosome and recycling endosomes. Indeed, localization of the mammalian target of rapamycin (mTOR) protein to lysosomes is critical for its downstream functions (Sancak, Peterson et al. 2008; Sancak, Bar-Peled et al. 2010). Therefore, v-Src may promote differential signaling activities through its interactions with the lysosome that are important during cellular transformation.
In conclusion, our data clearly demonstrate the importance of MICAL-L1 and EHD1 in regulating Src ERC-PM transport and activation. The finding that MICAL-L1-depletion affects Src-dependent processes such as focal adhesion dynamics, adhesion and migration underscores the *in vivo* relevance of the Src-MICAL-L1-containing tubular endosomes, and highlights the potential significance of MICAL-L1 and EHD1 in regulating non-receptor kinases.
Figure 2.1 Src structure and regulation. A) Schematic diagram of the linear domain arrangement of c-Src. B) Table showing key amino acids within each domain, and the function or post-translational modification of these residues. C) Model depicting the inactive, autoinhibitory conformation ('OFF') of Y527 c-terminal phosphorylated Src and the active, open conformation ('ON') of Y416 phosphorylated Src. Note that modular domain color-coding is synchronized with that shown in Figure 2.1 A.
Figure 2.2 Partial co-localization between MICAL-L1 and Src in mammalian cells. A-C) Immunofluorescence demonstrating partial co-localization of endogenous Src (A) along endogenous MICAL-L1-decorated tubular endosomes (B) in HeLa cells as marked by arrows in inset. D-F) Overexpressed Src-GFP (D) partially co-localizes with HA-MICAL-L1 (E) in SYF fibroblasts (arrows). G) Co-immunoprecipitation demonstrates that a small amount of endogenous MICAL-L1 immunoprecipitated with endogenous Src following EGF stimulation. Blue=DAPI, scale bar=10 µm. SS=serum-starved.
A) Western blotting showing MICAL-L1, Src-pY419, t-Src, and pan-actin levels under control and MICAL-L1 siRNA conditions with 5 min and 15 min EGF treatment.

B) Control siRNA treatment showing Src ERC fluorescence with 15 min EGF.

C) MICAL-L1 siRNA treatment showing Src ERC fluorescence with 15 min EGF.

D) Serum-starved cells showing Src ERC fluorescence.

E) 15 min EGF treatment showing Src ERC fluorescence.

F) Control siRNA treatment with 15 min EGF.

G) MICAL-L1 siRNA treatment with 15 min EGF.

H) Control siRNA treatment with 15 min EGF.

I) MICAL-L1 siRNA treatment with 15 min EGF.

J) Bar graph showing Src ERC fluorescence (arbitrary units) for control and MICAL-L1 KD conditions with 15 min EGF.
Figure 2.3  MICAL-L1-depletion in HeLa cells impairs EGF-induced Src activation and translocation out of the ERC.  A) Immunoblot of control (lanes 1-3) and MICAL-L1-siRNA (lanes 4-6) treated HeLa cells demonstrating reduced EGF-induced Src activation as measured by Src autophosphorylation (Src-pY419) upon MICAL-L1-depletion.  B,C) Control (B) and MICAL-L1 KD (C) cells were stimulated with EGF for 15 minutes, fixed and labeled with Src-pY419 (green) and paxillin (red). Profile analysis of two individual focal adhesions demonstrates recruitment of active Src in control (B) but not MICAL-L1 KD cells (C).  D-G) Under serum-starved (SS) conditions, total-Src (green) localizes to the perinuclear region (arrows) in control (D) and MICAL-L1 siRNA-treated cells (F). In response to EGF, Src translocates from the ERC to the plasma membrane in control cells (E) but is largely retained in the ERC in MICAL-L1 KD cells (G; arrows).  H-J) Cells were stimulated with EGF and stained for Src (green) and transferrin receptor (TfR; red). Note the increased overlap of Src and TfR at the ERC in MICAL-L1 KD cells (I; yellow arrows) compared to control (H).  (J) ImageJ was used to quantify Src fluorescence in the TfR-positive ERC after EGF stimulation and one-way ANOVA showed that there was significantly more Src in MICAL-L1 KD compared to control (p<.01) Error bar= S.E.M. Blue=DAPI, scale bar=10 µm.
**Figure 2.4** MICAL-L1-depletion in HeLa cells does not affect EGF binding capacity, EGFR internalization or EGFR activation. A,B) Control- (blue) and MICAL-L1 siRNA-treated (orange) HeLa cells were labeled with EGF-Rhodamine as described in the materials and methods and EGF surface binding was quantified using flow cytometry (red= unlabeled, background control). The histogram and graph are a representative done with ~10,000 cells C-F) Control (C,D) and MICAL-L1 KD (E,F) were serum starved (SS) and then pulsed with unlabeled EGF to stimulate EGFR internalization. Immunofluorescence demonstrates that EGFR internalization is similar in control (D) and MICAL-L1 siRNA cells (F). G) Immunoblot analysis of EGFR autophosphorylation in control and MICAL-L1 siRNA cells (additional siRNA oligonucleotides are to demonstrate specificity). MICAL-L1 KD has no effect on EGF-induced EGFR phosphorylation (compare lane 1-4 to 5-8).
**Figure 2.5** MICAL-L1 co-localizes with Src and focal adhesion proteins along circular dorsal ruffles (CDR) in human foreskin fibroblasts (BJ). A-F) BJ cells were serum-starved (SS) (A-C) or stimulated with 15 ng/ml PDGF (D-F) for 10 min. Immunofluorescence of Src (green) and MICAL-L1 (red) demonstrates their co-localization along CDR (arrows; see inset). G-L) BJ cells were stimulated with PDGF and stained for MICAL-L1 (green) and pY397FAK (red; G) or pY118paxilllin (red; J). Arrows depict co-localization along CDR. Arrowheads mark co-localization of MICAL-L1 and pY397FAK on macropinocytic-like vesicles. Blue=DAPI, scale bar=10 μm.
Control siRNA

MICAL-L1 siRNA

A

B

10 min. PDGF

10 min. PDGF

C

Src/Actin (Fluorescence)

0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

Control
MICAL-L1 KD

D

E

F

G

Control 50 nM
MICAL-L1 10 nM
MICAL-L1 25 nM
MICAL-L1 50 nM

H

I

J

K

Cells with macroinosomes/100 cells (%)

0
20
40
60
80

Control
MICAL-L1 KD

Rabankyrin-5
Rab 5
Merge

Actin

MICAL-L1

Scale bar: 10 μm
Figure 2.6 MICAL-L1 regulates Src recruitment to CDR. A-D) Control (A) and MICAL-L1 KD cells (B) were stimulated with PDGF for 10 minutes, fixed and stained with Src (green) and phalloidin to show F-actin (red). Src recruitment to CDR was then quantified using prolife analysis (C). Efficiency of MICAL-L1-depletion was measured by immunoblot (D). E-K) Control (E-G) and MICAL-L1 KD cells (H-J) were stimulated with PDGF for 20 minutes and stained with rabankyrin-5 (green) and Rab5 (red) to mark macropinosomes (yellow boxes). Cells containing macropinosomes were manually counted in 100 cells/experiment (K). Error bar= S.E.M. Blue=DAPI, scale bar=10 µm.
Figure 2.7 MICAL-L1-depletion affects CDR closure. A-E) Control cells were stimulated with PDGF and followed for times indicated in upper left corner of image. Yellow arrows denote progressive CDR closure. F-J) MICAL-L1 cells were treated and imaged as described above. White arrows denote impaired CDR closure.
Figure 2.8 MICAL-L1-depleted BJ cells show altered distribution of focal adhesion proteins at steady-state. A-F) Distribution of active Src (A,B) vinculin (C,D) and paxillin (E,F) in MICAL-L1-depleted fibroblasts compared to control cells. Arrows mark the accumulation of Src in intracellular vesicles in MICAL-L1-depleted cells. Blue= DAPI Scale bar= 10 µm.
**Figure 2.9** MICAL-L1 regulates PDGF-induced focal-adhesion turnover A-F) Representative images of control and MICAL-L1-depleted fibroblasts stimulated with PDGF for 10 min and stained for vinculin (green) and Src (red). Arrows denote co-localization of Src and vinculin at CDR in control cells while arrowheads mark the ERC-localized Src in MICAL-L1 depleted cells. G-L) Control and MICAL-L1-depleted fibroblasts were serum-starved (SS) (G and J) or stimulated with PDGF for 10 min. (H and K) or 15 min. (L and L). Focal adhesions are labeled with vinculin. M) The number of focal adhesions in SS or PDGF-stimulated cells was quantified using ImageJ. Error bars= S.E.M from three independent experiments. Total number of cells and focal adhesions are shown in Figure 2.10. For rescue, focal adhesions were quantified from 45 cells from three independent experiments (15/experiment). Tukey test was used to calculate statistical significance between treatments. Number of focal adhesions were significantly (p<.01) different between control ss and MICAL-L1 ss but not between control and MICAL-L1 rescue SS. There was also a significant difference between control PDGF and MICAL-L1 PDGF. MICAL-L1 rescue was significantly less than MICAL-L1 KD but was also significantly different than control PDGF (p<0.5). Blue=DAPI, scale bar=10 µm.
Threshold

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<th>Focal Adhesion Area</th>
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<tr>
<td>1-5 µm²</td>
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Total focal adhesions

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<td>8853.0</td>
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Total number of cells

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<tr>
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<td>161.0</td>
</tr>
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<td>122.0</td>
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**F**

<table>
<thead>
<tr>
<th>Control siRNA</th>
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<tr>
<td><strong>FAK-pY925</strong></td>
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<tr>
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<td><strong>Src-pY419</strong></td>
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<td><strong>t-Src</strong></td>
</tr>
<tr>
<td><strong>pan-actin</strong></td>
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**Figure 2.10** MICAL-L1-depletion impedes focal-adhesion turnover and leads to increased focal adhesion size. A-D) Images from Figure 2.9 were used to demonstrate size distribution quantification of focal adhesions in control and MICAL-L1-depleted BJ cells. Images were imported into ImageJ and fluorescence levels were thresholded to show focal adhesions. Focal-adhesion area was calculated using the ‘measure particles function’ and size distributions were set to group focal adhesions into three size categories: small (1-5 µm²), medium (6-10 µm²) and large (11-30 µm²). E) Breakdown of focal adhesion number and size distribution in serum starved (SS) and PDGF-stimulated cells. Data are presented as percent of focal adhesions within each area category for three independent experiments. Tukey Test was used to calculate statistical significance. For small focal adhesions, there were significantly less focal adhesions in PDGF treated MICAL-L1 KD cells compared to control. In contrast, MICAL-L1 KD cells had significantly more medium and large focal adhesions compared to control cells (p<.01). Scale bar=10 µm. F) Representative immunoblot of BJ cells stimulated with PDGF in the presence (lanes 1-4) or absence of MICAL-L1 (lanes 5-8). T-FAK, t-Src and actin were used as loading controls.
Actin

Control
MICAL-L1
siRNA

MICAL-L1
siRNA

Cell area μm²

Control  |  MICAL-L1 siRNA
--- | ---
0  | 1000  | 2000  | 3000  | 4000  | 5000

Focal Adhesions/Cell

Control  |  MICAL-L1 siRNA
--- | ---
0  | 50  | 60  | 70

G

Control siRNA  |  MICAL-L1 siRNA
---  |  ---
Suspension  |  15 min. FN  |  45 min. FN  |  90 min. FN  |  Suspension  |  15 min. FN  |  45 min. FN  |  90 min. FN

MICAL-L1
FAK-pY925
FAK
FAK-pY925
Src-pY419
t-Src
pan-actin
**Figure 2.11** MICAL-L1 is required for proper cell spreading on fibronectin and optimal integrin-induced Src activation. A-D) Control (A) and MICAL-L1-depleted cells (B-D) were serum starved (SS) in suspension for 1 h and then plated onto 10 µg/ml fibronectin-coated coverslips for 90 min and stained with phalloidin-488 (green) and vinculin (red). In spreading MICAL-L1-depleted cells, prominent short actin cables (B; arrows) and dorsal actin arcs (D; arrowhead) were observed. Scale bar=10 µm. E) Average cell area was quantified from three independent experiments (control n=65, MICAL-L1 siRNA n=66) using Pascal LSM image examiner. Error bars= S.E.M. One-way ANOVA demonstrated a significant increase in MICAL-L1 KD cell area compared to control cells (p<.01). F) Average number of focal adhesions/cell were quantified from three independent experiments (control n=40, MICAL-L1 siRNA n=53). There were significantly more focal adhesions/cell in MICAL-L1 KD cells (p<.01) G) Representative immunoblot of BJ cells held in suspension or plated onto fibronectin-coated plates in the presence (lanes 1-4) or absence of MICAL-L1 (lanes 5-8). Arrow denotes Src band. T-FAK, t-Src and actin were used as loading controls.
Figure 2.12 MICAL-L1 is required for cell migration. A-F) BJ cells were grown to confluence on fibronectin-coated coverslips and a scratch wound was applied. Monolayers were washed and incubated in low-serum medium for the times indicated, fixed, and stained with phalloidin. Images were taken with a 10X objective and are representative of three independent experiments. Yellow line denotes scratch boundary. G-I) Cells were wounded, allowed to migrate for 6 h, and then stained for Src (G) and MICAL-L1 (H). Arrowhead marks co-localization along the leading edge. Arrows in the inset show co-localization along tubulo-vesicular structures. J-N) Loss of MICAL-L1 impairs wound polarization. Cell monolayers were wounded and allowed to migrate for 6 h prior to fixation and then stained for phalloidin and GM130 (J,K) or vinculin (L,M). + and – denote proper polarization of the Golgi apparatus towards the wound, or lack of it, respectively. Representative images of three independent experiments are shown (J-M) and quantified (N, statistically significant p<.05). Asterisk= direction of wound. Error bars= S.E.M. Blue= DAPI, scale bar= 10 µm.
Figure 2.13 EHD1 is required for EGF-induced Src translocation and activation in HeLa cells. A-G) Control cells (A-C) and EHD1 siRNA cells (D-F) were stimulated with EGF and stained with Src (green) and TfR (red). EHD1 siRNA results in retention of Src in the ERC (overlap between Src and TfR; compare C to F). (G) The level of Src fluorescence in the ERC was quantified from three independent experiments and found to be significantly higher in EHD1 KD cells (One-way ANOVA, p<.01). H) Immunoblot demonstrating impaired Src activation in EHD1 siRNA cells (lanes 4-6) compared to control cells (lane 1-3). Pan-actin and t-Src were used as loading controls. Error bars= S.E.M. Blue= DAPI, scale bar= 10 µm.
Steady State

Serum Starved

15 min. EGF

Control siRNA

EHD1 siRNA

**MICAL-L1 tubular area (µm²)**

<table>
<thead>
<tr>
<th></th>
<th>Control siRNA SS</th>
<th>Control siRNA EGF</th>
<th>EHD1 siRNA SS</th>
<th>EHD1 siRNA EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average</strong></td>
<td>5.0</td>
<td>10.0</td>
<td>15.0</td>
<td>20.0</td>
</tr>
<tr>
<td><strong>Standard Error</strong></td>
<td>±0.5</td>
<td>±1.0</td>
<td>±1.5</td>
<td>±2.0</td>
</tr>
</tbody>
</table>
Figure 2.14 EHD1 is required for EGF-induced MICAL-L1 tubule vesiculation. A-I) Compared to steady state (A-C) and serum-starved cells (SS) (D-F), EGF treatment (G-I) induces increased recruitment of EHD1 to MICAL-L1-positive tubules (arrows) resulting in the cleavage and vesiculation of MICAL-L1 tubules. J-N) EHD1 siRNA impairs vesiculation of MICAL-L1-decorated tubular endosomes in response to EGF. In control cells, EGF stimulation leads to a reduction in the MICAL-L1 tubular area (compare K to J; quantified in N). EHD1 siRNA results in a modest increase in the MICAL-L1 tubular area in SS cells (L). In the absence of EHD1, EGF stimulation does not induce vesiculation of MICAL-L1-decorated tubular endosomes (M; quantified in N). Tubule area was quantified from three independent experiments (at least 30 cells/experiment). Tukey test demonstrated that there were significant increases in MICAL-L1 tubular area in SS and EGF treated EHD1KD cells compared to control (p<.01). There was not a significant difference between SS EHD1KD and EGF treated EHD1 KD cells. Error bars= S.E.M. Blue= DAPI, scale bar= 10 µm.
Figure 2.15 Schematic model depicting the proposed roles of MICAL-L1 and EHD1 in mediating Src translocation from the ERC to the plasma membrane in response to growth factor or integrin stimulation as well as potential recycling or degradation pathways.
Chapter 3

Novel functions for the endocytic regulatory proteins MICAL-L1 and EHD1 in mitosis

This chapter was derived from:

9. Introduction
   9.1 The cell cycle

Many tissues in the adult human body rely on continuous cell proliferation to replace damaged or dying cells. The replenishment of cells requires cell division, a step-wise process wherein a proliferation competent diploid cell in G1 phase, which contains 2 copies of each of the 23 chromosomes, replicates the entirety of its genome (S phase and G2 phase) and then equally segregates the replicated DNA into two daughter cells (M phase). The fidelity of cell division is crucial, as the inability to correctly replicate DNA or segregate DNA into two daughter cells leads to chromosome mutations, chromosomal loss, or deviations from diploidy (aneuploidy); all of which are collectively contribute to genomic instability. Accumulation of these deleterious events is thought to lead to cancer (Sandal 2002). Thus, understanding the mechanisms that regulate each phase of the cell cycle has important implications in both understanding the pathogenesis of diseases such as cancer as well as for finding novel therapeutic strategies. While endocytic trafficking is a key component of each phase of the cell cycle, it functions directly in regulating the M phase, specifically cytokinesis (Barr and Gruneberg 2007; Montagnac, Echard et al. 2008). Cytokinesis is the final step of the cell cycle and is the mechanism by which two daughter cells are created from one mitotic cell. Prior to discussing the important functions of endocytic proteins during cytokinesis, it is necessary to first discuss how ‘the three M’s of cytokinesis: microtubules, motors, and microtubule associated proteins (MAPs)’ (Glotzer 2009) function to construct the central spindle.
9.2 Stages of cytokinesis, central spindle assembly, the three M’s of cytokinesis

Cytokinesis can be split into several distinct stages: 1) assembly of the tubulin-rich central spindle; 2) furrow ingression via actin and actino-myosin-dependent contractility, which leads to the compression of the central spindle into the intercellular bridge (ICB) and mid-body; 3) stabilization of the ICB and mid-body; 4) abscission, which is a poorly understood process that promotes the destruction of the ICB and termination of cytokinesis. A schematic representation of cytokinesis progression as well as the mechanisms that lead to cytokinetic failure is shown in Figure 3.1 A. Immunofluorescent images shown in Figure 3.1 B depict actin and microtubule cytoskeletons changes during cytokinesis progression in vivo. While each step in the process of cytokinesis is essential, the first step of central spindle assembly is arguably the most important. The assembly of the central spindle dictates all downstream events; thus, I will now focus on the intricacies of the central spindle organization and the key protein machinery that is involved in the assembly and stability of the central spindle. Then I will highlight the role of the central spindle in coordinating furrow ingression before concluding the introduction by explaining the roles of endocytic recycling proteins during cytokinesis.

The central spindle is a bipolar structure that consists of highly bundled microtubules (Fig. 3.1 B; yellow arrow). The minus ends are focused just distal to the chromosomes with the plus ends of each half of the central spindle inter-digitating in an anti-parallel manner at the central spindle equator (marked by
the negative black space found between the central spindle halves). Despite the importance of the central spindle, the exact origin(s) of the microtubules that compose the central spindle is/are unclear. The centrosome is one possible origin given that it is the primary microtubule nucleation center. Interestingly, central spindle microtubules are not attached to the centrosome, suggesting that microtubules are transported through an undefined mechanism (Rusan and Wadsworth 2005). In addition to centrosomally-derived microtubules, de novo microtubule polymerization occurs distal to the chromosomes (Uehara and Goshima 2010).

By comparison, the proteins that regulate central spindle bundling and stability have been well characterized in mammalian and invertebrate systems (reviewed in (Glotzer 2009)). Several MAPs and kinesins accumulate at the overlapping central spindle plus ends and are required for microtubule bundling. The MAP Protein Regulator of Cytokinesis 1 (PRC1) and its binding partner KIF4a are absolutely required for central spindle assembly and microtubule bundling at the plus end terminal (Jiang, Jimenez et al. 1998; Mollinari, Kleman et al. 2002; Kurasawa, Earnshaw et al. 2004). In addition to PRC1/KIF4a, the hetero-tetrameric centralspindlin complex, which is composed of the atypical kinesin protein Mitotic Kinesin Like Protein1 (MKLP1) and the Rho family GTPase RacGap1, also promote microtubule bundling (Mishima, Kaitna et al. 2002). As one might expect, the centralspindlin complex controls Rho family activation. The centralspindlin complex promotes furrow ingression through the precise activation of RhoA at the central spindle equator by recruiting the RhoA

9.3 Endocytic recycling proteins and cytokinesis

Recycling endosomes regulate cytokinesis mainly by two mechanisms: 1) Delivery of membranes to the ingressing furrow and then the ICB, thereby stabilizing these structures; and 2) Controlling the transport of regulatory proteins that influence cytoskeletal and lipid dynamics to the furrow and ICB. Initial studies in *Drosophila* embryos demonstrated that endocytic regulatory proteins such as the GTP-binding protein Rab11 and its interacting partner Nuclear Fallout (the *Drosophila* homolog of the mammalian Rab11 Family Interacting Proteins 3, FIP3) are required for cellularization (Pelissier, Chauvin et al. 2003), a process akin to cytokinesis. In mammalian cells, depletion of Rab11 or FIP3 results in cytokinesis failure, leading to the accumulation of tetraploid cells with multiple nuclei (Wilson, Fielding et al. 2005).

A key component of endocytic trafficking is the tethering of endosomes at their target organelles. The exocyst, an octameric protein complex that mediates the tethering of secretory vesicles to the plasma membrane during cytokinesis (Gromley, Yeaman et al. 2005), is also required for the tethering of Rab11/FIP3 endosomes at the ICB (Fielding, Schonteich et al. 2005). FIP3 interacts with both Rab11 and the GTP-binding protein Arf6, forming a tertiary complex. Arf6 mediates the tethering of FIP3 endosomes to the ICB by interacting with the exocyst component Exo70. Expression of a dominant-
negative, GDP-locked Arf6 or siRNA-depletion of Exo70 impairs FIP3 recruitment to the ICB and thus disrupts cytokinesis (Fielding, Schonteich et al. 2005). Additionally, Syntaxin 16, a soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) protein, has been implicated in vesicle fusion during cytokinesis (Neto, Kaupisch et al. 2013).

Recent studies have shed new light on additional factors that recruit FIP3 to the ICB and the role of FIP3 endosomes in regulating ICB actin dynamics. For example, FIP3 directly binds to RacGAP1 (Simon, Schonteich et al. 2008). During late cytokinesis RacGAP1 acts as a tether for FIP3 containing endosomes and is required to prevent FIP3 endosome displacement from the ICB (Simon, Schonteich et al. 2008).

Changes in microtubule stability are also required for FIP3 localization to the ICB. Microtubule bending and depolymerization precede FIP3 endosome fusion at the ICB (Schiel, Park et al. 2011), which enhances ICB plasma membrane dynamics leading to secondary ingression, a rapid constriction of the ICB from a thickness of approximately 1-2 µm to 0.1 µm. While decreases in microtubule content are required for thinning of the ICB, depolymerization of cortical actin filaments is also required for both ICB thinning and abscission. FIP3 endosomes mediate the delivery of p50RhoGAP/ARHGAP1 and SCAMP2/3, which coordinate actin depolymerization at the ICB, allowing for secondary ingression (Schiel, Simon et al. 2012), and abscission. There are two separate, but likely inter-connected models regarding the molecular machinery that mediates abscission (Schiel and Prekeris 2011). The first
models suggests that FIP3 endosome-dependent secondary ingression mediates ICB thinning that creates a suitable structure wherein the ESCRT is able to assemble and mediate abscission via a mechanism analogous to its function in membrane scission during multivesicular body formation or retroviral budding (Carlton and Martin-Serrano 2007; Wollert, Wunder et al. 2009; Elia, Sougrat et al. 2011). CEP55, a centrosomal protein that localizes to the midbody, binds to and recruits the ESCRT component tumor susceptibility gene 101 (TSG 101) and the ESCRT accessory protein Alix. Alix and TSG101 then recruit ESCRT III component CHMP4B, which mediates membrane constriction and abscission. Alternatively, FIP3 endosomes may not be required for the terminal event of abscission. The ESCRT complex alone, through CHMP4B polymerization into long spiral filaments (Hanson, Roth et al. 2008), may mediate abscission. Thus, abscission may be ESCRT-dependent or recycling endosome-dependent. It is likely that the two models are not mutually exclusive (Schiel and Prekeris 2011).

Rab35 recycling endosomes are also required for abscission (Kouranti, Sachse et al. 2006; Chesneau, Dambournet et al. 2012). Rab35 binds to the phosphoinositide phosphatase OCRL and regulates its delivery to the ICB (Dambournet, Machicoane et al. 2011). OCRL dephosphorylates phosphatidylinositol-4,5-bisphosphate, thereby causing actin depolymerization on the ICB. Decreased ICB phosphatidylinositol-4,5-bisphosphate levels likely attenuate the activity of Arp 2/3, an actin polymerization promoting complex. SiRNA-mediated depletion of Rab35 or OCRL impairs abscission, leading to
daughter cells that remain connected by abnormally long and stable ICB with increased actin content.

Given the importance of recycling endosome delivery in controlling cytokinesis, it is crucial to understand the regulatory proteins involved in recycling endosome dynamics during cytokinesis. Given the established role of MICAL-L1 and EHD1 in regulating recycling during interphase and the necessity of recycling endosome delivery to the ICB for the completion of cytokinesis, we hypothesized that MICAL-L1 and EHD1 are required for cytokinesis and perhaps pre-cytokinesis events in light of recent findings that suggest mitotic functions for Rab11/FIP3 endosomes prior to cytokinesis (Hehnly and Doxsey 2014).

10. Materials and Methods

Reagents and Antibodies

Fibronectin, monastrol and MG132 were purchased from Sigma. Transferrin conjugated to AlexaFluor 568, Phalloidin-568, DAPI, as well as all secondary antibodies used for immunofluorescence were purchased from Molecular Probes. The following primary antibodies were used: EHD1 (Caplan, Naslavsky et al. 2002), MICAL-L1 (Novus Biologicals), affinity-purified rabbit polyclonal peptide antibody directed against the C-terminus of EHD2 (VERGPDEAMEDEDGEEGSDDEA) (AnaSpec), α-tubulin (Molecular Probes), anti-human anticentromere (ACA; Antibodies Inc.), pericentrin, giantin, MKLP1 and PLK1 (Abcam), Pan-actin (Millipore), Rab11 (US Biologicals), Rab35
(Protein Tech), Aurora B (Abnova; gift from Jixin Dong UNMC) and EEA1 (Cell Signaling Technologies).

*Cell Culture, siRNA Transfection and Rescue*

Cervical cancer cell line HeLa (ATCC-CCL2) and Normal human foreskin fibroblasts (BJ; ATCC-2522) were grown in DMEM (high glucose) containing 10% fetal bovine serum, penicillin/streptomycin (Invitrogen) and 2 mM glutamine. All siRNA and plasmid transfections were done in the absence of antibiotics. Pooled and individual oligonucleotides targeting human MICAL-L1, EHD2, Rab11a, Rab35, custom EHD1 siRNA (Sharma, Giridharan et al. 2009) and non-targeting control siRNA were obtained from Dharmacon. HeLa cells were transfected with 50 nM of SmartPool oligonucleotides (MICAL-L1, EHD2, Rab11a, Rab35) or 100 nM of individual oligonucleotides (EHD1) using Lipofectamine RNAiMAX (Invitrogen). Efficiency of protein knockdown was measured 48-72 h post-transfection by immunoblot or immunofluorescence. For EHD1 rescue experiments, HeLa cells at ~60% confluency were transfected with siRNA-resistant-GFP-EHD1 construct (Cai, Giridharan et al. 2013) using Lipofectamine 2000. After 2.5 h, DNA complexes were removed and fresh antibiotic-free medium was given to cells. Cells were then transfected with EHD1 siRNA as described above for 48 h and processed for immunofluorescence or immunoblot as described below. Control and MICAL-L1-depleted cells were transfected with FIP3-GFP or OCRL-GFP (gifts from Rytis Prekeris-University of Colorado Denver and Claudio Aguilar-Purdue
University, respectively) 24 h after siRNA transfection using Lipofectamine 2000.

**Immunoblotting**

Cell lysates were prepared by washing cells two times in ice-cold PBS. Cells were then scraped off the plate with a rubber policeman into ice-cold RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton-100, 0.5% sodium deoxycholate, 0.1% SDS, 1.8 mg/ml iodoacetamide, 1 mM orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM PMSF). Lysates were then clarified by centrifugation at 13,000 RPM at 4°C. Protein levels were quantified using the BCA assay (Biorad) and 20-30 µg protein lysate/sample was separated by 8% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in TBST (TBS +0.1% Tween 20) plus 5% dry milk (TBST-B) and then incubated overnight in primary antibody in TBST-B at 4 degrees Celsius. Membranes were washed with TBST and then incubated with HRP-conjugated goat anti-mouse (Jackson Research Laboratories) or donkey anti-rabbit (GE healthcare) secondary antibody for 1 h at room temperature. After three washes in TBST, membranes were incubated in SuperSignal West Chemiluminescence Substrate (Pierce) and developed using standard film-based techniques.

**Immunofluorescence**

Cells were treated as indicated in the text and then fixed in 3.7% paraformaldehyde in PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2
mM MgCl$_2$, pH 7.0) for 15 minutes at room temperature. For MICAL-L1
kinetochore fiber staining, cells were pre-extracted in PHEM buffer + 0.1%
Triton-X-100 (warmed to 37 degrees Celsius) for 1 minute. For calcium
treatment, cells were incubated in calcium buffer containing 100 mM PIPES, 1
mM MgCl$_2$, 1 mM CaCl$_2$, pH 7.0 for 10 minutes. Cells were then fixed as
described above. Cells were rinsed three times in PHEM and permeabilized in
0.5% Triton-X-100/PHEM for 15 minutes. The cells were incubated with
primary antibody in PHEM/1% BSA/0.02% Triton-X-100 for 1 h at room
temperature. After three washes in PHEM, cells were incubated with
appropriate fluorochrome-conjugated secondary antibodies (Molecular Probes)
plus DAPI for 1 h at room temperature. Cells were washed 3X in PHEM and
mounted in Fluoromount G.

Single plane or z-stack (slice size indicated in text) confocal images were
collected using Zeiss LSM5 Pascal laser confocal microscope with a Plan-
Apochromat 63X/1.4 oil objective or Plan-Apochromat 100X/1.4 oil objective (for
kinetochore images). For quantification, images were imported into ImageJ or
LSM Pascal Image Examiner and quantified as described in text. Images
presented in figures were imported into Adobe Photoshop CS, where they were
re-sized and formatted to 300 dpi resolution with minimal image manipulation
(whole-image adjustment of brightness was done using ‘levels’ function).

**Structured Illumination Microscopy (SIM)**

Cells were fixed and immunostained as described for confocal microscopy, and
mounted with Vectashield H-1000 mounting solution (Vector Laboratories). SIM
images were collected with a Zeiss ELYRA PS.1 illumination system (Carl Zeiss MicroImaging) using a 63X oil objective lens with a numerical aperture of 1.46 at room temperature. Three orientation angles of the excitation grid were acquired for each Z plane, with Z spacing of 110 nm between planes. SIM processing was performed with the SIM module of the Zen BLACK software (Carl Zeiss MicroImaging).

*Live cell imaging*

Cells growing in 6-well plastic dishes were imaged in an environmental chamber (19% oxygen, 5% carbon dioxide, 37 degrees Celsius) using an Olympus 1X81 inverted microscope with a 20X objective (NA 0.45). Images were gathered using a Hamamatsu Orca-ER camera every 10 min. for 36 hours. Slidebook 5.5.2 was used for image acquisition and processing.

*Statistics*

Data from ImageJ or LSM 5 Pascal Image Examiner were imported into Microsoft Excel. Mean and standard error of the mean were calculated from data obtained from three independent experiments. Statistical significance was calculated by One-way ANOVA and Tukey test (when comparing more than two samples) or Student’s T-test using Vassar stats program (www.vassarstats.net).

11. Results

11.1 MICAL-L1- or EHD1-depletion in HeLa cells impairs normal cell cycle progression

MICAL-L1 and EHD1 were depleted from HeLa cells using siRNA to test if they are required for cell-cycle progression. By immunoblot analysis, we
demonstrated >90% depletion of MICAL-L1 (Fig. 3.2 A) and EHD1 (Fig. 3.2 B) after 48 and 72 h of transfection. Control-siRNA cells contained a single, oval-shaped nucleus (Fig. 3.2 C, and D), while MICAL-L1-depleted cells displayed several abnormal nuclear phenotypes including bi-nucleation (Fig. 3.2 E and F; red arrow) and micro-nuclei/nuclei separated by ‘chromatin bridges’ (Fig. 3.2 E and F; purple arrows). EHD1-depletion led to a significant increase in bi-nucleated and multi-nucleated cells (Fig. 3.2 G and H, with quantitation shown in I; red arrows). By quantifying nuclear area, we observed that MICAL-L1-depleted cells contained a significantly smaller nuclear area than control nuclei. On the other hand, EHD1-depleted cells often contained one or more large nuclei and thus had a significantly larger nuclear area/cell (Fig. 3.2J), suggesting that MICAL-L1 or EHD1 KD perturbed DNA content.

We depleted MICAL-L1 using two different individual oligonucleotides to rule out off-target effects associated with siRNA pools. As shown, both MICAL-L1 siRNA oligonucleotides significantly depleted MICAL-L1 (Fig. 3.3 A) and increased the number of bi-nucleated and micro-nucleated cells compared to control-siRNA cells (Fig. 3.3 B-E). Introduction of a siRNA-resistant EHD1 construct partially but significantly rescued the bi-nucleation phenotype induced upon EHD-depletion (Fig. 3.3 F-M; quantified in L). As an additional control, we analyzed the impact of EHD2-depletion. EHD2 is a paralog that displays ~67% amino acid identity to EHD1, but localizes to the plasma membrane (Simone, Caplan et al. 2013) and regulates caveolar motility rather than membrane recycling (Hansen, Howard et al. 2011; Moren, Shah et al. 2012; Stoeber,
Stoeck et al. 2012). As demonstrated, EHD2-depletion did not induce bi-nucleation (Fig. 3.4 A-D). Overall, these data indicate a specific function for MICAL-L1 and EHD1 in the regulation of cell cycle.

11.2 MICAL-L1 and EHD1 are required for cytokinesis and transport of recycling endosomes to the ICB

Live imaging was then used to observe the phase in which EHD1 and MICAL-L1 KD promoted cell cycle defects. Control cells entered mitosis and completed cytokinesis in approximately 2-3 hours (Fig. 3.5 A). MICAL-L1-depleted cells also entered mitosis but remained attached via an elongated ICB for ~5 hours (Fig. 3.5B; arrow). Remarkably, some MICAL-L1 KD cells remained connected until the next mitotic division (Fig. 3.5C; arrows at 60 min mark the daughter cells from distinct mitoses that fuse to form a single bi-nucleated cell at 540 min). EHD1-depleted cells also displayed abnormal cytokinesis. Many cells displayed asymmetric furrow ingression early in cytokinesis (Fig. 3.5D; asterisks). EHD1-depletion also led to cytokinesis failure resulting in bi-nucleated cells (Fig. 3.5D; arrow).

We next used immunofluorescence to assess the localization of MICAL-L1 and EHD1 during cytokinesis given that they are required for cytokinesis completion. Both proteins localized near the ingressing furrow during early cytokinesis and to the ICB during late cytokinesis (Fig. 3.6 A-F; yellow arrows; see insets). MICAL-L1 also partially co-localized with Rab11 on the ICB during late cytokinesis (Fig. 3.6 G-I).
The delivery of recycling endosomes to the ICB is essential for cytokinesis (Wilson, Fielding et al. 2005). Since MICAL-L1 and EHD1 are both required for efficient recycling during interphase (Sharma, Giridharan et al. 2009), we assessed the localization of recycling endosomes marked by fluorescently labeled transferrin (Tf) during cytokinesis. We found that Tf-568 localized to the ICB during late cytokinesis in control cells (Fig. 3.6 J-L; yellow arrows; see inset). These Tf-labeled endosomes localized distal to a narrowing of the ICB, known as the site of secondary ingression (Fig. 3.6L; asterisks). Recycling endosome delivery to the distal ICB is required for secondary ingression and abscission (Schiel, Park et al. 2011; Schiel, Simon et al. 2012). Depletion of either MICAL-L1 or EHD1 caused Tf-endosomes to be retained at the base of the ICB (Fig. 3.6 M-R; red arrows; see insets). The lack of recycling endosome delivery to the distal ICB correlated with impaired secondary ingression in MICAL-L1 and EHD1 KD cells. Overall, our data implicated EHD1 and MICAL-L1 in the transport of recycling endosomes to the ICB, a process that is required for secondary ingression and the completion of cytokinesis.

11.3 Recruitment of MICAL-L1 to ICB is independent of EHD1, Rab11 and Rab35

Although MICAL-L1 and EHD1 are required for the completion of cytokinesis and the transport of Tf-containing endosomes to the ICB (this study), Rab11/FIP3 (Wilson, Fielding et al. 2005) and Rab35 (Kouranti, Sachse et al. 2006) have been previously implicated in the regulation of cytokinesis. How MICAL-L1/EHD1, Rab11 and Rab35 coordinate membrane recycling is not well
understood, although EHD1 does directly bind to FIP2 (Naslavsky, Rahajeng et al. 2006), a Rab11 effector that is not required for cytokinesis. Rab35 binds to MICAL-L1 through the MICAL-L1 C-terminal CC domain. Studies in HeLa cells as well as neuronal cells suggest that MICAL-L1 acts as a membrane hub for Rab35 and other GTP-binding proteins to coordinate recycling during interphase (Rahajeng, Giridharan et al. 2012; Kobayashi and Fukuda 2013; Kobayashi, Etoh et al. 2014).

We used siRNA-based genetic epistasis experiments to examine the order of recycling protein recruitment to the ICB. Immunoblot analysis demonstrated the specificity and efficacy of each siRNA treatment (Fig. 3.7 A). In control cells, MICAL-L1 localized to the distal ICB (Fig. 3.7 B). MICAL-L1 depletion resulted in a loss of MICAL-L1 signal at the ICB, demonstrating the specificity of the MICAL-L1 antibody (Fig. 3.7 C). EHD1-, Rab11-, or Rab35-depletion did not affect the recruitment of MICAL-L1 onto the ICB (Fig. 3.7 D-F) although all knockdowns affected MICAL-L1 distribution on the ICB, likely because of ICB morphology changes. Thus, we conclude that MICAL-L1 localizes to the ICB independent of Rab11, Rab35 and EHD1 during cytokinesis.

Endogenous Rab11 was concentrated at the distal end of the ICB near the midbody in control cells (Fig. 3.7G), in agreement with previous findings (Wilson, Fielding et al. 2005). However, MICAL-L1 KD impaired recruitment of Rab11 to the distal ICB (Fig. 3.7 H). Due to technical difficulties, we could not assess the localization of endogenous Rab35, thus, we used GFP-OCRL as a
marker for Rab35 endosomes, as its localization to the ICB is dependent on Rab35 (Dambournet, Machicoane et al. 2011). Under these conditions, MICAL-L1-depletion did not drastically impair the recruitment of GFP-OCRL to the distal ICB (Fig. 3.7 I and J).

Overall, we stress that our evaluation of the relationship between each recycling protein must be interpreted with caution. Without a mechanistic link between MICAL-L1 and Rab11, we cannot conclude if MICAL-L1-depletion directly impairs Rab11 localization to the ICB (see Discussion). Moreover, our analysis was done on fixed cells. While we staged cytokinetic cells based on previously reported morphological traits (Simon, Schontech et al. 2008), the use of live-cell imaging will be crucial to assess the temporal recruitment of each recycling protein to the ICB.

11.4 EHD1- but not MICAL-L1-, Rab11- or Rab35- depletion affects central spindle formation

Our live cell imaging analysis demonstrated that EHD1-depletion frequently resulted in asymmetric cell divisions (Fig. 3.4D; asterisk), suggestive of a defect during the furrowing process. Given that the centralspindlin complex is key to regulating the site of RhoA-mediated contraction during the early parts of cytokinesis (White and Glotzer 2012), we performed immunofluorescence analysis of MKLP1 localization during early cytokinesis in EHD1-depleted cells.

In control cells, punctate MKLP1 staining was observed in a narrow zone at the center of the central spindle (Fig. 3.8 A). While EHD1-depletion did not impair the localization of MKLP1 to central spindle microtubules, it did result
in a drastic widening of MKLP1 staining (Fig. 3.8 B). Importantly, this phenotype is specific to EHD1-depletion, as MICAL-L1-, Rab11- or Rab35-depletion did not significantly impair MKLP1 localization (Fig. 3.8 C-E), which is in agreement with previous studies showing that Rab11/FIP3 and Rab35 are required for the later stages of cytokinesis rather than early cytokinesis (Wilson, Fielding et al. 2005; Kouranti, Sachse et al. 2006; Simon, Schonteich et al. 2008; Dambournet, Machicoane et al. 2011). We found similar localization patterns for other central spindle components such as Aurora B and Polo-Like-Kinase 1 (Fig. 3.9 A-J). Using Structured Illumination Microscopy (SIM), we observed that MKLP1 puncta are found at the terminal ends of bundled microtubules at the central spindle equator (Fig. 3.8 F; single 0.110µm z-section shown). EHD1 KD resulted in disorganized and unbundled plus ends that did not terminate at the equator (Fig. 3.8 G). As a consequence, MKLP1 staining is elongated. This phenotype is reminiscent of KIF4-depletion (Hu, Coughlin et al. 2011). Further work is required to discern if EHD1 is directly involved in microtubule plus end dynamics or if EHD1 regulates the localization of KIF4 or other microtubule regulators to the central spindle (see Chapter 4). We speculate that the abnormal furrowing phenotype we observed upon EHD1-depletion is a consequence of elongated centralspindlin localization, which would likely broaden the RhoA activation zone. In support of this hypothesis, we found that bright cortical actin staining is concentrated at the ingressing furrow in control cells (Fig. 3.9 K; white arrow), however, EHD1-depleted cells displayed uniform cortical actin along the plasma membrane (Fig. 3.9L).
11.5 EHD1 and Rab35 regulate mitotic spindle orientation while MICAL-L1 controls spindle length

Recent findings have highlighted pre-cytokininetic roles for Rab11; therefore, we hypothesized that EHD1 or MICAL-L1 might also have pre-cytokinesis functions given that Rab11 localization and function require EHD1/MICAL-L1 during cytokinesis (Hehnly and Doxsey 2014). We used a well-characterized assay (Toyoshima and Nishida 2007) to test if MICAL-L1, EHD1 and Rab35 are required for spindle orientation. Control-, MICAL-L1-, EHD1- and Rab35-depleted cells were plated on fibronectin-coated coverslips for 48 h and then arrested in metaphase using MG132 for 2 h. MG132 is a proteasome inhibitor and, as such, inhibits the ubiquitin-dependent degradation of CyclinB and CDK1 by the ubiquitin E3 ligase APC/C (Potapova, Daum et al. 2006). The destruction of CyclinB and CDK1 is required for the metaphase-anaphase transition (Wolf, Sigl et al. 2007). Cells were then fixed and the centrosomes were labeled using anti-pericentrin antibodies. Confocal microscopy was used to acquire 0.5 µm optical sections through metaphase cells (Fig. 3.10 A; Z sections), and LSM5 Pascal imaging software was used to measure the angle between centrosomes (Fig. 3.10 B and C; α-angle) and the distance between centrosomes (Fig. 3.10D; pole-pole distance). Note that only bipolar cells with congressed chromosomes were analyzed and that the presence of other centrosomes in the XZ images are from surrounding interphase cells. While the majority of control cells and MICAL-L1-depleted cells had spindle angles of less than 5 degrees (Fig. 3.10 B and C), EHD1- and Rab35-depleted cells displayed
an increase in the number of cells with spindle angles greater than 10 degrees. The significant increase in spindle angle for EHD1- and Rab35-depleted cells suggests a general role for recycling proteins in regulating spindle orientation. Unexpectedly, MICAL-L1 KD significantly increased the length of the mitotic spindle but did not affect spindle orientation (Fig. 3.10 D). EHD1- and Rab35-depletion led to a small, but significant decrease in spindle length.

11.6 Effect of MICAL-L1-, EHD1-, and Rab35-depletion on kinetochores, inter-kinetochore tension and kinetochore fibers

Doxsey and colleagues recently demonstrated that Rab11 is required for chromosome alignment (Hehnly and Doxsey 2014). Unsynchronized control cells displayed an average inter-kinetochore distance of 1.02 +/- 0.013 µm (Fig. 3.11 A; quantified in E). MICAL-L1 KD metaphase cells had significantly shorter inter-kinetochore distances compared to control cells 0.89 +/- 0.015 µm (Fig. 3.11 B). Interestingly, EHD1-depleted cells had significantly wider inter-kinetochore distances (1.25 +/- 0.017 µm) compared to control cells (Fig. 3.11 C) while Rab35-depletion had no significant effect (1.05 +/- 0.012 µm; Fig. 3.11 D). In line with the putative function of MICAL-L1 on kinetochore fibers, we found that endogenous MICAL-L1 localized to detergent-resistant microtubules on the mitotic spindle in HeLa and BJ cells (Fig. 3.11 F-I).

Inter-kinetochore distance is maintained by dynamic interactions between kinetochores and microtubules (Cheeseman 2014; Sarangapani and Asbury 2014). We hypothesized that the effects of MICAL-L1- and EHD1-depletion on inter-kinetochore distance might be a result of altered kinetochore
fiber stability. We used a high calcium buffer to depolymerize all non-kinetochore microtubules (Weisenberg and Deery 1981). Fig. 3.12 A depicts the metaphase microtubule cytoskeleton prior to calcium-induced depolymerization in a cell treated with control-siRNA. Control, MICAL-L1-, EHD1-, or Rab35-depleted cells were then subjected to high calcium buffer for 10 min. prior to fixation and processing for immunofluorescence (Fig. 3.12 B-E). Compared to control cells, which had an average kinetochore fiber length of 3.05 +/- 0.06 µm, MICAL-L1-depleted cells had significantly longer kinetochore fibers (4.3 +/- 0.08 µm) while EHD1-depleted cells had significantly shorter kinetochore fibers (2.73 +/- 0.05 µm). Rab35-depleted cells did not display a significant difference in kinetochore fiber length (3.25 +/- 0.05 µm). We also found that MICAL-L1-depletion significantly increased cold-stable microtubules in the metaphase mitotic spindle (Fig. 3.13 A-H). Cold treatment effectively stops microtubule dynamics and thus inhibits growth. Therefore, microtubules that are static survive the cold-treatment. Given that increased stability of kinetochore attachments can impede the correction of merotelic chromosome attachments, we used SIM to visualize kinetochore/microtubule interactions. In control cells treated with calcium buffer as described above, punctate kinetochore structures localized to the ends of kinetochore fibers in an ‘end-on’ orientation (Fig. 3.13 G; white arrow). However, MICAL-L1 KD cells often displayed merotelic attachments, characterized by kinetochores attached to microtubules from both spindle poles (Fig. 3.13 H; yellow arrow). Lateral kinetochore attachments, where microtubules failed to terminate at
kinetochores, were also observed in MICAL-L1 KD cells (Fig. 3.13 H; white arrowheads).

12. Discussion and figures

The timing and precision of mitotic progression is paramount to insure genetic stability. Endocytic pathways are well suited to provide stringent spatio-temporal control over localization of mitotic regulators during each phase of mitosis. A variety of endocytic regulatory proteins control the steps of mitosis, and proteins such as Rab11/FIP3, Rab35, Arf6, ARH and dynamin are crucial for the successful completion of cytokinesis (Fielding, Schonteich et al. 2005; Wilson, Fielding et al. 2005; Kouranti, Sachse et al. 2006; Lehtonen, Shah et al. 2008; Ishida, Nakamura et al. 2011; Chesneau, Dambournet et al. 2012).

Rab11/FIP3, Rab35, and Arf6 are all regulators of membrane recycling, and by interacting with and localizing tethering proteins such as the exocyst, SNARE, or the ESCRT complex, influence membrane and cytoskeletal dynamics at the ICB.

Given that MICAL-L1 and EHD1 are required for transport of recycling endosomes to the ICB, we speculate that the MICAL-L1/EHD1 pathway is responsible for the delivery of membranes and cytokinetic regulators to the ICB. We show that MICAL-L1 localizes to the ICB independent of EHD1, Rab11 or Rab35. MICAL-L1-depletion impairs recruitment of Rab11 onto the ICB, while having no effect on the Rab35-dependent pathway, which is in agreement with studies in interphase cells showing that MICAL-L1 is downstream of Rab35 (Rahajeng, Giridharan et al. 2012; Kobayashi and Fukuda 2013). Further
studies will be required to elucidate the molecular mechanisms of how MICAL-L1 affects Rab11 recruitment. We stress that without direct evidence for an interaction between MICAL-L1 and Rab11, the impaired recruitment of Rab11 to the ICB may be secondary to defective microtubule depolymerization on the ICB or the presence of lagging chromosomes. Indeed, siRNA-depletion of the microtubule-severing enzyme Spastin increases ICB microtubule content and impairs FIP3 recruitment onto the ICB (Schiel, Park et al. 2011). As shown in Fig. 3.5, MICAL-L1 (and EHD1)-depleted cells often had enlarged, abnormally formed ICB. Furthermore, abscission (and possibly the recruitment of endosomes to the distal ICB) is inhibited in cells with lagging chromosomes in an Aurora B-dependent manner (Steigemann, Wurzenberger et al. 2009; Thoresen, Campsteijn et al. 2014). Given that we frequently observed lagging chromosomes in MICAL-L1-depleted cells, it is tempting to speculate that Rab11 transport is inhibited by an Aurora B-dependent mechanism in the presence of lagging chromosomes.

Our live cell imaging and morphological analysis of microtubules and the centralspindlin complex suggests that EHD1 is required for proper central spindle formation (Fig. 3.8). Currently, it is thought that central spindle microtubules emanate from non-centrosomal locations distal to the separating chromosomes (Uehara and Goshima 2010). Recent work by Doxsey and colleagues suggests that recycling endosomes may serve as sites for central spindle microtubule nucleation (Hehnly and Doxsey 2014). If EHD1-depletion affects recycling endosome localization during early cytokinesis, this may
explain the abnormal morphology of the central spindle microtubules.

Alternatively, we predict that EHD1 may regulate the localization of the kinesin protein KIF4. KIF4-depletion results in a similar early cytokinetic phenotype characterized by unorganized and improperly bundled central spindle microtubules and elongation of the central spindle components MKLP1, Aurora B and PLK1 (Hu, Coughlin et al. 2011).

In addition to the new roles for MICAL-L1 and EHD1 in cytokinesis, we found that EHD1 and MICAL-L1 are involved in pre-cytokinetic events. Why EHD1- and Rab35-depletion affects spindle orientation, whereas MICAL-L1-depletion elongates the mitotic spindle, presumably through stabilizing kinetochore microtubules (Fig. 3.13), requires further study. MICAL-L1 may perform non-endocytic ‘moonlighting’ functions (Royle 2013). MICAL-L1 is well-suited to perform non-endocytic functions given that it has a CH domain, a LIM domain as well as CC domains. Several mitotic microtubule regulators (i.e., Nuf2, Hec1, NDC80, EB1) that affect the stability and/or dynamics of kinetochore microtubules (Varma and Salmon 2012) have similar domains. MICAL-L1 has no known enzymatic activity, thus, MICAL-L1 likely recruits proteins such as microtubule de-polymerases to improperly attached kinetochores. MCAK is one such polymerase that is recruited to and required for correcting merotelic kinetochore attachments (Knowlton, Lan et al. 2006; Domnitz, Wagenbach et al. 2012). MCAK is also part of the machinery required to fix lateral kinetochore attachments (Shrestha and Draviam 2013), which we frequently saw in MICAL-L1-depleted cells. Without MICAL-L1, kinetochore
fibers become hyper-stable and the cells are unable to resolve abnormal, merotelic attachments, which occur frequently early in mitosis but are often corrected prior to the onset of anaphase (Cimini, Moree et al. 2003).

Although the precise mechanisms by which MICAL-L1 and EHD1 regulate mitosis remains unclear, we provide evidence that both proteins affect cytokinesis and pre-cytokinetic events. We suggest a model whereby MICAL-L1 performs ‘moonlighting’ functions during early mitosis. At the transition from early-to-late cytokinesis, MICAL-L1 resumes its more typical function as an endocytic regulatory protein that controls the delivery of recycling vesicles to the ICB. MICAL-L1, which is likely directly attached to microtubules, would then recruit EHD1 to the base of the ICB. EHD1 would then promote the vesiculation and release of recycling endosomes from the base of the ICB. We propose that MICAL-L1 and EHD1 then transport these endosomes to the distal ICB, where they control the fusion of recycling endosomes to the plasma membrane. This is supported by recent work demonstrating that EHD1 interacts with Snapin (Wei, Xu et al. 2010), a SNARE protein with a well-described role in cytokinesis (Gromley, Yeaman et al. 2005). Furthermore, EHD1 interacts with the exocyst (unpublished observations), which is also required for vesicle fusion at the ICB. Overall, our findings support roles for both EHD1 and MICAL-L1 at distinct steps of mitosis, widening their roles as cellular regulatory proteins.
Morphology of actin (green) and microtubule (red) cytoskeletons during cytokinesis progression

**Figure 3.1** Introduction to cytokinesis. A) Schematic representations of correct cytokinesis progression (left) and cytokinesis failure (right). Blue microtubules are mitotic spindle microtubules, green are central spindle microtubules, red marks the centrosome, while black denotes the chromosomes/DNA. B) Fixed HeLa cells were stained with tubulin and phalloidin to mark microtubules and actin, respectively, to demonstrate changes that occur during cytokinesis progression. Yellow area=central spindle while ‘C’ denotes area of chromosomes.
**A**

IB: MICAL-L1
IB: Actin

**B**

IB: EHD1
IB: Actin

**C**

DAPI

**D**

Tubulin/DAPI

**I**

Multi-nucleated cells (% of total)

**J**

Micro-nuclei/Bridged Nuclei (% of total)

**K**

Percent of cells in each size range
Figure 3.2 Depletion of MICAL-L1 or EHD1 in HeLa cells causes cell cycle defects. A and B) Immunoblots demonstrating the efficacy of MICAL-L1 (A) and EHD1 (B) siRNA-mediated knockdown. C-H) HeLa cells were transfected with control-siRNA (C and D), MICAL-L1-siRNA (E and F) or EHD1-siRNA (G and H) for 72 h and stained with α-tubulin (green) and DAPI (Blue). Purple arrows show micro-nucleation and bridged nuclei while red arrows mark bi-nucleated cells. I and J) Quantification of nuclear phenotypes from three independent experiments (n=300/experiment) expressed as a percentage of cells/experiment in each phenotypic category. K) Nuclear area per cell was quantified using the ImageJ 'analyze particles' function. n=100 cells/experiment, from three independent experiments. One-way ANOVA *p<0.05, **p<0.01. Scale bar=10 µm.
**Figure 3.3** Specificity of MICAL-L1- and EHD1-siRNA knockdown phenotypes. A-E) HeLa cells were transfected with control-siRNA or MICAL-L1-siRNA individual oligonucleotides for 72 h. Immunoblotting demonstrates the efficacy of MICAL-L1-depletion by oligo #1 and oligo #2. Immunofluorescence analysis of control- (B), MICAL-L1-siRNA oligo #1- (C) or MICAL-L1-siRNA oligo#2- (D) transfected cells stained with α-tubulin (red) and DAPI (blue). (E) Nuclear phenotypes were quantified from three independent experiments (150 cells/experiment). Data is presented as percentage of abnormal nuclei (micro-nucleated and bi-nucleated). F-L) HeLa cells were either mock transfected (Lipofectamine 2000 alone in F, I, and G, J) or transfected with siRNA-resistant-GFP-EHD1 (siResis-GFP-EHD1) for 2 h (H and K). Cells were then washed and transfected with control-siRNA (F and I) or EHD1-siRNA (G, J and H, K) for 48 h. Cells were fixed and stained with α-tubulin (red) and DAPI (blue). Yellow arrows mark multi-nucleated cells in G and H. (L) Quantification of multi-nucleation in control-siRNA, EHD1-siRNA and EHD1-siRNA + siRNA-Resis-GFP-EHD1 cells. Data are presented from three independent experiments, 200 cells/experiment. (M) Immunoblot analysis demonstrating depletion of endogenous EHD1 and exogenous expression of siResis-GFP-EHD1 in EHD1-depleted cells (black arrows). One-way ANOVA *p<0.05, **p<0.01. Scale bar= 10 μm.
**Figure 3.4** Depletion of the EHD family member, EHD2, has no gross effect on cell cycle. A) HeLa cells were transfected with control- or EHD2-siRNA for 48 h and subjected to immunoblot analysis. B-D) Cells treated as in A were processed for immunofluorescence (B and C) and multi-nucleated cells were quantified (D). Data are from three independent experiments (150 cells/experiment). Scale bar= 10 µm.
Figure 3.5 MICAL-L1- or EHD1-depletion leads to cytokinesis failure. A-D) HeLa cells growing on plastic dishes were transfected with control-siRNA (A), MICAL-L1-siRNA (B and C) or EHD1-siRNA (D) for 24 h. Live cells were then imaged by phase contrast every 10 minutes for ~36 h to follow progression through mitosis. (B) An arrow marks delayed abscission while in C, arrows (C) mark cytokinesis failure in MICAL-L1-depleted cells. In D, the asterisk denotes asymmetric division where there is unequal cell spreading during cytokinesis and the arrow points to cytokinesis failure in EHD1-depleted cells.
Early cytokinesis

Late cytokinesis

Late cytokinesis

Control

MICAL-L1-siRNA

EHD1-siRNA
Figure 3.6 MICAL-L1 and EHD1 are required for delivery of transferrin-containing recycling endosomes to the intercellular bridge (ICB). A-F) HeLa cells were co-stained with antibodies to endogenous EHD1 (green) and MICAL-L1 (red). EHD1 and MICAL-L1 localized near the ingressing furrow during early cytokinesis (A-C, yellow arrows; see inset) and on the ICB during late cytokinesis (D-F, yellow arrows; see inset). G-I) Hela cells co-stained with MICAL-L1 (green) and Rab11 (red) antibodies during late cytokinesis. J-R) HeLa cells were pulse-labeled with transferrin-568 for 1 h to reach equilibrium, fixed and then stained with α-tubulin antibody to mark the ICB (green) and with DAPI to mark the nucleus (blue). In control-siRNA treated cells (J-L), transferrin-568 localized to the tubulin-rich ICB (yellow arrows in insets, asterisk in L marks secondary abscission). In contrast, depletion of MICAL-L1 (M-O) or EHD1 (P-R) caused transferrin-568 retention at the base of the ICB (red arrows in insets). Maximum projections of 1 µm optical sections are shown. Scale bar= 10 µm.
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<th>EHD1</th>
<th>Rab11</th>
<th>Rab35</th>
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</table>

**siRNA:**
- Control-siRNA
- MICAL-L1-siRNA

**ICB (arrow):**
- ![Image](image26) | ![Image](image27) | ![Image](image28) | ![Image](image29) | ![Image](image30) |

**Rab 11:**
- ![Image](image31) | ![Image](image32) | ![Image](image33) | ![Image](image34) | ![Image](image35) |

**Rab 35:**
- ![Image](image36) | ![Image](image37) | ![Image](image38) | ![Image](image39) | ![Image](image40) |

**GFP-OCRL:**
- ![Image](image41) | ![Image](image42) | ![Image](image43) | ![Image](image44) | ![Image](image45) |
**Figure 3.7** Recycling regulatory protein requirements for their recruitment to the ICB. A) Immunoblot analysis demonstrating efficacy and specificity of siRNA-knockdown of indicated recycling proteins. B-F) HeLa cells were transfected with indicated siRNAs for 48 h, fixed and stained with MICAL-L1 (red), tubulin (green) antibodies, and DAPI (blue). G and H) HeLa cells were transfected with control or MICAL-L1-siRNA for 48 h, fixed and stained with Rab11 (green), tubulin (red) antibodies and DAPI (blue). I-J) HeLa cells were transfected with control or MICAL-L1-siRNA for 24 h and then transfected with the GFP-OCRL construct and incubated for another 24 h prior to fixation and immunostaining with tubulin antibody (red) and DAPI (blue). Yellow arrows mark the position of midbody on the ICB and the region of interest some in the insets. Scale bar=10 µm.
Figure 3.8 Depletion of EHD1 but not MICAL-L1, Rab11 or Rab35 affects central spindle formation. A-E) HeLa cells were transfected with the indicated siRNA for 48 h and then fixed and stained with MKLP1 (green), tubulin (red) antibodies and DAPI (blue). Maximum projections of 0.5 μm optical sections are shown. F and G) HeLa cells were transfected with control or EHD1-siRNA for 48 h and then fixed and stained with MKLP1 (green) and tubulin (red) antibodies and imaged by Structured Illumination Microscopy. Scale bar=10 μm.
siRNA:

<table>
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<th>MICAL-L1</th>
<th>Rab 11</th>
<th>Rab 35</th>
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<td>A</td>
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| A | Aurora B |
| B | EHD1     |
| C | MICAL-L1 |
| D | Rab 11   |
| E | Rab 35   |

Figure 3.9 Recruitment of Aurora B or PLK1 is not affected by depletion of MICAL-L1, EHD1, Rab11 or Rab35. Cells were transfected with the indicated siRNAs, fixed, and stained with Aurora B antibody (A-E) or PLK1 antibody (F-J). Control (K) and EHD1-depleted (L) cells stained with tubulin (green), phalloidin-568 (red) and DAPI (blue). Scale bar= 10 µm.
**A**

Control-siRNA-XZ

Pericentrin

DAPI

Merge

MICAL-L1-siRNA-XZ

EHD1-siRNA-XZ

Rab35-siRNA-XZ

**B**

![Graph showing spindle angle](image)

**C**

![Graph showing pole to pole distance](image)

**D**

![Graph showing spindle angle](image)
Figure 3.10 Role of recycling proteins in regulating mitotic spindle orientation and length. A-D) HeLa cells were plated onto fibronectin-coated coverslips and transfected with control-, MICAL-L1-, EHD1-, or Rab35-siRNA for 48 h. Cells were treated with the proteasomal inhibitor MG132 (10 µM) for 2 h to prevent metaphase-anaphase transition and then fixed and stained with pericentrin (red) to mark centrosomes and DAPI (blue). 0.5 µm optical sections were acquired through the entire depth of metaphase cells. LSM5 Pascal software was used to measure the α-spindle angle (B and C) and the distance between the two pericentrin labeled centrosomes (D, pole-pole distance). Results are from three independent experiments, 45 cells/experiment. One-way ANOVA **p<0.05.
siRNA: Control MICAL-L1 EHD1 Rab 35

A B C D

CREST/Tubulin

E

Inter-Kinetochore Distance (µm)

Control-siRNA MICAL-L1-siRNA EHD1-siRNA Rab35 siRNA

Metaphase Anaphase/Telophase

F G

HeLa

MICAL-L1

H I

BJ human fibroblasts
**Figure 3.11** Role of recycling proteins in regulating inter-kinetochore tension. A-D) Cells were transfected with indicated siRNAs for 48 h, fixed and stained with anti-centromere/kinetochore antisera (CREST, green) and tubulin (red) antibody. A single 0.2 µm optical section is shown for each condition. E) Inter-kinetochore distance was measured in >100 kinetochores from 10 representative cells across three experiments. Asynchronous HeLa cells (F and G) or BJ human fibroblasts (H and I) were subjected to Triton X-100 extraction prior to fixation (see methods) and then stained with anti-MICAL-L1 antibody (green) and DAPI (blue). Maximum projections of 1 µm optical sections are shown. One-way ANOVA **p<0.01.
siRNA: Control MICAL-L1 EHD1 Rab 35

**CREST/Tubulin**

Kinetochore Fiber Length (µm)

Control-siRNA MICAL-L1-siRNA EHD1-siRNA Rab35-siRNA

**G** Control-siRNA

**H** MICAL-L1-siRNA
Figure 3.12 MICAL-L1-depletion enhances kinetochore fiber length and leads to abnormal kinetochore-microtubule interactions while EHD1-depletion decreases kinetochore fiber length. A) HeLa cell fixed and stained with CREST anti-sera (green) and tubulin antibody (red) demonstrating presence of kinetochore and interpolar microtubules. B-E) HeLa cells were transfected with indicated siRNAs for 48 h, incubated in calcium containing buffer (Ca$^{2+}$) for 10 min, fixed and stained with CREST (green) and tubulin (red; a single 0.2 µm optical section is shown for each condition. F) Quantitation of kinetochore fiber length. >50 kinetochore fibers were measured from 10 representative cells across three experiments. G and H) Control and MICAL-L1-depleted cells were fixed and stained as above and analyzed by SIM. White arrow denotes ‘end-on’ attachment of microtubule to kinetochore. Yellow arrows in MICAL-L1-depleted cell shows abnormal kinetochore stretching while white arrowheads show ‘lateral’ microtubule-kinetochore attachment. One-way ANOVA**p<0.01.
Figure 3.13 MICAL-L1-depletion enhances kinetochore fiber cold-stability. A-F) HeLa cells were transfected with either control-siRNA (A-C) or MICAL-L1-siRNA (D-F) for 48 h and arrested in metaphase using MG132 (10 µM, 2 h). Cells were then placed on ice in ice-cold DMEM for 10 min. (A and D), 15 min. (B and E) or 30 min. (C and F) and then fixed and stained with α-tubulin (green), pericentrin (red) and DAPI (blue). Maximum projections of 1 µm optical sections are shown. G) Tubulin fluorescence of kinetochore fibers after 30 min. of cold-treatment was quantified using the ImageJ 'measure' function. Results are shown from three independent experiments (15 cells/experiment). H) Pole-pole distance was measured after cold treatment (three independent experiments, n=45 cells/experiment). Student's t-test **p<0.01. Scale bar= 10 µm.
Functions of MICAL-L1 and EHD1 in HeLa cell mitosis

Figure 3.14 Summary of MICAL-L1 and EHD1 functions during mitosis
Chapter IV

Summary and future directions
13. Summary

Overall, my work has uncovered several novel functions for the endocytic recycling regulators MICAL-L1 and EHD1. In my first body of work, we demonstrated that the non-receptor tyrosine kinase c-Src localizes to MICAL-L1-positive endosomes in HeLa and BJ (human fibroblast) cells. More importantly, we found that MICAL-L1 is required for Src localization and activation. Prior studies had shown that inactive Src localized to the perinuclear area and required transport from the perinuclear area to the cell surface for kinase activation and downstream signaling. Transient MICAL-L1 KD perturbed growth factor- and integrin-induced Src translocation and activation. Accordingly, Src-mediated events such as focal adhesion turnover, cell migration, cell spreading and actin rearrangements (CDR formation and closure) were all affected by MICAL-L1 KD. EHD1 was also required for EGF-induced Src activation in HeLa cells. Mechanistically, we provide evidence that EHD1 recruitment to TRE is enhanced by EGF-stimulation, leading to the vesiculation of MICAL-L1-positive TRE and the release of cargo such as Src from the ERC. Consequently, EHD1 KD abrogated EGF-induced TRE vesiculation. The dynamic changes to MICAL-L1-positive TREs following EGF stimulation was surprising, given that previous live imaging experiments had demonstrated that TREs are static structures. The molecular mechanism that mediates growth factor-induced and EHD1-dependent TRE vesiculation is unclear. The observation that EHD1 activity might be regulated by growth
factor stimulation suggests that post-translational modifications might play a role in regulating several facets of EHD1 biology. Analysis of high-throughput mass spectrometry data available on PhosphositePlus provides several candidate amino acid residues for phosphorylation and ubiquitination, most notably Y453 within the EH domain and several lysines K162 and K200 in the G-domain respectively. I hypothesize that tyrosine phosphorylation within the EH domain affects EH-mediated protein-protein interactions. Furthermore, ubiquitination or acetylation of lysine residues within the G-domain could affect EHD1 dimerization/oligomerization or ATPase activity. EGF stimulation is well known to induce global increases in tyrosine phosphorylation and ubiquitination, which are required to coordinate appropriate cellular responses to growth factor stimulation. Direct evidence for the existence of EHD post-translational modifications is limited, but our data suggests that EGF induces changes in localization and activity of EHD1, which strongly supports a role for post-translational modifications in modulating EHD1 activity.

In my second body of work, we found that EHD1 and MICAL-L1 play critical roles in the cell cycle. EHD1 and MICAL-L1, in coordination with Rab11 and Rab35 recycling pathways, are required for the successful completion of cytokinesis. MICAL-L1 or EHD1 KD increased the number of bi-nucleated cells, indicative of cytokinesis failure. Live imaging analysis confirmed that EHD1 and MICAL-L1 KD both led to post-furrowing cytokinesis failure. Rather surprisingly, we also found that MICAL-L1 and EHD1 participate in distinct functions during mitosis. MICAL-L1 KD enhanced kinetochore fiber stability and length, leading
to chromosome attachment defects and lagging chromosomes. On the other hand, EHD1 KD destabilized kinetochore fibers but was also required for proper central spindle assembly. The finding that EHD1 is required for the first step of cytokinesis distinguishes EHD1 from MICAL-L1, Rab11 and Rab35, which all play important roles during late cytokinesis but not early cytokinesis. EHD1 KD caused defects in central spindle bundling and also disrupted the correct targeting of the centralspindlin to the central spindle equator. Concentrating centralspindlin at the equator is required for limiting RhoA activation to the equatorial axis. Accordingly, EHD1 KD affected the furrowing process as demonstrated by the asymmetrical divisions we observed by live imaging. The function of EHD1 during central spindle assembly is unclear but it is likely that EHD1 directly modulates microtubule nucleation, stability and structure (see below).

14. Future directions: EHD functions beyond TRE biogenesis and vesiculation

14.1 EHDs as regulators of microtubule nucleation and release from centrosome

Work from our lab has demonstrated that EHD proteins, specifically EHD1 and EHD3, regulate TRE dynamics. EHD1 promotes TRE fission while EHD3 promotes TRE stability or fusion (Cai, Giridharan et al. 2013). One perplexing phenomenon regarding EHD biology is the apparent directionality to EHD mediated endocytic recycling, meaning EHD1 is required for transporting cargos out of the ERC and EHD3 is required for transporting cargos into the ERC. Interestingly, EHD1 and EHD3 also play opposing roles in the positioning
of other organelles such as LE/lysosomes (my unpublished observations), the retromer (McKenzie, Raisley et al. 2012; Zhang, Naslavsky et al. 2012; Zhang, Reiling et al. 2012) and the GA. EHD1 KD causes the clustering of LE and retromer in the perinuclear area while also causing the compaction of the GA. Conversely, EHD3 KD disperses LE and the GA. Collectively, the roles for EHD1 and EHD3 in recycling endosome, LE, retromer and GA positioning suggest a more general role for EHD1 and EHD3 in establishing organelle polarity relative to the perinuclear area.

In non-polarized cells, trafficking into and out of the perinuclear area is dependent on the centrosome and microtubules. The centrosome or microtubule organization center (MTOC), which consists of two centrioles and a pericentriolar matrix composed of a highly intricate proteinaceous meshwork of fibrils, coordinates microtubule growth and organization in many cell types (Bornens 2002; Lawo, Hasegan et al. 2012; Mennella, Keszthelyi et al. 2012). Microtubules are crucial for the cell cycle, for organelle positioning, and endocytic trafficking. While it is well established that the centrosome and microtubules are crucial for endocytic trafficking (Hopkins, Gibson et al. 1994), a direct connection between organelles such as recycling endosomes and the centrosome has only recently been elucidated. Rab11, the Rab11 GAP Evi5, and several exocyst components all localize to the mother centriole (Hehnly, Chen et al. 2012). The recruitment of Rab11 to the mother centriole regulates its GTP cycle and Rab11-mediated endocytic recycling of the transferrin receptor. How the centrosome coordinates other recycling proteins is unknown.
Alternatively, it is possible that recycling endosome proteins have functions in coordinating centrosome function and microtubule dynamics. Rab11 endosomes mediate the retrograde transport of microtubule nucleating components during mitosis and my work clearly implicates EHD1 in regulating microtubule dynamics during mitosis (Hehnly and Doxsey 2014; Reinecke, Katafiasz et al. 2015). Several recycling proteins, including EHD1 and EHD3, have been implicated in primary ciliogenesis (Knodler, Feng et al. 2010; Das and Guo 2011; Lu, Insinna et al. 2015). Primary cilia are specialized, immotile microtubule structures synthesized in non-proliferating (G0 phase) cells that protrude out into the extracellular milieu (Avasthi and Marshall 2012). Mitosis and ciliogenesis represent two specialized events during distinct phases of the cell cycle and thus prompt the question, are recycling proteins such as EHD1 and EHD3 general regulators of centrosome function and microtubule dynamics? My preliminary work suggests that EHD1 and EHD3 coordinate microtubule growth and release from the centrosome. In interphase cells, EHD1 KD promotes the growth of microtubules from the centrosomes but decreases microtubule release from the centrosome. On the other hand, EHD3 KD impairs microtubule anchoring at the centrosome. Interestingly, over-expressing wild-type EHD1 mimics EHD3 KD while over-expressing EHD3 mimics EHD1 KD. Thus, I hypothesize that EHD1 and EHD3 may inhibit the function of the other through hetero-oligomerization, suggesting that loss of one family member or the over-expression of one family member disrupts the stoichiometry of EHD hetero-oligomerization.
In addition to affecting microtubule dynamics, EHD1 and EHD3 might regulate centrosomal function. EHD3 KD leads to premature centriole separation and the formation of peripheral ectopic MTOCs while EHD1 KD impairs centrosome separation late in the cell cycle. In a cell-free based \textit{in vitro}, I provide evidence that purified EHD1 can bundle and perhaps induce severing of microtubule protofilaments in an ATP-dependent manner thus providing a potential mechanism for explaining the effects of EHD1 KD or over-expression on microtubules \textit{in vivo}. This observation is supported by the fact that addition of purified EHD1 G65R, which may impair ATP hydrolysis, leads to abnormally long, kinked microtubule filaments. Lastly, I use mitosis and ciliogenesis as models for demonstrating the physiological importance of the opposing roles of EHD1 and EHD3. In total, I expand on my data as well as others in answering what functions the EHD proteins are carrying out at the centrosome and on microtubules.

\textbf{14.2 EHD1 and mitochondrial fission}

Our lab has found that TRE biosynthesis and fission require dynamic changes to TRE lipid content. For instance, inhibiting phosphatidic acid production inhibits TRE synthesis while inhibiting phosphatidic acid metabolism inhibits TRE fission (Giridharan, Cai et al. 2013; Xie, Naslavsky et al. 2014). Phosphatidic acid is found on several organelles aside from TRE, most notably the outer mitochondrial membrane (Yang and Frohman 2012; Frohman 2015). Dynamic regulation of phosphatidic acid on mitochondria is also required for mitochondrial fission and fusion (Baba, Kashiwagi et al. 2014). Given that
EHD1 regulates the fission of phosphatidic-rich TREs, I postulate that it might also regulate the fission of other organelle membranes rich in phosphatidic acid, such as the mitochondria. Indeed, preliminary evidence suggests that EHD1 KD causes elongation of mitochondrial networks. Live imaging has confirmed that EHD1 KD reduces mitochondrial fission. Furthermore, we also have found that EHD1 tubules wrap around mitochondria just prior to mitochondrial fission. Drp1, a dynamin-like GTPase, is the only enzyme thus far that has been shown to directly mediate mitochondrial fission. Using super-resolution microscopy, we find, unexpectedly, that EHD1 KD does not impair the binding of Drp1 to mitochondria but rather affects the assembly of Drp1 on mitochondrial membranes. These results suggest that EHD1 may be downstream of Drp1 or alternatively, that EHD1 mediates membrane remodeling events required for Drp1-mediated fission (van der Bliek, Shen et al. 2013). A mitochondrial function for EHD1 is further supported by independent data from Howard Fox’s lab, which found that EHD1 is enriched on mitochondria isolated from murine neurons. Even more exciting is the finding that EHD1 levels on mitochondria decrease with both age and in disease processes such as Parkinson’s disease (PD). Increased age and PD are both associated with elongation of mitochondrial networks (Frank 2006). This preliminary work suggests that the loss of EHD1 may be involved in the pathogenesis of neurodegenerative diseases such as PD.
15. References


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