Plk4 Regulates Cell Motility through Arhgef1 and Rho GTPase Activation

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Laboratory Medicine and Pathobiology
University of Toronto

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Abstract

Cell migration is a fundamental process essential for embryogenesis, immune cell function and maintenance of homeostasis. It is driven primarily by polymerization of the actin cytoskeleton, which is regulated by a family of small Rho GTPases. Rho GTPases Rac1 and Cdc42 are known as the major regulators of actin polymerization and RhoA is mostly thought of as the regulator of actin stress fiber formation and actomyosin contraction. Rho GTPases are activated by GEFs and deactivated by GAPs. Polo-like kinase 4 (Plk4) is well-acknowledged as the master regulator of centriole duplication. Nevertheless, our laboratory has shown that Plk4 activates RhoA during cytokinesis to ensure proper cleavage furrow positioning. We also identified a novel role for Plk4 in regulation of spreading, migration and invasion of normal and cancer cells. However, the mechanisms through which Plk4 regulates these processes are not completely understood. I hypothesized that Plk4 regulates the activation of RhoA and possibly other Rho GTPases to induce cell migration and spreading. In support of this hypothesis, I show here that Plk4 acts as an activator of Rho GTPases RhoA and Rac1 in cycling cells. I also identify 12 GEFs that possess Plk4 consensus phosphorylation motif and show that Plk4 physically interacts with the GEFs Arhgef1 and P-Rex2. Plk4 functionally interacted with Arhgef1 to affect cell migration, showing at least partial dependence of Plk4 migration phenotype on Arhgef1. Further delineation of their interaction showed that Plk4 binds the DH/PH domains and phosphorylates the L-
DH/PH fragment of Arhgef1, and that Plk4 enhances Arhgef1 GEF activity. Finally, I show that Plk4 is not likely to activate Arhgef1-RhoA pathway to enhance cell spreading but may act through P-Rex2-Rac1. These results identify a novel mechanism through which Plk4 regulates cell migration. Plk4 has emerged as a potential oncogene, promoter of tumour progression and metastasis formation, prompting the creation of Plk4 inhibitors for therapeutic purposes. The data presented here deepen our understanding of the pathways that may be affected by the Plk4-targeted therapy.
Acknowledgments

I would like to thank my supervisor Dr. Carol Swallow, for her continuous support, guidance, patience and for taking a chance on me when I first applied to the lab as a fourth-year undergraduate student. Carol, your high expectations and encouragement have made me strive for and achieve my best in the lab and in life. Your kindness, sense of humour and compassion has created a truly wonderful and supportive environment in the lab. Throughout my future career I will aim to achieve the highest level of professionalism, integrity and scientific curiosity that you have always demonstrated. I will measure my work by your standards and always ask myself: “Would Carol approve?” before finalizing a draft. I am honoured to have you as my role model and you are the best mentor that anyone could ask for. A sincere thank you for guiding me, believing in me and supporting me all these years.

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Contributions

The author performed all experiments described in this thesis with the following contributions:

Dr. Karineh Kazazian: made the shPlk4, shLuciferase and U2OS T-REx YFP-Plk4 stable cell lines used in the experiments in this thesis; made the Plk4 truncation fragments used in the experiments described in figure 4.7 and 4.8a. Created figure 6.1 from the author’s data.

Qinghong Dan (under supervision of Dr. Katalin Szaszi): performed the active Arhgef1 pulldowns described in figure 4.9.
# Table of Contents

## Table of Contents

Acknowledgments ........................................................................................................ iv
Contributions ............................................................................................................. v
Table of Contents ....................................................................................................... vi
List of Tables ............................................................................................................. xi
List of Figures ......................................................................................................... xii
List of Abbreviations .............................................................................................. xiv

Chapter 1 .................................................................................................................... 1

1 Introduction ............................................................................................................. 1

1.1 Family of Polo-like kinases ............................................................................. 1
  1.1.1 Plk1 .............................................................................................................. 3
  1.1.2 Plk2, 3 and 5 ................................................................................................ 4
  1.1.3 Plk4 regulation and structure .................................................................... 5
  1.1.4 Plk4 in cell cycle ......................................................................................... 8
  1.1.5 Plk4 in centrosome duplication .................................................................. 8
  1.1.6 Plk4 in cell motility, invasion and spreading ........................................... 12
  1.1.7 Plk4 in carcinogenesis and cancer progression ........................................ 13

1.2 Cell Migration ................................................................................................... 21
  1.2.1 Individual cell migration .......................................................................... 22
  1.2.2 Collective cell migration .......................................................................... 32

1.3 Rho GTPases ..................................................................................................... 35
  1.3.1 Rho GTPase structure and guanine nucleotide switch regulation ............. 38
  1.3.2 Rho GTPase signaling in cell motility ...................................................... 40
  1.3.3 Rho GTPases in cell spreading and cell shape ........................................ 50
1.3.4 Rho GTPases in cancer ........................................................................................................54

1.4 GEFs for Rho GTPases ........................................................................................................57

1.4.1 GEFs structure ................................................................................................................57

1.4.2 Regulation of GEF activity ...............................................................................................59

1.4.3 Arhgef1 ..........................................................................................................................64

1.4.4 P-Rex2 ...........................................................................................................................71

Rationale ..................................................................................................................................77

Hypothesis and Aims ................................................................................................................78

Plk4 interacts with upstream regulators of Rho GTPases to promote their activation and cell migration. .................................................................................................................. 78

Chapter 2 ....................................................................................................................................79

2 Methods ..................................................................................................................................79

2.1 Cell lines and cell culture .....................................................................................................79

2.2 Antibodies ............................................................................................................................79

2.3 Plasmid constructs ...............................................................................................................80

2.4 RNA extraction and Real Time RT-PCR .............................................................................81

2.5 siRNA-mediated protein depletion .....................................................................................82

2.6 Transfections .......................................................................................................................82

2.7 Active Rac1 and Cdc42 pulldowns .....................................................................................83

2.8 Active RhoA pulldowns ........................................................................................................83

2.9 Active Arhgef1 pulldowns ....................................................................................................84

2.10 Immunoblotting ..................................................................................................................84

2.11 Screen for possible Plk4 substrates using Plk4 consensus phosphorylation motif ..........84

2.12 Immunofluorescence ..........................................................................................................85

2.13 Co-Immunoprecipitation ....................................................................................................85

2.14 In vitro kinase assays .........................................................................................................86
Chapter 2 – Plk4 regulates Rho GTPase activation

2.15 Scratch-wound assays .......................................................................................................... 86
2.16 Proliferation and viability assays ........................................................................................ 86
2.17 Spreading assays .................................................................................................................. 87
2.18 Morphology assays ............................................................................................................... 87
2.19 Statistical analysis .............................................................................................................. 87

Chapter 3 – Plk4 regulates Rho GTPase activation

3 Plk4 regulates Rho GTPase activation ...................................................................................... 88

3.1 Overview ................................................................................................................................ 88
3.2 Introduction ............................................................................................................................... 90
3.3 Results ..................................................................................................................................... 94

3.3.1 Lower expression of MMP-3 and MMP-13 in P15 Plk4+/− and in tumorigenic P3 Plk4+/− MEFs ................................................................................................................................. 94
3.3.2 Plk4 regulates active Rac1 levels ......................................................................................... 98
3.3.3 Plk4 may regulate active Cdc42 levels ................................................................................. 105
3.3.4 Plk4 regulates active RhoA levels ....................................................................................... 105
3.3.5 RhoA regulates directional cell migration in a manner consistent with Plk4 .......... 110
3.3.6 Rho GTPases do not have a consensus Plk4 phosphorylation motif ...................... 117

3.4 Discussion ............................................................................................................................... 118

Chapter 4 – Plk4 regulates cell migration through Arhgef1

4 Plk4 regulates cell migration through Arhgef1 ..................................................................... 123

4.1 Overview ................................................................................................................................ 123
4.2 Introduction ............................................................................................................................... 124
4.3 Results ..................................................................................................................................... 126

4.3.1 12 small Rho GTPase GEFs have the Plk4 consensus phosphorylation motif .......... 126
4.3.2 Arhgef1 and P-Rex2 GEFs localize to the cell edge similar to Plk4 and physically interact with Plk4 .......................................................................................................................... 130
4.3.3 Arhgef1 but not P-Rex2 affects cell migration in a manner consistent with Plk4 ..................................................................................................................135

4.3.4 Plk4 physically interacts with the DH/PH domain and phosphorylates L-DH/PH domain of Arhgef1 ........................................................................................................................................140

4.3.5 Plk4 activates Arhgef1 ..................................................................................................................................................................................147

4.3.6 Arhgef1 activates Rac1 ..................................................................................................................................................................................147

4.3.7 Plk4 functionally interacts with Arhgef1 to affect cell motility .............................................................................................................152

4.4 Discussion ..............................................................................................................................................................................................................162

Chapter 5......................................................................................................................................................................................................................173

5 Plk4 may regulate cell spreading via P-Rex2-Rac1 pathway ................................................................................................................................................173

5.1 Overview ..............................................................................................................................................................................................................173

5.2 Introduction ......................................................................................................................................................................................................174

5.3 Results ......................................................................................................................................................................................................................176

5.3.1 P-Rex2 but not Arhgef1 affects cell spreading in a manner consistent with Plk4 ........................................................................................................................................................................176

5.3.2 Rac1 but not RhoA affects cell spreading in a manner consistent with Plk4 ..........................................................................................183

5.3.3 Plk4 effect on cell shape is consistent with that of Rac1 but not RhoA, Arhgef1 or P-Rex2 ................................................................................................................................................187

5.4 Discussion ..............................................................................................................................................................................................................195

Chapter 6......................................................................................................................................................................................................................199

6 Discussion ..............................................................................................................................................................................................................199

6.1 Overview of the results ..................................................................................................................................................................................................199

6.2 Discussion ..............................................................................................................................................................................................................200

6.2.1 Plk4, microtubules and actin cytoskeleton ...........................................................................................................................................................................200

6.2.2 Cell migration vs cell spreading ...........................................................................................................................................................................203

6.2.3 Plk4 and EMT ..........................................................................................................................................................................................................206

6.2.4 Plk4 and Rho GTPases as therapeutic targets in tumour progression and metastasis ...............................................................................209
6.3 Conclusions ......................................................................................................................... 215

6.4 Future directions .................................................................................................................. 216

6.4.1 The mechanism of Plk4 regulation of Rho GTPases ..................................................... 216

6.4.2 Further delineation of Plk4 interaction with Arhgef1 .................................................. 218

6.4.3 The mechanism of Plk4 regulation of cell spreading .................................................. 219

6.4.4 The effect of Plk4 on immune cell migration and function ......................................... 220

6.4.5 Effect of Plk4 interaction with Arhgef1 on migration and invasion in 3D and 
in vivo ........................................................................................................................................ 220

6.4.6 Targeting Plk4–Arhgef1 pathway as a therapeutic strategy ......................................... 222

References ................................................................................................................................ 224

Copyright Acknowledgements ................................................................................................. 267
List of Tables

Table 1.1 Plk4 expression in human cancers known to date.

Table 1.2 Phosphorylation of the Dbl family and DOCK family GEFs and their role in cell migration.

Table 5.1. The effect of GEFs Arhgef1 and P-Rex2 and Rho GTPases RhoA and Rac1 on cell spreading and shape as compared to Plk4.

Table 5.2 The effect of GEFs Arhgef1 and P-Rex2 and Rho GTPases RhoA and Rac1 on cell size and shape as compared to Plk4.
List of Figures

Figure 1.1 Schematic representation of the expression and localization of polo-like kinases throughout the cell cycle.
Figure 1.2 Plk4 structure and roles of Plk4 domains in its activation and protein stability.
Figure 1.3 Overview of the centrosome cycle and Plk4 signaling in centrosome duplication initiation.
Figure 1.4 Regulation of actin nucleation and polymerization.
Figure 1.5 Modes of cell migration.
Figure 1.6 Evolutinal relationship and protein structure of Rho GTPases.
Figure 1.7 Regulation of Rho GTPase activity.
Figure 1.8 Rho GTPase signaling.
Figure 1.9 Rho GTPases in cell migration and spreading.
Figure 1.10 Arhgef1 structure and signaling.
Figure 1.11 P-Rex2 structure and signaling.
Figure 3.1. Plk4 regulates MMP-3 and MMP-13 expression levels in MEFs.
Figure 3.2. Lower expression of Plk4 reduces the level of active Rac1 in P3 MEFs.
Figure 3.3 Depletion of Plk4 expression reduces active Rac1 levels in MEFs and HeLa cells.
Figure 3.4 Plk4 overexpression does not alter active Rac1 levels in HEK293T cells.
Figure 3.5. Inconsistent levels of active Cdc42 in MEFs from different embryos.
Figure 3.6 Depletion of Plk4 expression reduces active RhoA levels.
Figure 3.7. Plk4 regulates directional cell migration in wound-healing assays.
Figure 3.8. Transient overexpression of CA-Rac1 and CA-RhoA does not alter directional cell migration in wound-healing assays in HeLa cells.
Figure 3.9. Inhibition of RhoA reduces directional cell migration in wound-healing assays in U2OS cells.
Figure 4.1. GEFs with Plk4 consensus phosphorylation motifs.
Figure 4.2. Arhgef1 and P-Rex2 accumulate at the cell edge together with Plk4. Figure 4.3. Arhgef1 and P-Rex2 interact with Plk4.
Figure 4.4. The effect of siArhgef1 and siP-Rex2 on cell migration.
Figure 4.5. Transient overexpression of GFP-Arhgef1 or V5-P-Rex2 does not affect cell migration.
Figure 4.6. Plk4 interacts with the DH/PH domains of Arhgef1.
Figure 4.7. Arhgef1 interacts with the polo-box1-2 and kinase domains of Plk4.
Figure 4.8. Plk4 phosphorylates Arhgef1.
Figure 4.9 Plk4 activates Arhgef1 towards RhoA.
Figure 4.10 Arhgef1 positively regulates active Rac1 levels in U2OS cells.
Figure 4.11. Arhgef1 depletion reduces U2OS cell migration.
Figure 4.12. Individual siArhgef1 constructs reduce U2OS cell migration.
Figure 4.13. Plk4 may regulate cell motility through Arhgef1.
Figure 4.14. Plk4 regulates cell motility through Arhgef1.
Figure 4.15. Schematic of Plk4 and Arhgef1 interaction in cell motility.
Figure 5.1. Plk4 depletion decreases cell spreading and increases cell roundness.
Figure 5.2. Arhgef1 negatively regulates cell spreading and roundness.
Figure 5.3. P-Rex2 regulates cell spreading and shape.
Figure 5.4. Constitutively active RhoA and Rac1 regulate cell spreading and shape.
Figure 5.5. Arhgef1 negatively regulates cell size.
Figure 5.6. P-Rex2 regulates cell size and shape.
Figure 5.7. Constitutively active RhoA and Rac1 regulate cell size and shape.
Figure 6.1. Plk4 regulates Cdc42 activity in U2OS cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>βPix</td>
<td>PAK-interacting exchange factor beta</td>
</tr>
<tr>
<td>Abi</td>
<td>Abl interactor</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>ADF</td>
<td>Actin depolymerizing factor</td>
</tr>
<tr>
<td>aPKCζ</td>
<td>Atypical protein kinase C isoform zeta</td>
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<tr>
<td>Arf1</td>
<td>ADP ribosylation factor 1</td>
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<td>ARHGAP22</td>
<td>Rho GTPase activating protein 22</td>
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<tr>
<td>ARHGEF</td>
<td>Rho guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>Arp</td>
<td>Actin-related protein</td>
</tr>
<tr>
<td>ARPC</td>
<td>Actin-related protein 2/3 complex</td>
</tr>
<tr>
<td>ATP/ADP</td>
<td>Adenosine triphosphate / adenosine diphosphate</td>
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<td>ATRA</td>
<td>All-trans retinoic acid</td>
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<tr>
<td>AURKA/B</td>
<td>Aurora kinase A/B</td>
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<td>BALB/c</td>
<td>Bagg albino genotype c (mouse strain)</td>
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<td>BRAF</td>
<td>Serine/threonine-protein kinase B-Raf</td>
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<tr>
<td>BTB</td>
<td>BR-C, ttk and bab domain</td>
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<tr>
<td>CA</td>
<td>Constitutively active</td>
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<td>CCR2</td>
<td>C-C motif chemokine receptor 2</td>
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<td>Cdc</td>
<td>Cell division cycle</td>
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<td>Cdkn2a</td>
<td>Cyclin-dependent kinase inhibitor 2a</td>
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<td>Cyclin-dependent kinase 1</td>
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<td>CENPJ</td>
<td>Centromere protein J</td>
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<tr>
<td>Cep</td>
<td>Centrosomal protein</td>
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<td>CHIP</td>
<td>Carboxy terminus of Hsp70-interacting protein</td>
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<td>Chk2</td>
<td>Checkpoint kinase 2</td>
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<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
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<tr>
<td>CP110</td>
<td>Centriolar coiled-coil protein of 110 kDa</td>
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<td>CPAP</td>
<td>Centrosomal P4.1-associated protein</td>
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<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
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<td>CRIB</td>
<td>Cdc42/Rac interactive binding motif</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>CtIP</td>
<td>C-terminal-binding protein (CtBP)-interacting protein</td>
</tr>
<tr>
<td>Dbl</td>
<td>Diffuse B-cell lymphoma</td>
</tr>
<tr>
<td>Db's</td>
<td>Dbl’s big sister</td>
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<td>DEP</td>
<td>Dishevelled, Egl-10, and Pleckstrin</td>
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</tr>
<tr>
<td>DHR</td>
<td>DOCK-homology region</td>
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<tr>
<td>Dia</td>
<td>Diaphonous</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DOCK</td>
<td>Dedicator of cytokinesis</td>
</tr>
<tr>
<td>DRE</td>
<td>Downstream regulatory element</td>
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<tr>
<td>DRF</td>
<td>Diaphonous-related formin homology</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>Ect2</td>
<td>Epithelial cell-transforming sequence 2 oncogene</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>ELMO</td>
<td>Engulfment and motility</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<tr>
<td>Ena</td>
<td>Protein enabled</td>
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<td>ERM</td>
<td>Ezrin, Radixin, Moesin</td>
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<td>FAK</td>
<td>Focal adhesion kinase</td>
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<td>Formin homology 2</td>
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<td>Filamin-A-associated</td>
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<td>Fibroblast growth factor-inducible kinase</td>
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<td>Frg/Yes-relared novel protein</td>
</tr>
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<td>Globular actin</td>
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<td>GTPase-activating protein</td>
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<td>GBD</td>
<td>GTPase protein-binding</td>
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<td>GDI</td>
<td>Guanosine nucleotide dissociation inhibitor</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GIT</td>
<td>G Protein-Coupled Receptor Kinase Interacting ArfGAP</td>
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<td>Description</td>
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</tr>
<tr>
<td>GNMT</td>
<td>Glycine N-methyltransferase</td>
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<td>GPCR</td>
<td>G protein coupled receptor</td>
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<td>GPR124</td>
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<td>Glycogen synthase kinase β</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>Hepatocellular carcinoma</td>
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<td>Homologous to E6AP carboxyl terminus homologous protein 9</td>
</tr>
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<td>Human embryonic kidney cells 293 transformed</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks cells</td>
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<td>HPV</td>
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<td>IQ motif containing GTPase activating protein 1</td>
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<td>Jak2</td>
<td>Janus kinase 2</td>
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<td>KAT</td>
<td>Lysine acetyltransferase</td>
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<td>KLF</td>
<td>Krüppel-like factor</td>
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<td>K-Ras</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
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<td>LARG</td>
<td>Leukemia-associated RhoGEF</td>
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<td>LIMK</td>
<td>Lin11, Isl-1, Mec-3 (LIM) domain kinase</td>
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<td>LPA</td>
<td>Lysophosphatidic acid</td>
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<td>LRAP35a</td>
<td>Leucine repeat adaptor protein 35a</td>
</tr>
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<td>Lsc</td>
<td>Lbc’s second cousin</td>
</tr>
<tr>
<td>MCP1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MDA-MB</td>
<td>M.D. Anderson - metastasis breast cancer cells</td>
</tr>
<tr>
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<td>miR</td>
<td>Micro RNA</td>
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<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRCK</td>
<td>Myotonic dystrophy kinase-related Cdc42-binding kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organizing center</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic target of Rapamycin</td>
</tr>
<tr>
<td>MZB</td>
<td>Marginal zone B cells</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Nap1</td>
<td>Nucleosome assembly protein</td>
</tr>
<tr>
<td>Nedd1</td>
<td>Neural precursor cell expressed developmentally down-regulated protein 1</td>
</tr>
<tr>
<td>Net1A</td>
<td>Protein NETWORKED 1A</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Nonhomologous end joining</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>National institutes of health 3-day transfer, inoculum $3\times10^5$ fibroblasts</td>
</tr>
<tr>
<td>NMIIA</td>
<td>Non-muscle myosin IIA</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>Non-obese diabetic- severe combined immunodeficiency mice</td>
</tr>
<tr>
<td>NPM</td>
<td>Nucleophosmin</td>
</tr>
<tr>
<td>NRAS</td>
<td>Neuroblastoma RAS viral oncogene homolog</td>
</tr>
<tr>
<td>NT</td>
<td>Non-tumorigenic</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural WASP</td>
</tr>
<tr>
<td>P</td>
<td>Passage</td>
</tr>
<tr>
<td>P₁</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinases</td>
</tr>
<tr>
<td>Par6</td>
<td>Partitioning defective protein 6</td>
</tr>
<tr>
<td>PB</td>
<td>Polo box</td>
</tr>
<tr>
<td>PCC</td>
<td>Pheochromocytomas</td>
</tr>
<tr>
<td>PCM</td>
<td>Pericentriolar material</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet-derived growth factor receptor α</td>
</tr>
</tbody>
</table>
PDZ    Post-synaptic density protein, *Drosophila* disc large tumour suppressor, and zonula occludens-1 protein
PH     Pleckstrin-homology
PIP₃   Phosphatidylinositol (3,4,5)-trisphosphate
PIR121 Transcription activator PIROGI 121
PI3K   Phosphoinositide 3-kinase
PKC    Protein kinase C
Plk    Polo-like kinase
Plo1   Polo-related kinase 1
P-loop Phosphate-binding loop
Plx1   Xenopus polo-like kinase 1
PM     Plasma membrane
PMA    Phorbol 12-myristate 13-acetate
PP     Protein phosphatase
P-Rex  Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger
Prk2   Protein kinase N2
PTEN   Phosphatase and tensin homolog
p115   Protein 115 kDa
p190GAP Protein 190 kDa
p41-Arc Protein 41 kDa- Arp2/3 complex
p53    Protein 53 kDa
p53BP1 p53-binding protein 1
p67phox 67kDa NADPH oxidase factor
Rac    Ras-related C3 botulinum toxin substrate
Ras    Rat sarcoma viral oncogene homolog
RGS    Regulator of G protein signaling
RH     RGS-homology
Rho    Ras homolog gene family
RNA    Ribonucleic acid
RNAi   RNA interference
ROCK   Rho-associated coiled-coil containing kinase
ROS    Reactive oxygen species
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAK</td>
<td>SNK akin kinase</td>
</tr>
<tr>
<td>SAS</td>
<td>Spindle assembly abnormal protein</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp, Cullin, F-box complex</td>
</tr>
<tr>
<td>SCRIIB</td>
<td>Protein scribble homolog</td>
</tr>
<tr>
<td>SAK</td>
<td>SNK akin kinase</td>
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<td>SAS</td>
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<td>SCF</td>
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</tr>
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<td>SCRIIB</td>
<td>Protein scribble homolog</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family of tyrosine kinases</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology 3</td>
</tr>
<tr>
<td>sh</td>
<td>Short hairpin</td>
</tr>
<tr>
<td>Snk</td>
<td>Serum-inducible kinase</td>
</tr>
<tr>
<td>Sos1</td>
<td>Son of sevenless homolog 1</td>
</tr>
<tr>
<td>Sra1</td>
<td>Steroid receptor RNA activator 1</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma proto-oncogene, non-receptor tyrosine kinase</td>
</tr>
<tr>
<td>STIL</td>
<td>SCL-interrupting locus protein</td>
</tr>
<tr>
<td>T</td>
<td>Tumorigenic</td>
</tr>
<tr>
<td>TIE2</td>
<td>Tunica interna endothelial cell kinase 2</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TOCA</td>
<td>Transducer of Cdc42-dependent actin assembly protein</td>
</tr>
<tr>
<td>TRKA/B</td>
<td>Tyrosine kinase receptor A/B</td>
</tr>
<tr>
<td>U2OS</td>
<td>U-2 osteosarcoma</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VCA</td>
<td>Verprolin, cofilin, acidic domain</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP-family verprolin-homologous</td>
</tr>
<tr>
<td>WH-2</td>
<td>WASP-homology 2 domain</td>
</tr>
<tr>
<td>WRC</td>
<td>WAVE regulatory complex</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZYG-1</td>
<td>Zygote defective protein 1</td>
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Chapter 1

1 Introduction

1.1 Family of Polo-like kinases

Figure 1.1 and some of the writing in this section have been published in part in:


Polo-like kinases (Plks) are a family of five structurally conserved serine-threonine kinases that play critical roles in cell cycle progression/regulation, response to DNA damage and oxidative stress. Polo-like kinases have been identified in most eukaryotes, apart from plants. In particular, Plk1 is present in most eukaryotes (Cdc5 in budding yeast, Plo1 in fission yeast), while Plk2-4 are found only in higher organisms such as worm C. elegans (Plk1, Plk2, Plk3, ZYG-1: structurally unrelated functional homolog of Plk4), zebrafish D. rerio (Plk1, Plk2b, Plk3, Plk4), fly D. melanogaster (Polo, Sak/Plk4), frog X. laevis (Plx1, Plx2, Plx3, Plx4), mouse M. musculus (Plk1, Plk2/Snk, Plk3/Fnk, Plk4) and humans H. sapiens (Plk1, Plk2/hSnk, Plk3/Prk, Plk4) (reviewed in (1)). Plk5 has so far been identified only in mouse and humans (2, 3). The Polo-like kinases have a conserved structure, with N-terminal kinase domain which is mostly responsible for substrate phosphorylation, and polo-box motifs which are responsible for substrate binding and protein localization. The expression and/or activation of Plks is tightly regulated throughout the cell cycle (Figure 1.1a). Loss of this tight control can disrupt a variety of fundamental cellular processes leading to genomic instability and potentially carcinogenesis. In addition to precise temporal regulation, Plk activity is subject to spatial control. As the cell moves through the various stages of the cell cycle, localization of Plks changes allowing them to perform different and often unique functions (Figure 1.1b) (Reviewed in (4)). Some of the fundamental functions of each member of the polo-like kinase family are described below.
Figure 1.1 Schematic representation of the expression and localization of polo-like kinases throughout the cell cycle. The function of Plks is controlled temporally (a and b) and spatially (b). a The mRNA levels of Plk2 and Plk3 are highest in G1 and decrease in S phase, while Plk1 and Plk4 mRNA is first detected in S phase and keeps increasing, reaching their peak in G2 and M. Plk5 is only expressed in quiescent cells. b In S and G2 phases Plk2 localizes to the centrosomes where it helps to initiate centrosome duplication. Plk3 localizes to the nucleus in G1-G2 where it regulates G1/S and G2/M transition and plays a role in DNA synthesis. Plk4 localizes to the centrosomes in interphase at the beginning of mitosis, where it plays an essential role in centriole biogenesis. Plk1 localizes to the centrosomes in G2 and early mitosis, controlling centrosome maturation and spindle assembly. In metaphase Plk1 localizes to kinetochores aiding attachment of microtubules to kinetochores and later, chromosome segregation. At later mitotic stages Plk1, Plk3 and Plk4 localize to midbody where they play a role in cytokinesis. Figure adapted from Kazazian et al., 2015 (5).
1.1.1 Plk1

Plk1 is the most studied member of the Plk family due to many important functions it has throughout the cell cycle, especially during mitotic entry and mitosis. Plk1 promotes G2/M transition by phosphorylation of Cdc25C on Ser198 which allows for its release from 14-3-3 proteins and thus activates it (6). Plk1 also phosphorylates the inhibitor of Cdc25C Wee1 on Ser53 which targets it for the proteasome-mediated degradation and further activates Cdc25C (7). Active Cdc25C then dephosphorylates Cyclin-dependent kinase 1 (Cdk1) on Tyr15 allowing for activation of Cdk1-CyclinB complex and its translocation to the nucleus, thereby signaling for nuclear envelope breakdown and initiation of mitosis.

Plk1 is a major kinase that coordinates the cell cycle with the centrosome cycle, by playing an important role in centrosome maturation. Centrosome cycle begins with the initiation of centriole duplication in G1/S which is followed by centrosome maturation from G2 and throughout mitosis. Plk1 initiates centrosome maturation by phosphorylation of pericentrin and recruitment of γ-tubulin, Nedd1, Centrosomal protein (Cep) 192 and other important proteins that make up pericentriolar material (PCM) (8, 9). Recruitment of γ-tubulin to the centrosome initiates microtubule nucleation, turning centrosomes into microtubule organizing centers (MTOC), which are integral part of the mitotic spindle. Mitotic spindle is a cellular structure responsible for proper chromosome segregation, another process regulated by Plk1. Plk1 plays a role in cleaving Cohesin, which holds two sister chromatids together during mitosis, thus aiding chromosome segregation (10). The final stage of mitosis, known as cytokinesis, also depends on Plk1 activity. Plk1 phosphorylates MgcRacGAP at the central spindle, which in turn attracts Ect2, a Ras homolog gene family, member A (RhoA) guanine nucleotide exchange factor (GEF), and allows for its binding (11). Ect2 then activates RhoA, a small Rho GTPase that is essential for proper cleavage furrow positioning.

Aside from its functions in mitosis, Plk1 plays an important role in DNA replication. In S phase, through phosphorylation of its downstream targets, Plk1 aids formation of pre-replicative complex (12). In case of DNA damage, Plk1 is inhibited by Chk2 which initiates G2 block and allows for DNA damage repair (13). Once repair is complete, Plk1 is activated again by Aurora A kinase. Phosphorylation of Plk1 downstream targets such as p53BP1, Cdc25C, Wee1 and
others aids the recovery of the cell from cell cycle arrest induced by DNA damage checkpoint and promotes mitotic re-entry (14).

1.1.2 Plk2, 3 and 5

Plk2, Plk3 and Plk5 kinases are thought to be immediate early response genes since their expression increases upon DNA damage and oxidative stress (2, 15-17).

The role of Plk3 in regulating DNA damage checkpoint is best understood. Upon DNA damage and oxidative stress, Plk3 expression is increased, which allows for its binding and phosphorylation of p53 leading to its translocation into the nucleus (16, 17). In the nucleus p53 induces G1-S transition arrest and DNA damage repair through its downstream effectors. During G2/M checkpoint, Plk3 accumulates in the nucleus and phosphorylates Cdc25C on Ser216 which facilitates Cdc25C relocation from the nucleus to the cytoplasm, effectively halting mitotic entry (18). Recently, a more direct role for Plk3 in DNA damage repair, specifically nonhomologous end joining (NHEJ) double strand break repair, has been suggested. Barton et al. show that Plk3 phosphorylates CtIP upon DNA damage response and together these proteins are required at various stages of NHEJ (19). Therefore, Plk3 is an important regulator of DNA damage repair at various stages of the cell cycle.

Overexpression or ectopic expression of Plk3 and Plk5 lead to G1 arrest and apoptosis, suggesting a role for these kinases in G1/S transition (2, 20). Indeed, it has been shown that although Plk3 is expressed throughout the cell cycle, its activity peaks in G1 where it positively regulates G1/S transition and promotes DNA replication (17, 21-23). The role of Plk5 and Plk2 in DNA damage response or DNA replication remains to be determined.

Plk2 is mostly expressed in G1 and S phases where it plays a role in centriole duplication (24, 25). In particular, Plk2 phosphorylation of Nucleophosmin (NPM) leads to its binding to Rho-associated coiled-coil containing kinase 2 (ROCK2) at the centriole. ROCK2 then acts directly or indirectly on Plk4 and thereby initiates centriole biogenesis (26, 27). Plk3 was also reported to localize to the centrosomes, mitotic spindle and midbody but its function at the centrosome and during cytokinesis is still unclear (20, 28, 29). One possibility is that Plk3 may play a role in regulation of microtubular dynamics during these processes (29, 30).
Plk5 expression is confined predominantly to the brain and it’s mostly expressed in quiescent cells at G0. Human Plk5 lacks kinase domain compared to mouse Plk5 and in humans it has been shown to be involved in neuron differentiation and formation of neuritic processes (2, 3).

1.1.3 Plk4 regulation and structure

Plk4 was first identified in a screen for genes regulating sialylation and was initially named Sak (Snk akin kinase) (31). It is regulated at transcriptional and post-transcriptional levels. Transcriptionally, Plk4 expression has been shown to be negatively regulated by p53 (32). The positive effectors of its transcription are poorly understood but Ledoux et al. have shown that Plk4 transcription is directly regulated by the NFκB (33). In addition, a study by Fischer et al. proposed that Plk4 expression is regulated through similar regulatory elements as that of other cell-cycle regulated genes (34). More recently, it has been suggested that Plk4 transcription is repressed by Krüppel-like factor (KLF) 14, a transcription factor whose own transcription is often downregulated in cancer (35). KLF14 knockout resulted in Plk4-driven centrosome overduplication in mouse embryonic fibroblasts (MEFs) derived from KLF<sup>-/-</sup> mice, suggesting a direct role of this transcription factor in Plk4 regulation. Even less is known about the regulation of Plk4 translation, but the level of Plk4 protein has been reported to mirror its transcript levels (36, 37). Studies of Plk4 structure have led to better understanding of the regulation of Plk4 protein stability and kinase activity as Plk4 itself is directly involved in this regulation. The N-terminal Plk4 kinase domain is highly similar to that of other Plk members, with the exception of Plk5 which lacks most of the kinase domain in humans (Figure 1.2a) (3, 31). It functions mainly in substrate phosphorylation and regulation of Plk4 protein stability. Mutations in the ATP-binding domain of Plk4, such as K41M, make the kinase catalytically inactive, while T170D mutation in the activation T loop enhances Plk4 kinase activity (38). Recently, Fournier et al. have discovered that human lysine acetyltransferases KAT2A and KAT2B acetylate Plk4 kinase domain at K45 and K46 residues, thereby causing a shift in kinase conformation, thus reducing its activity (39). In Plk4 constructs where these residues were mutated to disable their acetylation, Plk4 autophosphorylation decreased. Overexpression of these constructs did not result in centrosome overduplication, like overexpression of wild type Plk4 does, suggesting that Plk4 acetylation is important for its function. Plk4 differs from other members of the polo-like kinase family in that it has three polo-box domains, while other Plks have two (40). Tandem
polo-boxes PB1 and PB2 (also known as cryptic polo-box) allow for Plk4 homodimerization, a distinguishing feature of Plk4 in Plk family (40, 41). These polo-boxes are also necessary for Plk4 substrate binding and are essential for its localization to the centrosome (40-44). When first produced, Plk4 is autoinhibited by the interaction of its kinase domain with the L1 region (Figure 1.2b) (45).

Upon dimerization, polo-box 3 plays a role in relieving the autoinhibitory loop by a yet unidentified mechanism. This may be the main function of PB3, as to date only SCL-interrupting locus protein (STIL) has been shown to bind PB3 to affect centriole duplication (46). The release of the autoinhibitory loop allows for Plk4 trans-autophosphorylation on multiple sites leading to a number of consequences described below. Phosphorylation of L1 further decreases Plk4 autoinhibition, while phosphorylation of the kinase domain at the autoinhibitory loop enhances its kinase activity (45). Plk4 trans-autophosphorylation of its phosphodegron motif (residues 282-305), known as downstream regulatory element (DRE), results in the Skp, Cullin, F-box complex (SCF) complex recruitment, PB1 ubiquitination and ultimately protein degradation (45, 47, 48). Phosphorylation of the L2 region of Plk4 leads to the dimer separation (45). This rapid degradation of Plk4 upon its activation is important to ensure that centrosomes replicate only once per cell cycle and prevents multipolar spindle formation and genomic instability.
Figure 1.2 Plk4 structure and roles of Plk4 domains in its activation and protein stability. 

**a** Plk4 kinase domain is highly similar to the other members of the family, but Plk4 differs at the C-terminus, as it possesses three polo-box domains as compared to two in the other members. 

**b** 

1. **Autoinhibition**

2. **Dimerization**

3. **Autoinhibition relief**

4. **Autophosphorylation**

5. **Ubiquitination**

6. **Dimer separation**

7. **Degradation**

**KD** = kinase domain  
**DRE** = downstream regulatory element (including phosphodegron)  
**AL** = activation loop  
**L1** = linker region 1  
**L2** = linker region 2  
**PP2A** = protein phosphatase 2A

**i** When first expressed Plk4 is autoinhibited by the binding of the kinase domain to L1.  
**ii** Plk4 homodimerizes via interactions of polo boxes 1 and 2, at which point polo box 3 disrupts KD to L1 binding by a yet unidentified mechanism, which exposes the activation loop.  
**iii** Plk4 then trans-autophosphorylates at the activation loop, enhancing its kinase activity and at the phosphodegron motif, thus attracting the SCF/Slimb complex. PP2A dephosphorylates Plk4 phosphodegron motif to maintain Plk4 activity.  
**iv** The SCF/Slimb complex binds Plk4 and ubiquitinates it at polo box 1, targeting it for degradation.  
**v** Plk4 trans-autophosphorylation of L2 leads to dimer separation. Figure 1.2b modified from Klebba et al., 2015 (45).
1.1.4 Plk4 in cell cycle

Plk4 expression is first detected in G1 and peaks in the S phase, where it plays an essential role in centriole biogenesis, as described below (36, 49, 50). Plk4 may also play a role in G2/M transition by phosphorylation of Cdc25C causing its subsequent translocation to the nucleus (51). This observation however, is yet to be confirmed. The expression levels remain high in mitosis where Plk4 facilitates mitotic spindle formation and centriole duplication (52, 53). During cytokinesis Plk4 localizes to the midbody and it has been suggested that it may play a role in proper cleavage furrow positioning through a mechanism similar to that of Plk1 (52, 54). This claim however, remains controversial (55).

1.1.5 Plk4 in centrosome duplication

During G2-M stages of the cell cycle, centrosomes move to the opposite poles where they act as MTOCs. Proper organization of microtubules around the poles is important for ensuring correct cell symmetry and even division of chromosomes. It is therefore essential to ensure that centrosomes duplicate only once per cell cycle. Centrosome is made up of two orthogonally assembled centrioles and surrounding proteins collectively known as the PCM. Canonically, the centrosome cycle goes hand in hand with the cell cycle (Figure 1.3). Centrioles begin to duplicate in G1-S and elongate throughout S and G2 stages.

The centriole along which the new centriole is formed during mitosis is known as the mother, while the newly formed centriole is known as the daughter. For most organisms which have centrosomes, each centriole is composed of nine microtubule triplets to form a cylindrical shape (reviewed in (56)). The mother and daughter centrioles are connected by filaments and only the mother centriole has distal and subdistal appendages. New centriole assembly starts at the proximal end of the mother centriole with the formation of a cartwheel structure. The cartwheel has a hub in the center and nine spokes coming out of it, each connecting to one of the microtubule triplets, thus forming a scaffold for the new centriole and ensuring the nine-fold symmetry. Once the assembly of the new centriole is complete, the cartwheel is disassembled, leaving the centriole hollow.
Figure 1.3 Overview of the centrosome cycle and Plk4 signaling in centrosome duplication initiation. 

**a** During G1/S transition the daughter centriole matures by acquiring appendages at the distal end. Centriole duplication is initiated when Cep192 and Cep152 bind to polo-boxes 1 and 2 of Plk4 thus localizing it to the centrosome. In turn, Plk4 binds SAS-6 and STIL and phosphorylates STIL, localizing these proteins to the proximal end of each mother centriole. 

**b** In S stage SAS-6 initiates the cartwheel formation, which acts as a scaffold for the new centriole. Both SAS-6 and STIL provide a scaffold for microtubule docking thus initiating a procentriole formation. Plk4 interacts with and phosphorylates CP110 to initiate centriole assembly. It also interacts with CPAP, which stabilizes microtubules and defines their number and length, and with Cep135, which binds SAS-6 and acts as a scaffold for other proteins necessary for centriole elongation and maturation. 

**c** In G2 the daughter centrioles continue to elongate, the mother centrioles are separated by separase and the two centrosomes start to move to the opposite poles of the cell. 

**d** In mitosis, the two centrosomes form mitotic spindles, made up of microtubules that attach to the kinetochores of the chromosome pairs and in late mitosis one chromosome from each pair is pulled towards each centrosome, thus ensuring equal chromosome distribution to each daughter cell. Figure modified from Gonszy, 2015 (57).
Plk4 has become known as the master regulator of centriole biogenesis and similar to Plk1 plays an important role in mitotic spindle formation and centriole duplication. Cells overexpressing Plk4 or deficient in the SCF ubiquitin-protein ligase exhibit supernumerary centrosomes and multipolar spindles, and cells exhibiting lower levels of Plk4 or treated with Plk4 kinase inhibitor have fewer than 2 centrosomes (47, 49, 53, 58-62). While low overexpression of Plk4 results in overduplication of centrioles along the mother centriole, high overexpression of Plk4 can lead to de novo centriole formation, highlighting the centrosome as a concentrator of Plk4 activity (63). This also helps explain the role of Plk4 in favoring the canonical centriole duplication. Besides Plk4, a few other proteins have been identified as essential for centriole duplication and biogenesis in mammalian cells: Cep192, Cep152, STIL, spindle assembly abnormal protein 6 (SAS-6), Cep135, centrosomal P4.1-associated protein (CPAP) and centriolar coiled-coil protein (CP) 110 (Figure 1.3) (64-70). In G1 Cep192 and Cep152 cooperate to recruit Plk4 to the centrosome by binding to PB1 and PB2 (71, 72). Once at the centrosome, Plk4 interacts with STIL and SAS-6, proteins that help determine the positioning and number of the new centrioles, as well as begin their assembly (Figure 1.3a). Plk4 binding to SAS-6 initiates the formation of a cartwheel, the first structure in the centriole assembly (73-76). Plk4 aids the interaction of SAS-6 with STIL by phosphorylating STIL and recruiting it to the centrosome (46, 77-79). At the centrosome, SAS-6 and STIL provide a scaffold for microtubule docking, thus initiating the daughter procentriole formation (43, 76, 78, 80). Therefore, Plk4-SAS-6-STIL form a core complex essential for the centriole biogenesis. Similar to Plk4, overexpression of SAS-6 and STIL also leads to supernumerary centrioles and vice versa (67, 68). Interestingly, although most of the mechanisms for centriole assembly are conserved between most species, recently Cottee and colleagues have shown that unlike in humans, in flies STIL does not interact with Plk4 by binding to PB3 (46, 81). Therefore, while key proteins in centriole duplication and biogenesis are conserved, the exact interactions between them may be species-specific. The exact positioning of the new centriole as well as the number of the new centrioles is controlled by both Plk4 protein-protein interactions and Plk4 activity and abundance (reviewed in (82)). Plk4 also regulates centriole assembly by interacting with CPAP (CENPJ) which acts in stabilizing and defining the number and length of centriolar microtubules by binding and capping tubulin (68, 83); Cep135, which acts as a scaffold for binding of other centrosomal proteins necessary for centriole elongation and maturation as well as binds microtubules and promotes the formation of
microtubule bundles; and CP110 which is phosphorylated by Plk4 to induce centriole assembly and control centriolar length (Figure 1.3b) (69, 83-87). Plk4 is rapidly degraded upon autophosphorylation of its phosphodegron motif to prevent centriole overduplication (88). However, high enough levels of Plk4 need to be maintained to allow for initiation of the centriole duplication in S phase and its elongation in G2/M phases of the cell cycle. Brownlee and colleagues have demonstrated that in Drosophila, Protein Phosphatase 2A (PP2A) is responsible for dephosphorylation of Plk4 in mitosis to promote centriole duplication and this is at least one mechanism by which high enough Plk4 levels are maintained (89). Similarly, in C. elegans PP2A regulates the protein levels of Plk4 homolog ZYG-1 (90). Therefore, the mechanism of dephosphorylation of Plk4 by a phosphatase may be conserved between species but whether PP2A is responsible for Plk4 dephosphorylation in humans remains to be determined. The cell cycle and the centrosome cycle need to be synchronized in order to maintain centrosome number and chromosomal stability. A recent study by Zitouni et al. uncovered a mechanism for prevention of centrosome re-duplication during the same cell cycle. They show that during mitosis STIL is bound by Cdk1-CyclinB, thus preventing its interaction with and phosphorylation by Plk4, thereby restricting centrosome duplication to once per cell cycle (91). Cdk1-CyclinB are inactivated upon exit from mitosis and therefore centrosome duplication can be initiated again in G1. It is important to note that besides its role in centriole duplication and biogenesis, Plk4 plays an important role in spindle formation. In mouse embryos, the first five divisions occur without centrioles and bipolar spindles assemble around the chromosomes. Coelho et al. have shown that mouse embryos lacking parental Plk4 show abnormal, monopolar spindle formation due to the lack of microtubule nucleation, which then leads to cytokinesis failure and arrest in embryo development (92). Therefore, Plk4 role in spindle formation may be at least in part independent from its role in centriole duplication.

Aberrant centriole numbers, largely amplifications, have been reported in many human cancers and have been associated with errors in mitotic spindle formation, microtubule-kinetochore attachment and chromosome segregation, resulting in chromosomal instability (CIN) (Reviewed in (93, 94)). Centrioles are also essential for cilia formation (95). Cilia are microtubule-based structures that arise on the surface of most cell types and are essential for extracellular signal sensing and transduction (Reviewed in (96)). Errors in centriole duplication often lead to serious pathologies collectively known as ciliopathies (96). Therefore, as a major regulator of centriole
duplication, abnormalities in Plk4 expression have been linked to carcinogenesis and cilia defects, highlighting the need to better understand Plk4 regulation and molecular interactions.

1.1.6 Plk4 in cell motility, invasion and spreading

Cultured Plk4+/+ MEFs senesce by passage 9 (P9), while Plk4+/− MEFs continue to divide and become immortalized (54). To determine what secondary changes in gene expression are associated with immortalization of Plk4+/− MEFs, Rosario et al. performed a mouse genome-wide expression array, comparing P3 Plk4+/+ MEFs, P3 Plk4+/− MEFs, and P15 Plk4+/− MEFs (101). There were no significant differences in expression identified between P3 Plk4+/+ MEFs and P3 Plk4+/− MEFs (except for the neo cassette present in the latter), whereas in P15 Plk4+/− MEFs, 659 genes were differentially expressed compared to P3. When grouped by biological function, the majority of differentially expressed genes were found to be involved in cell growth, cell death, and cellular movement pathways. Somewhat surprisingly, the difference in expression pattern predicted a decrease in cellular movement in late passage Plk4+/− MEFs, according to Ingenuity pathway analysis of biological function. A second array analysis comparing gene expression between early passage Plk4+/− MEFs that went on to be tumorigenic (T) or non-tumorigenic (NT) at late passage identified 54 differentially expressed genes. When grouped by biological function, genes associated with cellular movement were identified as significantly overrepresented, and the differences in expression predicted reduced cellular movement in tumorigenic Plk4+/− MEFs. When these genes were further categorized by whether they increase or decrease cellular movement and their relative expression levels in T and NT Plk4+/− MEFs, the majority fell into the category of genes that increase cell migration but whose expression is decreased in T Plk4+/− MEFs. Functional validation of the array results showed that late passage Plk4+/− MEFs are significantly defective in invasion when compared to early passage cells of either genotype. Furthermore, even early passage Plk4+/− MEFs are impaired in directional migration and invasion compared to wild type MEFs. In addition, HEK293T cells transfected with FLAG-Plk4 showed a significant increase in migration through a porous membrane compared to FLAG-alone transfected cells in a transwell migration assay (101). Kazazian et al. went on to show using both in vitro experiments and in vivo models, that Plk4 enhances the migration and invasion of cancer cells (102). Plk4 also enhances cell spreading and protrusion.
formation in MEFs and cancer cells (101, 102). Plk4\(^{+/−}\) MEFs exhibited a delay in cell spreading, as measured by cell area (101). As well, transfection of Plk4\(^{+/−}\) MEFs with FLAG-Plk4 resulted in a significant increase in number of protrusions per cell compared to neighboring non-transfected cells, and to cells in cultures transfected with FLAG alone (101). In a scratch-wound assay Plk4\(^{+/−}\) MEFs have a delay in actin rearrangement towards the site of wounding compared to Plk4\(^{+/+}\) MEFs. Interestingly, Plk4 localized to the edge of the protrusions and this correlated with the ability of the cell to rearrange actin filaments perpendicularly to the wound (101). The same Plk4 spreading and actin rearrangement phenotype was observed in cancer cells (102). Together, these findings suggest that Plk4 is required for faster actin rearrangement following a stimulus for cell movement. Kazazian et al. have uncovered one of the mechanisms by which Plk4 affects actin rearrangement. They demonstrate a novel interaction between Plk4 and actin-related protein (Arp) 2, a member of the Arp2/3 complex, which binds actin filaments and initiates actin branching. (102, 103) Plk4 phosphorylates Arp2 on T237/T238 activation site (104, 105) and this phosphorylation is necessary for Plk4 induced cell spreading and motility. Importantly, manipulation of Arp2 protein level did not affect the centrosome number, suggesting that Plk4 role in spreading and motility is separate from its role in centrosome duplication (102).

1.1.7 Plk4 in carcinogenesis and cancer progression

Because centrosomes play a major role in spindle formation and chromosome segregation, altered levels of Plk4 expression may lead to mitotic errors, aberrant chromosome segregation and aneuploidy, all common occurrences in cancer. Indeed, early studies in mice indicated a role for Plk4 as haploinsufficient tumour suppressor. By 18-24 months of age, 50% of Plk4 heterozygous mice have developed spontaneous liver, lung and/or soft tissue tumors, amongst which the majority are primary hepatocellular carcinomas (HCC) (60). NOD-SCID mice injected with late passage (P15) Plk4\(^{+/−}\) MEFs develop a high-grade tumour in 60% of cases, with a high mitotic index consistent with rapid proliferation. Neither early passage (P3) Plk4\(^{+/+}\) nor (P3) Plk4\(^{+/−}\) MEFs were tumorigenic in vivo (54). In human HCC 50% of specimens show loss-of-heterozygosity at the Plk4 locus, accompanied by reduced Plk4 expression at the mRNA level (54, 106). In addition, low Plk4 expression in human HCC correlates with poor survival (107). In certain hematological malignancies, Plk4 promotor has been shown to be hypermethylated
resulting in lower Plk4 expression (108). However, whether this is the cause, or the consequence of the malignancy remains to be determined.

Emerging evidence also suggests that viral infection may alter Plk4 levels, thereby affecting tumorigenesis. In particular, chronic Hepatitis B Virus (HBV) infection, a known driver of HCC, is associated with lower Plk4 expression (109). In contrast to HBV infection, overexpression of the high-risk human papilloma virus (HPV)-16-encoded E7 oncoprotein in human keratinocytes led to increased Plk4 expression and centrosome amplification (110). Together, these results indicate that Plk4 expression may be commonly altered upon viral infection, possibly contributing to the induction of carcinogenesis by these viruses.

Centrosome amplification has long been known to be the hallmark of cancer and is often associated with the advanced tumour grade and poor prognosis. However, whether supernumerary centrosomes are the cause or the outcome of cancer is still debated. Early evidence for Plk4 overexpression in tumor progression and initiation came from studies in Drosophila. When cells from sak overexpressing larval brains were transplanted into wild type (WT) fly abdomens, 36% of the flies formed tumours, many of which then metastasized to the eye (111). It is interesting to note, that although sak overexpressing cells did possess supernumerary centrosomes, they clustered to the two poles of the cell and did not cause multipolar cell division. This study suggests that the tumorigenesis was rather due to the extra centrosomes causing the loss of balance in brain stem cell division. Conversely, in mouse brains overexpressing Plk4, amplified centrosomes did not cluster efficiently. Inefficient centrosome clustering led to multipolar spindles, aneuploidy, cell death and subsequently microcephaly (112). However, in humans suffering from microcephaly Plk4 was found to be frequently mutated, usually resulting in truncation of the protein and subsequent decrease in centrosome number (113). Thus, it appears that disruption in centrosome number via Plk4 level alteration, whether up or down, may lead to microcephaly and carcinogenesis.

Plk4 expression has been found to be increased in a number of human cancers and this list continues to grow. The first study to suggest a role for Plk4 overexpression in cancer was done by Macmillan and colleagues. Using RT-PCR to compare mRNA levels in primary human colorectal cancer (CRC) tumor tissue to adjacent normal mucosa, they showed that Plk4 expression was on average increased by 66% in tumour tissue (114). As might be expected, Plk4
expression level is also significantly higher in liver metastases from CRC than in adjacent
normal liver. Other investigators found that Plk4 expression is increased in breast cancer,
especially the basal-like subtype (115-117). The depletion of Plk4 in breast cancer cells using
siRNA was anti-proliferative (61). In addition, increased levels of Plk4 expression are associated
with poor survival in breast cancer patients (115, 117). High levels of Plk4 expression were also
found to be associated with increased incidence of lymph node metastasis, distant metastasis and
local recurrence in breast cancer patients (118). In support of this finding, in NOD-SCID mouse
xenograft model, tumours formed by MDA-MB-231 shPlk4 cells did not invade through the
peritoneum and did not form lung metastasis as efficiently as those formed by the control cells
(102). In our laboratory we have shown that Plk4 regulates spreading, motility and invasion of
primary and cancer cells (including breast cancer cell lines) (101). We also showed that Plk4
localizes to cell protrusions during directional cell migration and spreading (101). The above
findings implicate Plk4 as a driver of not only tumorigenesis but also tumour invasion and
metastasis. Importantly, Plk4 has the highest overexpression in trastuzumab-insensitive Her-2-
overexpressing breast cancer cells (117), while Plk4 overexpression in breast cancer patients was
linked to poor response to taxane-based neoadjuvant therapy (118). Therefore, Plk4 may also
play a role in and act as a marker for resistance to certain therapies. Plk4 mRNA upregulation
has also been reported in gastric cancer cell lines and primary tumours (100), Hodgkin
lymphoma cell lines (119), lung cancer (100), pancreatic cancers (115, 120), cervical
and bladder cancers (115), glioblastoma (115) and other brain tumours (121) (Table 1.1).
Together, these findings show that Plk4 expression needs to be tightly regulated and its aberrant
expression may play a causative role in a number of diseases and in particular, tumour initiation
and progression.
Table 1.1 Plk4 expression in human cancers known to date.

<table>
<thead>
<tr>
<th>Low Plk4 Expression</th>
<th>High Plk4 Expression</th>
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<tbody>
<tr>
<td>Hepatocellular Carcinoma (54)</td>
<td>Colorectal Cancer (114)</td>
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<tr>
<td>Lymphoma (108)</td>
<td>Breast Cancer (115-117)</td>
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<tr>
<td>MDS/Leukemia (108)</td>
<td>Gastric Cancer (100)</td>
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<td>Pancreatic Cancer (115, 120)</td>
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<td>Bladder Cancer (115)</td>
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<td>Cervical Cancer (115)</td>
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<td>Lung Cancer (100)</td>
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<td>Glioblastoma (115)</td>
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<td>Rhabdoid Cancer (121)</td>
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<td>Hodgkin Lymphoma (119)</td>
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Although Plk4 is overexpressed in variety of human cancers, and such cancers often exhibit high centrosome overduplication, until recently it was unclear whether Plk4 and centrosome overduplication alone can cause tumorigenesis or are the secondary effect thereof. Given that centrosome duplication is tightly regulated to occur only once per cell cycle and if something goes wrong with this process, one of the mitotic checkpoints gets activated, it seems intuitive that proliferation of cells with aberrant centriole number would require a primary mutation in a checkpoint or a checkpoint-triggering protein. Indeed, as mentioned previously, early studies showed that Plk4 expression is controlled by p53, a regulator of several cell cycle checkpoints and one of the most commonly mutated proteins in cancer. MEFs derived from p53 deficient mice exhibit supernumerary centrosomes and aberrant spindle formation, consistent with Plk4 overexpression, although this effect may also be due to overexpression of other mitotic proteins controlled by p53 (122). However, Plk4 overexpression-induced supernumerary centrosomes trigger p53 dependent cell cycle arrest and apoptosis (32). In cells lacking p53, supernumerary centrosomes may bypass the p53-dependent cell cycle arrest and continue to proliferate, thus increasing multipolar spindle formation, chromosome instability and as a result accumulation of further tumorigenic mutations and chromosome aberrations (123). This was not the case in a study by Kulukian et al., which conditionally overexpressed Plk4 in mouse epidermis (124). They showed that although Plk4 overexpression resulted in supernumerary centrosomes, aneuploidy and chromosomal aberrations, these cells were largely cleared in a p53-dependent manner. However, reducing p53 expression did not result in the significant increase in extra-centrosome bearing or aneuploid cells compared to Plk4 overexpression alone, suggesting that other mechanisms preventing proliferation of cells with supernumerary centrosomes exist. Of note, the authors did not observe spontaneous tumorigenesis in mice overexpressing Plk4, even at old age. Similar observations were made by Vitre et al., who showed that although Plk4 overexpression results in significant increase in Plk4 levels and as a result, centrosome number in liver and lungs of p53 null mice, these mice get tumours at an identical rate and have the same survival rate as p53 null mice (125). This supported the idea that other mutations are required and that Plk4 overexpression or centrosome overduplication on its own is not sufficient to drive tumorigenesis, even in the absence of p53. However, studies by Sercin et al. and Coelho et al. which utilized inducible Plk4 overexpression to study the effect of supernumerary centrosomes in mouse skin epidermis showed that in the absence of p53, Plk4 overexpression does accelerate
tumorigenesis (97, 126). The differences between these studies may be due to the shorter exposure to and different timing of Plk4 overexpression in the latter two studies. The first of the two latter groups turned off Plk4 overexpression postnatally. Once the Plk4 overexpression was turned off, the cells carrying supernumerary centrosomes were cleared by P3 (126). The second group induced Plk4 overexpression at 8 weeks postnatally, eliminating the exposure of mouse embryos to Plk4 overexpression and centrosome amplification. This perhaps explains why these mice developed tumours, while the ones with prolonged Plk4 overexpression did not. In particular, in mice with prolonged Plk4 overexpression, the cells with supernumerary centrosomes were cleared by both p53 dependent and p53 independent mechanisms (when p53 was absent or depleted), halting the propagation of any chromosomal aberrations that might have occurred. However, when Plk4 is overexpressed only for a short period of time, cells that initially carried supernumerary centrosomes go back to normal centrosome number in a few divisions (126), which allows them to continue proliferating while propagating any chromosomal aberrations that might have been acquired. Finally, recent work by Levine et al. through an elegant design of a mouse model conditionally overexpressing Plk4 upon doxycycline induction showed that even in the presence of p53, Plk4-driven centrosome overduplication results in spontaneous formation of lymphomas, squamous cell carcinomas and sarcomas (98). They created a mouse with only a single additional copy of Plk4 and induced Plk4 overexpression from 1 to 2 months of age chronically or only for one month. Strikingly, mice developed similar tumours in both cases, suggesting that even transient overexpression of Plk4, followed by centrosome amplification is enough to cause prolonged centrosome amplification and tumorigenesis. It is important to note, that the tumour cells from these mice harbored only one to two extra chromosomes, which perhaps allowed them to escape the cell cycle arrest seen in previous studies upon induction of centriole overduplication. In addition, the authors note that upon examination of lymphomas formed in Plk4 overexpressing mice on prolonged doxycycline treatment, a wide range of p53 expression was observed but on average it was lower compared to mice not exposed to doxycycline, suggesting that this pathway is at least partially compromised. It is therefore significant that mice with short exposure to doxycycline still developed tumours, since short exposure to Plk4 overexpression minimizes the potential non-centrosome related effects. Hence, this study established Plk4 as an oncogene and showed that centrosome amplification alone can drive tumorigenesis. Together these studies suggest that while chronic
Plk4 overexpression and centrosome amplification results in cell cycle arrest and apoptosis, mild increase in Plk4 levels and centrosome number can be tolerated but leads to aneuploidy and tumorigenesis. Additionally, compromised p53 pathway was either a prerequisite for or the result of Plk4-driven tumour progression or initiation.

Plk4 has also been shown to interact with another commonly mutated or inhibited tumor suppressor- phosphatase and tensin homolog (PTEN). First, Brough and colleagues discovered a synthetic lethal genetic interaction of Plk4 and PTEN in breast cancer cell lines (127). A dependency of PTEN negative breast cancer xenografts on Plk4 was then demonstrated by Mason et al. (61). When these tumours were treated with Plk4 inhibitor CFI-400945, their growth was reduced to a significantly greater extent than that of PTEN wild type xenografts. Therefore, PTEN status can be used as a marker to predict the response to Plk4 inhibitor therapy. However, more studies to better understand how exactly the PTEN and Plk4 pathways interact are necessary.

Plk4 role in centriole duplication, spindle assembly and cell cycle progression, as well as its structure which is the most divergent from other Plk family members makes it an attractive target for small molecule inhibitors that can be used for anti-cancer therapy. Indeed, two Plk4 small molecule inhibitors, CFI-400945 and centrinone, have been generated to date (61, 62). Plk4 was selected as a target for inhibitor design by Laufer et al., based on the RNAi screen of kinases and kinase-related genes that affect breast cancer cell viability, as well as due to its overexpression in most breast cancer cell lines (115, 128). To confirm that Plk4 depletion would decrease tumour growth in vivo, a xenograft model of MDA-MB-468 shPlk4 and control shRNA was established. Tumours formed by the cells with reduced Plk4 expression were significantly impaired in growth compared to controls (61). A screen of ligand-based library of potential kinase inhibitors as well as molecules identified through Plk4-homology model yielded a promising candidate which was then modified to enhance potency and specificity leading to the development of Plk4 inhibitor CFI-400437 (128). This was further modified to increase the drug’s oral bioavailability and activity, resulting in the development of CFI-400945 inhibitor (129). CFI-400945 has been shown to differentiate between Plk4 and other members of the Plk family, however it also inhibits off-target kinases, namely Aurora A (AURKA), Aurora B (AURKB), TIE2, TRKA and TRKB, albeit to a lesser degree than Plk4 (129). Somewhat surprisingly, the treatment of cancer...
cells (U2OS, MDA-MB-231 and MDA-MB-468) with lower doses of this inhibitor led to centriole overduplication, consistent with overexpression of Plk4 (58, 61). This may be due to only partial inhibition of Plk4. In this case, kinase inactivated Plk4 would bind and stabilize uninhibited Plk4 due to reduced trans-autophosphorylation, similar to overexpression of kinase dead Plk4 (130). This is supported by the fact that at higher dose of the inhibitor, and hence more complete Plk4 kinase inhibition, only one centriole per pole was observed. The centrosome number was not followed for sufficient amount of time to determine whether prolonged exposure to high doses of CFI-400945 would result in complete centrosome loss. With both doses however, the authors observed multipolar spindle formation that led to cell cycle arrest or cell death (61). In case of higher dose of CFI-400945 the formation of multipolar spindles was due to cytokinesis failure, which the authors attributed to the AURKB inhibition. In a human breast cancer xenograft model in mice, treatment with CFI-400945 resulted in significant reduction of tumour growth at a well-tolerated dose (61). These encouraging results led to the initiation of a clinical trial for this Plk4 inhibitor in breast cancer patients. Further studies carried out by the same group suggest that CFI-400945 may also be used in treatment of pancreatic cancer (120). They show reduced tumour growth in patient-derived pancreatic cancer xenograft models treated with this inhibitor.

Given that CFI-400945 also inhibited AURKA and AURKB, another group set out to create a more specific Plk4 inhibitor to study the role of centrosome overduplication in cancer and in cell proliferation. They started with a pan-Aurora kinase inhibitor VX-680, which targets the ATP-binding site of these kinases and modified it to be Plk4 specific (62, 131). They show high selectivity of the resulting inhibitor centrinone (and less potent centrinone B) towards Plk4, as compared to Aurora kinase family, Plk family and a panel of 422 human kinases. Centrinone inhibited centriole formation in a number of primary and cancer cell lines (62). They confirmed that the loss of centrosomes was due to Plk4 inhibition, by rescuing the centrosome duplication with overexpression of drug-insensitive Plk4 mutant. Interestingly, while primary cells lacking centrosomes arrested at G1, cancer cells were able to continue to proliferate, albeit at a slower rate and with reduced mitotic fidelity. This suggests that while centrosomes are essential for normal cell proliferation, cancer cells have additional mutations that allow them to bypass the G1/S checkpoint. It is important to note that upon centrinone washout, the centrosome number returned to pre-treatment level in cancer cells (62). Together these findings suggest that cancer
cells with increased number of centrosomes are not addicted to them, irrespective of the extent of the overexpression, but they do have a certain cell line-specific centrosome number that can be tolerated without triggering cell cycle arrest.

In sum, the above findings show that tight control of Plk4 level is essential and that manipulation of Plk4 expression, whether up or down, may lead to carcinogenesis and other serious disorders. In order to create new Plk4-based anti-cancer agents and to enable selection of the patients that would benefit most from this therapy, a better understanding of the mechanisms of Plk4-driven tumorigenesis, tumour progression, invasion and metastasis is required.

1.2 Cell Migration

Cell migration is an essential process during embryo development and maintenance of homeostasis. Cell migration occurs throughout embryogenesis. One example is the formation of gastrula which is dependent on cell migration from a single-layered blastula to different parts of the embryo to create multilayered structure, with each layer later forming different organs. During immune response, immune cells migrate from the blood stream to the surrounding tissues and back, to neutralize pathogens. Cell migration is also necessary during wound healing and tissue regeneration. Given the importance of these processes, it is not surprising that deregulation of cell migration may lead to serious developmental abnormalities and autoimmune diseases. In addition, when cancer cells that arise in non-motile tissues gain the ability to move through a process called epithelial mesenchymal transition (EMT), they gain metastatic potential. Once the primary tumour metastasizes, it becomes harder to treat by surgery and more resistant to therapy. Thus, it is the metastasis that most often leads to the death of cancer patients, not the primary tumour per se. Therefore, many studies focus on determining the modes and mechanisms of cell motility. Cell motility is usually triggered by an external signal of either physical or chemical nature. This signal is then picked up by the transmembrane cell receptors which initiate a chain of intracellular signaling leading to cytoskeletal rearrangement and thus the initiation of cell movement. Cytoskeleton coordinates cell movement through the re-shaping of the cell membrane and providing the mechanical force necessary to move forward. It consists of three biopolymer types: actin filaments (composed of actin), microtubules (composed of tubulin) and intermediate filaments (various structural proteins). Microtubules play a role in cell polarity and vesicular trafficking, as well as forming the bulk of mitotic spindle. In turn, intermediate
filaments mostly act as cell “scaffolding” and therefore determine cell shape by bearing tension that otherwise would deform it. In addition, they also play a role in cell-cell and cell-matrix adhesions. Although microtubules and intermediate filaments often play important roles in cell migration, actin filaments are the main drivers of this process and their polymerization alone can be sufficient for the cell movement (132). Hundreds of other proteins are involved in coordinating the many complex processes that occur during cell motility, but even though there are different modes and types of cell motility, a lot of the underlying mechanisms guiding the mechanical aspects are similar. Broadly speaking, cell motility can be divided into individual cell motility, such as that of immune cells and often cancer cells, and collective cell motility, such as the collective movement of a sheet of cells during embryogenesis or wound healing. Here, the focus will be on mesenchymal cell motility, the most common and well described type and only the differences with the other types of motility will be mentioned in their respective sections.

1.2.1 Individual cell migration

As mentioned previously, individual cell migration occurs most often during immune response but also may often be seen in cancer cell metastasis. At least two major types of individual cell migration have been described to date: mesenchymal and amoeboid, the latter more commonly seen in cancer cells travelling in 3D matrix/environment.

1.2.1.1 Mesenchymal cell migration

Most eukaryotic animal cells that are motile, utilize mesenchymal cell migration, also known as cell crawling. Upon receiving the signal to move, the cell becomes polarized, with a wider, protrusive front, known as the leading edge; and a thinner, retractive back, known as the trailing edge. Three major stages of mesenchymal cell motility are: protrusion formation, protrusion attachment and de-attachment of the cell rear, and contraction of the trailing edge.

Protrusions begin to form by means of actin polymerization in the direction of cell migration. Actin is a 42kDa protein that is widely expressed in all eukaryotic cells, except for the nematode sperm (133). Actin family of proteins is comprised of α-actin (3 isoforms), β-actin and γ-actin (2 isoforms). The α-actin isoforms and the γ-smooth-actin isoform are expressed mainly in smooth muscle, cardiac and skeletal cells, while the remaining two cytosolic isoforms are ubiquitously expressed. All actin isoforms have no less than 93% sequence similarity and due to this similarity in structure, their functions often overlap (134). However, unique functions for each
isoform have also been reported and are often determined by their localization and isoform-specific interactions with other proteins (135-137). In the cell, actin exists as a free monomer, known as globular or G-actin, or it can assemble into filaments which are known as filamentous or F-actin (Figure 1.4). G-actin monomers are ATPases which usually exist in an ADP or more commonly an ATP-bound form, while nucleotide free actin is rapidly degraded. Filamentous actin is physiologically more relevant, as it is capable of binding myosin, thus creating the contractile force necessary for motility. Actin filaments are polymers that are built from dimers of globular actin molecules and are organized into a double helix. Upon the addition of the actin monomer to the growing filament, ATP is hydrolysed to ADP+Pi. Hydrolysis of ATP is not necessary for the monomer binding, but it contributes to the filament stability, as ATP is rapidly hydrolysed upon binding and while ADP remains bound to the actin molecule, Pi is slowly released (138, 139). Therefore, through the process of ATP hydrolysis, the actin polymer becomes polar in nature with a fast-growing “barbed” or “plus” end, where ATP is hydrolysed upon monomer incorporation, and a slow-growing “pointed” or “minus” end which consists mostly of ADP-bound actin monomers (140). The surrounding concentration of actin monomers determines the pace of the growth of the actin filaments. Therefore, it is important for motile cells to maintain a high enough concentration of monomeric actin. This is achieved by binding of the large pool of G-actin to the sequestering proteins profilins and β-thymosins. Both of these proteins bind G-actin in 1:1 ratio and relatively high concentrations of each are required to maintain adequate pool of free actin (141). Profilin binds actin more effectively than β-thymosin and facilitates the ADP to ATP exchange on G-actin to form ATP-G-actin:profilin complexes (141, 142). Profilin can bind only the barbed end of the actin filament, thus participating in its elongation. Therefore, profilin-bound G-actin represents the main source of free actin for building of the actin filament. β-thymosin on the other hand, preferentially binds to ATP-G-actin and has low affinity to actin filaments, hence ensuring the availability of adequate free actin pool (143, 144). The interaction between β-thymosin and G-actin is weak and is frequently broken and reformed, allowing the unbound actin to be added to the filament, when necessary (141). As actin monomers are incorporated into the filament, the concentration of the G-actin decreases, which in turn causes actin depolymerization at the pointed end, thus maintaining the actin equilibrium. The process of actin polymerization at the barbed end and depolymerization at the pointed end is known as treadmilling.
Figure 1.4

Figure 1.4 Regulation of actin nucleation and polymerization.

a Sufficient concentration of monomeric (G) actin is maintained by 1:1 binding of actin monomers to profilin, coflin or β-thymosin.

b Actin filaments are shaped as a double helix and grow fast at the barbed end (B) and slow at the pointed end (P). Actin monomers can polymerize spontaneously but this reaction is unfavored as the actin dimers and trimers are unstable and thus require actin nucleation proteins.

c Formins bind actin monomers and aid dimer and trimer formation, a process known as nucleation. They remain attached to the barbed end of the filament and aid further polymerization, forming straight actin filaments.

d Nucleation-promoting proteins, such as WASP/Scar, aid Arp2/3 complex formation. Arp2/3 complex then nucleates daughter actin filaments at a 70° angle to the mother filament, by acting as an actin dimer. This results in branched actin filaments.

e Capping proteins bind the barbed end of the filament to stop its growth. Severing proteins, such as Coflin and Gelsolin, sever actin filaments. Crosslinking proteins bind actin filaments in anti-parallel fashion, creating actin “mesh” networks or in a parallel fashion, creating actin bundles, which form stress fibers.

Figure adopted from Pollard and Cooper, 2009 (145).
This dynamic nature of the actin filaments allows for the flexibility of the cytoskeleton and enables the cell to react quickly to the changing signals or environment. The formation of actin filaments begins with a process known as nucleation, where three or four actin monomers are assembled thus forming a nucleus onto which the rest of the filament is built. Actin nucleation can be initiated spontaneously but this reaction is extremely unfavoured due to the instability of the actin dimers and the fact that most G-actin is bound to actin-sequestering proteins (146).

Instead, nucleation is usually initiated with the aid of nucleating proteins the best characterized of which are Arp2/3 complex and some proteins of the formin family. Two types of protrusions occur at the leading edge: filopodia, which are long, thin, finger-like protrusions, and lamellipodia, wide and sheet-like protrusions (Figure 1.5a). Filopodia are mostly formed by the cell to sense signals and probe for changes in microenvironment, while lamellipodia are the protrusions that drive cell migration. Different arrangement of actin filaments is what gives distinct shape to these protrusions. To form lamellipodia, actin filaments branch out and interlink, creating a wide mesh that extends the cell membrane forward. The formation of the actin branches is initiated by the Arp2/3 complex. The complex consists of Arp2 and Arp3 actin-related proteins and it is aided by five other protein subunits (ARPC1-5) in the initiation of branching. Arp2/3 mimics the actin dimers and binds the existing actin filament at the site where the branch is to be formed at a characteristic 70° angle, where it acts as a template for the actin filament formation (147, 148). However, until the signal to move is received by the cell, the Arp2, Arp3 and the ARPCs are inactive and the binding of actin by the Arp2/3 complex is unfavoured (148-150). The formation of this complex therefore requires assistance from a set of proteins known as nucleation-promoting factors. The most common and well-characterised nucleation promoting factors in cell motility are Wiskott-Aldrich syndrome protein (WASP), neural WASP (N-WASP) and WASP-family verprolin-homologous (WAVE). These are WASP-homology 2 (WH-2) domain containing proteins that allow for binding of G-actin monomer to the Arp2/3 complex, which mimics an actin dimer (151, 152). This creates what looks like an actin trimer or nucleus, which sets the stage for further polymerization. This branched actin is then cross-linked by filamin family of proteins to form a mesh and still other proteins such as ERM (ezrin/radixin/moesin) and spectrins cross link the actin filaments to plasma membrane (PM). In filopodia protrusions actin filaments are not branched. Instead, multiple straight filaments are bundled by bundling proteins such as fascin, to create a rod-like shape. A different
set of proteins, those from the formin family, is responsible for the nucleation of actin during filipodia formation (153). The most common actin nucleator of the formin family is mammalian Diaphonous (mDia). To initiate the nucleation, formin dimers are formed, where the formin homology (FH) 2 domains of each monomer form a ring-like structure (reviewed in (154)). Two actin monomers are then bound by the formin dimer and when they pass through this ring-like structure, the actin monomers are forced to form a dimer, hence creating an actin nucleus. Interestingly, the formin dimer remains attached to the barbed end of the growing actin filament and acts as an actin elongating protein (155). Formins and other F-actin elongating proteins, such as Ena/VASP, are multidomain proteins that bind G-actin with one domain and the barbed end of the actin filament with a different domain, thus delivering the actin monomer to the growing end of the filament. The force created by the elongation of F-actin is what drives the plasma membrane forward, creating a protrusion (156, 157). The length of the actin filament is controlled by the capping proteins. They bind to the barbed end of the actin filament and prevent both the addition and the dissociation of actin monomers, keeping the length of the filament constant. Very few proteins cap the pointed end of the actin filament, most of which are tropomodulins. When the protrusion formation needs to stop or change direction, actin depolymerization can be aided by the severing proteins of the actin depolymerizing factors (ADF)/cofilin complex or gelsolin-like proteins, some of which can also act as capping proteins.

While the protrusion is forming, it also needs to attach to the extracellular matrix (ECM), otherwise it becomes unproductive and retracts due to the retrograde flow (158). First, at the front of the protrusion a very transient adhesion is formed, known as a nascent adhesion. It can then become bigger through binding of multiple structural proteins and mature into a more stable focal complex. Further maturation leads to focal adhesion formation, a relatively stable type of adhesion, found mostly towards the rear of the cell. The different stability of the adhesions is necessary, as without adhesions a lamellipodium cannot be maintained but with only stable adhesions it cannot extend further. Although adhesions vary in stability, the core structural components which form them are the same. Adhesions are usually formed by connection of actin filaments to the ECM by adhesion receptors. Adhesion receptors are transmembrane proteins of which integrins are the most common kind in protrusions. Integrins can bind ligands at both ends, the extracellular and the cytosolic, therefore establishing the communication between the intracellular signaling and the microenvironment. Inside the cell, integrins bind linker proteins.
that attach them to the actin filaments. For example, talin can directly bind F-actin through its C-terminal and integrins through its N-terminal (159-162). Other proteins such as vinculin bind F-actin and talin, providing additional layer of regulation of adhesion formation and strength (162-164). In filopodia, myosin-X is at least in part responsible for bringing the integrins to the protrusion, thus regulating its attachment (165). Upon receiving an extracellular signal, integrins also bind intracellular proteins, such as focal adhesion kinase (FAK), which initiate signaling cascades, including Rho GTPase activation, leading to further actin polymerization and activation of other pathways. Overall, the strength of the adhesion depends on the cell type, the type of the ECM and the type of the extracellular signal.

Behind lamellipodia, which themselves are only 1-3 µm from the cell edge, is a much more extensive structure known as lamellum (166). Lamellum spans about 5-15 µm from lamellipodium towards the cell interior, and it is less dynamic than lamellipodium due to more stable attachments (focal complexes), slower actin polymerisation and actin rearrangement (166). In lamellae, the Arp2/3 complex is practically absent and therefore, the actin is not branched (167, 168). Instead, the actin is arranged into straight filaments that are antiparallel and are cross-linked by α-actinin and class II myosin. The actin filaments can also be arranged in a parallel manner and cross-linked into bundles by tropomyosins and filamins to form what are known as stress fibers. Phosphorylation of the myosin in a Rho GTPase-dependent manner, leads to the myosin conformation change, thus inducing filament sliding, and therefore creates a contractile force (169). Since lamellum helps stabilize the cell protrusion, this structure is more common and more prominent in the slower-moving cells, such as fibroblasts, as compared to the fast-moving cells, such as neutrophils. There is no precise border where the lamellipodium ends and the lamellum begins, since the two structures overlap (170).

Towards the rear of the cell, the actin filaments are cross-linked in an anti-parallel fashion less often and rather organized into parallel bundles. In the cell body, the cell-ECM attachments grow into stable focal adhesions, which inhibit protrusion formation at that site. The actomyosin complexes start to form in the cell body and become more extensive at the cell rear. Actomyosin complexes consist of F-actin bundles interlinked with myosin II minifilaments. Myosin II dimerizes and in turn, the dimers assemble into at least hexamers, with all the globular myosin domains (or “heads”) pointing in one direction and the heavy chain “tails” in the other.
Therefore, like actin, myosin is polarised. To form minifilaments, several myosin hexamers are assembled in a tail-to-tail fashion, thus generating a bipolar filament. Each pole of the minifilament binds a barbed end of F-actin, creating an actomyosin complex. ROCK-dependent phosphorylation and ATP hydrolysis at the head region of myosin II, leads to its conformational change, thus bringing the actin filaments together and resulting in contraction (171). This contraction brings the cell rear and the cell body forward. It is interesting to note that although actomyosin contractility is the most common mechanism of rear end contraction, it is not the sole mechanism. Other mechanisms such as the force of cell protrusion and the force of actin depolymerization and cross-linking contraction are also able to retract the cell rear (172). The predominant mechanism of cell rear retraction is often cell-type specific. To allow for contraction to occur, the focal adhesions at the cell rear are disassembled and their components are recycled to the leading edge.

Actin cytoskeleton generates the force for the cell migration by growing protrusions at the front and providing actomyosin contraction force at the cell rear. However, in order to start this process, the cell first needs to establish where its front and rear will be. This process is called cell polarization and it is essential for directional cell migration. In a non-polarized cell, membrane receptors, cytoskeletal components and other players involved in cell motility are distributed equally throughout the cell. Cell polarization is the partitioning of these components between the front and the rear of the cell. Cells can polarize spontaneously but more often polarization is induced by an extracellular cue of chemical or mechanical nature (173-175). For example, a gradient of chemoattractant induces the re-distribution and clustering of the appropriate receptors, often G-protein-coupled receptors (GPCRs), at the point of the highest chemoattractant concentration (176). This re-distribution of the receptors is followed by the equivalent re-distribution of various adaptor and signaling proteins that interact with GPCRs, creating an asymmetry where the point of the highest concentration of these biomolecules becomes the cell front, and that of the lowest concentration, the cell rear. For example, Rho GTPases cell division cycle (Cdc42) and Ras-related C3 botulinum toxin substrate 1 (Rac1) are activated downstream of nascent adhesions at the cell front where they regulate further cell polarization, actin polymerization and protrusion formation. In contrast, RhoA is activated predominantly toward the cell rear, where it regulates actomyosin contractility and adhesion maturation. In addition, cells polarize in response to the rigidity of the extracellular matrix, with
the front forming towards the more rigid matrix (177). This is in part due to the higher tension exerted on the cell by the rigid ECM, which favours the formation of the focal adhesions, in turn leading to stress fibre formation and other actomyosin rearrangements (178, 179).

Besides the various cytoskeletal rearrangements, mesenchymal cell migration is characterized by the ECM degradation at the leading edge to make way for the cell. This is achieved by the secretion of the matrix degrading enzymes, matrix metalloproteinases (MMPs). There are 24 MMPs in humans, some of which are specific to certain substrates, while others can degrade multiple substrate types. However, together the MMPs can degrade all types of the ECM (reviewed in (180)). Aside from degrading the ECM, MMPs can also degrade cell-cell and cell-matrix adhesion proteins, thus playing an important role in EMT, which significantly enhances cancer cell migratory potential (181, 182).

1.2.1.2 Amoeboid cell migration

Many circulating immune cells, stem cells and cancer cells utilize a mode of cell migration known as amoeboid cell migration in 3D environment (183). This type of migration is characterized by low matrix adhesion, no stress fibre formation, no MMP expression and high actomyosin activity (Figure 1.5a) (184). Due to low adhesion to the ECM this mode of motility is much faster than mesenchymal motility. Instead of crawling by the means of lamellipodia extension and ECM degradation, amoeboid movement is achieved by squeezing through the ECM (185). There are two types of amoeboid migration. One is α motility, which is characterized by rounded but elongated cell shape that is polarized, with the cell front known as the pseudopod and the cell rear known as the uropod and very weak ECM adhesions (186). Unlike lamellipodia, which are very thin sheet-like structures, pseudopodia are thick 3D structures. Pseudopodia are filled with branched actin filaments, formation of which depends on the WASP and WAVE activation of the Arp2/3 complex (186). Actin polymerization creates a retrograde flow in the front of the cell that helps propel it forward (187). Since the same proteins are responsible for lamellipodial extension, it is still unclear how the formation of one vs. the other is regulated. Uropods are thicker than the trailing edge of cells undergoing mesenchymal migration, however they also utilize actomyosin contractility to retract the cell rear (188). The second type of amoeboid motility is motility by blebbing. In this mode of motility cells are mostly round, lack ECM attachment and move by forming round membrane protrusions or blebs.
in the direction of the movement. There is a high amount of cortical actin and myosin II throughout the cell that is tethered to the plasma membrane by ERMs (189). RhoA/ROCK pathway driven cortical actomyosin contraction of the cell body creates a hydrostatic pressure which can rupture the actomyosin network (190, 191). When the plasma membrane detaches from actomyosin network, internal cell pressure creates a membrane protrusion in the form of bleb (189). Once formed, the bleb keeps expanding until the pressure inside the cell is stabilized. At this point formin-driven actin polymerization and ERM-dependent PM-tethering starts to occur in the bleb (189). Next, myosin is attached to the polymerized actin and actomyosin contraction then causes a retraction (189). Unlike in α motility, actin polymerization together with myosin II contractility create a retrograde flow in the entire cell, which in turn stabilizes the bleb mode (192). As there is evidence that membrane tension changes during bleb formation and that it may regulate Rho GTPase activation, it is likely that it regulates the expansion and retraction of blebs (193, 194). In turn, this suggests a mechanism for contact-driven direction of amoeboid migration. When the cell encounters an obstacle, the membrane tension increases, causing the initiation of bleb retraction, while blebs that do not encounter an obstacle and are under low membrane tension continue to expand and therefore the direction of blebbing changes. As well, a study by Lorentzen et al. suggest that during blebbing, it is the rear of the cell that forms first, thus dictating the polarity of the cell and general direction of movement (195).

Cells can switch between mesenchymal and amoeboid migration. Which type of migration to use is often cell type dependent, as some cell types are less adhesive than others or lack regulators necessary for a certain type of migration (192). Substrate also plays a big role in dictating the mode of motility, as amoeboid cell migration is more common on soft substrates and mesenchymal on hard substrates (196).
polymerized and the filaments are less branched, while the adhesions grow into focal complexes.

At the cell body, straight actin filaments are gathered into actin bundles or stress fibers, which bind myosin. Here, the adhesions mature into focal adhesions. Actomyosin bundles are concentrated in the cell rear and their contraction helps bring the rear forward, while adhesions are disassembled. (ii) During amoeboid migration the cell does not adhere to the ECM nor secrete MMPs to degrade the matrix. Instead, the contraction of the actomyosin cortex increases the pressure inside the cell, leading to the break in the actomyosin cortex, which gives rise to a membrane bleb protrusion. By means of these blebs, the cell squeezes through the ECM. Once the bleb increases in size and the pressure inside the cell starts to equalize, the actomyosin cortex is rebuilt inside the bleb. b In collective cell migration, cells with a free edge become leader cells and behave similar to the mesenchymally migrating individual cells: they form lamellipodia and filopodia protrusions and secrete MMPs. The leader and the follower cells are connected by the cell-cell adherence junctions, which also link the actin cytoskeletons of the neighboring cells. Actin cables formed by the cells with a free edge help preserve its mechanical integrity.
1.2.2 Collective cell migration

Collective cell migration is essential for tissue morphogenesis and organ formation during developmental stage, as well as for wound-healing, tissue homeostasis and angiogenesis among other processes, in adulthood. It also plays a role in disease, being a common type of migration in cancer invasion and metastasis (197). The defining feature of collective cell migration is the migrating cells being stably or transiently interconnected by cell-cell adhesions, polarized in the same direction and thus acting as a single unit and migrating at the same speed (**Figure 1.5b**). This migration can occur in 2D “sheets” of either monolayers or multilayers, or 3D “clusters” organized into tubes or in case of cancer cells poorly organized. These cells can move on or though the ECM but can also use other cells as a substrate. Collective cell migration is always directional, where the cells are following a cue, such as a chemoattractant or a mechanical cue. Due to the constant remodeling of the ECM and other ongoing changes in the cell environment, studying collective cell migration *in vivo* has proven very challenging. While new *in vivo* techniques are being developed, a number of *in vitro* techniques have been well established to study collective migration. One of the most common such techniques is a 2D wound healing assay. In this assay, a gap is created in the monolayer of cells by means of cell removal, for example by a scratch, or by removal of a barrier separating the two monolayers of cells. Cells then move into the gap to try to close it and form a monolayer again. The cells facing the directional cue often form a “leading edge”, while the cells behind them form a “trailing edge”. Therefore, these cells act as one big cell with the “leader” cells in the leading edge creating a forward traction force and the “follower” cells being pulled behind the leader cells. The two major types of collective cell migration are epithelial and mesenchymal (198). In epithelial cell migration, the cells are tightly bound to each other and move as one collective front. In mesenchymal collective migration, the adhesions between the cells are transient and the leader cells can sometimes separate into single cells until new adhesions are formed. In both types, the leader cells adopt mesenchymal cell morphology, with lamellipodia forming towards the direction of movement and integrin-based adhesions that create the traction force necessary for the forward motion. Similar to mesenchymal migrating single cells, the leader cells degrade the ECM by MMP secretion and thus form a “path” for the follower cells (199). Consequently, the ECM degradation and the traction forces created at the leading edge, drive the cell collective forward. However, in some modes of collective cell migration, such as in tubular branching, the
leader cells are indistinguishable from the follower cells morphologically (200, 201). In this case, the leader cells can still be identified by the different gene expression patterns, which all leader cells exhibit, compared to the follower cells (202, 203). Not all cells with a free edge become leader cells. The factors determining which cells will become leaders are still under investigation, but a few factors influencing this decision are already known (203, 204). One of these is the proximity of the cell to the guidance cue, the closer the cell is to it, the more likely it is to become a leader cell. Another factor is the intracellular signaling, where the ability of the cells to activate the pathways needed for the leader cell formation is important for establishing the cell fate. However, once the cell becomes a leader it is not always committed to this fate. In some modes of collective cell migration, such as the one observed in angiogenesis, there is constant competition to become leader cells (203, 205). This way, if the guidance cue is moved for example, the cells that are now closer to it can become leaders, while those that were leaders can still switch to the follower position. The non-leader cells at the front, form thick actin cables at their free edge. The actin cables of each cell are interconnected with neighboring cells by adherence junctions, to preserve the leading edge mechanical integrity (206). These cables also exhibit actomyosin contractility and can drive cell migration by a purse-string mechanism (207). The leader cells relay the information on the speed and the direction of movement to the follower cells through the cell-cell adhesions. Through these adhesions, the follower cells sense the direction of the pull of the leader cell and that establishes the “front” of the follower cells. Therefore, cell-cell adhesions are essential for directed cell movement, a key feature of collective cell migration. The most common types of cell-cell adhesions involved in collective migration are adherence junctions which are cadherins-based (204). Cadherins are transmembrane receptors that link the actin cytoskeleton of one cell to the actin cytoskeleton of another cell. This relays the changes occurring in actin cytoskeleton of the leader cell to that of the follower cells. Highlighting the importance of adherence junctions in collective cell migration is the fact that inhibition of cadherins abolishes collective cell migration (208). In turn, the different types of collective cell motility are in part determined by the different types of cadherins involved in the adherence junctions. The follower cells do not adopt mesenchymal morphology, but they can form lamellipodia-like structures, that extend in the direction of movement underneath the cells that are in the front. Such lamellipodia are called “cryptic lamellipodia” and they are believed to help to propel the cell forward by generating more traction force (209).
Although the type of collective cell migration described above is most common and well-studied, recent advances in live-imaging have helped to uncover other types of collective cell migration, where the cells lack a clear leading edge or lack a free edge altogether (210). Studies to elucidate the precise mechanisms of these types of collective migration are ongoing.

Importantly, despite the added complexity of cell-cell adhesions, the intracellular mechanisms that regulate collective cell movement are the same as those that regulate single cell motility (211). The main drivers of these modes of motility are Rho GTPases, which control cell-cell and cell-matrix adhesion formation, cytoskeleton rearrangement and expression of MMPs and other genes. This is highlighted by the fact that collectively moving cells can switch to individual cell migration and vice versa (212). However, the distribution of Rho GTPases may be different in leader vs. follower cells. For example, cell-cell contacts inhibit protrusion formation by activation of RhoA, while Rac1 and Cdc42 Rho GTPases accumulate at the contact free edge, where they induce actin polymerization and protrusion formation, thus forming a leader cell (213, 214). Since follower cells usually do not have a free edge, they do not form extensive protrusions. In contrast, in mesenchymal migration of individual cells, the absence of cell-cell contacts always results in leader-cell type morphology and Rac1 and Cdc42 activation in the front and RhoA activation mostly at the rear of the cell. The pathways regulated by the Rho GTPases in cell motility will be discussed in more detail in the next section.
1.3 Rho GTPases

GTPases are proteins with a highly conserved G domain that binds guanosine triphosphate (GTP) and hydrolyzes it into guanosine diphosphate (GDP) and inorganic phosphate (P$_i$). They act as molecular switches to affect almost every process in the cell, including but not limited to protein biosynthesis, cell proliferation and differentiation, vesicular transport, protein shuttling through membranes and signal transduction from the transmembrane receptors. GTPases are divided into three large superfamilies: heterotrimeric G proteins (also known as large GTPases), GTPases in protein biosynthesis and translocation and rat sarcoma viral oncogene homolog (Ras) superfamily (also known as small GTPases) (215). Rho GTPases are one of the five subfamilies of the Ras superfamily that regulate cell polarity and motility, cytoskeleton rearrangements, cell cycle progression, vesicular transport and axon guidance (216). Rho GTPase family consists of over 20 members in mammals. They are further organized into 8 subfamilies the best characterized of which are Rho (RhoA, RhoB, RhoC), Rac (Rac1, Rac2, Rac3, RhoG) and Cdc42 (Cdc42, RhoQ, RhoJ) (Figure 1.6a) (217).

Rho GTPases are small (~21 kDa) molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state (Figure 1.7). GTPase activating proteins (GAPs) facilitate the GTP hydrolysis into GDP, while GEFs facilitate the exchange of the GDP to GTP, thus activating the GTPase. There are over 80 Rho GEFs and 70 Rho GAPs identified in humans, which allows for precise regulation of activation of the Rho GTPases (218). In addition, Rho GTPases are regulated by the guanine dissociation inhibitors (GDIs), which bind the inactive (GDP-bound) Rho GTPases and prevent their translocation from the cytosol to the membrane, where they can be activated. Only three Rho GDIs have been described to date (RHOGDI1-3). Such variety of these regulators may in part be explained by tissue specific expression, specific localization within the cell and pathway specificity of some of these GEFs, GAPs and GDIs. In turn, it allows for the ability of a few Rho GTPases to precisely control multiple, diverse and very complex processes in the cell.
Figure 1.6

Evolutional relationship and protein structure of Rho GTPases a. Phylogenetic tree of the Rho GTPase family of proteins. The 20 members of the family are divided into 8 subfamilies, which are colour-coded here. Figure modified from Murali and Rajalingam, 2013. b. General protein structure of RhoA, Rac1 and Cdc42 Rho GTPases. G-box motifs are mostly responsible for the nucleotide binding, while Switch regions 1 and 2 are responsible for the GTP to GDP hydrolysis. G1 region is also known as the P-loop, the major nucleotide-binding site that contains the magnesium ion. The effector domain is mainly responsible for the effector binding, while Rho insert region binds both effectors and GEFs. Hypervariable region is responsible for both regulator (GEFs and GAPs) and effector binding, where polybasic region also plays a role in membrane binding. Finally, CAAX box is the main site of the posttranslational lipid modifications of the Rho GTPases, such as geranylgeranylation.
Regulation of Rho GTPase activity

Extracellular signals activate G-coupled receptors, integrins and other transmembrane receptors, which directly or indirectly activate GEFs. GEFs facilitate the GDP to GTP exchange on Rho GTPase, thus activating it. Active Rho GTPases (indicated by the yellow star) are anchored to the plasma membrane by geranylgeranylation of their C termini where they signal to their downstream effectors. GAPs facilitate the Rho GTPase hydrolysis of GTP, thus inactivating the Rho GTPase. GDI binding to the inactive Rho GTPases masks the lipid modification of the enzyme, thus keeping it in the cytoplasm and preventing its activation.
1.3.1 Rho GTPase structure and guanine nucleotide switch regulation

Rho GTPases have a very conserved domain structure (Figure 1.6b) (217, 219, 220). They all possess a G domain, which spans most of the protein length. The G domain includes five G-box motifs (G1-5) which are responsible for the nucleotide binding. Of these, G1 is known as the phosphate-binding (P)-loop and it is the main nucleotide binding site. A magnesium ion (Mg\(^{2+}\)) that is associated with the phosphate-binding loop (P-loop), acts as a co-factor for the high-affinity binding of the nucleotides and for the GTPase reaction. However, the magnesium ion is not essential for the GTPase reaction of Rho GTPases like it is for most other members of the Ras superfamily (221). The G domain also includes two functional elements switch I and switch II. The switch I region is essential for the binding of the downstream effectors and therefore this region plus surrounding residues constitute the effector region of the Rho GTPases (222). However, other regions of Rho GTPases are also capable of effector binding. Mutation of certain residues in the effector region impairs the binding of many effectors (222). For example, mutations in Ala27 and Gly30 of Rac1 impair its binding to the p67phox region of an important Rac1 effector nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (223). Similarly, F39A mutation of RhoA inhibits its binding to two of its effectors Prk2 and ROCK (224). For most Rho GTPases both switches are necessary for the efficient binding of the GEFs, GAPs and GDIs and catalysis of the nucleotide exchange (225-227). In addition, switch II has a mechanistic role in both intrinsic and GAP-mediated GTP hydrolysis (218). The switches I and II also bind the γ-phosphate of the GTP and therefore can differentiate between the active and inactive states of the Rho GTPase. Upon sensing the bound GTP, the switch I and II regions change their conformation, which signals the active nucleotide status of the Rho GTPase to the downstream effectors (228). Interestingly, Rnd, Rho BTB and RhoH subfamilies have a modification in the G domain which deprive them of the GTPase activity. Towards the end of the G domain, Rho GTPases harbour a Rho insert region (10-15 residues), the defining feature of the Rho GTPases in the Ras superfamily (229). Similar to the effector region, the insert region is mainly responsible for the effector binding and activation and is also involved in the binding of the GEFs. The sequence of this region varies greatly between Rho GTPases and even between the closely related Rho GTPase isoforms. It is therefore thought that the differences in the Rho insert region determine the effector specificity of the different Rho GTPases and their isoforms (220, 222). At the C terminal most Rho GTPases have a hypervariable region (~20 residues), which is
mainly involved in protein-protein interactions, including those with the regulatory proteins and with the effector proteins. The hypervariable region also contains a polybasic region, comprised of lysine and arginine residues. Aside from its role in effector and regulator binding, this region mediates the membrane binding of Rho GTPases (230). Finally, a CAAX-box (where C is a cysteine, A is an aliphatic residue and X is any residue) follows the hypervariable region. Most post-translational lipid modifications of Rho GTPases, the most common of which is geranylgeranylation, occur in this region (231). These posttranslational modifications are very important for the proper function of the Rho GTPases as they mediate the binding of the Rho GTPases to the membranes and thus their interaction with the upstream regulators and the downstream effectors (232, 233). For example, blocking geranylgeranylation of RhoA results in its loss of function (234, 235). This region together with the hypervariable region are also the cites of the interaction with the GDI regulators. GDIs bind the lipid region of the Rho GTPase and insert it into their hydrophobic pocket, thus preventing its interaction with the membranes (236).

The GTPase activity of Rho GTPases is quite low, making the GTP hydrolysis a very slow process. GAPs stabilize the charged intermediate of the GTP hydrolysis and properly position the essential hydrolytic water molecule, thereby reducing the activation barrier for this reaction (237). Like GTP hydrolysis, the exchange of the GDP to GTP is also slow, as the nucleotide free GTPase is unstable, making the creation of this intermediate unfavoured. This exchange is therefore facilitated by the GEFs. GEFs disrupt the interaction of switch I with the nucleotide binding site by steric hinderance and form extensive contacts with the switch II region, thus stabilizing the nucleotide free GTPase (237). Different GEFs employ diverse means of facilitating the GDP release (230). One mechanism is the insertion by the GEF of an acidic residue into the phosphate binding site, which creates an electrostatic repulsion that causes the expulsion of the GDP. Second possibility is the lowering of Mg\(^{2+}\) affinity for its binding site, which also lowers the affinity of the GDP binding. Finally, some GEFs remodel the switch II motif in a way that also creates an electrostatic repulsion of GDP. Since the concentration of the GTP in cells is higher than that of GDP, it gets loaded preferentially onto the Rho GTPase. Some GEFs and GAPs interact only with a specific Rho GTPase, while others have been shown to affect multiple Rho GTPases, still others can act as GEFs towards one Rho GTPase and as GAPs toward another (222, 238). Aside from their role in regulating the membrane localization of the
Rho GTPases, RhoGDIs also inhibit their GTPase activity. They do so by binding the switch I and switch II domains of the GDP bound Rho GTPase and restricting their flexibility (236). This in turn, inhibits the ability of the switches to undergo the conformational changes necessary for the nucleotide exchange.

Due to the large number of the Rho GTPases, the focus of the following sections will be on the best studied family members: Cdc42, Rac1 and RhoA, and their roles in regulation of actin dynamics.

1.3.2 Rho GTPase signaling in cell motility

Rho GTPases are the main regulators of actin rearrangement, and therefore regulate cell motility, shape and attachment. In the classical model of the mesenchymal cell migration, Rac1 and Cdc42 were thought to be active at the leading edge of the cell, where they regulate the formation of lamellipodia and filopodia, respectively. RhoA was thought to be active only at the trailing edge, where it controls the trailing edge retraction through the regulation of the actomyosin contraction. This simple paradigm has since been challenged by reports showing that RhoA can also act at the leading edge of the cell to promote cell motility (239). In addition, RhoA is the primary driver of the amoeboid cell migration (240, 241). Analogous to having specific as well as shared GEF activators, Rho GTPases also have specific and shared effectors, underlining the complex interplay between the family members in actin rearrangement (Figure 1.8a). Some of the most important effector interactions of Rac1, Cdc42 and RhoA in cell motility are described below.

Cdc42

Initial studies of the role of Cdc42 in cell migration described it as the master regulator of cell polarity. Cells expressing the dominant negative Cdc42 mutant lost the ability for directional movement as a consequence of the loss of cell polarity (242). It was shown that accumulation of the active Cdc42 at a certain area of the membrane led to the re-positioning of the MTOC and the Golgi apparatus in front of the cell nucleus and toward the Cdc42 activation, thus establishing the cell’s leading edge (242, 243). The main effector of Cdc42 regulating this process is partitioning defective protein 6 (Par6). Mammalian Par6 is the scaffold protein that forms a complex with atypical protein kinase Cζ (aPKCζ). The binding of Cdc42 to Par6 induces a
conformational change that in turn activates aPKCζ (244). One of the targets of this activated complex is glycogen synthase kinase β (GSK3β) whose kinase activity is inhibited by the aPKCζ phosphorylation (245). In turn, this allows for the association of the adenomatous polyposis coli (APC) protein with the plus ends of the microtubules, a required step for the MTOC reorientation (245). Inactive GSK3β also cannot phosphorylate dynein, resulting in its activation (246). Active dyneins are required for the microtubule reorientation, as they are molecular motors that travel along microtubules and convert ATP energy to mechanical work (247). Cdc42 also binds and recruits to the plasma membrane IQGAP1, which itself interacts with the proteins that are associated with the microtubule plus ends (248). This effectively tethers the microtubules to the plasma membrane, which may create the necessary force to reorient the microtubule cytoskeleton (249). Thus, the regulation of the microtubule cytoskeleton by the Cdc42 is one major way through which it regulates cell polarity and motility but Cdc42 is also a major regulator of cell motility through its regulation of the actin cytoskeleton.

Cdc42 was first shown to be involved in actin rearrangement when the protein was microinjected into fibroblasts which caused filopodia formation (250). Later, Cdc42 was shown to regulate filopodia formation through actin polymerization and actin turnover regulation. The main effectors through which Cdc42 performs these functions are WASP, p21 activated kinases (PAK1-6), Diaphanous-related formins (DRFs) and myotonic dystrophy-related Cdc42-binding kinase (MRCK).

Cdc42 induces actin nucleation through the interaction with WASP and neural WASP (N-WASP), two homologous proteins with identical domain structure. During resting state, the WASP proteins are autoinhibited by the binding of the C-terminal region collectively known as the Verprolin, coflin, acidic domain (VCA) region, to the central GTPase protein-binding/ Cdc42/Rac interactive binding (GBD/CRIB) motif (251). Active Cdc42 binds the GBD/CRIB motif and causes a change in its conformation, which releases the VCA region. The VCA region is then free to bind G-actin and the Arp2/3 complex, aiding the formation of a nucleation dimer (252). Therefore, WASP functions as a nucleation promoting factor. Although active Cdc42 can interact with WASP directly, it has recently been shown that other proteins, such as the TOCA family of proteins, can facilitate this interaction (253).
PAK family of the serine/threonine kinases are involved in the establishment of cell polarity, as well as filopodia, lamellipodia and focal adhesions formation. They are highly homologous proteins, especially in their kinase domain region and as a result their functions often overlap. They are divided into two types: type I (PAK1-3) are inactive and rely on the interaction with a Rho GTPase for activation, while type II (PAK4-6) are constitutively active and only rely on Rho GTPases for localization of their activity. Both Cdc42 and Rac1 can activate type I PAK proteins. In unstimulated cells PAK exists as homo and sometimes heterodimer, with the autoinhibitory region of one dimer partner inhibiting the kinase activity of the other (254, 255). Upon stimulation, active Cdc42 or Rac1 binds one of the PAK dimer partners, releasing its kinase autoinhibition (256). The active PAK then phosphorylates its dimer partner, activating it and allowing for more trans-autophosphorylation (257). PAK trans-autophosphorylates at Thr402 and Thr423, further activating its own kinase activity (258, 259). The dimers then separate and bind their downstream targets (256). Active PAK can induce protrusion formation via two main pathways. First, active PAK phosphorylates Lin11, Isl-1, Mec-3 (LIM) kinase (LIMK), which leads to LIMK phosphorylation of actin filaments severing proteins ADF/cofilin (260). Phosphorylated ADF/cofilin can not bind actin filaments leading to actin polymerization and protrusion formation. Second, active PAK phosphorylates myosin light chain kinase (MLCK), which inhibits its activity to phosphorylate myosin light chain (MLC) (261). Unphosphorylated MLC cannot induce stress fiber formation and myosin contractility, making the cell less rigid and therefore, more motile. Somewhat surprisingly, PAK can directly phosphorylate MLC, thereby actually inducing stress fiber formation and actomyosin contraction (262). The phosphorylated MLC localizes to both the leading edge and the retracting rear to affect actomyosin contraction, necessary to propel the cell forward (263). These seemingly contradicting roles of PAK highlight the need for tight regulation of PAK activation and localization. In addition, PAK1 can phosphorylate a subunit of the Arp2/3 complex, p41-Arc (264). Phosphorylated p41-Arc enhances the formation of the Arp2/3 complex and thus facilitates actin nucleation and polymerization. A few other downstream targets of PAK directly or indirectly affect actin cytoskeleton and have been reviewed by Szczepanowska (265).

As mentioned in the cell motility section of this thesis, formins are the F-actin nucleation and elongation factors that drive filopodia formation. As the main initiator of the filopodia formation, Cdc42 has been shown to regulate the activation of a number of the DRFs (Reviewed in (266)).
As seen with many effectors of the Rho GTPases, DRFs are autoinhibited by the interaction of their N and C terminus domains and this autoinhibition is relieved by the binding of the active Rho GTPase. This allows the formin to bind actin monomers and nucleate and elongate the F-actin filament.

Although Cdc42 binds N-WASP and formins directly, the help of adaptor proteins that bring the Rho GTPase and its effector closer together at the plasma membrane is often required. One such adaptor protein that is itself a key effector of Cdc42, is IRSp53 (insulin receptor substrate protein 53 kDa). Again, the binding of the N-terminal inverse -Bin, Amphiphysin, Rvs (I-BAR) and the C-terminal Src homology 3 (SH3) domains of this protein autoinhibits it and following the interaction with the active Cdc42 this autoinhibition is relieved (267). The activation exposes the I-BAR and the SH3 domain of IRSp53. The I-BAR domain then binds to the membrane and drives the membrane tubular protrusion (reviewed in (268)). In turn, the SH3 domain binds N-WASP, WAVE and a number of mDia formins among other Rho GTPase effectors that regulate actin polymerization (269). These effectors are then more readily available to be activated by the Rho GTPase. Importantly, the I-BAR domain has an actin-binding site which may be used to localize the polarized actin to the deformed membrane, therefore aiding the filopodia formation (268). Thus, IRSp53 has been shown to be essential for the Cdc42 driven filopodia formation, where IRSp53 null MEFs or neuroblastoma cells with IRSp53 mutations that inhibit its binding to Cdc42 or IRSp53 effectors, cannot form filopodia (269, 270).

Other important effectors of Cdc42 are MRCKα,β,γ. They have recently emerged as the main regulators of the actomyosin contraction together with the RhoA effector ROCK, to which they are structurally and functionally related (271). MRCKs dimerize or oligomerize and intramolecular binding of their C-terminal to the N-terminal kinase domain keeps them inactive, but whether the binding of the active Cdc42 to their CRIB domain contributes to the activation of MRCKs is still unclear (272). However, it has been shown that this interaction is necessary for the proper localization of the MRCK to the actomyosin filaments in lamella (273). Depletion of the Cdc42 by siRNA in HeLa cells abolished proper localization of the MRCK to these structures. At the lamella, MRCK promotes cell protrusion possibly by increasing actin-myosin retrograde flow, which allows for generation of traction forces necessary for the cell movement (273). MRCKα has also been shown to interact with Rac1 but any functional consequences of
this interactions are yet to be determined (274). One of the main effectors of MRCKs is MLC, phosphorylation of which is necessary for the actomyosin contraction. Although MRCK can phosphorylate MLC in a cell-free system, whether it does so in vivo is still unclear (275, 276). Besides the possible direct MLC phosphorylation, MRCKs lead to increased MLC phosphorylation by phosphorylation of the MLC phosphatases (MLCPs). Phosphorylated MLCPs are inactive and therefore MLC remains phosphorylated (275, 277). Like PAK, MRCKs also phosphorylate LIMK, leading to the stabilization of actin filaments (278). Finally, work by Nakamura and colleagues has suggested that MRCK can phosphorylate ERM proteins, which contributes to the Cdc42-dependent filopodia formation (276). In addition to being necessary for cell motility, the actin-myosin retrograde flow induced by the MRCKs plays an important role in establishing cell polarity. In particular, it aids the re-orientation of the cell nuclei behind the MTOC, thus defining the cell rear and front respectively and establishing directionality of the migrating cell (279). Given the important roles that MRCKs targets play in actomyosin regulation, it is not surprising that inhibition of MRCKs by a specific small molecule inhibitor was shown to impair cancer cell migration and invasion in vitro (280).

1.3.2.1 Rac1

The pioneering work of Ridley and Hall et al. was the first to link Rac1 to actin rearrangement. They microinjected the recombinant Rac protein into fibroblasts and observed membrane ruffling and the formation of actin filaments at the plasma membrane (281). Subsequently, it was determined that Rac1 regulates actin cytoskeleton rearrangement to affect actin polymerization and lamellipodia formation, as well as actin turnover (282). The main effectors through which Rac1 performs these functions are WAVE1-3 and PAK family of proteins.

Since WASP and WAVE proteins are homologs, it is not surprising that the mechanism of their activation and their functions are similar. Like WASP, WAVE protein is autoinhibited in the unstimulated cells and active Rac1 relieves this autoinhibition. However, unlike Cdc42, Rac1 does not bind WAVE directly. In the unstimulated cells, WAVE is kept inactive by the binding of the VCA region to steroid receptor RNA activator 1 (Sra1), which conceals the G-actin binding site (283). Sra1 is part of the WAVE regulatory complex (WRC) which consists of the N-terminal domain of WAVE bound to PIR121/Sra1, Nap1, Abi1/2/3 and HSPC300. All the components of this complex have been shown to be essential for the Rac1-mediated actin
remodeling and lamellipodia formation, as siRNA of each individual component inhibited these processes (284-286). Upon receiving the signal for migration, Rac1 gets activated, allowing it to bind its effector Sra1. It has been shown that Rac1 and VCA bind Sra1 in a competitive fashion (283). The interaction of Rac1 and Sra1 results in the release of the VCA region from the WRC, making it available for the G-actin and Arp2/3 binding (283). The WAVE VCA domain promotes actin nucleation and branching through the same mechanism as the WASP VCA domain, outlined above. Of note, a recent study by Chen et al., has demonstrated that Rac1 binds two distinct sites on the Sra1, one with higher affinity than the other. However, the binding of Rac1 to both of these sites is necessary for the activation of WAVE, suggesting that two active Rac1 molecules need to bind each WRC (287). Such a mechanism may be used by the cell to sense the amount of active Rac1 and initiate the lamellipodia formation only once a certain threshold is reached. Upon activation, the WRC complex does not dissociate. This may be due to the role that it plays in the interaction with the transmembrane proteins, which ensure the activity of the complex only at the specific areas of the cell membrane. One such interaction is that of WRC and cell adhesion proteins protocadherin 10 and 19 which is mediated by Nap1 (288, 289). Examination of this interaction led to the identification of a conserved peptide motif that interacts with WRC and was found in up to 120 other membrane-associated proteins (290). A study by Suetsugu et al., demonstrated that WRC isolated from the cytosol is inactive, while that isolated from the membrane is active, giving further evidence for the role of the WRC in proper localization and activation of WAVE, as Rac1 is also active only at the membrane (291). In addition, like Cdc42, Rac1 can bind WAVE through its effector IRSp53 (292). IRSp53 interaction with WAVE brings it closer to Rac1, enhancing WAVE activation (291). Interestingly, IRSp53 has also been shown to bind some Rac1 GEFs, thus acting both upstream and downstream of Rac1 activation (293, 294). Further highlighting the crosstalk between the GTPases is the fact that Arf1, a member of the Arf family of Ras-related GTPases, also binds WRC. However, the activation of WAVE by Rac1 or by Arf1 GTPase alone is weaker than when both Rho GTPases cooperate (295). This shows that many processes controlled by the Ras superfamily of GTPases need to cooperate in order to control such complex process as cell migration. As mentioned above, Rac1 activates type I PAKs in a similar fashion to Cdc42. Of note, PAK binds PAK-interacting exchange factor beta (βPix), a Rac1 GEF, and activates it by phosphorylating it (296, 297). Therefore, like IRSp53, PAK can act both downstream and
upstream of Rac1 activation, creating a positive feedback loop. Rac1 also interacts with DRFs to affect actin cytoskeleton. In particular, its interaction with the FHOD1 formin has been shown to induce actin stress fiber formation and promote cell migration through activation of the Rho-ROCK cascade (described below) (298, 299). Rac1 also regulates other processes besides actin rearrangement to induce cell motility, including promotion of epithelial to mesenchymal transition through disruption of cell-cell junctions, nascent adhesion formation and promotion of MMP expression (300-302).

1.3.2.2 RhoA

RhoA is the first human Rho GTPase to be identified and the other family members were later identified based on homology (303). It was first noted that inhibition of RhoA results in the loss of stress fibers, while microinjection of RhoA into cells induces their formation (304, 305). RhoA is now known as the master regulator of stress fiber formation, actomyosin contraction, especially at the cell rear, and amoeboid motility. It performs these functions through its main effectors: ROCK and DRFs.

As with most Rho GTPase effectors already described, ROCK is autoinhibited by the binding of its C-terminal to the N-terminal kinase domain (306). It is thought that the binding of RhoA to ROCK interrupts its autoinhibition, thus activating this serine/threonine kinase (307-309). However, this notion has recently been challenged by Truebestein et al., who propose that ROCK kinase activity is governed by its localization, and interaction with RhoA may be the means for spatial control of this kinase (310). There are a lot of parallels between ROCK and MRCK activity, as both kinases inhibit MLCP by phosphorylation, both can phosphorylate the MLC directly to affect actomyosin contraction, and both phosphorylate LIMK to induce actin filament stability (311-313). The differences in functions of these kinases lie in their unique cellular localization due to interactions with unique adaptor proteins and different target specificity. While active MRCK is mostly found in the lamella, active ROCK localizes mostly to the focal adhesions and the stress fibers in the cell midbody, as well as retracting cell rear during migration (314, 315). This difference in localization is partially controlled by the adaptor proteins with which these kinases interact. MRCK interacts with LRAP35a, which itself is located at the lamella, and through this adaptor protein it can interact with lamellar myosin (273). ROCK on the other hand, is likely targeted to the cell rear by its interaction with RhoA and
possibly yet unidentified adaptor proteins (316). An example of different target specificity is the phosphorylation of the MLC; ROCK phosphorylates it at Thr18 and Ser19, while MRCK can only phosphorylate it on Ser19 (317). This differential phosphorylation results in different regulation and localization of myosin, where the mono-phosphorylated form goes to the peripheral stress fibers and the di-phosphorylated form localizes to the central stress fibers (318). In addition to differential regulation of the same targets, the two kinases have different target specificity. For example, Gally et al. demonstrated that ROCK has targets that are different from MRCK in C. elegans development (275). They inhibited both kinases and attempted a rescue of this inhibition with the phosphorylated MLC but this only rescued the MRCK and not the ROCK inhibition (275). RhoA/ROCK pathway is an important regulator of cell polarity during the cell movement, as it establishes the cell rear by localization of phosphorylated myosin II to the back of the cell, inhibition of lamellar proteins and by destabilization of microtubules at the cell rear (319-321). Also, as a major regulator of the actomyosin contractility, RhoA/ROCK pathway has been shown to play an important role in regulating cell attachment, spreading and shape (243, 322-324).

Like Cdc42 and Rac1, RhoA also interacts with DRFs to affect actin polymerization. One of the best characterized family of formin effectors of RhoA in mammals is mDia (mDia1-3). RhoA interaction with mDia causes a conformational change that relieves the mDia autoinhibitory C and N terminal binding (325). Active mDia then binds the barbed ends of F-actin and promotes its polymerization to form stress fibres (326, 327). Since focal adhesions are often connected to the stress fibers, it is not surprising that mDia has also been implicated in focal adhesion turnover (328). In addition to its effect on the actin cytoskeleton, RhoA regulates microtubule stability and therefore, cell polarity and directional migration through mDia (329-331). Although RhoA activity and function are often thought to be confined to the cell rear, recent studies indicate that RhoA is also active at the cell front (332, 333). Here it has been suggested to initiate protrusion formation through formins such as mDia which initiate actin polymerization and regulate cell polarity, while Rac1 and Cdc42 expand and stabilize the protrusion (327, 332).

As the major regulator of the actomyosin contractility through ROCK and of mDia formins, RhoA is the driver of the amoeboid cell motility, through the mechanisms described in the previous section (240, 241). In melanoma cells Cdc42 activation of N-WASP is also necessary
for the amoeboid cell migration, perhaps through the nucleation of branched actin filaments in the pseudopod (334). Interestingly, the activation of Cdc42 in this mode of migration occurs through the dedicator of cytokinesis 10 (DOCK10) GEF, which has not been reported to activate Cdc42 in mesenchymal migration, meaning that upstream signaling and different downstream effectors are likely to be the main determinants of the mode of motility (334).

1.3.2.3 RhoA-Rac1 crosstalk

As can be appreciated from the previous section, Rho GTPases often have targets that overlap. Nevertheless, RhoA is mostly active towards the cell rear, while Rac1 is active at the cell front. Even the brief RhoA activation at the cell front that precedes protrusion formation is spatially and temporally separate from Rac1 activation (332). How this spatial and functional separation is achieved has been a subject of rigorous investigation. It has now been established that RhoA and Rac1 are involved in the mutual inhibitory feedback loops (Figure 1.8b).

Active RhoA activates ROCK kinases which in turn can phosphorylate and therefore activate Fil-GAP, a GAP for Rac1 (335). In melanoma cells ARHGAP22 was shown to be required for the ROCK-dependent Rac1 inhibition but it was not through its direct phosphorylation by ROCK (336). This shows that Rac1 suppression through activation of its GAPs may be a more general mode of action of the RhoA/ROCK pathway and that which GAPs are activated and by what pathway may be cell type or stimulus dependent. In addition, ROCK inhibits Rac1 GEF βPix, leading to further Rac1 inhibition (337).

Active Rac1 on the other hand, activates PAK which has been shown to inhibit a number of RhoA GEFs (reviewed in (338)). One well studied example is inhibition of GEF-H1 by PAK phosphorylation. Once phosphorylated, GEF-H1 is localized to the microtubules where it is unable to activate RhoA (339). In addition, active Rac1 binds to a RhoA GAP, p190GAP and activates it (340). This direct activation of p190GAP is reinforced by its indirect activation through the initiation of the reactive oxygen species (ROS) signaling cascade by Rac1 (341).
Figure 1.8 Rho GTPase signaling. a. Downstream signaling of Rho GTPases to their effectors to affect actin rearrangement. Rac1 through WAVE and Cdc42 through WASP activate Arp2/3 nucleation complex thus leading to branched actin polymerization in lamellipodia. Through PAK activation they also enhance actin turnover. All three Rho GTPases can activate formin family of proteins (DRFs) and thus initiate unbranched actin filament formation, resulting in filopodia formation downstream of Cdc42 signaling, and actin stress fiber formation downstream of RhoA signaling. RhoA activates ROCK and through its downstream signaling leads to actomyosin contraction and retraction of the cell rear or to bleb formation in amoeboid motility. b. Inhibitory (black arrows) and activation (red arrows) loops between Rac1 and RhoA. Through ROCK, RhoA activates Rac1 GAPs or inhibits its GEFs, leading to inhibition of Rac1 activation. Rac1 acts in a similar manner to inhibit RhoA activation. In turn, RhoA activates Rac1 by formin (mDia) activation and Rac1 activates RhoA by activating its GEF Dbs.
Our understanding of the crosstalk between the Rho GTPases is further complicated by the existence of the positive feedback loops between RhoA and Rac1 \((\text{Figure 1.8b})\) (281). A study by Tsuiji et al. has shown that RhoA may induce Rac1 activation through mDia, although the precise mechanism of this is still unknown (342). Conversely, in NIH 3T3 fibroblasts, active Rac1 binds to and activates the RhoA GEF Dbl’s big sister (Dbs) (343). Interestingly, this effect might be either cell type or disease state specific as in breast cancer cells, Dbs was found to also activate Rac1 and Cdc42 (344).

From these complex interactions, it is evident that a precise balance of both upstream regulators and downstream effectors of the Rho GTPases is necessary for efficient cells migration. Some advances have been made in understanding of the interplay of RhoA and Rac1 in cell motility by design of \textit{in silico} computational models of their feedback loops, which were partially tested \textit{in vitro} (345). However, much remains to be learned about the regulators and effectors of Rho GTPases to fully understand the crosstalk between them.

\subsection*{1.3.3 Rho GTPases in cell spreading and cell shape}

Most unattached adherent cells assume a spherical morphology in suspension. However, upon encountering a substrate, the cells attach and start to flatten out and expand their membrane outward, a process known as cell spreading. Cell spreading is intimately coupled with the cell adhesion and the two processes are essential for relaying the information about the surrounding environment to the cell’s internal machinery. In turn, being able to sense and respond to the environment is critical for the establishment of the cellular homeostasis (346). Depending on the environment, there are two modes of cell spreading: isotropic and anisotropic. Isotropic spreading happens when there are enough ligands for the cells to attach to (347). In this case, the cell spreads out evenly in all directions. When ligands are scarce on the substrate surface, the cell spreads out anisotropically, where it sends out filopodia extensions to probe the environment for higher ligand concentration (347). Importantly, most cell types can undergo both modes of spreading, depending on the environment. The forces governing the adhesion formation, the cytoskeletal rearrangement needed to extend the cell membrane, and the machinery behind them are the same for cell spreading and cell motility. Thus, cell spreading is often thought of as a simpler tool to study motility. Dobereiner and colleagues identified three main phases of the cell spreading based on the traction forces exerted and the rate of the cell area increase (348, 349).
During the early phase, the traction forces are low and thus the rate of spreading is low as well. Upon first encountering the substrate, the cell flattens out like a drop of liquid on a flat surface, making this process largely passive (350). The rate at which this happens, depends on membrane and cytoskeleton rigidity (350). A faster rate of spreading is achieved in the cells with low membrane rigidity and low levels of polymerized actin and actomyosin network. As Rho GTPases regulate the latter two processes, their inactivation at this stage increases the rate of spreading. Once the first contact with the substrate has been established, the cell starts to form nascent cell-matrix adhesions, largely through integrin signaling. Activated integrins induce an intracellular signaling cascade, where paxillin and talin bind integrin intracellular tails and recruit FAK and sarcoma proto-oncogene, non-receptor tyrosine kinases (Src) (351). FAK and Src increase each-other’s kinase activity and form a signaling complex (351). Signaling downstream of FAK-Src signaling complex leads to the Rac1 and Cdc42 GEF recruitment to the nascent adhesions. Some of the Rac1 GEFs that have been shown to localize to nascent focal adhesions and play an important role in spreading are β-Pix (352), DOCK1 (353), Trio (354), Tiam1 (355) and Vav family of GEFs (356, 357). The precise GEFs activated often depend on the cell type. These GEFs activate Rac1 and Cdc42, which leads to actin polymerization by the mechanisms described in earlier sections, and formation of the cytoskeletal protrusions such as membrane blebs and filopodia (358). Src also activates a Rho GAP, p190RhoGAP, leading to RhoA inactivation (359, 360). Increased Rac1 and Cdc42 activation leads to the second phase of cell spreading (348, 349, 358).

During the intermediate phase, the traction force increases significantly and is followed by the rapid increase in the cell area. As the cell spreads, more integrin attachments are formed, which in turn result in further activation of Rac1 and Cdc42, creating a positive feedback loop (361). High Rac1 and Cdc42 activity results in rapid membrane expansion through WAVE and WASP activation. Eventually, the more mature adhesions, now located towards the center of the cell, begin to disassemble due to low RhoA activity and prolonged FAK activation (362, 363). The integrins from these adhesions get recycled to the cell periphery, where they help form new adhesions and facilitate further membrane expansion (364). As the cell spreads and its cytoskeleton network grows, the tension inside the cell is increased (365). This in turn increases the tension at the adhesion sites leading to activation of RhoA GEFs GEF-H1 (366), LARG.
(366), p190RhoGEF (367) and Arhgef1 (368). RhoA activation by these GEFs brings about the last phase of the cell spreading.

During the late phase, when the cell has reached its critical spread area, the traction forces level off, resulting in minimal further increase in the cell area (348, 349, 369, 370). Active RhoA through activation of ROCK and subsequent activation of the non-muscle myosin IIA (NMIIA) enhances actomyosin contractility of the cytoskeleton. In turn, contractile forces exerted on the adhesion sites initiate a signaling cascade that results in reinforcement of the cell-matrix adhesions (371). Through activation of the formins, RhoA promotes non-branched actin polymerization, crosslinking and bundling of which with myosin, leads to stress fiber formation. The amount of RhoA activation and actomyosin contractility of the cell is determined by the substrate stiffness, ligand availability and focal adhesion size, among other factors (358, 369, 370). The external stimuli such as substrate stiffness, ligand availability and microenvironment, together with the internal components such cell-matrix and cell-cell adhesions, actomyosin contractility, cortical actin, stress fibers and other components of the cytoskeleton, affect the cell force generation (346). The balance of the forces exerted on the cell and by the cell determines its final shape.
**Figure 1.9**

**a Individual cell migration**

Mesenchymal  
Amoeboid

- **RhoA**
- **Cdc42**
- **Rac1**

**Direction of migration**

**b Collective cell migration**

- **RhoA**
- **Cdc42**
- **Rac1**

**C Cell spreading**

- **Early phase**
- **Intermediate phase**
- **Late phase**

**Figure 1.9 Rho GTPases in cell migration and spreading.**

**a** In mesenchymal migration, RhoA activation precedes the activation of Rac1 and Cdc42 at the leading edge. Active Rac1 facilitates lamellipodia formation, while active Cdc42 induces filopodia formation. RhoA is also highly active at the trailing edge, where it facilitates the actomyosin contraction. Amoeboid cell migration is primarily driven by the active RhoA and actomyosin contraction. **b** In collective cell migration, all three Rho GTPases are active at the leading edge of the leader cells and act in a similar manner to mesenchymal cell migration. RhoA is also active in the follower cells, where it regulates actomyosin contraction, and especially at the free edge, where it facilitates the actomyosin cords assembly. **c** In the beginning of the early phase of cell spreading Rho GTPases are inactive, which reduces the cytoskeleton rigidity and allows for faster passive spreading. Next, the cell forms nascent ECM adhesions, which leads to Rac1 and Cdc42 activation. In the intermediate phase, high levels of active Rac1 and Cdc42 result in rapid actin polymerization and rapid cell spreading. In the late phase, Rac1 and Cdc42 remain active at the cell edges, while in the cell center RhoA activity starts to increase, which facilitates adhesion maturation.
1.3.4 Rho GTPases in cancer

As they are the major regulators of actin rearrangement, cell proliferation, polarity, motility, cell-ECM and cell-cell adhesions, it is not surprising that deregulation of Rho GTPases can lead to tumorigenesis and increased tumour cell metastasis. The first studies to implicate Rho GTPases in tumorigenesis used constitutively active RhoA and Rac1 and fast-cycling Cdc42 mutant to show that they have weak transforming activity (372-374). Until recently, few mutations in Rho GTPase genes have been described in cancer. However, with the use of new sequencing technologies it is becoming clear that mutations in Rho GTPases are more frequent than previously thought. How relevant these mutations are to tumorigenesis and tumour progression remains to be determined. Still, the most common deregulation of Rho GTPases in cancer is their overexpression and/or increased activity due to the changes in the expression or activation of their regulators or effectors. As such, Rho GTPases are mostly thought to play an oncogenic role in tumour initiation and progression. However, recent studies describing reduced Rho GTPase expression and activation in cancer are starting to challenge that notion.

Although somatic mutations in the Cdc42 gene have been detected in different tumour tissues, they appear to be random and none have been implicated in tumorigenesis or tumour progression so far (375). However, overexpression and/or increased activity of Cdc42 has been described in breast cancer (376), colorectal cancer (377), HCC (378), testicular cancer (379), melanoma (380), cervical squamous cell carcinoma (381), and non-small cell lung cancer (382). This overexpression has been associated with either a higher grade or poor outcome in many of these studies. Although complete Cdc42 knockdown is embryonic lethal (383), in mouse models of CRC conditional knockdown of Cdc42 had a negative effect on cell tumorigenicity (384). Interestingly, constitutively active Cdc42 mutants actually inhibit cell growth, while “fast-cycling” mutants which have enhanced GDP to GTP exchange increase it (374). This suggests that the changes in Cdc42 regulators that lead to its enhanced cycling between “on” and “off” states may be pro-oncogenic, while those that lock Cdc42 in the active state, might in fact be tumour suppressive. In addition to Cdc42 itself, mutations or deregulation of its effector WASP have been linked to breast cancer (385, 386) and other malignancies (387). Intriguingly, a few recent studies have indicated that Cdc42 may also act as tumour suppressor. Its depletion in mouse bone marrow resulted in myeloproliferative disorder (388), while in mouse hepatocytes it
led to HCC (389), and finally its deletion in the mouse intestine led to epithelial cell hyperplasia (390). This suggests that precise regulation of Cdc42 expression and activity is necessary, and any changes in them whether up or down, may facilitate tumour formation and progression.

Rac1 mutations, most of which are activating, have been identified in many human malignancies including prostate cancer (391), melanoma (392), testicular cancer (393), brain cancers (394), and head and neck carcinoma (391). However, like with Cdc42, overexpression of Rac1 is more common than mutations in tumours. It has been reported in breast (376), HCC (395), lung (382), testicular (379) cancers, oral squamous cell carcinoma (396), gastric cell carcinoma (397), and leukemia (398). Increased amount of Rac1 correlates with tumour progression in breast (399), gastric (397) and testicular cancers (379). In addition, Rac1 has been implicated in tumour cell invasion and metastasis, as suppression of Rac1 expression or activity in breast cancer (400), lung cancer (401), gastric cancer (402), pancreatic cancer (403) and glioma (404), resulted in reduced cell invasiveness and, where tested, metastasis formation. Aside from its role in cytoskeletal rearrangement and protrusion formation, Rac1 stimulation of cancer cell migration can partially be attributed to its role in MMP regulation. In particular, Rac1 downstream signaling results in activation of MMP gene transcription and protein activation in primary and cancer cells (405-407). In turn, MMPs facilitate the EMT, enhancing cancer cell motility and metastatic potential. Rac1 deletion in mice is embryonic lethal (408), but its conditional knockdown in Kirsten rat sarcoma viral oncogene homolog (K-Ras) driven mouse model of lung cancer showed that Rac1 function is required for tumour cell proliferation and tumorigenesis (409). Rac1b, a splice variant of Rac1 that acts like a fast-cycling Rho GTPase, is often described as cancer-specific, as its expression is low in normal tissues but it is overexpressed in a growing number of tumour types, most well characterized of which are those of the breast and colon (399, 410). Rac1 downstream effectors have also been frequently implicated in tumor initiation and progression. One of the most notable examples is PAK, whose expression is commonly upregulated in human cancers (411). As a downstream effector of both Rac1 and Cdc42, it has been shown that PAK activity is necessary for the cell transformation induced by these Rho GTPases (412). Inhibition of PAK1 in pancreatic adenocarcinoma model reduced tumour growth and metastasis (413). Similarly, in a K-Ras induced skin cancer model depletion of PAK1 significantly impaired tumour formation and progression (414). Finally, inhibition of
PAK activity sensitizes a number of tumour types to treatment (415-417). Another Rac1 effector frequently deregulated in cancer is WAVE. WAVE is overexpressed in most cancers such as breast (418), ovarian (419), HCC (420), prostate (421), and melanoma (422) to name a few. In all of these tumours higher expression correlates with increased invasion and metastasis and/or poor prognosis. Intriguingly, there are conflicting reports on the expression of WAVE in gastric cancer, with some reporting it overexpressed (423), while others downregulated (424). This is likely due to the dual roles of WAVE in promotion of cell migration, where on the one hand it promotes motility through actin polymerization, and on the other it promotes cell-cell adhesions which reduce cell motility. Which function of WAVE is predominant may be cell type or cancer specific.

Recent cancer sequencing studies revealed that RhoA is mutated in a wide range of human cancers, with the highest frequency in B- and T-cell lymphomas and leukemias (up to 70%) and gastric cancers (up to 25%) (425). In lymphomas and leukemias high frequency of mutations is observed in the GTP/GDP binding region. In some subtypes, such as angio-immunoblastic T-cell lymphoma, these mutations lead to the inability of RhoA to bind GTP and thus inactivate it (426). In others, such as adult T-cell lymphomas and leukemias, in addition to inactivating mutations, mutations that lead to the fast-cycling GDP/GTP RhoA and thus its increased signaling are also observed (427). In gastric cancer however, most mutations occur in the effector-binding domains and prevent RhoA signaling to its effectors (425). These mutations were shown to result in poor cell differentiation, decrease in cellular cohesion and permeative cell growth (428). Like for other Rho GTPases, RhoA overexpression has been reported in many human cancers such as: breast (376), ovarian (429), colon (376), lung (376, 382), HCC (430), gastric (397), bladder (431), testicular (379) cancers and head and neck (432) and esophageal (433) squamous cell carcinoma and in most of them have been associated with cancer progression or poor prognosis. In a mouse model of lung adenocarcinoma that have a K-Ras mutation and are Cdkn2a null, inhibition of RhoA led to the cell death only in tumour cells, suggesting that RhoA signaling is necessary for tumour maintenance (434). Although the majority of the evidence so far points to RhoA as being pro-oncogenic, a few recent studies have found that it can also have a tumour-suppressive role. For example, in mouse model of colorectal cancer, expression of the dominant negative RhoA resulted in more frequent and larger
adenomas, as well as increased metastasis to the lung (435). Whether RhoA expression may be decreased in certain types of colon cancer or whether its activity may be inhibited through inactivating mutations remains to be determined. In parallel to other Rho GTPases, deregulation of RhoA effector ROCK is also common in cancer. Activating mutations of ROCK family members have been reported in breast (436, 437) and gastric cancers (437), non-small cell lung carcinoma (436, 437), and melanoma (437). High level of ROCK protein has been detected in breast (438), colon (439), and bladder (431) cancer, HCC (440), and osteosarcoma (441) and correlated with increased tumour grade and poor overall survival in patients with these tumours.

1.4 GEFs for Rho GTPases

As described in the previous section, GEFs and GAPs regulate the GTP/GDP exchange of Rho GTPases. There are about 81 Rho GEFs and 66 Rho GAPs known to date (442). Some GEFs can activate more than one Rho GTPase, while others are GTPase specific. In addition, a few GEFs have been reported to activate one Rho GTPase but act as GAPs for another Rho GTPase. Such multifunctionality may in part be explained by the differing structure among the GEFs, although GEFs often share some of the functional domains. Based on their domain structure GEFs are divided into two families, the diffuse B-cell lymphoma (Dbl) family of about 70 members and the dedicator of cytokinesis (DOCK) family of 11 members (DOCK1-11). While Dbl family GEFs can activate all Rho GTPases, DOCK GEFs are Rac1 and/or Cdc42 specific.

1.4.1 GEFs structure

1.4.1.1 Dbl GEFs

The first mammalian Rho GEF to be identified was Dbl, a transforming oncogene in diffuse B-cell lymphoma (443). As more GEFs with homologous domains emerged, it became the prototypical member of the Dbl family. The Dbl family of GEFs is characterised by the highly conserved tandem Dbl-homology (DH) and Pleckstrin-homology (PH) domains. The DH domain interacts extensively with the Switch regions of the Rho GTPases and therefore is responsible for the binding to and the GDP dissociation from the Rho GTPases (444). It is thus the minimal unit necessary for the GEF activity. The PH domain can play varying roles, depending on the GEF but is mostly known to regulate GEF localization to the membrane, where Rho GTPases are
located, therefore facilitating GEF-GTPase interactions. There is evidence that PH domain may also promote a direct interaction between the Rho GTPase and the GEF, as well as enhance the nucleotide exchange reaction (444). Finally, PH domains can act as the docking sites for other proteins, such as the effectors of the Rho GTPases and therefore facilitate the Rho GTPase signaling. Highlighting the importance of the PH domain in proper GEF function is the fact that purified DH domain can bind and activate purified Rho GTPases, while *in vitro* the DH domain is insufficient, and the addition of the PH domain is required for proper Rho GTPase activation and signaling (445). However, aside from the DH/PH domains, the rest of the structure of the Dbl GEFs varies significantly and therefore, only the structures of the GEFs relevant to this thesis will be discussed in further sections.

1.4.1.2 DOCK GEFs

While the DOCK family of GEFs performs the same function, of activating Rho GTPases, as the Dbl family, it is somewhat surprising that they bear little, if any, sequence homology. Instead of the DH/PH domains, DOCKs have DOCK-homology regions 1 and 2 (DHR1-2) which are highly conserved among all DOCK family members. DHR2 is a functional homolog of DH domain. However, although it performs the same function as DH domain of aiding the release of GDP form the Rho GTPase, it does so by a different mechanism. Specifically, a Valine residue that is highly conserved among all DOCKs can sense the bound GDP and then acts on the magnesium ion to displace it from the P-loop (446). As the Mg$^{2+}$ stabilizes the nucleotide binding, its displacement from the P-loop results in the GDP release. The DHR1 domain targets GEFs to the membranes through its interaction with the phospholipids, thus regulating GEF localization and activity, by bringing it closer to the Rho GTPases (447). DOCKs are divided into four subgroups (DOCK-A-D) based on their structural elements, besides DHR1 and 2, and on Rho GTPase specificity (reviewed in (447)). DOCK-A subgroup includes DOCK1,2,5 which are Rac1 specific. In addition to the DHR1 and 2 domains they possess: N-terminal SH3 domain, which mediates their interaction with the Engulfment and motility (ELMO) scaffolding proteins, followed by a helical region, and a C-terminal polybasic region (only in DOCK 1 and 2), which binds signaling lipid phosphatidic acid. The polybasic region is followed by the PxxP region, which binds adaptor proteins that also contain the SH3 domain. DOCK-B group includes DOCK 3 and 4 and is Rac1 specific. It differs from the A group by lacking the helical and polybasic
regions. DOCK-C group contains DOCK6-8 and acts on both Rac1 and Cdc42. This group does not have any other domains besides DHR1 and 2. Finally, DOCK-D group includes DOCK9-11 and is Cdc42 specific. Besides the DHR1 and 2 domains, it has an N-terminal PH domain, which plays a similar role to Dbl GEFs PH domain of phosphoinositide binding to facilitate membrane translocation.

1.4.2 Regulation of GEF activity

Like the effectors of the Rho GTPases, GEFs are often autoinhibited through intra- or inter-molecular interactions until the activation signal is received. The different ways through which GEF activity may be regulated are described in this section.

1.4.2.1 Intra- and inter-molecular regulation

A large body of evidence suggests that many of the Dbl and DOCK GEFs are autoinhibited through intramolecular interactions in uninduced cells. This is supported by the fact that the truncation of the N-terminus of many GEFs leads to their constitual activation. The interaction of the amino terminus with other parts of the protein obscures the DH or the DHR2 domains of the GEFs, prohibiting its interaction with the Rho GTPases. For example, in both DOCK1 and 2, the SH3 N-terminal domain interacts with the DHR2 domain which inhibits its GEF activity (448, 449). Importantly, the SH3 interaction with the ELMO adaptor proteins relieves this autoinhibition (450). Similarly, in the three regulator of G protein signaling (RGS) domain containing GEFs, post-synaptic density protein, Drosophila disc large tumour suppressor, and zonula occludens-1 protein (PDZ)-RhoGEF, leukemia-associated RhoGEF (LARG) and p115 RhoGEF, the region known as the autoinhibitory loop, located between the N-terminal RGS domain and the DH domain, interacts with the DH domain, thus inhibiting it (451, 452). Interestingly, these three GEFs can oligomerize through the RGS domain and this oligomerization inhibits their GEF activity (453). This is supported by the fact that the removal of the RGS domain enhances the GEF activity of these GEFs.

1.4.2.2 Receptor-coupled activation

Signaling through the GPCRs is one of the most common signaling pathways leading to the GEF and Rho GTPase activation. One clear example of the direct activation of Rho GEFs by GPCRs
is that of the activation of the RGS domain GEFs. The RGS domain directly interacts with the
G\textsubscript{12/13} α-subunit of the receptor and acts as a GAP for this subunit. In turn, the association with
the G\textsubscript{12/13} α-subunit releases the autoinhibition of these GEFs and allows for their activation of
RhoA (451, 452, 454). Recent studies have also implicated GPCRs in the regulation of the
DOCK family of GEFs. For example, Hernandez-Vasquez et al. have shown that the ELMO
protein in complex with the DOCK1 binds to the GPR124 GPCR and this interaction activates
DOCK1 and promotes cell adhesion (455). Other DOCKs are also activated by the GPCRs but
whether it’s through a similar mechanism, remains to be determined. Another very important
group of receptors regulating the activation of Rho GEFs is receptor tyrosine kinases (RTKs).
Numerous RTKs have been shown to activate both families of the Rho GEFs, often by
facilitating their phosphorylation, to affect actin rearrangement and cell motility/adhesion. For
example, epidermal growth factor receptor (EGFR) has been shown to activate such Dbl GEFs as
Vav family (456), βPix (457), and Sos1 (458) to affect cytoskeletal rearrangements and cell
migration. Similarly, DOCK1 is activated through the platelet-derived growth factor receptor α
(PDGFRα) (459).

1.4.2.3 Regulation of GEF activity and protein stability by post-translational
modifications

The most common post-translational modification of GEFs is phosphorylation, which often
results in their activation. Phosphorylation of GEFs can activate them in two ways: by altering
their conformation and thereby relieving their catalytic domain from inhibition or by facilitating
GEF interaction with the scaffolding or adaptor proteins which bring the GEF together with its
activators or effectors. However, sometimes phosphorylation can inhibit GEF interactions with
its activators, thus reducing its activity. This is the case for the Rac1 GEF phosphatidylinositol
3,4,5-trisphosphate-dependent Rac exchanger 2 (P-Rex2), which is part of the Rac1 negative
feedback loop. Barrows and colleagues have shown that while P-Rex2 activates Rac1, the
subsequent activation of PAK by the Rac1 leads to the PAK-dependent phosphorylation of P-
Rex2 (460). This phosphorylation reduces its GEF activity by inhibiting its interaction with the
upstream activators phosphatidylinositol (3,4,5)-trisphosphate (PIP\textsubscript{3}) and Gβγ. In addition to
phosphorylation, many GEFs have been shown to be ubiquitinated, leading to their degradation
by the proteasome complex. An example of that is the interaction of the Dbl GEF with the
protein-ubiquitin ligase CHIP and two chaperone proteins, which results in the rapid ubiquitination and degradation of the Dbl (461). Finally, recently a RhoA GEF Net1A has been shown to be acetylated near its nuclear localization signal sequences, which allows for the GEF translocation into the cytoplasm and therefore activates it (462). Known phosphorylation sites and their effects on GEF activity for the GEFs relevant to this thesis are described in Table 1.2.

1.4.2.4 Regulation of GEF activity by localization

Finally, one of the important ways of regulating Rho GEFs activity is through the regulation of their localization. GEFs are mostly active at the cell membrane and at the sites of adhesion, where the Rho GTPases are most often located. The localization of the GEFs can be regulated by the posttranslational modifications or through the interactions with other proteins. Some examples of the former have already been mentioned in the previous sections, such as the activation of Net1A by relocation from the nucleus to the cytoplasm upon acetylation and inhibition of GEF-H1 by PAK phosphorylation which sequesters it to the microtubules, keeping it away from RhoA. An example of the latter is the interaction of the βPix GEF with PAK and the scaffolding protein SCRIB. The interaction of βPix with PAK and GIT proteins localizes βPix to the nascent adhesions in the lamellipodia, where it acts to activate Rac1 and RhoJ to stimulate adhesion turnover (463, 464). However, the binding of βPix to SCRIB localizes it to the leading edge of the migrating cell, where it activates Cdc42 to affect astrocyte polarization (465).
<table>
<thead>
<tr>
<th>GEF</th>
<th>Alternative name</th>
<th>Rho GTPase</th>
<th>Location (Kinase)</th>
<th>Phosphorylation</th>
<th>Function in motility</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS2</td>
<td>Alsin, ALS2CR6</td>
<td>Rac1</td>
<td>Ser277,492,1335,1464, Thr510 (466)</td>
<td>No effect on GEF activity (466)</td>
<td>ND ND</td>
</tr>
<tr>
<td>Arhgef1</td>
<td>p115 RhoGEF</td>
<td>RhoA, Rac1?</td>
<td>Tyr738 (Jak2) (467) Ser/ThrX (PKCa) (468)</td>
<td>Enhances GEF activity (467) Enhances GEF activity (468)</td>
<td>ND Endothelial cell retraction (468)</td>
</tr>
<tr>
<td>PLEKHG6</td>
<td>MyoGEF</td>
<td>RhoA, RhoG, RhoC</td>
<td>Thr544 (AuroraB) (474) Thr547 (Plk1) (475)</td>
<td>Enhances GEF activity; creates a docking site for Plk1 (474) GEF activation, central spindle localization (475)</td>
<td>ND ND</td>
</tr>
<tr>
<td>P-Rex2</td>
<td>DEPDC2</td>
<td>Rac1</td>
<td>Ser1107 (PAK) (460) 18 other Ser/Thr sites (460)</td>
<td>Inhibits GEF activity through reduced PIP3 and Gβγ binding, PM localization (460) ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rgnef</td>
<td>p190RhoGEF, ARHGEF28, RIP2</td>
<td>RhoA</td>
<td>TyrX (FAK?) (480)</td>
<td>Enhances GEF activity (480)</td>
<td>ND</td>
</tr>
</tbody>
</table>

Regulates attachment, polarity, migration and invasion of normal and cancer cells (471-473)  
Bleb retraction in amoeboid motility (476) Breast cancer cell polarity and invasion (477)  
Cell motility, invasion, proliferation of normal and cancer cells (478, 479)  
Cell adhesion, polarity, motility and invasion (481, 482) Protrusion formation (483)  
Enhances cell spreading (486), migration, and invasion (487) Adhesion to laminin (488) Stabilizes cell-cell junctions (489)
<table>
<thead>
<tr>
<th>GEF</th>
<th>Alternative name</th>
<th>Rho GTPase</th>
<th>Phosphorylation</th>
<th>Function in motility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Location (Kinase)</td>
<td>Effect on GEF</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DOCK2</td>
<td></td>
<td>Rac1</td>
<td>TyrX (Jak) (490)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOCK3</td>
<td>MOCA, PBP</td>
<td>Rac1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>DOCK5</td>
<td></td>
<td>Rac1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
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<tr>
<td>DOCK6</td>
<td>ZIR1</td>
<td>Rac1, Cdc42</td>
<td>Ser1194 (Akt) (498)</td>
<td>Decreases GEF activity (498)</td>
</tr>
<tr>
<td></td>
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<tr>
<td>DOCK7</td>
<td>ZIR2</td>
<td>Rac1, Cdc42</td>
<td>Tyr1118 (ErbB2) (501)</td>
<td>Enhances GEF activity (501)</td>
</tr>
<tr>
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</tr>
<tr>
<td>DOCK8</td>
<td>ZIR8</td>
<td>Rac1, Cdc42</td>
<td>TyrX (Pyk2) (503)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Table 1.2 Phosphorylation of the Dbl family and DOCK family GEFs and their role in cell migration.* Where there is an X instead of a number next to the phosphorylated amino acid, the exact residues that are phosphorylated have not been determined. The kinase responsible for the phosphorylation of each site, where known, is listed in the brackets and is underlined. ND = not determined.
In sum, there are multiple layers to the regulation of the GEF activity to ensure the precise and timely activation of the Rho GTPases. The differing structures of the GEFs outside the DH/PH or DHR1/DHR2 domains is what determines the interaction of the GEFs with their regulators. Given their important functions in regulating cell migration, adhesion, polarity and proliferation, among other processes, it is not surprising that GEFs are often mutated or deregulated in cancers. Most often, GEF deregulation results in their increased or ectopic activity, however recent studies indicate that GEF inhibition may also play a role in carcinogenesis. The functions of some of the GEFs in cell migration and their role in carcinogenesis are outlined in the following sections.

1.4.3 Arhgef1

Human Arhgef1 was first discovered as an interacting partner of the nucleotide-free RhoA, a version of RhoA that has a much higher affinity for the GEFs than the nucleotide-bound RhoA (509). Indeed, it was shown to activate RhoA specifically and this activation led to the transformation of the NIH 3T3 cells. As the molecular weight of the newly discovered GEF protein was 115 kDa, it was first named p115 RhoGEF. A mouse homolog of p115 GEF that was named Lbc’s second cousin (Lsc) was discovered at about the same time (510). Although Arhgef1 is expressed ubiquitously, increased expression has been noted in the hematopoietic and lymphoid tissues (510, 511). Interestingly, two isoforms of the Arhgef1 have been identified in the spleen, both lacking the C-terminal region and therefore more active than wild type protein (512). In the central region of the Arhgef1 there are the tandem DH/PH domains typical of the Dbl family GEFs, and thus it belongs to the Dbl GEF family (Figure 1.10a). Additionally, Arhgef1 is one of the three RGS-homology (RH)-domain containing subfamily of GEFs. Although the amino terminal RH domain of RH-Rho GEFs has low sequence homology to that of other RGS proteins, the structures of these domains are similar and therefore, have been shown to perform similar functions (513). Specifically, the RH domain of Arhgef1 has been shown to be responsible for the binding to and acting as a GAP for Gα12/13 subunits of the guanine nucleotide binding proteins (G proteins), proteins that relay the signal from the GPCRs (514). Thus, Arhgef1 was the first protein to directly link the GPCR signalling to the RhoA activation (515). Although Arhgef1 can bind to and act as a GAP for both Gα12 and Gα13, only Gα13 has been shown to stimulate its GEF activity towards RhoA (515). In resting cells, Arhgef1
resides primarily in the cytoplasm, but upon activation of the GPCRs with the lysophosphatidic acid (LPA) or thromboxane it rapidly relocates to the plasma membrane (516, 517). Overexpression of the constitutively active Gα13 is enough to target Arhgef1 to PM and induce its GEF activity, suggesting that GPCR signaling and subsequent Gα13 activation are an important mechanism of activation of the Arhgef1 GEF activity (518, 519). Thus, many studies have focused on delineating the Gα13 and the Arhgef1 interaction, which has now been described in great detail. In general, the N-terminal of Gα13 is responsible for its GAP activity while the C-terminal is responsible for the activation of the GEF activity of Arhgef1 (520). The precise residues of both Gα13 and Arhgef1 responsible for their interaction have been uncovered through point mutation experiments (521-524). The RH domain was shown to be required for the Gα13 binding, but it is not sufficient for the GAP activity of the Arhgef1 (515, 525). Various point mutation and deletion experiments have shown that the residues immediately N-terminal and C-terminal of the RH domain enhance the interaction with the Gα13 and are required for the GAP activity (524, 526, 527). Mutation of E27 and E29 in the RH domain abolished Arhgef1 binding to the Gα13 and Arhgef1 GAP activity (524). Interestingly, this mutant could still be recruited to the PM and the mutation did not affect the GEF activity of the Arhgef1. This suggested that the GAP function of the Arhgef1 does not affect its GEF activity and raised the possibility that membrane recruitment alone can activate Arhgef1. Indeed, two groups have shown that tethering Arhgef1 to the membrane activates its GEF activity and this is independent of the Gα13 stimulation (519, 528). Somewhat surprisingly, this also required the presence of the active RhoA, as its inhibition abolished Gα13-dependent PM recruitment. In turn, addition of the Arhgef1 RH domain to the two Rac1-specific GEFs activated them in a Gα13-dependent manner, suggesting that the RH domain acts as an independent sensor of Gα13 activation and does not confer specificity to the Rho GTPases (519). The DH domain of the Arhgef1 possesses the GEF switch which also includes a few residues N-terminal to this domain and is directly involved in the GEF activity of the Arhgef1 towards the Rho GTPases (452, 527). This domain also binds Gα13 and residues 503-507 have been shown to be important for this binding (527). Point mutations in these residues reduce Gα13 binding and as a result, Gα13-dependent stimulation of the Arhgef1 GEF activity. Of note, although the DH domain of Arhgef1 is predicted to interact with RhoA (527), neither purified DH domain alone nor the purified N-terminal region of the Arhgef1 up to the PH domain, interacted with the RhoA that was immobilized on beads (516).
Whether the DH domain interacts with RhoA in cells is still unclear and the crystal structure of the Arhgef1 bound to RhoA could help answer this question. Importantly the DH/PH module of Arhgef1 bound RhoA with high affinity, suggesting that the PH domain is essential for this interaction (516). Consequently, the PH domain is also essential for the Arhgef1 GEF activity (516, 517). In addition, the PH domain of Arhgef1 has been shown to target the protein to the PM and this domain alone is sufficient for its PM translocation (516, 517). The linker region between the RH and the PH domains, as well as the C-terminal of the Arhgef1 negatively regulate the Arhgef1 GEF activity (452, 516). Removal of the amino acids 353-394, which comprise the linker region, dramatically increased the basal GEF activity of the Arhgef1, irrespective of the presence or absence of the RH domain (452). The crystal structures of the DH/PH domains with and without the linker region suggest that the linker region may modulate the GEF switch of the Arhgef1. Hence, one way that Gα13 may induce Arhgef1 GEF activity is by binding the Arhgef1 which then relieves the linker’s inhibitory effect. The C-terminal of the Arhgef1 has a coiled-coil motif and is the site of the Arhgef1 homo-oligomerization (mostly tetramers) (453, 512). In turn, the oligomerization negatively regulates the Arhgef1 GEF activity, as oligomerization disruption results in the higher GEF activity (512, 529). Another way the C-terminal can inhibit the GEF activity is by binding to the inhibitory linker region. The idea that these two regions may cooperate in the GEF inhibition is supported by the fact that the removal of both regions did not have an additive effect to the removal of either one region alone (516).

All of these observations together suggest a possible mechanism of the GPCR signaling to RhoA through Arhgef1 (Figure 1.10b). In resting cells, Arhgef1 is autoinhibited by the linker region and oligomerization and localizes mostly to the cytoplasm. Upon activation of the Gα13 by the GPCRs, it sequesters the Arhgef1 to the plasma membrane by binding the RH and then the DH domains. This binding relieves the Arhgef1 autoinhibition of its GEF activity and brings the Arhgef1 closer to its effector-RhoA. Arhgef1 binds RhoA through the DH/PH domains which facilitate the exchange of the GDP to the GTP on RhoA and thus activate it.
Figure 1.10 Arhgef1 structure and signaling. a Schematic of the Arhgef1 domain structure. RH domain binds to and is a GAP for Gα13; it is responsible for the Arhgef1 membrane recruitment and enhancement of its GEF activity. The linker (L) region inhibits Arhgef1 GEF activity in unstimulated cells by the regulation of the GEF switch (GS). The DH domain possesses the GEF activity and most of the GEF switch which also includes a few upstream residues. The PH domain is responsible for RhoA binding and specificity and plasma membrane recruitment. The coiled coil (CC) motif inhibits Arhgef1 GEF activity and is the site of the Arhgef1 oligomerization. aa = amino acids. b GPCR signaling to the RhoA through the Arhgef1. In unstimulated cells, Arhgef1 homo-oligomerizes and is autoinhibited in the cytoplasm. Upon GPCR stimulation and the subsequent Gα13 activation, Arhgef1 homo-oligomerization is disrupted and the autoinhibition is relieved. Arhgef1 binds Gα13 and relocates to the plasma membrane, where its GEF activity is fully activated. Arhgef1 then binds and activates RhoA.
Besides being regulated by the autoinhibition, the homo-oligomerization and the localization, Arhgef1 has been shown to be regulated by phosphorylation. Wells and colleagues were the first to report that Arhgef1 is phosphorylated and that its phosphorylation is not dependent on the Gα13 activation (516). As RhoA activation is dependent on the PKCα, Holinstat et al. have tested whether PKCα activates RhoA through Arhgef1. Indeed, they have shown that upon thrombin stimulation, PKCα binds to the Arhgef1 and phosphorylates it (468). In turn, inhibition of the PKCα-dependent Arhgef1 phosphorylation resulted in the inhibition of the RhoA activation, suggesting that phosphorylation enhances Arhgef1 GEF activity. However, activation of both the PKCα and the Gα13 pathways by thrombin was necessary for the Arhgef1 dependent RhoA activation, subsequent stress fiber formation and loss of the endothelial cell barrier function. The specific residues that the PKCα phosphorylates remain to be determined. Another group showed that tumour necrosis factor (TNF)-α can also induce the PKCα-dependent Arhgef1 phosphorylation to control the endothelial cell barrier function in the brain (530). In addition, Guilluy et al. have demonstrated that similar to the other RH-GEFs, Arhgef1 is regulated by tyrosine phosphorylation (467). They have shown that Jak2 phosphorylates Arhgef1 on Tyr738 downstream of the angiotensin II signalling. This phosphorylation results in the activation of the Arhgef1 GEF activity and the subsequent RhoA activation, which in turn regulates the vascular tone and the blood pressure. Additionally, it was recently reported that the Src family of tyrosine kinases (SFKs) regulate the Arhgef1 activation, as their inhibition by a small molecule inhibitor decreased Arhgef1 activity towards RhoA (531). However, whether the SFKs phosphorylate Arhgef1 is still unclear. Mass spectrometry analysis of the Arhgef1 phosphorylation showed that serines 14, 330, 786 and 833 are phosphorylated irrespective of the all-trans retinoic acid (ATRA) or the phorbol 12-myristate 13-acetate (PMA) activation, while Ser863 was only phosphorylated after the treatment (469). When the Arhgef1 constructs bearing point mutations in each phosphorylated residue were overexpressed in HeLa cells, only the Ser330 mutant resulted in a significant reduction of the RhoA activation and GEF activity, which persisted even after the Gα13 activation. Curiously, both the phospho-mimicking (S330D) and the phospho-defective (S330A) mutations had the same effect of reducing the GEF activity. The mechanism through which Ser330 may regulate the Arhgef1 GEF activity and whether other phosphorylation sites have any regulatory roles, remains to be uncovered. As well, it is still unclear which kinases may be responsible for the phosphorylation of these residues. One obvious candidate is PKCα, as
it is a serine/threonine kinase and has been shown to phosphorylate Arhgef1. Another serine/threonine kinase that has been reported to bind and phosphorylate Arhgef1 is PAK1, effector of the Rac1 (470). This interaction did not alter the Arhgef1 GEF activity but the inhibition of the PAK1 dramatically enhanced thrombin-induced RhoA activation mediated by the Arhgef1. This suggests that other mechanisms of the Arhgef1 inhibition by the PAK1, such as the regulation of localization, might be at play. Therefore, although it has been shown that Arhgef1 is phosphorylated on multiple residues and phosphorylation of some of these sites may alter its activity, the precise roles of these post-translational modifications, mechanisms of their action and kinases phosphorylating them remain to be elucidated.

As a GEF for RhoA, Arhgef1 has been shown to regulate some of its functions such as cell-substrate and cell-cell attachment, cell motility and invasion. Since Arhgef1 is most highly expressed in cells of the hematopoietic and the lymphoid lineages, it has been shown to be involved in the regulation of migration of these cells, including B cells (532), thymocytes (533), neutrophils (471), lymphocytes (532), monocytes and macrophages (534). It is therefore not surprising that it plays an important role in immune response and inflammation. Knockout of the mouse homologue of the Arhgef1, Lsc in 129/Sv mice produced viable and apparently healthy progeny (532). However, a closer examination of cells of the hematopoietic lineage of the Lsc+/− mice showed disrupted homeostasis in the peripheral lymphoid compartments, impaired humoral immunity and altered cell motility compared to the wild type mice. Interestingly, while T and B cells from the Lsc−/− mice showed reduced motility, the marginal zone B cells (MZB) cells showed increased basal and directional motility, suggesting that Arhgef1 may be regulated differently or signals to different effectors in the different cell types. Knockout of the Lsc in BALB/c background mice also resulted in the thymic hyperplasia due to the increased number of thymocytes in the thymus and the lymph nodes (533). Lsc was then shown to be involved in the thymocyte apoptosis downstream of the thromboxane signaling. A different group has shown that the Lsc is enriched in the leading and the trailing edge of the stimulated neutrophils, while the neutrophils from the Lsc null mice do not form pseudopods properly (471). They went on to show that Lsc is required for the normal speed, directionality and integrin attachment of the neutrophils and that this is mostly mediated through the Lsc’s role as a RhoA activator. Of note, while the Lsc deficient neutrophils moved faster than the wild type controls, they lacked
directionality when stimulated to move toward a chemoattractant. This may be due to the recently uncovered role of the Arhgef1 in the regulation of the front-rear cell polarity which is essential for the directional migration (535). As Arhgef1 regulates immune cell motility and therefore, inflammation which in turn may lead to atherosclerosis, it has recently been proposed as therapeutic target for the atherosclerosis (536). Besides regulating cell motility and invasion through the regulation of the actin cytoskeleton, Arhgef1 also affects cell invasion by the regulation of the MMP expression and activity. Specifically, Hartney et al. have found that seeding on fibronectin induced MMP-2 and MMP-9 expression and MMP-9 activity in macrophages and monocytes. However, in Arhgef1 null cells the induction was much stronger than in the wild type controls (472, 537). This suggested that normally Arhgef1 attenuates the expression and activity of the MMPs in monocytes and macrophages. In addition, there are multiple reports of the Arhgef1 regulation of the neuronal cell motility (528, 538, 539). *In vivo*, Lsc deficient mice were impaired in esophageal neuronal innervation which led to the reduced thickness of the esophageal muscle layer and its motor dysfunction (540). Interestingly, recently Arhgef1 was also proposed to play a role in the amyotrophic lateral sclerosis (541). Finally, Arhgef1 is involved in the pathway that mediates breast (473) and pancreatic (542) cancer cell growth and invasion.

Arhgef1 has also emerged as an important regulator of the cell-cell and the cell-matrix adhesions. Cell-cell adhesions and efficient actin rearrangement are very important in the regulation of the epithelial and endothelial cell barrier functions. Multiple groups have shown that the Go13-Arhgef1-RhoA pathway plays an important role in the regulation of these functions (530, 543-547). Birukova and colleagues have further demonstrated that one mechanism by which Arhgef1 regulates this function is through the regulation of the microtubule assembly and the cell-cell junctions (544, 548). As endothelial cell barrier dysfunction can lead to asthma, sepsis, acute respiratory distress syndrome, hypertension and other cardiovascular disorders as well as the blood-brain barrier dysfunction, it is not surprising that the Arhgef1 has been suggested to play a role in many of these conditions (467, 548-551). Arhgef1 has also been shown to regulate the cell-matrix adhesions, in particular downstream of the fibronectin (368) and collagen IV (552) adhesion. It has recently been implicated in mechanotransduction, where it
was shown that the tension applied to the cells leads to the Arhgef1 and the GEF-H1 activation and therefore results in the RhoA-dependent increase in the cell stiffness (531, 553).

Like its effector RhoA, overexpression or increased activity of the Arhgef1 causes cell transformation (453, 509). Not surprisingly, deregulated Arhgef1 expression or activity has been reported in a number of cancers. Arhgef1 expression or activity is increased in breast (554), bladder (554, 555) and prostate (556) tumours, while it is decreased in pheochromocytoma (554). Additionally, Arhgef1 expression is regulated by miR-143, a microRNA that functions as a tumour suppressor and is deregulated in the pancreatic and other solid tumours (542, 557). So far, mutations in Arhgef1 have been reported only in lymphoma, all of them deleterious and most of them result in premature stop codons (558). In addition, high Arhgef1 expression was shown to be a predictor of poor prognosis in renal cancer, while in breast cancer it predicted a favourable prognosis (559). Together, these data suggest that Arhgef1 may act to promote tumorigenesis in some cancer types and suppress it in others. A more detailed look at the Arhgef1 mutation status and expression or activation in different cancer types would further aid our understanding of the role this GEF may play in cancer initiation and progression.

1.4.4 P-Rex2

P-Rex2 is a 183kDa protein that belongs to the Dbl family of GEFs. It is a homolog of the P-Rex1 Rac1 GEF, with which they share identical domain structure and 59% nucleotide and amino acid sequence homology (560, 561). Both are activated by the PI(3,4,5)P3 downstream of the phosphoinositide 3-kinase (PI3K) signaling and by the Gβγ subunits downstream of the GPCR receptors (561, 562). However, the two family members have different tissue distributions. Whereas P-Rex1 is mostly expressed in the brain and neutrophils (562), P-Rex2 is widely expressed but is absent from the neutrophils (561). Like P-Rex1, P-Rex2 is a Rac1 GEF with a slight GEF activity towards Cdc42 (560, 561). Besides being a GEF for the Rac1, P-Rex2 also interacts with the tumour suppressor PTEN and the two proteins mutually inhibit each other (479, 563, 564). However, the interaction between P-Rex1 and PTEN has been suggested but has not been reported. P-Rex2 null mice are born at Mendelian ratios, are viable, fertile and apparently healthy (565). Upon further examination, these mice had altered morphology of the Purkinje cells, where the main dendrite was thinner and tended to be shorter than in the wild type
mice. Since the cerebellum, where the Purkinje cells are concentrated, is responsible for the motor coordination, P-Rex2 null mice were examined for the motor function. These mice exhibited symptoms consistent with the cerebellar dysfunction such as mild motor coordination defects that worsened with age and were more pronounced in females.

P-Rex2 consists of the N-terminal DH-PH module, followed by the two tandem Dishevelled, Egl-10, and Pleckstrin (DEP) domains, the tandem PDZ domains, and an inositol polyphosphate 4-phosphatase (IP4P) domain at the C-terminal (Figure 1.1a). P-Rex2 has at least two splice variants, where the full-length version is known as P-Rex2a and the splice variant that lacks the C-terminal IP4P domain is known as P-Rex2b (560). The DH domain of P-Rex2 is mostly responsible for the Rac1 binding and GEF activity of the protein (560, 566). The PH domain interacts with the Gβγ subunits, which in turn enhance the DH GEF activity (478). While the PH domain of P-Rex1 is also the site for the PI(3,4,5)P_3 binding (566), direct interaction of PI(3,4,5)P_3 with P-Rex2 has not been reported. The PH domain was also shown to be required for the substrate specificity and recognition (567), although by similarity with the P-Rex1 it is not predicted to impact the activation of Rac1 by the DH domain (568). Additionally, the PH domain interacts with the catalytic region of the PTEN, thus inhibiting it (569). DEP and PDZ domains are at least in part responsible for the autoinhibition of the P-Rex2 GEF activity, as the N-terminal fragment of the P-Rex2 that includes only the DH-PH domains is more active (478, 570). Besides playing a role in the P-Rex2 autoinhibition, DEP domains also bind downstream effectors, such as the mechanistic target of Rapamycin (mTOR) (571). In turn, PDZ domains function as adaptors in other proteins that contain these domains and are needed for the PTEN interaction with the P-Rex2 (570). The IP4P domain has been shown to bind PTEN with high affinity (569) and is required for the PTEN inhibition of the P-Rex2a (563). Interestingly, IP4P domain of the P-Rex proteins has no known phosphatase activity (562, 566). The C-terminal domain of the P-Rex1 has been shown to interact with its DH-PH domains, thereby autoinhibiting its GEF activity and it is thought that the P-Rex2 is autoinhibited in a similar fashion (568, 572). Indeed, P-Rex2b which lacks the IP4P domain has higher GEF activity towards Rac1. However, the removal of the DEP and PDZ domains of P-Rex2b increased its GEF activity even further, suggesting that these domains also participate in the P-Rex2
autoinhibition (478). The mechanism of the relief of this autoinhibition is still unclear but may involve post-secondary modifications and/or binding to the upstream regulators.

Like many other GEFs, P-Rex2 has been shown to be regulated by the phosphorylation and the localization. Mass spectrometry analysis of the P-Rex2a phosphorylation sites uncovered 19 serine/threonine sites, 12 of which were only phosphorylated in response to the insulin activation (460). Out of 19 sites, 12 were also conserved between the P-Rex2a and the P-Rex1. Barrows and colleagues further examined the Ser1107, which is phosphorylated upon activation, is conserved in P-Rex1 and was shown to be phosphorylated in growth-factor dependent fashion in P-Rex1. They showed that this residue is de-phosphorylated by the phosphatases PP1α and PP2A, similar to the conserved residue of the P-Rex1. Furthermore, the Ser1107 phosphorylation prevented the P-Rex2 localization to the plasma membrane where its target Rac1 is located, effectively abating P-Rex2 GEF activity. The lack of PM localization of the P-Rex2 with Ser1107 phosphorylation was likely due to the reduction of P-Rex2 binding to the PIP3 and Gβγ. In addition, reduced PM translocation was the most likely cause of the reduced GEF activity as both Rac1 and PTEN bound Ser1107 phosphorylated and de-phosphorylated P-Rex2 with equal affinity. Importantly, Barrows et al. further showed that Rac1 effector PAK was responsible for the Ser1107 phosphorylation and thus implicated P-Rex2 in the Rac1 negative feedback loop. However, the authors did not exclude the possibility that other kinases may also be involved in the phosphorylation of the Ser1107 and other residues on P-Rex2.

Finally, P-Rex2 has been shown to be regulated at a transcript and protein levels. The P-Rex2 mRNA is a target of micro RNA miR-338-3p (573). This micro RNA has been shown to be downregulated in metastatic neuroblastoma compared to the primary tumour. Depletion of P-Rex2 by siRNA, which mimics the effect of miR-338-3p, decreased the neuroblastoma cell proliferation, migration and invasion. In addition, miR-338-3p is downregulated in HCC and gastric primary tumours and cell lines (574). miR-338-3p is also one of the genes under the control of the transcriptional suppressor MECP2, which is commonly de-regulated in cancer (575). On the protein level, P-Rex2 has been shown to be a target of the ubiquitin ligase, which targets P-Rex2 for the proteasome degradation (576).
P-Rex2 structure and signaling. a Schematic of the P-Rex2a domain structure. Rac1 binding and GEF activity are regulated by the DH domain, while the PH domain mediates GPCR signaling by binding Gβγ. PH domain is also required for the substrate recognition and specificity; it binds and inhibits PTEN. DEP domains bind mTOR, aiding its activation. PDZ domains are adaptor domains that facilitate PTEN binding. The IP4P domain binds PTEN and mediates its inhibition of P-Rex2. It may also mediate P-Rex2 autoinhibition. aa = amino acid. b P-Rex2 signaling in cell motility. In unstimulated cells P-Rex2 is kept inactive in the cytoplasm, possibly by autoinhibition. In turn, PTEN inhibits PI3K signaling by PIP3 dephosphorylation. P-Rex2 GEF activity can be stimulated in two ways. First upon GPCR activation, the activated Gβγ subunits bind to and activate P-Rex2. Second, GPCR and RTK activation induces PI3K signaling, resulting in PIP3 production and PIP3 can activate P-Rex2 directly. PIP3 also activates the Akt signaling which results in the enhanced cell motility and proliferation. Active P-Rex2 can then translocate to the PM where it activates Rac1. P-Rex2 also binds mTORC2, leading to further Akt pathway activation. In addition, P-Rex2 inhibits PTEN, further activating PI3K pathway. PTEN, in turn, inhibits P-Rex2 GEF activity. How the interaction between P-Rex2 and PTEN is regulated requires further investigation.
As a Rac1 GEF, P-Rex2 can regulate cell migration, invasion and proliferation through multiple pathways downstream of RTKs and GPCRs (Figure 1.1b) (Reviewed in (577)). Both RTKs and GPCRs activate PI3K, which phosphorylates PI(4,5)P2 to make PI(3,4,5)P3. P-Rex2 mediates the PI3K signaling by stabilizing the PI(3,4,5)P3. It does so through the inhibition of the PTEN, which is a phosphatase for the PI(3,4,5)P3. In turn, the PI(3,4,5)P3 and the Gβγ subunits downstream of the GPCR signaling activate P-Rex2 GEF activity towards Rac1 and thus mediate actin rearrangement, MMP expression and cell proliferation through the Rac1 downstream targets. Active P-Rex2 also interacts with and activates mTOR, which in turn activates Akt, thus resulting in the cell growth, proliferation, migration and enhanced glucose metabolism. In addition, the PI(3,4,5)P3 directly activate the Akt pathway. Through these pathways, P-Rex2 has been shown to regulate the migration, invasion and proliferation of MEFs (563), endothelial cells (478) and many types of cancer cells which will be discussed below.

Given the role of P-Rex2 as Rac1 activator and regulator of the mTOR and PTEN signalling, all pathways very commonly deregulated in tumorigenesis, it is not surprising that the list of cancer types where P-Rex2 is deregulated is large and continues to grow. P-Rex2 was shown to be overexpressed in breast (479), prostate (479), pancreatic (479, 578), HCC (579, 580), ovarian (479) and brain (479, 573, 581) cancers, as well as in leukemia (582) and osteosarcoma (583) and this often correlated with the higher P-Rex2 protein levels. As P-Rex2 and PTEN inhibit each other, expression of the P-Rex2 is increased 3-fold in PTEN negative breast cancer tumours compared to the PTEN positive ones (479). Importantly, in all P-Rex2 overexpressing tumours, siRNA of P-Rex2 led to a decrease in cell migration, invasion and proliferation. In osteosarcoma, the reduction of the cell proliferation, migration and invasion in P-Rex2 depleted cells was at least in part due to the reduced levels of MMP-2 and MMP-9, suggesting that P-Rex2-Rac1 pathway regulates MMP expression (583). In glioma, higher P-Rex2 expression correlates with an increased tumour grade (581). In HCC and perhaps other cancer types, P-Rex2 overexpression is likely to be the result of the inhibition of miR-338-3p (574). Another mechanism of P-Rex2 overexpression in HCC is the common downregulation of the glycine N-methyl transferase (GNMT) in this cancer type. GNMT was shown to enhance the interaction between P-Rex2 and HectH9, thereby promoting P-Rex2 ubiquitination and degradation (576). Therefore, reduced GNMT expression in HCC leads to the higher P-Rex2 protein levels and enhanced downstream signaling to the Akt. Higher expression of the P-Rex2 in HCC correlated
with the tumour portal vein invasion, metastasis, poor differentiation and poor survival (576, 579). Additionally, P-Rex2 is located in a region of high amplification in breast, prostate and colorectal cancers that has been linked to an aggressive phenotype and metastasis formation in these tumour types (479). P-Rex2 is also emerging as one of the most commonly mutated genes in cancer. Somatic mutations in P-Rex2 have been reported in melanoma (584), colorectal (479), lung (479) and pancreatic cancers (479). In a small cohort (n=11) study of breast cancer patients no P-Rex2 mutations were identified (479). However, in a larger group of 560 breast cancer tumours, P-Rex2 was shown to be mutated in 7.7% of the cases (585). In melanoma, P-Rex2 was third most frequently mutated gene behind BRAF and NRAS (584). There are at least 28 non-synonymous P-Rex2 mutations in melanoma which are distributed throughout the gene and functional consequences of 7 of them have been described (577). The majority of them result in the higher P-Rex2 GEF activity and hence increase the cell proliferation, migration, invasion, tumour formation and decrease survival in xenograft mouse model (577). The increased GEF activity was often due to a mutation that causes a truncation of the C-terminal of the P-Rex2 protein. The effect of one of these truncating mutations E824, which results in the absence of the IP4P domain, was examined in detail in an NRAS mutant mouse model of melanoma (570). Mice conditionally expressing the mutant version of P-Rex2 in melanocytes upon doxycycline induction, exhibited accelerated tumour onset and penetrance compared to the uninduced mice. Other truncating mutations of the P-Rex2 in the same mouse model were also shown to accelerate tumour formation. Importantly, when the differentially expressed genes in induced vs uninduced melanocytes were examined, the majority were shown to regulate cell adhesion and actin filament organization, cell cycle and mitotic checkpoint regulation, and ribosomal and mitochondrial biogenesis pathways. These results are consistent with the P-Rex2 role of the Rac1 GEF and the PI3K signaling mediator. Aside from this model, the role of the P-Rex2 in tumorigenesis, tumour progression and metastasis formation in vivo has not been examined, thus further studies are needed to fully elucidate the role of P-Rex2 in these processes.
Rationale

Plk4 expression is increased in a variety of human cancers and it has been shown to promote breast cancer progression of human xenografts in mice. Two small molecule Plk4 inhibitors have been created, one of which is in phase I clinical trials for patients with advanced solid tumours (511). Yet the mechanisms of how Plk4 promotes tumour initiation and progression are not well defined. One suggested mechanism is through centrosome overduplication leading to chromosome instability. In addition, our laboratory has identified a novel role for Plk4 in spreading, migration and invasion of normal and cancer cells. We have also shown that Plk4 localizes to cell protrusions in spreading and migrating cells, where it induces protrusion formation through facilitating actin rearrangement. However, how Plk4 regulates actin rearrangement to induce cell motility is incompletely understood. Rho GTPases are the major regulators of actin rearrangement and cell motility. They cycle between an active state, mediated by GEFs, and an inactive state, mediated by GAPs and GDIs. Our previous study identified Plk4 as an activator of one of the three major Rho GTPases, RhoA, during cytokinesis to regulate proper cleavage furrow positioning in MEFs. We also showed that the activation of RhoA by Plk4 is dependent on Plk4 interaction with the Ect2 GEF, a known regulator of RhoA activity in cleavage furrow positioning. Therefore, we had evidence that Plk4 regulates cell migration and that it can regulate the activation of RhoA, one of the major regulators of cell migration. Thus, the work presented in this thesis aims to determine whether Plk4 regulates the activity of RhoA and possibly other Rho GTPases during cell migration and spreading and identify the underlying mechanisms of Rho GTPase activation. In chapter 3, I examine the effect of Plk4 on three major Rho GTPases: Rac1, Cdc42 and RhoA and compare the effect of these Rho GTPases on cell migration to that of Plk4. In the following chapter, I examine the mechanism of Rho GTPases on cell migration to that of Plk4. In the following chapter, I examine the mechanism of Plk4 regulation of Rho GTPase activation. Rho GTPase regulators were screened for the Plk4 consensus phosphorylation motif and promising candidates were tested for physical and functional interaction with Plk4 in cell migration. Finally, in chapter 5, I test the effect of candidates from chapter 4 on cell spreading and compare it to that of Plk4.
Hypothesis and Aims

Hypothesis

Plk4 interacts with upstream regulators of Rho GTPases to promote their activation and cell migration.

Specific aims

1) Determine the effect of Plk4 on the activation of Rho GTPases RhoA, Rac1 and Cdc42 and compare the effect of these Rho GTPases and Plk4 on cell migration.

2) Determine the mechanism whereby Plk4 affects Rho GTPase activation and how it pertains to cell motility.

3) Determine the effect of Plk4 candidate targets on cell spreading and shape.
Chapter 2

2 Methods

2.1 Cell lines and cell culture

MEFs were generated and cultured as previously described (52, 54). HeLa cells were obtained from ATCC in 2009. HEK293T cells were a gift from the Tony Pawson laboratory (obtained in 2010; Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Canada). U2OS cells were a kind gift from the laboratory of Laurence Pelletier (obtained in 2013; Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Canada). Generation of the U2OS T-Rex YFP-Plk4, shPlk4 and shLuciferase stable HeLa cell lines was previously described (102).

Cells were grown at 37°C in Dulbecco's modified Eagle Medium (DMEM: MEFs, HeLa, HEK293T) supplemented with 10% fetal bovine serum (FBS), or McCoy’s 5A medium (U2OS) supplemented with 10% tetracycline free FBS and 1% GlutaMAX (Thermo Fisher, 35050-061). Stable cell lines were cultured with 200µg/ml of puromycin.

2.2 Antibodies

Antibodies used for immunofluorescence in this thesis were anti-FLAG M2 (Sigma, F1804), anti-GFP (Abcam, Ab290), anti-V5 (Abcam, Ab9116), anti-p115 RhoGEF (Cell Signaling, 3669). Secondary antibodies were conjugated to Alexa Fluor 488 or 546 (Life Technologies). DNA was detected using Hoechst (Invitrogen, H3570). Actin was detected using Phalloidin Alexa 546 (Invitrogen, A22283) or 488 (Invitrogen, A12379). The antibodies used for immunoblotting were: anti-γ-tubulin (Sigma, T6557), anti-FLAG M2 (Sigma, F1804), anti-GFP (Abcam, Ab290), anti-HA (Sigma, H6908), anti-p115 RhoGEF D25D2 (Cell Signaling, 3669), anti-RhoA (Cytoskeleton, ARH04), anti-Rac1 (Millipore, 05-389).
2.3 Plasmid constructs

GFP-ALS2, GFP-Arhgef1, GFP-DOCK2, GFP-PLEKHG6, GFP-Rgnef and GFP-Trio constructs cloned into Creator™ system were obtained from Rick Bagshaw (Tony Pawson’s Laboratory). V5-P-Rex2 pcDNA3.1 V5/His construct was a gift from Ramon Parsons (Addgene plasmid #41555). Vectors were constructed using Invitrogen’s Gateway system, with pDONR223 donor vector and pcDNA 5/FRT/TO C-terminal HA tag or N-terminal 3xFLAG tag. Arhgef1 deletion mutants (RH Domain 1-207, L-DH/PH 207-733, DH/PH 362-733, C term 733-879) were generated by PCR using Phusion High Fidelity DNA Polymerase (New England Biolabs, M0530) according to manufacturer’s instructions. All constructs were validated by sequencing.

List of primers for Arhgef1 truncation mutant cloning

<table>
<thead>
<tr>
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<th>Forward attB1 (5'-3')</th>
<th>Reverse attB2 (5'-3')</th>
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<td>Arhgef1 RH domain (1-207)</td>
<td>GGGGACAAAGTTGTACAAAAA AGCAGGGTCCACCATGGAAGA CCTCGCCCGA</td>
<td>GGGGACCACCTTTGTACAAGAAAAGCTG GGTCTTACCGGAAAGTTCCCTCC</td>
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<td>Arhgef1 L-DH/PH domain (207-733)</td>
<td>GGGGACAAAGTTGTACAAAAA AGCAGGGTCCACCATGAGTGG AGACAAGAAGTCC</td>
<td>GGGGACCACCTTTGTACAAGAAAAGCTG GGTCTTACCTAGGGCGAGAGGAGCAGG</td>
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<td>GGGGACCACCTTTGTACAAGAAAAGCTG GGTCTTACTTTAGGGCGAGAGGAGCAGG</td>
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<tr>
<td>Arhgef1 C Terminal (733-879)</td>
<td>GGGGACAAAGTTGTACAAAAA AGCAGGGTCCACCATGCTCTG AAAGTCCCTG</td>
<td>GGGGACCACCTTTGTACAAGAAAAGCTG GGTCTTACGAGCAGCCAGGACG</td>
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2.4 RNA extraction and Real Time RT-PCR

Cells were pelleted and RNA was extracted using RNeasy mini kit (QIAGEN) per manufacturer’s instructions. RNA was quantified using NanoDrop ND-1000 spectrophotometer and 800ng from each sample was used for the reverse transcription. RNA was treated with DNase I (Invitrogen, 18068-015) before reverse transcription using Random Primers (Invitrogen, 48190-011) and SuperScript II reverse transcriptase (Invitrogen, 18064-014). SYBR Green PCR Mastermix (Applied Biosystems, 4309155) was used for the real time RT-PCR, which was run on an ABI 7900HT apparatus and data was generated by PCR software (SDS2.2.2, Applied Biosystems). The expression in test samples was normalized to that of HPRT (for MEFs experiments) or GAPDH (for human cell lines) using $2^{(-\Delta\Delta C_t)}$ method as previously described (586). Primers used for the PCR were as follows:

<table>
<thead>
<tr>
<th>Mus musculus</th>
<th>RT-PCR Primer (5’-3’)</th>
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<tbody>
<tr>
<td>HPRT</td>
<td>F: AAACAATGCAAAACTTTTGCTTTCC; R: GGTCCTTTTTCAACCAGCAAGCT</td>
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<tr>
<td>MMP-3</td>
<td>F: ACATGGAGACTTTTGCCCTTTTG; R: TGGGCTGAGTGTTAGAGTCCC</td>
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<tr>
<td>MMP-13</td>
<td>F: CTTCTTCTTGTGAGCTGGACTC; R: CTTGGAGGTCACTGTAGACT</td>
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<td>Plk4</td>
<td>F: AAAAGGCTGGTGAGCATCC; R: AAGGCCTCTTCCATCGTTCCG</td>
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<tr>
<td>Homo sapiens</td>
<td></td>
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<tr>
<td>Arhgef1</td>
<td>F: GCACGACCTCTTCTTCCAGCCCA; R: CTCCGTGGGGATCATGTCCCTTCAG</td>
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<tr>
<td>GAPDH</td>
<td>F: AAGGTGAAGTGGTGGAGTAAC; R: GGGGCATTGATGGCAACATAA</td>
</tr>
<tr>
<td>Plk4</td>
<td>F: AATCAAGGACTCTCTCAAATC; R: TGGTCTCTCTGCAAATC</td>
</tr>
<tr>
<td>P-Rex2</td>
<td>F: ACCATGAGAAGGACAAAAA; R: CTTGCATATTCTTGTATGTTG</td>
</tr>
</tbody>
</table>
2.5 siRNA-mediated protein depletion

SMARTpool siRNA or the individual constructs (Dharmacon) were transfected using Lipofectamine RNAiMax reagent (Invitrogen, 13778) to a final concentration of 30nM. Transfection mixes were diluted in OptiMEM (Gibco, 31985-070). A reverse-transfection was performed, where cells were plated into plates that already contained the siRNA mix. Media was changed, and siRNA was applied each day for a total of 48-72h. Sequences for siRNA constructs were as follows:

<table>
<thead>
<tr>
<th>Homo sapiens</th>
<th>siRNA sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arhgef1</td>
<td>1. AGAACGAGCUGGAGACAAA (siArhgef1-1)</td>
</tr>
<tr>
<td></td>
<td>2. CAACGUCGCCUUGAACU (siArhgef1-2)</td>
</tr>
<tr>
<td></td>
<td>3. GGUGUCUCUCAUCACUGA (siArhgef1-3)</td>
</tr>
<tr>
<td></td>
<td>4. UGACGUGGCGGGUGACUA (siArhgef1-4)</td>
</tr>
<tr>
<td>Plk4</td>
<td>1. GAAUGAAACAGGGUACUA 2. GAAACAUCCUUUCUACUGA 3. GGACCUAAGUACCAGUUA 4. GUGGAAGACUAAUUGUA</td>
</tr>
<tr>
<td>P-Rex2</td>
<td>1. CUGAUAGACUGGUUAUUG 2. UAGAAACAUCAUGCUAAUG 3. GAACGGCGAAGGUUUA 4. GAAGCGGACUCCACGGAA</td>
</tr>
<tr>
<td>RhoA</td>
<td>1. AUGGAAGACAGGUAGAGUU 2. GAACUAUGUGGCAGAUAUC 3. GAAGACAUUGCUUGCUCAU 4. GAGAUAUGGCCAACCAGGA</td>
</tr>
<tr>
<td>Photinus pyralis</td>
<td>1. CGTACGGGAATACTTCGA</td>
</tr>
</tbody>
</table>

2.6 Transfections

Cells were transfected 24h after seeding or for active Rac1 pulldown HEK293T cells were grown to a confluency of 50% at which point they were transfected. For transient transfection, 2mg/ml PEI (Polyethylenimine; Sigma, 408727) reagent was used for Plk4 and Arhgef1 truncation mutants co-immunoprecipitation and for the in vitro kinase assay for a total transfection time of 24h; and Lipofectamine 2000 (Invitrogen, 11668) reagent was used for the rest of the experiments for a total transfection time of 16h, per manufacturer’s instructions. Transfection mixes were diluted in OptiMEM (Gibco, 31985-070).
2.7 Active Rac1 and Cdc42 pulldowns

For pulldowns with siRNA treatment, the cells were treated for 72h prior to pulldown, with re-application of siRNA every 24h. For pulldowns with transient overexpression, cells were transfected for 16h prior to the pulldown. For all pulldowns cells were grown to 80% confluency, placed on ice and lysed with 0.8ml of Rac1/Cdc42 buffer (Initial buffer: 25mM Hepes, 150mM NaCl, 1% NP40, 10% Glycerol, 10mM MgCl2. Added before use: 1mM EDTA, 1mM Na3VO4, 1mM PMSF, 25mM NaF, 1x Protease inhibitor cocktail tablet (Roche, 11836145001). Cells were quickly scraped, lysate collected and cleared by centrifugation at 13000rpm for 3 min at 4°C. Next, 40μL of lysate was used for total protein and another 40μL for protein quantification. 20μL of 3X loading dye per sample was added to the totals. The rest of the cleared lysate was placed on dry ice. Total amount of protein was determined using the BCA assay. Lysates were thawed on ice and equal amounts were incubated on orbital shaker with 10μL (1mg/ml) PAK-GST beads (Cytoskeleton, PAK02) for 45 min at 4°C. Samples were then washed 3 times with 500μL wash buffer per sample (wash buffer is the same as initial lyses buffer plus the same amount of NaF and for Rac1 Na3VO4 as in lyses buffer). Each time the samples were spun at 2000rpm for 30 seconds to precipitate the beads. Wash buffer was then removed and 20μL of 3X loading dye per sample was added. Samples with loading dye were then boiled for 5 min at 96°C and subsequently placed at -80°C until ready for immunoblotting.

2.8 Active RhoA pulldowns

For induction of Plk4 expression in U2OS T-Rex YFP-Plk4 cells, they were treated with 0.01µg/ml of tetracycline for 18h prior to pulldown. This assay was performed the same way as Rac1/Cdc42 pulldown assay with the following exceptions. RhoA lysis buffer: Initial buffer: 50mM Tris pH 7.6, 100mM NaCl, 10 mM MgCl2, 1% Triton X. Added before use: 0.1% SDS, 0.5% Sodium Deoxycholate, 1mM Na3VO4, 1mM PMSF, 25mM NaF and Protease inhibitor cocktail tablet (Roche, 11836145001). Wash buffer was the same as initial lyses buffer plus 25mM NaF and 1mM Na3VO4. Pulldown was performed with 20µl Rhotekin RBD beads (Cytoskeleton, RT-02B).
2.9 Active Arhgef1 pulldowns

Active Arhgef1 pulldowns were performed as described previously (587). Briefly, U2OS T-REx YFP-Plk4 cells were treated with 0.01µg/ml tetracycline for 16h and grown to 100% confluency. The pulldown was performed in the same way as active Rac1/Cdc42 pulldown with the following exceptions. Lysis buffer and wash buffer: 20 mM HEPES pH 7.5; 150 mM NaCl; 5 mM MgCl2; 1% TX-100; 1 mM DTT; 1 mM PMSF; 1 Complete Mini Protease Inhibitor tablet. For pulldown 20µl of G-Sepharose beads coupled to RhoA(G17A) per sample were used.

2.10 Immunoblotting

Protein samples were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% milk and 0.1% Tween in phosphate buffered saline. They were then probed with primary antibodies at 4°C overnight with continued shaking, then with HRP-linked secondary antibodies (GE Healthcare). SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, 34095) was used to detect the signal. Where appropriate, band intensity was quantified using ImageJ. For active Rho GTPases and active Arhgef1 pulldown quantification, a ratio of pulldown (active) to total protein was created. Where indicated on the y axis as Relative Active Rac1/RhoA/Arhgef1, the values of test conditions were normalized to controls, the value of the active/total ratio of control was designated as 1.

2.11 Screen for possible Plk4 substrates using Plk4 consensus phosphorylation motif

A library of 149 small Rho GTPase related proteins (71 GEFs, 65 GAPs, 8 Rho GTPases, and 5 Rho GTPase or GEF/GAP interacting proteins) was searched for the presence of the consensus Plk4 phosphorylation motif (19,20). The most complete protein sequences, including isoforms, were obtained for all 149 proteins from the NCBI protein database and examined manually. Initially, a screen for the core Plk4 phosphorylation motif was performed. Stringent criteria were applied, with only phenylalanine, tyrosine and tryptophan considered as large hydrophobic residues. A secondary assessment for the complete Plk4 phosphorylation motif was then performed.
2.12 Immunofluorescence

Cells were plated on glass cover slips and transiently transfected the following day. After 16h of transfection cells were fixed using 4% paraformaldehyde for 20 min, permeabilized using 0.5% NP40 for 20min, and blocked with 10% FBS for 1h. Next, the slides were stained with anti-FLAG and anti-GFP antibodies (1:800) for 30 min followed by respective fluorescent secondary antibodies and Hoechst (1:1000) for another 30min. Then the glass cover slips were mounted on glass slides with Immuno-Mount reagent (Thermo Scientific, 2860060) and kept at 4 degrees overnight in the dark. For actin rearrangement in siArhgef1 treated cells, the normal transfection and scratch-wound protocols were followed, except the cells were plated on the glass cover slips. At 15 min post scratch the cells were fixed and permeabilized as per protocol above. They were then stained with Phalloidin Alexa 488 and Hoechst for 1h and mounted on glass slides. Olympia Deconvolution fluorescence microscope with 20X objective was used to visualize the staining.

2.13 Co-Immunoprecipitation

After 16h of transient transfection, cells were lysed with TNTE lyses buffer (50mM Tris-HCl, pH7.5, 150mM NaCl, 5% TritonX-100, 1mM EDTA) supplemented with protease inhibitors (Roche, 11836145001) and 25mM NaF. The lysate was cleared by centrifugation at 13000rpm for 5 min. Beads were pre-washed 3 times with the lyses buffer. Lysate was divided equally for GFP or HA antibody and FLAG (anti-FLAG M2 beads, Sigma, F1804) pulldowns. Lysates were incubated at 4°C, while mixing, for 1h. Then G-Sepharose beads (GE Healthcare, 17061801) were added to the GFP or HA IP and all samples were incubated at 4°C, while mixing, for additional 30 min. IPs were then spun down at 1200rpm for 1 min and lysate removed. Beads were washed 3 times with wash buffer (TNTE, 150mM NaCl, 25mM NaF), transferred to a new Eppendorf and washed 2X with TNTE and 25mM NaF. Then, all wash buffer was removed, beads were resuspended in 80μL lyses buffer and 15μL of 5X Laemmli buffer was added. Samples were then boiled for 5 min at 96°C, followed by immunoblot.
2.14 In vitro kinase assays

HEK293T cells were lysed using TNTE lysis buffer with protease inhibitor cocktail, 5mM NaF and 2mM Na3VO4, extracts centrifuged at 13,000rpm for 5min, and supernatants immunoprecipitated with anti-FLAG M2 affinity beads (Sigma, F1804). The beads were washed five times with lysis buffer with 300mM NaCl. For FLAG-Arhgef1 or its derivatives the protein was eluted with 15µg 3XFLAG-Peptide (APExBIO, A6001) with gentle mixing for 30min.

FLAG-Plk4 was then incubated with 3µg Arhgef1 protein in 30µl kinase buffer (25mMTris-HCl pH7.5, 25mM MgCl2, 15mM sodium glycerolphosphate, 0.5mM Na3VO4, 2mM EDTA, 25mM NaF, 1mM DTT, 1.25µg BSA) containing 100µM ATP with 10µCi [γ-33P]ATP (PerkinElmer, NEG602H). Kinase reactions were performed at 30°C for 30 min and terminated by adding Laemmli sample buffer. Proteins were separated by SDS–PAGE, stained with Colloidal Blue Staining Kit (Invitrogen, LC6025), and dried using Bio-Rad gel dryer. Phosphorylation was visualized by autoradiography (Typhoon FLA9500, GE Healthcare).

2.15 Scratch-wound assays

The cells were treated as indicated and grown to 100% confluency. At that point they were scratched with a P10 pipette tip (3 scratches per well), washed with PBS and media with 1% FBS was added. Healing was then monitored for 8-16h using time lapse microscopy via an inverted microscope (DMIRE2; Leica Microsystems) with a motorized stage and live cell chamber. Images were captured every 1h using ORCA Hamamatsu camera with a 10X lens. Image analysis was carried out by Volocity 3D Image Analysis Software by measuring the total remaining wound area in 3 fields per condition.

2.16 Proliferation and viability assays

For each cell line, 1300 cells/well were seeded into 96-well plates. Cells were treated the same way as they would be up until the scratch in the scratch-wound assay and proliferation and viability were assessed at 8h after addition of low serum media. Cells were incubated at room temperature with Hoechst (1:800) and Propidium Iodide (PI, Invitrogen, P3566) (1:400, 1mg/ml stock) for 15 minutes. The plates were then imaged with the Celigo Cell Imaging Cytometer using the Blue (377/447nm) and Red (531/629nm) channels, respectively. Proliferation was
measured from brightfield images by determining the difference in the total surface area covered by the cells at 0h and 8h timepoints. Viability was measured as a difference in the percentage of PI positive cells at 0h and 8h timepoints.

2.17 Spreading assays

250,000 HeLa cells were plated per well of a 6 well plate. Transfections and siRNA treatment were performed according to the above protocols. Cells were then trypsinized and plated into 96 well plate, 2500 cells per well, 8 wells per treatment. The plate was left in the tissue culture fume hood for 30min and then transferred to the 37°C incubator for an additional 3h. The cells were then fixed and stained as per immunofluorescence protocol. Images were captured by the INCell Analyzer 2000. Columbus 2.3 (PerkinElmer) software was used to analyze the images and determine cell area and cell axial ratio (length/width), where 1 is a perfectly circular cell.

2.18 Morphology assays

A 48h siRNA transfection was performed according to the above protocol. 2500 HeLa cells were plated into each well of a 96-well plate (8 wells per treatment). For overexpression experiments 2500-3000 HeLa cells were plated per well of a 96 well plate. Cells were then transfected with 0.2μg/well of each plasmid for 16h at which point they were fixed and stained as per immunofluorescence protocol. Images were captured by INCell Analyzer 2000. Columbus 2.3 (PerkinElmer) software was used to analyze the images and determine cell area and cell axial ratio (length/width), where 1 is a perfectly circular cell.

2.19 Statistical analysis

Statistical analysis was done either by Student t-test (one sample student t-test for normalized pulldowns) or two-way ANOVA using Prism software (GraphPad Software, La Jolla, CA).
Chapter 3

3 Plk4 regulates Rho GTPase activation

The data presented in this chapter were published in part in:


3.1 Overview

Plk4 has been suggested to have both tumour suppressive and oncogenic functions. On the one hand, Plk4 heterozygous mice develop lung and/or soft tissue tumours, the majority of which are hepatocellular carcinomas (60), while in humans 50% of HCCs exhibited loss of heterozygosity at Plk4 locus (54), which correlates with poor survival (107). On the other hand, Plk4 overexpression has been reported in a wide variety of different human cancer types and predicts inferior survival in breast cancer (61) and neuroblastoma patients (588). However, more studies are needed to elucidate the role of Plk4 in tumorigenesis.

Plk4 is a serine/threonine kinase whose role in centriole duplication has been well established and extensively studied. Nevertheless, non-centriolar functions of Plk4 are beginning to emerge. Among them is its role in trophoblast differentiation (589), cytokinesis (54) and spindle assembly in acentriolar cells (92). In addition, our laboratory has recently shown that Plk4 regulates spreading, migration and invasion of MEFs and cancer cells in vitro and in vivo (101, 102). In mouse breast cancer xenograft model, Plk4 regulates tumour invasion into peritoneum and lung metastasis formation (102). Yet, the mechanisms of Plk4 regulation of cell migration and invasion are poorly understood. Here, I show that Plk4 regulates the expression of MMP-3 and -13 in MEFs, suggesting one possible mechanism of Plk4 effect on cell motility and migration. I also show that Plk4 regulates the amount of active Rac1 and RhoA in MEFs and HeLa cells. RhoA and Rac1 belong to the Rho GTPase family of proteins that are the main regulators of actin rearrangement, cell motility and invasion. Reduction of RhoA expression results in delayed directional cell migration as determined by a scratch-wound assay. In turn, this
phenotype is consistent with that of reduced Plk4 expression. These results suggest a mechanism for Plk4 regulation of cell migration and may further our understanding of the role of Plk4 in cancer progression.
3.2 Introduction

Polo-like kinase 4 (Plk4) is one of the five members of the Polo family of serine/threonine kinases. Plk4 is expressed from G1 to M phase of the cell cycle with the peak of expression occurring during the S phase (36). It plays an essential role in centriole duplication (40, 53) and is also required for mitotic progression (52). Illustrating the latter, Plk4+/- murine embryos exhibit a significant increase in the percent of cells “stuck” in late stages of mitosis, and arrest at E7.5 (52). Plk4+- mice, by contrast, are born at a normal Mendelian ratio and appear to have a grossly normal phenotype at birth and throughout the first 12-18 months of life. However, by 18-24 months, spontaneous liver, lung and/or soft tissue tumors were observed in 50% of Plk4 heterozygous mice (60). Of these, the majority were HCC. Further supporting the role of Plk4 in HCC is our lab’s finding that 50% of human HCC specimens show loss-of-heterozygosity at the Plk4 locus. When compared to the adjacent normal liver the HCC samples also exhibited reduced Plk4 expression at the mRNA level (54). Another group later showed that the low Plk4 expression correlates with poor prognosis in HCC patients (107). This evidence suggests that Plk4 may act as a haploinsufficient tumor suppressor in some contexts. However, numerous recent studies suggest an oncogenic role for Plk4. Plk4 is reported to be overexpressed in a variety of common human cancer types (61, 100, 114, 115, 119, 588) and predicts inferior survival in breast cancer (61) and neuroblastoma patients (588). High levels of Plk4 expression in triple negative breast cancer and neuroblastoma, both of which are notoriously difficult to treat with conventional therapies, make Plk4 an intriguing therapeutic target. One mechanism through which Plk4 may induce tumour formation is centrosome overduplication in cells overexpressing Plk4. Centrosome overduplication and aneuploidy have long been known as the hallmarks of cancer (590, 591) and are consistent with Plk4 overexpression phenotype (49, 58). However, it is still debated whether these phenotypes are the cause or the result of carcinogenesis (124, 125). A recent study by Levine et al. has shed some light on this matter. They demonstrated that even transient overexpression of Plk4 in mice, which leads to modest increase in centrosome number, can cause tumorigenesis (98). This provided direct evidence that Plk4 is an oncogene and that centrosome overduplication can cause tumour formation. However, this study did not completely exclude other effects of Plk4 overexpression that may contribute to its oncogenic potential.
Plk4\(^{+/-}\) MEFs spontaneously immortalize in culture by P9 and become tumorigenic by P15 (54). Specifically, high grade tumours with high mitotic index, indicative of rapid proliferation, were observed in 60% of the NOD-SCID mice that were subcutaneously injected with P15 Plk4\(^{+/-}\) MEFs. No tumours were formed by the injection of either Plk4 wild type or heterozygous P3 MEFs. We investigated the secondary genetic alterations associated with the tumorigenicity of P15 Plk4\(^{+/-}\) MEFs. P3 Plk4\(^{+/-}\), P3 Plk4\(^{+/-}\) and P15 Plk4\(^{+/-}\) MEFs were compared by genome-wide expression array (54). When differentially expressed genes were organized by biological function, increased cell proliferation and death and decreased cell motility in the P15 MEFs were predicted. An independent array analysis comparing tumorigenic (T) to nontumorigenic (NT) P3 Plk4\(^{+/-}\) MEFs showed a pattern of altered gene expression predictive of decreased motility in the T MEFs (54). Among the genes involved in motility that were shown to be significantly downregulated in T MEFs were MMP-3, and -13. To functionally validate the array, we performed transwell invasion assays through Matrigel of P3 Plk4\(^{+/-}\), P3 Plk4\(^{+/-}\) and P15 Plk4\(^{+/-}\) MEFs. As predicted, the invasion of the late passage Plk4\(^{+/-}\) MEFs was significantly impaired when compared to early passage cells of either genotype (101). The surprising finding was that even early passage Plk4\(^{+/-}\) MEFs were deficient in invasion compared to wild type MEFs. Additionally, overexpression of FLAG-Plk4 in HEK293T cells led to an increase in their migration through a porous membrane compared to FLAG transfected cells (101). Using real-time transwell invasion assays, our laboratory then showed that Plk4 enhances the migration and invasion of cancer cells (102). Finally, we recently showed that Plk4 also increases cell migration and invasion in vivo (102). When MDA-MB-231 cells depleted of Plk4 were subcutaneously injected into NOD-SCID mice, they formed tumours at a slower rate than controls and these tumours did not penetrate the peritoneum efficiently. Importantly, the incidence of lung metastasis was significantly lower in mice injected with the Plk4 depleted cells. In addition, we used scratch-wound assays, to examine the efficiency of actin rearrangement in Plk4\(^{+/-}\) and Plk4\(^{+/-}\) MEFs, as well as in HeLa cells (101, 102). Plk4\(^{+/-}\) MEFs or siPlk4 HeLa did not rearrange their actin filaments perpendicularly to the wound as efficiently as controls and this correlated with the delayed Plk4 localization to the cell protrusions in Plk4\(^{+/-}\) MEFs. Together, these findings suggest that Plk4 is required for efficient actin rearrangement following a stimulus for cell movement. However, the mechanisms by which Plk4 induces actin rearrangement, cell invasion and migration are poorly understood. In our laboratory we have
shown that one such mechanism is Plk4 interaction with and phosphorylation of the Arp2/3 complex (102). Plk4 localizes with the Arp2/3 complex in cell protrusions where this complex initiates the branched actin polymerization and lamellipodia protrusions formation. In turn, in a scratch-wound assay, in Plk4 depleted cells the Arp2/3 complex does not localize to cell protrusions, which is consistent with deficient actin rearrangement in these cells. However, this may not be the only mechanism by which Plk4 regulates cell migration.

Small Rho GTPases are the main regulators of actin rearrangement and cell motility (592). They cycle between an active GTP-bound state and an inactive GDP-bound state. GTP binding is facilitated by guanine nucleotide exchange factors (GEFs), and inactivation is enhanced by GTPase activating proteins (GAPs) (592). Increased expression and more often increased activity of Rho GTPases has been reported in many human malignancies (216), while the expression of their constitutively active variants in primary cells leads to cell transformation (372-374). Moreover, increased activity of Rho GTPases often correlates with increased metastasis formation and poor survival. RhoA, Rac1 and Cdc42 are the most studied members of the family of small Rho GTPases. One of their best studied functions is in regulation of actin and microtubule rearrangement as they pertain to cell motility (592). In the conventional model of cell motility, Rac1 and Cdc42 act at the leading edge of the cell, promoting formation of lamellipodial and filopodial protrusions, respectively, that help move the cell forward (184). RhoA, on the other hand, has been described as acting at the trailing edge of the cell, controlling cell contractility to effect retraction, also necessary for cellular movement (184). This simple paradigm has recently been challenged by reports showing that RhoA can also act at the leading edge of the cell to promote cell motility (329). Moreover, RhoA is the main driver of the amoeboid cell motility, a mode of motility often employed by tumour cells with high metastatic capacity (241). Aside from its role in cell motility, RhoA activation has been shown to be required for proper cleavage furrow positioning and ingestion (593). Plk4+/− MEFs exhibit a defect in cytokinesis, resulting in multinucleated daughter cells (54). This cytokinetic defect can be attributed, at least in part, to the failure of Plk4+/− MEFs to properly position the cleavage furrow (54). When G2/M synchronized Plk4+/+ and Plk4+/− MEFs were released from a nocodazole-induced block, activation of RhoA was impaired in Plk4+/− as compared to Plk4+/+ MEFs (54). A pulldown assay on unsynchronized MEFs showed that Plk4+/− MEFs had
significantly lower amount of GTP-bound (i.e. activated) RhoA than Plk4+/+ MEFs. In addition, when HEK293T cells were subjected to a similar pulldown assay, FLAG-Plk4 transfected cells showed increased GTP-bound RhoA compared to FLAG-alone transfected cells (54). Our laboratory has further shown that the effect of Plk4 on RhoA activation is through phosphorylation of a RhoA GEF Ect2 (54).

As our laboratory has shown that Plk4 regulates actin rearrangement, cell motility and RhoA activation, and since Rho GTPases, including RhoA, are the main drivers of actin rearrangement and cell motility, I hypothesized that Plk4 regulates cell motility by regulating the activation of Rho GTPases. The experiments described in this chapter were aimed at determining the role of Plk4 in the activity of Rho GTPases RhoA, Rac1 and Cdc42 in MEFs and cancer cells and the role of these Rho GTPases in cell migration as compared to Plk4.
3.3 Results

3.3.1 Lower expression of MMP-3 and MMP-13 in P15 Plk4+/− and in tumorigenic P3 Plk4+/− MEFs

Microarray that compared the gene expression between P3 Plk4+/+, P3 Plk4+/− and P15 Plk4+/− MEFs identified 659 differentially expressed genes, of which 61 genes involved in cellular movement were predicted to be decreased in P15 vs. P3 Plk4+/− MEFs (101). No significant differences in gene expression between P3 Plk4+/+ and Plk4+/− MEFs were found. In a different microarray that compared P3 Plk4+/− MEFs that went on to become tumorigenic (T) in vivo to those that did not (NT), 54 differentially expressed genes were identified (101). Of these, 17 genes are known to be involved in cellular movement and 9 of them were genes that normally increase cell motility but were downregulated in T MEFs. To validate the microarray results, MMP-3 and MMP-13 genes were chosen, as the microarrays predicted them to be significantly downregulated in both P15 Plk4+/− and P3 T MEFs. RNA from the same samples of 4 embryos/genotype that were utilized for the microarrays was used to compare the expression of MMP-3 and MMP-13 by real time RT-PCR. In agreement with the first microarray results, real time RT-PCR showed that the expression of MMP-3 and MMP-13 was lower in P15 Plk4+/− MEFs as compared to P3 Plk4+/− and P3 Plk4+/+ MEFs (Figure 3.1a). Furthermore, according to the results of this microarray, although there was a trend to lower expression of the MMP-3 and MMP-13 in the P3 Plk4+/− MEFs as compared to P3 Plk4+/+ MEFs, this difference was not statistically significant. However, upon examination of the expression of these MMPs by real time RT-PCR, it was determined that the expression of MMP-3 and MMP-13 is in fact significantly lower in early passage Plk4+/− MEFs as compared to early passage Plk4+/+ MEFs (Figure 3.1a). This result is consistent with the functional validation of this microarray. The second microarray was also validated by real time RT-PCR, where P3 tumorigenic MEFs were shown to have significantly lower expression of MMP-3 and MMP-13 as compared to the P3 non-tumorigenic MEFs (Figure 3.1b).

Since the lower expression of MMPs in P3 Plk4+/− as compared to P3 Plk4+/+ MEFs was detected by the real time RT-PCR but not by the microarray, the expression of MMP-3 and MMP-13 was further tested by real time RT-PCR in MEFs from embryos that were not part of the array (8 embryos per genotype). Again, decreased expression of MMP-3 and MMP-13 in P3 Plk4+/− as
compared to P3 Plk4<sup>+/−</sup> MEFs was observed (Figure 3.1c). However, although statistically significant, this decrease was modest (13% for MMP-3 and 24% for MMP-13). Therefore, the reduced MMP expression alone is not likely to fully account for the significant delay in migration and invasion of Plk4<sup>+/−</sup> MEFs. This suggested that Plk4 regulates other pathways to affect cell motility.

Rho GTPases are major regulators of actin rearrangement and cell motility, whose upstream regulation mostly occurs not at the mRNA or protein level, but at their activation level. Our laboratory has previously shown that transient Plk4 overexpression results in increased level of active RhoA (54). I therefore went on to further test the effect of Plk4 on activity of RhoA and two other major Rho GTPases Rac1 and Cdc42.
Figure 3.1

(a) Relative expression of MMP-3 and MMP-13 in P3 Plk4+/+, P3 Plk4+/-, and P15 Plk4+/- mice.

(b) Relative expression of MMP-3 and MMP-13 in NT and T conditions.

(c) Relative expression of MMP-3 and MMP-13 in P3 Plk4+/+ and P3 Plk4+/- conditions.
Figure 3.1. Plk4 regulates MMP-3 and MMP-13 expression levels in MEFs. a) Confirmation of expression array analysis that predicted decreased expression of MMP-3 (left panel) and MMP-13 (right panel) in Plk4+/+ vs. Plk4 +/- MEFs (4 embryos/genotype, n=2). b) Confirmation of expression array analysis that predicted decreased expression of MMP-3 (left panel) and MMP-13 (right panel) in tumorigenic (T) vs. non-tumorigenic (NT) P3 Plk4 +/- MEFs (2 embryos/genotype, n=3). c) Lower MMP-3 and MMP-13 mRNA expression in P3 Plk4 +/- vs. Plk4 +/- MEFs (8 embryos/genotype, n=3). For panels a) and c) the expression was normalized to that of P3 Plk4+/+ MEFs; for panel b) the expression was normalized to the NT MEFs using 2(-ΔΔCt) method. Data are mean + SEM, *p<0.05, **p<0.01, ***p<0.001.
3.3.2 Plk4 regulates active Rac1 levels

In order to determine whether Plk4 affects the activity level of Rac1, I performed active Rac1 pulldown assays on Plk4+/+ and Plk4+/− MEFs. The beads used in these pulldowns are coated with PAK protein binding domain (BPD). PAK is an effector of Rac1 that only binds the active or GTP-bound version of Rac1. The amount of Rac1 in pulldowns and total lysate were visualized by Western blot and quantified by densitometry analysis. For a more accurate comparison of the amount of active Rac1 I normalized it to total amount of Rac1 by creating the active Rac1/total Rac1 ratios for each sample. I compared Rac1 activity in 3 Plk4 wild type and 3 heterozygous embryos and observed a significant reduction in active Rac1 in Plk4+/− compared to Plk4+/+ MEFs (Figure 3.2). In order to test the effect of manipulation of Plk4 levels on Rac1 activation, I then performed a similar pulldown in Plk4+/+ MEFs treated with siPlk4 or siLuciferase as a negative control. Treatment with siPlk4 reduced Plk4 expression levels by about 80% (Figure 3.3a i) This depletion of Plk4 expression resulted in the significant reduction of active Rac1 in MEFs, as determined by the active Rac1 pulldowns (Figure 3.3a ii). Interestingly, siPlk4 treatment reduced active Rac1 levels by about 40%, which is comparable to the amount of active Rac1 in Plk4+/− MEFs. Since our laboratory has shown that Plk4 affects the motility of not only MEFs but also cancer cells (102), I wanted to determine whether Plk4 also affects Rac1 activation in cancer cells. For this, I performed active Rac1 pulldowns in HeLa cells transduced with and stably expressing shPlk4 or shLuciferase constructs. Plk4 expression in shPlk4 HeLa cells was reduced by about 75% (Figure 3.3b i), which is comparable to the 80% reduction seen in siPlk4 treated MEFs. Similar to MEFs, reduction of Plk4 mRNA levels leads to a significant decrease in active Rac1, while overexpression of Rac1 GEF P-Rex2 increases active Rac1 levels and is a positive control (Figure 3.3b ii). In order to test the effect of increased Plk4 levels on Rac1 activation, HEK293T cells were used, as MEFs have a very low transfection efficiency. Interestingly, although the level of Plk4 expression in FLAG-Plk4 transfected cells at the time of the pulldown increased substantially (Figure 3.4a), FLAG-Plk4 overexpression did not affect the active Rac1 levels (Figure 3.4b). Overall, these results suggest that Plk4 positively regulates Rac1 activation and this might be the mechanism through which Plk4 enhances cell migration and spreading.
Figure 3.2

a

P3 MEFs

b
Figure 3.2. Lower expression of Plk4 reduces the level of active Rac1 in P3 MEFs. 
a) Representative blot of active Rac1 pulldowns in P3 MEFs from one Plk4+/+ and one Plk4+- embryo, done in duplicates from different plates; images are from the same membrane and exposure. b) Quantification of active Rac1 in P3 MEFs that shows reduced active Rac1 levels in Plk4+- MEFs, normalized to Plk4+/+ (2 independent experiments, 3 embryos/genotype). Data are mean + SEM, *p<0.05.
**Figure 3.3**

a  
P3 WT MEFs

i)  
![Graph showing relative Plk4 expression in P3 WT MEFs with siLucif and siPlk4.](image)

ii)  
![Bar graph showing relative active Rac1 with siLucif and siPlk4.](image)

b  
HeLa

i)  
![Graph showing relative Plk4 expression in HeLa with shLucif and shPlk4.](image)

ii)  
![Bar graph showing relative active Rac1 with shLucif and shPlk4.](image)
Figure 3.3 Depletion of Plk4 expression reduces active Rac1 levels in MEFs and HeLa cells. 

a) i) Lower level of Plk4 mRNA expression prior to active Rac1 pulldown in siPlk4 treated P3 wild type (WT) MEFs, normalized to siLuciferase treated controls. ii) Representative blot of active Rac1 pulldown in P3 MEFs treated with siPlk4 or siLuciferase, done in duplicates from different plates (top panel) and quantification of 4 independent active Rac1 pulldown experiments that shows a decrease in active Rac1 levels as a result of siPlk4 treatment, normalized to siLuciferase treated controls (bottom panel). 

b) i) Lower level of Plk4 mRNA expression prior to active Rac1 pulldown in shPlk4 HeLa, normalized to shLuciferase treated controls. ii) Representative blot of active Rac1 pulldown in shPlk4 and shLuciferase HeLa cells (top panel) and quantification of 3 independent active Rac1 pulldown experiments that show decrease in active Rac1 level in shPlk4 cells, normalized to shLuciferase treated controls (bottom panel). P-Rex2 is a positive control as it is a Rac1 GEF (explained further in Chapter 4). Data are mean + SEM, *p≤0.05, **p≤0.01, ***p≤0.001.
Figure 3.4

a

Relative Active Rac1

b

Pulldown: PAK-PBD

Total Lysate
Figure 3.4 Plk4 overexpression does not alter active Rac1 levels in HEK293T cells a) Confirmation of overexpression of Plk4 in HEK293T cells transfected with FLAG or FLAG-Plk4. b) Representative blot of active Rac1 pulldown in HEK293T cells transiently transfected with FLAG-Plk4 or FLAG, done in duplicates from different plates (top panel) and quantification of 3 independent active Rac1 pulldown experiments that show no effect of Plk4 overexpression on active Rac1 levels, normalized to FLAG transfected controls (bottom panel). For each pulldown experiment images are from the same blot and exposure. Data are mean + SEM.
3.3.3 Plk4 may regulate active Cdc42 levels
To determine the level of active Cdc42 in Plk4+/+ vs. Plk4+/− MEFs, I performed the same pulldown as for Rac1, since active Cdc42 also binds PAK. It should be noted that these were separate experiments and not stripped and re-probed Rac1 blots. I performed the assay on MEFs from 3 wild type and 3 heterozygous embryos. Although there was a trend for an increased amount of active Cdc42 in Plk4+/− MEFs (Figure 3.5c), the amount of active Cdc42 was very inconsistent between the embryos of the same genotype (Figure 3.5b) and from day to day (Figure 3.5a), and therefore the difference in the active Cdc42 was not statistically significant. Hence, I did not pursue further experiments with Plk4 expression level manipulations.

3.3.4 Plk4 regulates active RhoA levels
Our laboratory had already demonstrated that transient Plk4 overexpression results in increased active RhoA levels (54). However, the 16-40-fold increase in Plk4 mRNA and protein levels in a transient system may not be physiologically relevant. Therefore, a tetracycline-inducible stable Plk4 overexpressing U2OS cells, where Plk4 expression increases only 2-8 fold upon tetracycline induction (Figure 3.6a i), were utilized for the active RhoA pulldown assays. The beads used in these pulldowns were coated in Rhotekin-RBD but otherwise the pulldowns were performed in the same way as the Rac1 pulldowns. Rhotekin is a RhoA effector that only binds active RhoA. The results showed no significant difference in the tetracycline treated vs. untreated cells. However, the results between the experiments done on different days were too inconsistent to draw any conclusion (Figure 3.6a ii).
To determine the effect of reduced Plk4 levels on active RhoA, HeLa cells stably expressing Plk4 shRNA or control Luciferase shRNA were utilised. Active RhoA pulldowns show that shPlk4 cells have significantly lower amount of active RhoA compared to the controls (Figure 3.6b). Taken together, these results suggest that Plk4 regulates active RhoA levels and may affect cell motility through RhoA pathway.
Figure 3.5

(a) P3 MEFs

<table>
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<tr>
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<th>22.2 Plk4+/+</th>
<th>22.3 Plk4+/+</th>
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<td>27kD</td>
<td>27kD</td>
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<tr>
<td>Total Cdc42</td>
<td>15kD</td>
<td>15kD</td>
</tr>
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</table>

Pulldown: PAK-PBD

(b) P3 MEFs

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<th>22.14 Plk4+/+</th>
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<tr>
<td>GTP-Cdc42</td>
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<td>27kD</td>
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<tr>
<td>Total Cdc42</td>
<td>15kD</td>
<td>15kD</td>
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Pulldown: PAK-PBD

(c) P3 MEFs

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<th>22.8 Plk4+/+</th>
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<tbody>
<tr>
<td>GTP-Cdc42</td>
<td>27kD</td>
<td>27kD</td>
</tr>
<tr>
<td>Total Cdc42</td>
<td>15kD</td>
<td>15kD</td>
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Pulldown: PAK-PBD

Relative Active Cdc42

<table>
<thead>
<tr>
<th></th>
<th>Plk4+/+</th>
<th>Plk4+/+</th>
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<td></td>
<td>2.0</td>
<td>1.5</td>
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Figure 3.5. Inconsistent levels of active Cdc42 in MEFs from different embryos. a) Immunoblots of active Cdc42 pulldown in MEFs (top panels) and their quantification (bottom panels) normalized to total Cdc42 in MEFs from embryos 22.2 Plk4+/+ and 22.3 Plk4+/-; single experiments done on two different days (Day 1-left panel and Day 2-right panel). b) Immunoblots of active Cdc42 pulldown in MEFs (top panels) and their quantification (bottom panels) normalized to total Cdc42 in MEFs from two Plk4+/+ and two Plk4+/- embryos, n=1 for each embryo. c) Summary of quantification of active Cdc42 in MEFs, normalized to Plk4+/+, where active/total ratio for Plk4+/- was taken as 1 (n= 3 embryos/genotype). Data are mean + SEM.
a  U2OS T-REx YFP-Plk4

i)

Figure 3.6

ii)

b  HeLa shRNA

Pulldown: Rhotekin-RBD
Figure 3.6 Depletion of Plk4 expression reduces active RhoA levels. a) i) Level of Plk4 mRNA expression prior to active Rac1 pulldown in U2OS T-REx YFP-Plk4 cells treated with tetracycline, normalised to untreated controls. ii) Quantification of active Rac1 pulldown experiments performed on 3 different days, normalized to untreated controls. b) Representative blot of active Rac1 pulldown in shPlk4 and shLuciferase, HeLa cells (left panel) and quantification of 5 independent active Rac1 pulldown experiments, normalized to shLuciferase controls (right panel). Data are mean + SEM, **p<0.01.
3.3.5 RhoA regulates directional cell migration in a manner consistent with Plk4

We have previously shown that Plk4 regulates cell migration during wound-healing, where decreasing Plk4 expression results in slower wound-healing and stable Plk4 overexpression results in enhanced wound-healing of MEFs and cancer cells. (101, 102) To confirm these findings, I performed wound-healing assays on siPlk4 vs. siLuciferase treated HeLa cells. Reduced Plk4 expression indeed resulted in reduced wound-healing (Figure 3.7a). Next, I utilised tetracycline induced vs. non-induced U2OS cells stably overexpressing Plk4 and confirmed significant enhancement in wound-healing in tetracycline induced cells at 4h post wounding (Figure 3.7b).

I then went on to examine the effect of overexpression of constitutively active mutants and depletion of Rho GTPases Rac1 and RhoA on wound-healing, with the aim to compare it to that of Plk4. In turn, this could help me determine which Rho GTPase may be more likely to mediate Plk4 effect on cell motility. I transiently overexpressed constitutively active (CA) FLAG-tagged Rac1 and GFP-tagged RhoA in HeLa cells and performed wound-healing assays. I did not observe a significant difference in wound healing for any of the Rho GTPases as compared to the respective controls (Figure 3.8). This could be, at least in part, due to the insufficient transfection efficiency for both Rac1 and RhoA (Figure 3.8 ai and bi). Since the reduction of the Plk4 expression levels by siRNA is more efficient and more consistent than transient transfection, I went on to test the effect of RhoA siRNA on wound healing. HeLa cells treated with siRhoA had impaired wound-healing ability compared to siLuciferase controls (Figure 3.9). This result is consistent with the effect of siPlk4 treatment on wound-healing and suggests that RhoA may mediate Plk4 effect on directional cell motility. However, it does not rule out Rac1 and Cdc42 as possible mediators.
Figure 3.7

a  HeLa

b  U2OS T-REx YFP-Plk4
Figure 3.7. Plk4 regulates directional cell migration in wound-healing assays. a) Representative time-lapse phase-contrast images of a wound healing assay in confluent HeLa cells treated with siPlk4 or siLuciferase (top) and quantification of 3 independent experiments (bottom) that demonstrates reduced wound healing in siPlk4 treated cells. b) Representative time-lapse phase-contrast images of a wound-healing assay in confluent U2OS T-REx YFP-Plk4 cells with and without the addition of tetracycline (top) and quantification of 4 independent experiments (bottom) that demonstrates enhanced wound healing in tetracycline induced cells. Data are mean ±SEM, *p<0.05, **p<0.01.
Figure 3.8

(a) i) FLAG vs. FLAG-CA-Rac1

ii) Graph showing % Wound Healed over Time Post Wound (h) for FLAG and FLAG-CA-Rac1.

(b) i) GFP vs. GFP-CA-RhoA

ii) Graph showing % Wound Healed over Time Post Wound (h) for GFP and GFP-CA-RhoA.
Figure 3.8. Transient overexpression of CA-Rac1 and CA-RhoA does not alter directional cell migration in wound-healing assays in HeLa cells. ai) Representative immunofluorescence images of HeLa cells transiently transfected with FLAG or FLAG-CA-Rac1 during spreading assay. ai) Representative time-lapse phase-contrast images of a wound healing assay in confluent HeLa cells transiently transfected with FLAG or FLAG-CA-Rac1 (left) and quantification of 5 independent experiments (right) that demonstrate no difference in wound healing in FLAG-CA-Rac1 and FLAG expressing cells. bi) Representative phase-contrast (left) and fluorescence (right) images of HeLa cells transiently transfected with GFP or GFP-CA-RhoA prior to a wound healing assay. bii) Representative time-lapse phase-contrast images of a wound healing assay in confluent live HeLa cells transiently transfected with GFP or GFP-CA-RhoA (left) and quantification of 5 independent experiments (right) that demonstrate no difference in wound healing in GFP-CA-RhoA and GFP expressing cells. Data are mean +SEM.
Figure 3.9

(a) Western blot images showing 
- siLucif
- siRhoA
- α RhoA
- α γ-Tubulin

(b) Images of wound healing over time:
- 0h
- 4h
- 6h
- 8h

Graph showing:
- % Wound Healed
- siLucif vs siRhoA
- Time Post Wound (h)

* indicates significant difference
Figure 3.9. Inhibition of RhoA reduces directional cell migration in wound-healing assays in U2OS cells. a) siRhoA treatment significantly reduces the amount of RhoA protein b) Representative time-lapse phase-contrast images of a wound healing assay in confluent U2OS cells treated with siRhoA or siLuciferase control (top) and quantification of 3 independent experiments (bottom) that demonstrate reduced wound healing in siRhoA as compared to siLuciferase treated cells. Data are mean ± SEM. *p<0.05.
3.3.6 Rho GTPases do not have a consensus Plk4 phosphorylation motif

In order to determine whether Plk4 can affect RhoA, Rac1 or Cdc42 activation by directly phosphorylating them, I looked for the Plk4 consensus phosphorylation motif (594) in the full amino acid sequences of the human versions of these Rho GTPases. The amino acid sequences were obtained from the NCBI. I started by looking for the core phosphorylation motif, which consists of serine/threonine residue, followed by the two large hydrophobic residues. As large hydrophobic residues I only considered phenylalanine, tyrosine and tryptophan. I searched for every possible combination of these amino acids in the Rho GTPase sequences using Microsoft Word “find” feature. I did not find the core phosphorylation motif in any of the three Rho GTPases. In addition, I also looked at 5 other small Rho GTPase family members (RhoD, RhoG, RhoH, RhoJ, Rnd3) and similarly did not find a core Plk4 phosphorylation motif in them. Since my criteria for the large hydrophobic residue were quite stringent, it is possible that I missed some candidate motifs. However, given these results I decided not to pursue this line of experiments any further.
3.4 Discussion

Plk4 is a serine/threonine kinase that has become known as the master regulator of centriole biogenesis and centrosome duplication. However, roles for Plk4 outside of the centrosome have been described by our laboratory and others. The first role for Plk4 outside the centrosome was reported by Martindill and colleagues. They show that Plk4 interacts with and phosphorylates the transcription factor Hand1 to affect trophoblast differentiation (589). Our laboratory has shown that Plk4 localizes to the protrusions of the motile and spreading cells and this localization is necessary for efficient actin rearrangement and protrusion formation (101). In addition, Plk4 regulates migration and invasion of MEFs and cancer cells (101, 102). The mechanisms of how Plk4 regulates these processes are poorly understood and require further investigation.

Microarray assays done in our laboratory predicted a role for Plk4 in cell motility, where P15 Plk4+/− and tumorigenic P3 Plk4+/− MEFs were predicted to have reduced cell motility (101). Three genes of the MMP family, MMP-3, -10, and -13, were shown to be downregulated in these cells. Here, I confirm the results of these microarrays for MMP-3 and MMP-13 genes by verifying their expression in the same samples used in microarrays by a more reliable method: real time RT-PCR. Moreover, I was able to show that the expression of MMP-3 and -13 is also lower in P3 Plk4+/− MEFs as compared to the P3 wild type controls and I confirmed this finding in 8 other embryos/genotype. This was consistent with the functional validation of the array reported by Rosario et al. which showed reduced cell spreading, migration and invasion in P3 Plk4+/− as compared to P3 Plk4+/+ MEFs (101). MMP-3 is a collagenase, capable of degrading triple-helical fibrillar collagen, a major part of bone, cartilage and ECM. MMP-13 belongs to the stromelysins family of MMPs and has a broad spectrum of ECM substrates, which excludes triple-helical fibrillar collagen. These MMPs have been shown to promote mesenchymal type of cell migration by cleaving the ECM and making a path for the migrating cell (595-598). Thus, Plk4 effect on MMP expression is a possible mechanism through which Plk4 may regulate cell migration. Further studies are needed to determine the mechanism of Plk4 effect on the expression of these MMPs. MMPs are usually secreted in a pro-enzyme, inactive form and need to be cleaved to become active. It would therefore be important to determine the effect of Plk4 on activity of these and possibly other MMPs. However, the reduction of MMP-3 and -13 expression, although significant, was only 13-24%. To me this suggested that regulation of MMP
expression by Plk4 may not be the main mechanism of Plk4 effect on cell migration. Moreover, our laboratory showed that Plk4 regulates actin rearrangement (101), while mounting evidence suggests that actin can regulate MMP expression (599). This further suggests that Plk4 effect on MMP expression may be an outcome of its effect on cell motility, and not the mechanism by which Plk4 regulates motility.

Plk4 heterozygous MEFs have a cytokinesis defect which was attributed to the improper localization of the cleavage furrow due to insufficient RhoA activity (54). RhoA and other members of the small Rho GTPases family are the major regulators of actin rearrangement and cell motility and invasion. Together with Plk4 effect on cell motility and invasion these data point at Plk4 regulation of the Rho GTPase activity as one of the possible mechanisms of regulation of cell motility and invasion.

Here I show that Plk4 regulates the active levels of Rac1 Rho GTPase in cycling MEFs and HeLa cells. Specifically, lower levels of Plk4 expression, whether in Plk4 heterozygous MEFs or siPlk4 treated cells, resulted in decreased active Rac1 levels. Interestingly, Plk4 overexpression in HEK293T cells did not affect active Rac1 levels. There can be several explanations for this. First, it is possible that the level of active Rac1 in control cells is near its peak already, since the experiments are performed on cells cultured in media with high (10%) serum is known to activate Rac1 (600). Second, RhoA and Rac1 are known to antagonize each other’s activity (338) and therefore, dramatic increase in active RhoA by Plk4 overexpression, observed by Rosario et al. (54), may lead to suppression of Rac1 activity. Finally, the effect of Plk4 on Rho GTPase activation can be cell type specific and whereas in HEK293T cells Plk4 overexpression results in increased active RhoA but not Rac1, this may not be true for other cell types. In support of this explanation, Godinho and colleagues showed an increase in active Rac1 levels in MCF10A breast cancer and two non-cancer, immortalized human cell lines (HaCaT, 16-HBE) stably overexpressing Plk4 upon tetracycline addition (601). Together with my results presented in this chapter, these data show that Plk4 regulates Rac1 activation in primary, normal and cancer cells. Although transient Rac1 overexpression did not alter the wound healing in HeLa cells, transient overexpression of dominant negative Rac1 mutant significantly decreased it (102), which is consistent with the decreased wound healing in Plk4 depleted cells. Hence, it is possible that Plk4 regulates cell invasion and migration through regulation of Rac1 activity. Additionally, it
was previously shown that Rac1 regulates the expression of MMPs, including MMP-3 (602). It is therefore plausible that the effect of Plk4 on MMP-3 and -13 expression may be mediated by its effect on Rac1 activity.

I was not able to determine the effect of Plk4 on Cdc42 activation in MEFs, due to the variation in active Cdc42 levels between the embryos of the same genotype. Using Plk4 depletion by siRNA or stable overexpression can prove more useful for the active Cdc42 pulldown experiments, due to the more consistent and more profound effect of these treatments on the Plk4 levels. Indeed, it was later shown by Kazazian et al. that depletion of Plk4 decreases active Cdc42 level while stable overexpression of Plk4 increases it in HeLa cells (102). Cdc42 is a known regulator of cell polarity, as its activity is necessary for MTOC and Golgi repositioning in front of the nucleus, towards the direction of migration (243). Our laboratory has also shown that Plk4+/− MEFs do not re-orient their Golgi towards the wound in a scratch-wound assay as efficiently as Plk4+/+ MEFs, further implicating Cdc42 as Plk4 effector (102). Cell polarization is an essential step in directional cell migration and defects in this process are known to result in impaired directional cell migration (535, 603, 604). Further experiments are required to elucidate whether Plk4 regulation of Cdc42 activity is the mechanism by which Plk4 regulates cell migration and invasion. For example, a scratch-wound assay in cells with induced Plk4 overexpression and treated with Cdc42 inhibitor could help answer that question.

Our laboratory has previously reported that Plk4+/− MEFs have lower amount of active RhoA as compared to Plk4+/+ cells upon release from nocodazole treatment (54). In addition, FLAG-Plk4 overexpression in HEK293T cells increases active RhoA levels (54). Here I show that Plk4 also regulates RhoA activity in cancer cells. Together these data identify Plk4 as a regulator of RhoA activity in primary and cancer cells. In addition, siRhoA treatment led to decreased wound-healing of U2OS cells, consistent with the siRNA Plk4 depletion, suggesting that Plk4 may affect cell migration by regulation of RhoA activity.

Mounting evidence suggests that RhoA and Rac1 are involved in a mutual inhibitory feedback loops during cell motility (338). In general, the activation of one of these Rho GTPases and their subsequent signaling to effectors results in activation of GAPs and inhibition of GEFs of the other Rho GTPase (338). This allows for spatial and temporal separation of the RhoA and Rac1
activities and raises the question of how Plk4 regulates Rac1 and RhoA activity in the same direction. During cell motility, RhoA and Rac1 activation is separated spatially with Rac1 active at the leading edge and active RhoA localized mostly at the trailing edge. Nevertheless, the activity of both Rho GTPases is necessary for efficient cell protrusion formation and rear retraction and thus for efficient cell motility (605). This also explains how Plk4 may affect cell migration in a similar fashion through activation of both RhoA and Rac1. However, our laboratory has shown that active Plk4 localizes to the cell protrusions and not to the cell rear during motility (101), raising the question of how it can activate RhoA. Recent studies have shown that active RhoA in fact also localizes to the lamellipodia, but its activation precedes Rac1 activation (332). This temporal segregation of Rac1 and RhoA activation is also controlled by the mutual negative feedback loops. However, active Plk4 is widely distributed throughout the protrusion for at least 90 minutes post induction of motility by scratch-wound (101), which suggests that it can activate both RhoA and Rac1 at this location. In addition, besides the negative feedback loop, a positive feedback loop between Rac1 and RhoA has also been suggested, but it may be either cell type and/or disease state specific (338). Simultaneous activation of RhoA and Rac1 has been reported previously. For example, in endothelial cells S1P treatment resulted in increased amount of both active RhoA and Rac1 (606), while in mouse pancreatic acini treatment with cholecystokinin not only resulted in the activation of both Rho GTPases but also induced their translocation from the cytosol to the membrane (607). Plk4 can be a downstream target of these or similar pathways. Some GEFs can also activate both Rac1 and RhoA. One of them is Vav2, which has been shown to activate RhoA, Rac1 and Cdc42 and resulted in enhanced migration of the CHO cells (608). We have previously shown that Plk4 regulates RhoA GEF Ect2 during cytokinesis (54), while Weeks et al. suggested a role for Ect2 in astrocytoma cell invasion and migration (238). It is therefore possible that Plk4 might regulate RhoA activity during cell migration through Ect2 and regulate other GEFs and/or GAPs to affect RhoA, Rac1 and Cdc42 activation. Plk4 is a serine/threonine kinase and both Rac1 and RhoA have been shown to be phosphorylated by other serine/threonine kinases and this phosphorylation often affects their activity (220, 609, 610). However, by examining their full amino acid sequences, I determined that RhoA, Rac1 and Cdc42 Rho GTPases do not have the Plk4 consensus phosphorylation motif. This diminishes but does not exclude the possibility that
Plk4 may phosphorylate these Rho GTPases, thereby directly affecting their activity. This can be a topic for future studies.

Overall, a delicate balance exists between the activity of Rho GTPases, which is maintained by the large array of upstream regulators, including but not limited to GEFs, GAPs, GDIs and membrane receptors. Since the amount and distribution of these regulators varies between cell types and with disease state, the balance is maintained at different thresholds of the Rho GTPase activity. Thus, alteration of this balance, such as by introduction of constitutively active or dominant negative mutants of Rho GTPases, may give different results in different cells and/or under different conditions. For example, there are numerous reports of manipulation of Rho GTPase activity having opposite effects on cell motility in different cell types, which complicates our understanding of their interactions (611-617). It is therefore significant that Plk4 affects cell motility and Rho GTPase activation in a similar fashion in different cell types.

Attempts at modelling the switch-like balance between the Rho GTPases have been made (618, 619), but so far they do not take into account important factors such as the ECM and cell-cell interactions. More studies to enhance our understanding of the Rho GTPase regulation and interactions are required to enable us to build better models. Determining the mechanism of Plk4 regulation of Rho GTPase activation will provide an important piece of this puzzle.

Taken together, these results demonstrate a role for Plk4 in regulation of Rho GTPase activity and suggest a possible mechanism for Plk4 regulation of cell motility. However, the mechanism of Plk4 regulation of these Rho GTPases remains to be elucidated and is the focus of the following chapter.
Chapter 4

4 Plk4 regulates cell migration through Arhgefl

4.1 Overview

Plk4 is a serine/threonine kinase whose role in centriole duplication and de novo biogenesis has been established (49, 58, 63, 71, 123). However, more and more functions for Plk4 outside the centrosome are beginning to emerge. Our laboratory and Pellman group have uncovered a role for Plk4 in regulation of migration and invasion in MEFs and cancer cells (101, 102, 601). Two mechanisms through which Plk4 regulates these processes have been suggested. One is the disruption of cell-cell junctions by activation of Rac1 through deregulation of microtubule dynamics caused by Plk4 overexpression (601). The other is regulation of actin cytoskeleton remodeling and protrusion formation by interaction of Plk4 with Arp2/3 complex and its phosphorylation (102). However, Plk4 also regulates activity of Rho GTPases RhoA, Rac1 and Cdc42, the major regulators of actin rearrangement and cell migration (54, 102). The mechanism of how Plk4 regulates the activation of these Rho GTPases in cell motility remains poorly understood. Rho GTPases are activated by GEFs and de-activated by GAPs. Our laboratory has shown that Plk4 interacts with and phosphorylates RhoA GEF Ect2 during cytokinesis, providing a clue for the possible mechanism (54). To elucidate the mechanism of Plk4 activation of Rho GTPases, I screened a library of 141 GEFs, GAPs and other Rho GTPase interactors for Plk4 consensus phosphorylation motif. Among them, I identified 12 potential Plk4 substrates, all of them GEFs. I further show that RhoA GEF Arhgefl and Rac1 GEF P-Rex2 localize to cell edges/protrusions, similar to Plk4 and Plk4 co-immunoprecipitates with these two GEFs. Moreover, I define the mechanism of Plk4 interaction with Arhgefl and show that Plk4 phosphorylates its L-DH/PH domain. Importantly, I found that efficient directional cell migration regulated by Plk4 is at least partially dependent on its interaction with Arhgefl. Based on my findings I propose a mechanism of how Plk4 interaction with Arhgefl may enhance cell migration. Positive regulation of cell migration by Plk4 contributes to tumour progression and metastasis formation (102). Thus, determining the downstream targets and pathways of Plk4 in cell migration will aid in optimization of patient selection for treatment with Plk4 inhibitors and possibly identify novel therapeutic targets.
4.2 Introduction

Our laboratory has previously shown that Plk4 regulates motility and invasion of MEFs and cancer cells (101, 102). Importantly, our in vitro observations held up in vivo, where Plk4 was shown to regulate breast cancer cell invasion into the peritoneum and subsequent metastasis to the lung (102). Kazazian et al. went on to propose a mechanism for the Plk4 effect on cell migration and invasion. They showed that Plk4 physically interacts with and phosphorylates Arp2/3 actin nucleating complex (102). This interaction and phosphorylation was necessary for the efficient actin rearrangement and in turn, efficient cell migration. However, this is likely not the only mechanism through which Plk4 regulates cell migration and invasion. For example, Godinho et al. have also reported a role for Plk4 in cell migration and invasion, which they attribute to the effect of Plk4 on centriole duplication (601). Plk4 is a master regulator of centriole duplication and its overexpression results in supernumerary centrosomes and multipolar spindle formation (49, 60), while its inhibition decreases the centrosome number (58, 62). Centrosomes are the major components of MTOC and play an important role in microtubule nucleation and polarization. Thus, Godinho et al. propose that increased Plk4 levels and centrosome number, both of which are often observed in cancer, lead to increased microtubule nucleation (601). Increased microtubule nucleation then leads to increased Rac1 activity by a yet unknown mechanism, thereby enhancing cell migration. In the previous chapter, I also show that Plk4 regulates Rho GTPase Rac1 activation (published in part in (5)). Moreover, previous research from our laboratory and my data presented in chapter 3 implicates Plk4 in regulation of activity of other Rho GTPases, namely RhoA and Cdc42 (5, 54). Until the studies presented in this chapter, the mechanism of Plk4 regulation of Rho GTPase activation, as it pertains to cell migration, has not been investigated.

Rho GTPases are among the main drivers of cell motility and invasion, through their well-established roles in actin rearrangement, cell polarity and gene expression. They cycle between an active GTP-bound state and an inactive GDP-bound state. GTP binding is facilitated by guanine nucleotide exchange factors (GEFs), and inactivation is enhanced by the GTPase activating proteins (GAPs) (592). Rho GTPases are activated at the cell membrane, where a lot of their upstream regulators and downstream effectors are also located (231). GDIs interact with inactive Rho GTPases and keep them in the cytoplasm, thus maintaining them inactive until the
activation signal is received. There are about 81 GEFs, 66 GAPs and 3 GDIs that have been described in mammals (442). This large variety of regulators is necessary for the precise regulation of Rho GTPase activation, as many of these regulators are Rho GTPase, cell type, signal, and localization specific. Not surprisingly, many of them have a reported function in cell motility (442). Many GEFs and GAPs are autoinhibited in unstimulated cells by the inhibitory loops within the protein, that facilitate the protein folding in a manner that obstructs their active site (218). Alternatively, the active site can also be inhibited by the homodimerization or oligomerization of the protein (620). When activating signal is detected, this autoinhibition is relieved by the interaction of the GEF/GAP with the upstream regulators and/or by the post-secondary modifications such as phosphorylation (620). Thus, kinases are often found to regulate the activity of GEFs and GAPs.

Our laboratory has shown that Plk4 interacts with and phosphorylates RhoA GEF Ect2 to affect proper cleavage furrow positioning and therefore completion of cytokinesis (54). In turn, Weeks and colleagues have shown that Ect2 aberrantly localizes to the cytoplasm and the leading edge of the migrating cells in astrocytoma, where it initiates a switch from mesenchymal-to-amoeboid mode of motility (238). Together, these results suggest that Plk4 may affect cell motility and invasion through the regulation of Rho GTPase regulators GEFs, GAPs and GDIs. Therefore, the goal of this chapter was to determine whether Plk4 may interact with and/or phosphorylate Rho GTPase regulators and if such candidates are identified, determine whether they mediate the Plk4 effect on cell migration.
4.3 Results

4.3.1 12 small Rho GTPase GEFs have the Plk4 consensus phosphorylation motif

A library of 141 small Rho GTPase related proteins (71 GEFs, 65 GAPs and 5 Rho GTPase or GEF/GAP interacting proteins) was searched for the presence of the consensus Plk4 phosphorylation motif (Figure 4.1a) (37, 594). The most complete protein sequences, including isoforms, were obtained for all 141 proteins from the NCBI protein database and examined manually. A screen for the core Plk4 phosphorylation motif, which consists of serine or threonine followed by two large hydrophobic residues, identified 67 candidates containing both GEFs and GAPs. Only phenylalanine, tyrosine and tryptophan were considered as large hydrophobic residues for this screen. A secondary assessment for the complete Plk4 phosphorylation motif identified 12 candidates (Figure 4.1b), all of which were GEFs. Stringent criteria for the large hydrophobic residues in the core Plk4 phosphorylation motif were applied in order to narrow the screen to only the most promising candidates, which is the reason why this screen did not identify Ect2, a GEF that was previously shown to be phosphorylated by Plk4. As such, some of the other candidates might have also been missed. Half of the 12 identified GEFs belong to the Dbl and the other half to the DOCK family. Of these, only the Rac1 GEF P-Rex2 is predicted to have two full Plk4 phosphorylation motifs. Three of the Dbl family GEFs are RhoA GEFs, two are Rac1 GEFs, and Trio can be a GEF for both RhoA and Rac1 (Figure 4.1b). DOCK2,3,5 are Rac1 specific GEFs, while DOCK6-8 activate both Rac1 and Cdc42. Interestingly, no Cdc42 specific DOCKs (DOCK9-11) had the Plk4 phosphorylation motif. There were no amino acids that are conserved in all 12 GEFs (Figure 4.1b). Considering a comparable amount of GEFs and GAPs was examined and on average GEFs were only moderately longer in sequence (1258aa) than GAPs (912aa), it is significant that the predicted Plk4 phosphorylation motif was only found in GEFs. Thus, I considered the possibility of the predicted Plk4 phosphorylation motifs occurring in the same domain or functional unit of these GEFs. The predicted location of the phosphorylation motif of the 12 GEFs was cross-referenced with their published domain structures. No pattern of localization of the predicted Plk4 phosphorylation motif was found in the Dbl family GEFs. In three DOCK family GEFs (DOCK-3,-7,-8) the predicted phosphorylation motif is located in the DHR2 domain. DHR2 domain is conserved between the DOCK family members and is responsible for the GEF activity of the
DOCKs. Thus, it is possible that the predicted phosphorylation site is located in a structurally conserved region. For DOCK2,-5,-6 the predicted Plk4 phosphorylation motif is located between the DHR1 and DHR2 domains. The amino acid sequence of predicted Plk4 phosphorylation motifs in the DOCK GEFs is not particularly conserved, save for the more frequent arginine at the -2 position, and more frequent tyrosine at the +1 position. Of note, none of the candidate DOCKs had tryptophan in their core motif. Overall, Plk4 phosphorylation motif did not seem to be preferentially located in any specific domain of either Dbl or DOCK family of GEFs. All candidate GEFs, besides ALS2, were reported to play a role in cell motility and therefore were considered candidates for Plk4 interaction that may mediate its effect on cell migration.
Figure 4.1

a  Plk4 consensus phosphorylation motif

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δ- charged
φ- large, hydrophobic
X- any

b

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Figure 4.1. GEFs with Plk4 consensus phosphorylation motifs. a) Plk4 consensus phosphorylation motif that was used to screen the proteins from the library. The core motif is highlighted in bold. b) GEFs that were determined to possess Plk4 phosphorylation motif; the exact amino acids in the motifs and their location in the protein are shown. The GEFs are sorted by those that activate RhoA and/or Rac1.
4.3.2 Arhgef1 and P-Rex2 GEFs localize to the cell edge similar to Plk4 and physically interact with Plk4

In order to narrow down which GEFs to focus on further, the localization of the GEFs was examined and compared to that of Plk4. HeLa cells were transiently transfected with a GFP (or V5 for P-Rex2)-tagged GEF together with FLAG-Plk4 and their localization was determined. Of the 7 GEFs examined, only RhoA GEF Arhgef1 and Rac1 GEF P-Rex2 localized to cell edges/protrusions together with Plk4 (Figure 4.2). Interestingly, after 16-18h of transfection, Plk4 localization to the cell edges/protrusions when cells were transfected with the FLAG-Plk4 construct alone, was rare. This suggested that interaction with the Arhgef1 and P-Rex2 GEFs may localize Plk4 to the cell edge. Therefore, these two GEFs were tested for physical interaction with Plk4 by co-immunoprecipitation (co-IP). HEK293T cells were transiently transfected with full length FLAG-Plk4 and either full length GFP-Arhgef1 or V5-Prex2. Both Arhgef1 and P-Rex2 co-IPd with Plk4 (Figure 4.3a,b). However, for P-Rex2 the respective negative controls also had a band in them, albeit weaker than the test band (Figure 4.3b), which suggested that the constructs also weakly interact with the beads themselves. Moreover, PLEKHG6 GEF that did not co-localize with Plk4 to cell edges/protrusions did not co-IP with Plk4 (Figure 4.3c). In the future, the DOCK GEFs that were not tested in the co-localization assays need to be tested for co-localization and co-immunoprecipitation. Taken together, these results suggest that Plk4 may affect cell migration through GEFs Arhgef1 and P-Rex2.
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<td>PLEKHG6</td>
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<td>Rgnef</td>
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</tr>
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<td>Trio</td>
<td>X</td>
</tr>
<tr>
<td>DOCK2</td>
<td>X</td>
</tr>
</tbody>
</table>

**Figure 4.2**

- **Panel a** shows a table listing GEF proteins and whether they localize to the cell edge.
- **Panel b** displays fluorescence images of various GEF proteins, including GFP-ALS2, GFP-DOCK2, GFP-Trio, GFP-PLEKHG6, and GFP-Rgnef, each co-expressed with different markers like FLAG-Plk4.
Figure 4.2. Arhgef1 and P-Rex2 accumulate at the cell edge together with Plk4. a) Summary of the GEF localization to the cell edge. b) Representative immunofluorescence images showing localization of each GEF that was tested and FLAG-Plk4 in co-transfected HeLa cells. Arrows indicate accumulation of the GEF and FLAG-Plk4. Bars: 50µm.
Figure 4.3

(a) FLAG-Plk4 + + - -
   FLAG - - + +
   GFP-Arhgef1 + - + -
   GFP - + - -

(b) FLAG-Plk4 + + - -
   FLAG - - + +
   V5-P-Rex2 + - + +
   V5 - + - -

(c) FLAG-Plk4 + - + +
   FLAG - + - -
   GFP-PLKHC6 + + - -
   GFP - - + -

Input IB: GFP
IP: FLAG IB: GFP
IP: GFP IB: FLAG
Input IB: FLAG

kDa 130 100
kDa 250 130 100
kDa 130 100
**Figure 4.3. Arhgef1 and P-Rex2 interact with Plk4.**  

a) Immunoblots from reciprocal co-immunoprecipitation showing GFP-Arhgef1 and FLAG-Plk4 interaction. The constructs were co-expressed in HEK293T cells and blots were stained with anti-GFP and anti-FLAG antibodies.  

b) Immunoblots from reciprocal co-immunoprecipitation showing V5-P-Rex2 and FLAG-Plk4 interaction. The constructs were co-expressed in HEK293T cells and the blots were stained with anti-V5 and anti-FLAG antibodies.  

c. Immunoblots from reciprocal co-immunoprecipitation showing no interaction between GFP-PLEKHG6 and FLAG-Plk4. The constructs were co-expressed in HEK293T cells. The blots were stained with anti-GFP, anti-V5 and anti-FLAG antibodies.
4.3.3 Arhgef1 but not P-Rex2 affects cell migration in a manner consistent with Plk4

To determine the effect of Arhgef1 and P-Rex2 on cell migration, scratch-wound assays were performed on HeLa cells that were treated with siArhgef1 or siP-Rex2 and compared to the siLuciferase controls. Three-day treatment with siArhgef1 significantly decreased the amount of Arhgef1 protein and resulted in delayed cell migration (Figure 4.4a). Similarly, three-day siP-Rex2 treatment significantly decreased P-Rex2 mRNA expression. However, somewhat surprisingly, depletion of P-Rex2 resulted in enhanced cell migration (Figure 4.4b). Transient overexpression of either GEF did not affect the rate of cell migration (Figure 4.5), which is consistent with the transient overexpression of Rac1 and RhoA (see chapter 3, section 3.8). Since the depletion of Plk4 with siRNA causes delayed wound-healing, it is unlikely that Plk4 acts through P-Rex2 to regulate cell migration, but it is possible that it does so through Arhgef1. Therefore, I focused on Arhgef1 for the rest of the experiments in this chapter.
Figure 4.4

(a) HeLa cells treated with siLucif or siArhgef1. Images show wound healing over time.

(b) HeLa cells treated with siLucif or siP-Rex2. Images show wound healing over time.

Graphs show the percentage of wound area healed over time, with significant differences indicated by stars. The y-tubulin and Arhgef1 Western blots are also included, showing expression levels.
Figure 4.4. The effect of siArhgef1 and siP-Rex2 on cell migration. a) Representative time-lapse phase-contrast images of a wound healing assay in confluent HeLa cells treated with siArhgef1 or siLuciferase (top) and quantification of 3 independent experiments (bottom left) that demonstrates reduced wound-healing in siArhgef1 treated cells. Immunoblot showing reduced Arhgef1 expression in siArhgef1 treated cells, stained with anti-Arhgef1 and anti-γ-tubulin antibodies (bottom right) b) Representative time-lapse phase-contrast images of a wound-healing assay in confluent HeLa cells treated with siP-Rex2 or siLuciferase (top) and quantification of 3 independent experiments (bottom left) that demonstrates enhanced wound healing in siP-Rex2 treated cells. Bottom right: real time RT-PCR results showing reduced P-Rex2 expression in siP-Rex2 treated cells, normalized to the expression in siLuciferase controls using 2^{-ΔΔCt} method. Data are mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.
Figure 4.5

**HeLa**

(a) 0h, 4h, 8h, 12h, 16h

GFP

GFP-Arhgef1

(b) 0h, 4h, 8h, 12h

V5

V5-P-Rex2

% Wound Area Healed

Time (h)

GFP

GFP-Arhgef1

% Wound Area Healed

Time (h)

V5

V5-P-Rex2

kDa 250

130

V5-P-Rex2
Figure 4.5. Transient overexpression of GFP-Arhgef1 or V5-P-Rex2 does not affect cell migration. a) Representative time-lapse phase-contrast images of a wound-healing assay in confluent HeLa cells transiently transfected with GFP-Arhgef1 or GFP (top) and quantification of 5 independent experiments (bottom left) that demonstrates no change in wound healing in GFP-Arhgef1 expressing cells. Immunoblot showing increase in GFP-Arhgef1 expression, stained with anti-GFP antibody (bottom right) b) Representative time-lapse phase-contrast images of a wound-healing assay in confluent HeLa cells transiently transfected with V5-P-Rex2 or V5 (top) and quantification of 3 independent experiments (bottom left) that demonstrates no change in wound healing in V5-P-Rex2 expressing cells. Immunoblot showing efficient transfection with V5-P-Rex2, stained with anti-V5 antibody (bottom right). Data are mean ±SEM.
4.3.4 Plk4 physically interacts with the DH/PH domain and phosphorylates L-DH/PH domain of Arhgef1

Full length Arhgef1 consists of an N-terminal RGS homology (RH) domain that interacts with the G12 class of heterotrimeric G proteins and acts as their GAP (514), and canonical Dbl homology (DH) and Pleckstrin homology (PH) domains that are responsible for the Rho GTPase binding and activation (452). Located between the RGS and DH/PH domains is an autoinhibitory loop (L), thought to be unfolded and relieved by the Gα12G13 interaction (452, 527). To further elucidate the nature of the Plk4 interaction with Arhgef1, deletion constructs containing RH domain, DH/PH domains with and without the autoinhibitory loop, as well as the C terminal (C) fragment of Arhgef1 were created (Figure 4.6a). Co-immunoprecipitation experiments performed using HEK293T cells co-transfected with these HA-tagged Arhgef1 fragments and full length FLAG-Plk4 showed that Plk4 interacts with the DH/PH domain of Arhgef1, irrespective of presence or absence of the autoinhibitory loop (Figure 4.6b,c). Furthermore, full length GFP-Arhgef1 interacts with the kinase domain and polo-box 1-2 domain of Plk4, but not its polo-box 3 domain (Figure 4.7). In an in vitro kinase assay kinase domain (KDwt) of Plk4 phosphorylated full length purified FLAG-Arhgef1 (Figure 4.8a). As expected, kinase dead kinase domain (KDK41M) of Plk4 did not autophosphorylate. However, some phosphorylation of the Arhgef1 in the KDK41M lane was observed. This may be due to some endogenous Plk4 that may have come down with the Plk4K41M in the IP, as endogenous Plk4 and KDK41M have been shown to interact previously (130). The predicted Plk4 phosphorylation motif lies within the DH domain of Arhgef1 (residues 471-477, S473); to determine whether Plk4 indeed phosphorylates Arhgef1 at the DH domain, in vitro kinase assays were carried out with the Arhgef1 deletion constructs used for co-IPs. Interestingly, Plk4 phosphorylated the L-DH/PH domain construct, but not the DH/PH domain construct (Figure 4.8b). When the sequence of the linker region was examined for the Plk4 core phosphorylation motif with less stringent criteria, where all hydrophobic amino acids (A,I,L,M,F,W,Y,V) were considered for +1 and +2 positions, two core motifs were found. One of them has a proline at the +4 residue but no charged residue at the -2 position, making an incomplete Plk4 phosphorylation motif. In the future it will be interesting to mutate this motif and the predicted phosphorylation motif in the DH domain to determine whether Plk4 phosphorylates them.
Figure 4.6

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(b)

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Input

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IB: FLAG

IP: HA

IB: HA

(c)

Arhgef1

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Plk4

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|   |   | C   |     |     |     |       |

Input

IP: FLAG

IB: HA

IP: FLAG

IB: FLAG

Input

IB: Flag
Figure 4.6. Plk4 interacts with the DH/PH domains of Arhgef1. a) Schematic representation of the full length Arhgef1 protein and truncation fragments that were used for the co-IP; included amino acids are listed on the right  b) Immunoblots showing reciprocal co-immunoprecipitation of Plk4 with the DH/PH and L-DH/PH fragments of Arhgef1, after co-expression of each HA-tagged fragment with full length FLAG-Plk4 in HEK293T cells. c) Summary of Plk4 interaction with full length Arhgef1 and its fragments; number of pluses represents the strength of the interaction.
Figure 4.7. Arhgef1 interacts with the polo-box1-2 and kinase domains of Plk4. a)
Schematic representation of the full length Plk4 protein and truncation mutants that were used for the co-IP; included amino acids are listed on the right b) Immunoblots showing reciprocal co-immunoprecipitation of Arhgef1 with the polo-box1-2 (PB1-2) and kinase domain (KD) fragments of Plk4, after co-expression of each FLAG-tagged Plk4 fragment with full length GFP-Arhgef1 in HEK293T cells. c) Summary of Arhgef1 interaction with full length Plk4 and its fragments; number of pluses represents the strength of the interaction.
Figure 4.8. Plk4 phosphorylates Arhgef1. a) FLAG-tagged wild type Plk4 kinase domain (KDwt) purified from HEK293T cells phosphorylates purified full-length FLAG-Arhgef1 to a greater extent than kinase dead KD mutant (KD^{K41M}) b) Purified full length FLAG-Plk4 phosphorylates the purified L-DH/PH fragment of Arhgef1.
4.3.5 Plk4 activates Arhgef1
To further elucidate the nature of Plk4 interaction with Arhgef1, it was important to determine whether Plk4 can activate its GEF activity. To test this, a GEF pulldown assay was performed. Since Arhgef1 is a RhoA GEF, beads coated with the nucleotide-free version of RhoA (RhoG17A) were used to pull down the active Arhgef1. Nucleotide free RhoA is unstable and therefore has a very high affinity for GEFs, nevertheless only the active GEFs can bind it. The amount of active Arhgef1 was then normalized to the total amount of Arhgef1 in the lysate. Using this assay, I show that cells induced by tetracycline to overexpress YFP-Plk4 have more active Arhgef1 compared to non-induced controls (Figure 4.9). Whether this activation is a result of Plk4 phosphorylation of Arhgef1 remains to be determined.

4.3.6 Arhgef1 activates Rac1
Since RhoA and Rac1 often antagonize each other, I wanted to determine the effect of Arhgef1 on Rac1 activity. Pulldown assays for active Rac1 were performed in siArhgef1 treated U2OS cells. Surprisingly, depletion of Arhgef1 resulted in lower amount of active Rac1. To test for the effect of Arhgef1 overexpression on Rac1 activity, U2OS cells were transiently transfected with GFP-Arhgef1 or GFP alone for 16h and the active Rac1 pulldown was performed. GFP-Arhgef1 overexpressing cells had more active Rac1 as compared to controls. Considering Plk4 also activates RhoA and Rac1, it is possible that it activates both through Arhgef1.
Figure 4.9

U2OS T-REx YFP-Plk4

a

b
Figure 4.9 Plk4 activates Arhgef1 towards RhoA. a) Representative blot of active Arhgef1 pulldown in U2OS T-REx YFP-Plk4 cells, showing increased amount of active Arhgef1 bound to nucleotide free RhoA in cells induced by tetracycline (0.01µg/mL) to overexpress YFP-Plk4. Blots were stained with the Arhgef1 antibody b) Quantification of 4 independent active Arhgef1 pulldown experiments, normalized to no tetracycline controls. Data are mean + SEM, ***p<0.001.
Figure 4.10

U2OS

a

\begin{figure}
\centering
\includegraphics[width=\textwidth]{a.png}
\end{figure}

b

\begin{figure}
\centering
\includegraphics[width=\textwidth]{b.png}
\end{figure}
**Figure 4.10 Arhgef1 positively regulates active Rac1 levels in U2OS cells**

a) Left: representative blot of active Rac1 pulldown in siArhgef1 treated cells. Right: quantification of 2 independent active Rac1 pulldown experiments that show lower amount of active Rac1 in Arhgef1 depleted cells, normalized to siLuciferase controls (bottom panel).

b) Left: representative blot of active Rac1 pulldown in cells transiently transfected with GFP-Arhgef1 or GFP. Right: quantification of 4 independent active Rac1 pulldown experiments that show increased active Rac1 levels in Arhgef1 overexpressing cells, normalized to GFP transfected controls. Data are mean + SEM. *p<0.05.
4.3.7 Plk4 functionally interacts with Arhgef1 to affect cell motility

Our laboratory has previously shown that tetracycline induction of Plk4 expression enhances wound healing in stably transfected U2OS cells (102), while Plk4 depletion reduces wound healing. Here I show that depletion of Arhgef1 by siRNA decreases wound healing in U2OS cells, without affecting their proliferation or viability (Figure 4.11). In addition, when each of the 4 constructs comprising siArhgef1 pool were tested separately, all but one construct significantly reduced cell motility and none significantly affected cell viability or proliferation (Figure 4.12). To determine whether Plk4 acts through Arhgef1 to promote cell migration, wound healing by U2OS cells stably overexpressing YFP-Plk4 and depleted of Arhgef1 by siRNApool was examined. As expected, tetracycline induction of Plk4 expression resulted in enhanced migration, while Arhgef1 depletion suppressed migration (Figure 4.13a). Importantly, overexpression of Plk4 failed to stimulate wound healing in Arhgef1-depleted cells (Figure 4.13a). Although depletion of Arhgef1 in uninduced cells did not affect Plk4 expression, the Plk4 expression in tetracycline induced cells depleted of Arhgef1 was lower than that of tetracycline induces cells (Figure 4.13b), which raised the possibility that this might account for the reduced motility of these cells. To test this, the effect of each of 4 constructs in the siArhgef1pool on Plk4 expression was examined in YFP-Plk4 overexpressing cell. All 4 constructs reduced Arhgef1 expression but surprisingly, none of the constructs decreased the Plk4 expression, while two constructs increased it (Figure 4.14a). It is possible that it is only the combination of all or certain constructs in the pool that results in the decrease of Plk4 expression. One of the constructs that I named siArhgef1-2, significantly decreased the Arhgef1 expression and cell migration, without significantly altering the Plk4 expression. It was therefore chosen for repeat of the functional interaction scratch-wound assay. As with the siArhgef1pool, siArhgef1-2 reduced U2OS cell migration and this could not be rescued by the tetracycline induced YFP-Plk4 overexpression (Figure 4.14b). Moreover, siArhgef1-2 treatment resulted in delay of protrusion formation towards the wound at 15 min post scratch (Figure 4.14c), which is consistent with our group’s previous results that showed siPlk4 delays actin rearrangement in a scratch-wound assay (102). This suggests that Plk4 may act through Arhgef1 to regulate efficient actin rearrangement and directional cell migration. Taken together, these results show that Arhgef1 is required for Plk4-dependent regulation of cell migration and for efficient actin rearrangement.
When transiently overexpressed in U2OS cells, Arhgef1 does not localize to the centrosomes (Figure 4.14d), as Plk4 does. This suggests that the interaction between Plk4 and Arhgef1 and subsequent phosphorylation and activation of Arhgef1 by Plk4 is more likely to occur at the cell edge/protrusions where they co-localize (Figure 4.2).
Figure 4.11

U2OS

(a) Time course images of siLucif and siArhgef1 treated U2OS cells at 0h, 2h, 4h, 6h, and 8h.

(b) Quantification of wound area healed and dead cells increase.

- % Wound Area Healed
- % Dead Cells Increase at 8h

Graph showing % Area Increase at 18h and % Dead Cells Increase at 8h for siLucif and siArhgef1.
Figure 4.11. Arhgef1 depletion reduces U2OS cell migration. a) Representative time-lapse phase-contrast images of a wound healing assay in confluent U2OS cells treated with siArhgef1 or siLuciferase (top) and quantification of 4 independent experiments (bottom left) that demonstrates reduced wound healing in siArhgef1 treated cells. Immunoblot showing reduced Arhgef1 expression in siArhgef1 treated cells (bottom right); 4 different constructs of siArhgef1 were used individually (see Figure 4.12) as well as a pool of all four which was used for the experiments described in this figure. Data are mean ±SEM, *p<0.05, **p<0.01. b) Quantification of 5 independent proliferation (left) and viability (right) experiments that demonstrates no effect of siArhgef1 treatment on cell proliferation or viability at 8h. Proliferation was measured by the change in area covered by the cells at 8h compared to 0h, using Celigo Cell Imaging Cytometer automated analyzer. Dead cells were stained with propidium iodide and viability was measured by calculation of the increase in dead cell number, at 8h compared to 0h using automated Celigo Cell Imaging Cytometer software. Data are mean ±SEM.
**Figure 4.12**

**U2OS**

(a) 

- **siLucif**
- **siArhgef1-1**
- **siArhgef1-2**
- **siArhgef1-3**
- **siArhgef1-4**

**% Area Increase**

- **siLucif**
- **siArhgef1-1**
- **siArhgef1-2**
- **siArhgef1-3**
- **siArhgef1-4**

**% Dead Cells Increase**

- **siLucif**
- **siArhgef1-1**
- **siArhgef1-2**
- **siArhgef1-3**
- **siArhgef1-4**

**Time (h)**

- **0h**
- **4h**
- **8h**
- **12h**

**% Wound Area Healed**

- **siLucif**
- **siArhgef1-1**
- **siArhgef1-2**
- **siArhgef1-3**
- **siArhgef1-4**

**α Arhgef1**

**α γ-Tubulin**
Figure 4.12. Individual siArhgef1 constructs reduce U2OS cell migration. a) Representative time-lapse phase-contrast images of a wound healing assay in confluent U2OS cells treated with four siArhgef1 constructs or siLuciferase (top) and quantification of 4 independent experiments (bottom left) that demonstrates reduced wound healing in cells treated with siArhgef1-1, -2, and -3. Immunoblot showing reduced Arhgef1 expression in siArhgef1-1, -2, -3, and -4 treated cells (bottom right). Data are mean ±SEM. b) Quantification of 5 independent proliferation (left) and viability (right) experiments that demonstrates no effect of siArhgef1-1, -2, -3 and -4 treatment on cell proliferation, no effect of siArhgef1-2, and -4 on viability, and siArhgef1-1, -3 enhancing cell viability at 8h. Proliferation was measured by the change in area covered by the cells at 8h compared to 0h, using Celigo Cell Imaging Cytometer automated analyzer. Dead cells were stained with propidium iodide and viability was measured by calculation of the increase in dead cell number, at 8h compared to 0h using automated Celigo Cell Imaging Cytometer software. Data are mean ±SEM. *p<0.05, **p<0.01, ***p<0.001.
Figure 4.13

a

U2OS T-REx YFP-Plk4

tet 0h 4h 8h 12h

siLucif

siArhgef1 pool

b

Relative Plk4 Expression

α Arhgef1
α γ-Tubulin
Figure 4.13. Plk4 may regulate cell motility through Arhgef1. a) Representative time-lapse phase-contrast images of a wound healing assay in confluent U2OS T-REx YFP-Plk4 cells treated with siArhgef1_{pool} or siLuciferase (top) and quantification of 5 independent experiments (bottom) that demonstrates reduced wound healing in siArhgef1_{pool} treated cells that cannot be rescued by Plk4 overexpression (0.01µg/mL tetracycline). b) Immunoblot showing reduced Arhgef1 expression in siArhgef1_{pool} treated cells (left). Real time RT-PCR results, representing 2 independent experiments, showing enhanced Plk4 expression in tetracycline induced cells (0.01µg/mL), normalized to the expression in siLuciferase treated cells using 2^{-ΔΔCt} method (right). The expression of Plk4 was induced not as efficiently in siArhgef1_{pool} as compared to siLuciferase treated cells. Data are mean +SEM. **p<0.01, ***p<0.001.
Figure 4.14

a) U2OS TREx YFP-Pik4

b) Tet 0h 4h 8h 12h

<table>
<thead>
<tr>
<th>siLucif</th>
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% Wound Area Healed

Time (h)

Relative Pik4 Expression

Relative Arhgef1 Expression

15 min post wound
Figure 4.14. Plk4 regulates cell motility through Arhgef1. a) Real time RT-PCR results showing reduced Arhgef1 expression in siArhgef1-1,-2,-3, and-4 treated cells (left, n=3) and Plk4 expression in tetracycline (0.01µg/mL) induced cells (right, n=5) all normalized to the expression in siLuciferase treated, tetracycline induced cells using 2^-ΔΔCt method. Data are mean +SEM. *p<0.01, **p<0.001. b) Representative time-lapse phase-contrast images of a wound healing assay in confluent U2OS T-Rex YFP-Plk4 cells treated with siArhgef1-2 or siLuciferase (left) and quantification of 3 independent experiments (top right) that demonstrates that reduced wound healing in siArhgef1-2 treated cells cannot be rescued by Plk4 overexpression. Real time RT-PCR results, representing 2 independent experiments, showing enhanced Plk4 expression in tetracycline induced cells and reduced Arhgef1 expression in siArhgef1-2 treated cells, normalized to the expression of siLuciferase cells using 2^-ΔΔCt method (bottom right). Data are mean ±SEM. *p<0.05, **p<0.01, ***p<0.001. c) Representative immunofluorescence images of HeLa cells showing markedly suppressed protrusion formation after scratch wound in siArhgef1-2 treated cells (direction of the wound indicated by white dashed line) at 15min post wound. Protrusions visualised by actin staining (green). Bar: 50µm d) GFP-Arhgef1 (green) does not localize to the centrosome when co-transfected with FLAG-Plk4 (red, labelled with FLAG antibody) in HeLa cells (blue is hoechst staining). White arrows represent the localization on the centrosomes. Bars: 50µm, inset: 25µm.
4.4 Discussion

To date, two mechanisms for Plk4 regulation of cell migration and invasion have been proposed. Pellman group has attributed this effect to supernumerary centrosomes formation which they induced by Plk4 overexpression or by blocking cytokinesis (601). In particular, they show that supernumerary centrosomes that result from Plk4 overexpression increase microtubule nucleation, which activates Rac1 by a yet unknown mechanism. In turn, active Rac1 decreases cell-cell adhesions, rendering the cells more motile and invasive. However, our group has shown that even though Plk4 heterozygous MEFs have increased number of centrosomes, they still migrate and invade slower than wild type MEFs (54, 101). Moreover, when Plk4 levels were manipulated in other cell types, the rate of motility did not always change proportionately to the centrosome number, but always mirrored the Plk4 levels (102). Godinho and colleagues also argue that it is only the centrosome number that affects cell migration, since overexpression of the Plk4\(^{1-608}\) truncation fragment which comprises the Plk4 kinase domain and does not cause centrosome amplification, did not alter cell migration or invasion (601). However, this truncation mutant is missing Plk4 polo-box1-2 and polo-box3 domains, which are essential for proper Plk4 localization and protein interaction. Therefore, it is likely that overexpression of the Plk4\(^{1-608}\) truncation fragment does not fully capture all aspects of full length Plk4 functionality. Taken together, this suggested that centrosome number regulation is not the sole mechanism by which Plk4 affects cell migration. Indeed, our laboratory has shown that another mechanism of Plk4 effect on cell migration is through regulation of actin rearrangement by interaction with and phosphorylation of Arp2, a component of the Arp2/3 actin nucleating complex (102). Interestingly, Arp2/3 is regulated and activated by the WASP family of proteins (WASP, N-WASP, Scar/WAVE) which themselves are regulated by the active Rho GTPases Rac1 and Cdc42. Although we have shown that Plk4 regulation of the Arp2/3 complex can be independent of the Rac1 and Cdc42 activity (102), it did not exclude the possibility of Plk4 regulation of Rac1 and Cdc42 activity as a mechanism for regulation of motility and invasion by Plk4. Moreover, while Arp2/3 inhibition by small molecule inhibitor CK-666 resulted in inhibition of migration in MEFs (621), it did not affect the migration of A2780 human ovarian carcinoma cells (622). This suggests that not all cells require the Arp2/3 complex for migration and other mechanisms are at play. Our group and my own work have shown that Plk4 affects the activity of RhoA Rho GTPase ((54) and chapter 1), which like Rac1 and Cdc42 plays a prominent role in
cell motility. The possibility of Plk4 effect on cell motility through regulation of RhoA activity has not been explored until the research described in this thesis. Our lab has also shown previously that Plk4 phosphorylates RhoA GEF Ect2 to affect proper cleavage furrow positioning during cytokinesis (54), further implicating Plk4 in Rho GTPase activity regulation. It also suggested that regulation of upstream regulators of Rho GTPases (GEFs, GAPs, GDIs) may be a more general mechanism of Plk4 regulation of Rho GTPase activity. To explore this possibility, I examined a library of 141 Rho GTPase regulators for Plk4 consensus phosphorylation motif and identified 12 GEFs that possess it. Since stringent criteria for core Plk4 phosphorylation motif was applied, some potential interactors might have been missed, exemplified by the fact that my screen did not identify Ect2 which does have the Plk4 phosphorylation motif. All of these GEFs besides ALS2 were shown to regulate cell migration and thus all have the potential to functionally interact with Plk4. A brief summary of the effect of these GEFs on cell migration is provided in Table 1.2, chapter 1.

To my knowledge this is the first study to utilize Plk4 phosphorylation motif to screen for its potential targets and it shows that this is a viable strategy provided a list of likely candidates can be compiled first. A similar strategy, combined with analysis of polo-box mediated interactions has previously been applied successfully to predict Plk1 targets at the centrosome (623). The candidates identified by this approach should be fairly specific to Plk4, as its consensus phosphorylation motif is different from that of Plk1 (594). However, this does not exclude the possibility of phosphorylation by other Plk family members. For example, Ect2 GEF that is phosphorylated by Plk4 is also a target of Plk1 (624). Moreover, one of the potential Plk4 substrates PLEKHG6 identified in my screen is phosphorylated by Plk1 but at a different residue than the one predicted for Plk4 (475). This phosphorylation leads to PLEKHG6 activation and localization to the central spindle. Given that Plk4 also localizes to the central spindle during cytokinesis, it is possible that it may interact with PLEKHG6 in a similar manner to Plk1.

Of the identified GEFs, Arhgef1 and P-Rex2 localize to the cell edge or protrusions together with Plk4. In motility, Rho GTPases are usually activated at the cell membrane and GEFs also often localize to the cell membrane, proximal to their targets. Consistent with its role as a RhoA GEF, Arhgef1 has been shown to localize not only to the protrusions of neutrophils stimulated to
move, but also to their trailing edge (471). In embryonic neural crest cells and in glioblastoma cell lines, Arhgef1 localized to lamellipodia and perinuclear region (535). Thus, while precise Arhgef1 localization may depend on the cell type or the signal, its localization to cell protrusions is well documented. Precise P-Rex2 subcellular localization during cell motility is not yet described. However, cell fractionation experiments show that P-Rex2 activation leads to its translocation to the cell membrane, where it activates Rac1 (460). In addition, P-Rex2 overexpression in endothelial cells results in Rac1-like lamellipodia formation (561). Thus, localization of Arhgef1 and P-Rex2 to the cell edge/protrusions observed in HeLa cells is consistent with previous observations. As the master-regulator of centriole duplication, Plk4 major localization site is at the centrosomes (41, 49, 58), although it also localizes to the cleavage furrow during cytokinesis (54). We have previously shown that active Plk4 also localizes to the protrusions of motile and spreading cells (101, 102). It was therefore curious that when transiently transfected into HeLa cells FLAG-Plk4 rarely localized to the cell edge or protrusions unless Arhgef1 or P-Rex2 were also present. One explanation for this is that these cells were not induced to move or spread and this may be necessary for Plk4 localization to protrusions. The mechanism of Plk4 localization to cell protrusions is still unknown. Thus, it is possible that these GEFs localize Plk4 to this location but further studies are needed to test this. It should be acknowledged however, that 5 of the identified GEFs have not been tested for co-localization or physical interaction with Plk4 and thus remain viable candidates for interaction with Plk4 in cell motility or other pathways.

Using Co-IP assays, I show that Arhgef1 and P-Rex2 not only localize similar to Plk4 but also physically interact with it. This provided further evidence that these GEFs and Plk4 might interact functionally in cell motility. Therefore, I determined the function of Arhgef1 and P-Rex2 on cell motility to compare it to that of Plk4. Depletion of Arhgef1 in HeLa cells leads to decreased directional cell migration in a scratch-wound assay, which is consistent with the effect of Plk4 depletion (102). Depletion of Arhgef1 has also been shown to decrease migration of B cells (532), lymphocytes (532), thymocytes (533), monocytes and macrophages (534). In stimulated neutrophils, knockout of Arhgef1 increased the speed of the cells but decreased their directionality, therefore resulting in inefficient directional migration (471). However, in marginal zone B cells depleted of Arhgef1, both basal and directional motility were increased (532).
Arhgef1 depletion in MCF7 and MDA-MB-231 breast cancer cells also resulted in enhanced migration and the authors propose this is due to loss of cell-cell adhesions and loss of protrusions (547). This suggests that the effect of Arhgef1 on motility is cell type specific. Most reports to date described P-Rex2 as a positive regulator of cell migration, where reduced P-Rex2 expression decreased migration of MEFs (563), endothelial cells (478), breast cancer (479), pancreatic cancer (578), brain cancer (573, 581) and cells of other cancer types. However, to my knowledge, the effect of P-Rex2 on HeLa cell migration has not been reported until now. I show that in a scratch-wound assay, depletion of P-Rex2 in HeLa cells led to an increase in cells migration. Therefore, like with Arhgef1, the effect of P-Rex2 on migration is likely cell type specific. Cell specific effects are common for GEFs, as different cells may lack or have more of the upstream GEF regulators or of their downstream effectors. A clear example of this is a Rac1 GEF TIAM1. In epithelial cells, TIAM1 promoted cell-cell adhesion, thereby reducing cell migration (625). However, in fibroblasts it enhanced cell migration by increasing lamellipodia extension (293). Hence, a certain balance exists between the different functions of GEFs and this balance is different in different cell types. Shifting this balance may therefore produce variable and sometimes even opposite results depending on cell type. Plk4 may functionally interact with P-Rex2 to affect other cell processes, such as cell spreading (which will be explored in the next chapter), attachment or proliferation. Potential functional interaction between Plk4 and P-Rex2 is even more interesting given that besides being a Rac1 GEF P-Rex2 also inhibits tumour suppressor PTEN (479, 563). Plk4 and PTEN connection has also been suggested (127) and Plk4 inhibitors have been shown to be more effective in PTEN negative tumours (61). Therefore, P-Rex2 may represent a link between Plk4 and PTEN and this should be explored in the future.

Nevertheless, in cell migration of HeLa cells Plk4 and P-Rex2 have the opposite effects, which led me to conclude that Plk4 is more likely to regulate cell migration through Arhgef1.

Further characterization of the interaction between Arhgef1 and Plk4 determined that the interaction occurs between the DH/PH domains of Arhgef1 and kinase domain and bolo-box1-2 (PB1-2) domains of Plk4. PB1-2 domain of Plk4, also known as cryptic polo-box is responsible for Plk4 homodimerization (40, 41), substrate binding (40, 42, 43) and its subcellular localization (40, 41). Hence, Arhgef1 binding to this domain is consistent with previous reports. Arhgef1 also bound to Plk4 kinase domain, although to a lesser extent than to the full-length protein or PB1-2.
Although rare, interactions of other proteins with kinase domain of Plk4 have also been reported. For example, Kratz et. al. have reported an interaction of Plk4 kinase domain with a well-characterized interactor and substrate of Plk4- STIL (77). This interaction was also weaker than that with the full-length protein. While no interaction with PB1-2 or PB3 were reported by these authors, another group reported that STIL interacts with PB3 of Plk4 but not the kinase domain (46). In general, the interactions of substrates with the Plk4 kinase domain are likely to be of a very transient nature, making them harder to detect and explaining why they appear weaker when they are detected. The DH/PH are the tandem domains conserved between all Dbl family GEFs. DH domain of Arhgef1 contains the GEF switch and is responsible for the GEF activity towards Rho GTPases (452), as well as Gα13 binding (527). PH domain targets Arhgef1 to the plasma membrane, where it also mediates its interaction with RhoA (516, 517). In general, PH domain can act as a docking site for the GEF interactors, and therefore likely mediates Arhgef1 interaction with Plk4. Importantly, Plk4 interacted with the DH/PH fragment of Arhgef1 irrespective of the presence of the linker region that lies N-terminal to the DH domain. This linker region has been shown to autoinhibit Arhgef1, possibly by modulating its GEF switch. Therefore, Plk4 can interact with Arhgef1 regardless of whether Arhgef1 is active or not.

GEFs activity is regulated through multiple mechanisms including protein-protein interactions, post-secondary modifications and localization. Phosphorylation is the most common post-secondary modification that has been shown to result in activation of multiple GEFs, among them Arhgef1. Arhgef1 is phosphorylated by both tyrosine and serine/threonine kinases. For example Jak2 has been shown to phosphorylate Tyr738 of Arhgef1 and this activates its activity towards RhoA (467). Serine/threonine phosphorylation of Arhgef1 by PKCα also results in its activation, although the precise residues that PKCα phosphorylates are still unknown (468). Here I show that kinase domain of Plk4 phosphorylates full length Arhgef1 in an in vitro kinase assay. Furthermore, Plk4 phosphorylated only the L-DH/PH fragment and not the DH/PH fragment. This was an interesting finding as Plk4 phosphorylation motif is predicted to be located in the DH domain, specifically residue S473. This suggests that while Plk4 can interact with both active and inactive form of Arhgef1, it phosphorylates only the inactive form, and perhaps this leads to Arhgef1 activation. Another possibility is that Plk4 may also phosphorylate a residue located in the autoinhibitory loop. In fact, when less stringent criteria for the core Plk4 consensus
phosphorylation motif were applied, two core Plk4 phosphorylation motifs were uncovered in the linker region. One of them only lacked a charged residue at the -2 position to make it a full Plk4 phosphorylation motif. Multiple phosphorylated serine/threonine residues of Arhgef1 have been identified by mass spectrometry and phosphorylation of S330, was shown to stimulate Arhgef1 GEF activity but the kinase responsible for phosphorylation of these sites was not identified (469). The S330 residue is located in the linker region of Arhgef1, and none of the other identified phosphorylation sites were in the DH/PH domains or the linker region. The S330 is not part of the Plk4 consensus phosphorylation motif, but it would still be important to test whether it may be phosphorylated by Plk4 given its location in the autoinhibitory loop. Some phosphorylation sites might also have been missed by the study, as certain sites may be phosphorylated only under specific conditions, in case of Plk4 when the cells are induced to move. Thus, to determine whether Plk4 in fact phosphorylates S473, a point mutation mutant should be tested by the kinase assay.

As phosphorylation often activates GEF activity, I tested whether Plk4 can activate Arhgef1 and I show that Plk4 increases Arhgef1 activity towards RhoA. However, further studies are needed to determine whether this increase in Arhgef1 activation is phosphorylation dependent. One way to determine this would be to overexpress the wild type or kinase dead Plk4 and perform the active GEF pulldown. Another approach is inhibition of Plk4 kinase activity by Plk4 inhibitor centrinone followed by the same assay. The surprising finding in this chapter was that Arhgef1 positively regulated Rac1 activity, similar to Plk4. Although traditionally Arhgef1 is thought of as a specific GEF for RhoA, emerging evidence indicates that it may also activate Rac1. The first study to report Rac1 regulation by the Arhgef1 looked at Rho GTPase activity in human pheochromocytomas (PCCs) (554). Here, the Rac1 activity was shown to be decreased in the PCCs compared to the adjacent normal tissues, while RhoA activity remained unchanged. The expression of Arhgef1 was also significantly lower in the tumour samples and this strongly correlated with the Rac1 activity. Transient knockdown of the Arhgef1 expression by siRNA in PC12 cells (derived from rat adrenal PCC) reduced Rac1 activity by 30% but did not affect RhoA activity. This suggested that at least in the PCCs, Arhgef1 acts as a Rac1 and not a RhoA GEF. A recent study by Singh and colleagues also reported that Arhgef1 activated Rac1 and not RhoA upon monocyte chemotactic protein 1 (MCP1) stimulation of human aortic smooth muscle
cells (626). Inhibition of the Arhgef1 by siRNA inhibited not only the Rac1 activation, but also the MCP1-induced cell migration and proliferation. MCP1 stimulation also resulted in Arhgef1 tyrosine phosphorylation and therefore potentially in Arhgef1 activation. Inhibition of the downstream signaling of the MCP1, specifically inhibition of the CCR2, G\textsubscript{1/0} or Fyn, inhibited Arhgef1 tyrosine phosphorylation and Rac1 activation, further elucidating the mechanism of the Arhgef1 regulation and of the subsequent Rac1 activation by the MCP1. Therefore, this was the first report to show that Arhgef1 activity can be stimulated by a member of the G proteins family (G\textsubscript{1/0}), that belongs to a different sub-type of G proteins than G\textsubscript{12/13}. Taken together, these studies suggest that Arhgef1 may act as a Rac1 or a RhoA GEF and which Rho GTPase is activated may depend on the stimulus and the specific GPCR it activates and on the cell type. Importantly, Plk4 is already known to phosphorylate one GEF that can activate both RhoA and Rac1. Ect2 is mostly considered a RhoA GEF that acts in proper positioning of the cleavage furrow by precise activation of RhoA at that location (627). Plk4 interacts with and phosphorylates Ect2 and this interaction is necessary for efficient RhoA activation during cytokinesis (54). In turn, Weeks and colleagues have shown that in astrocytoma, Ect2 aberrantly localizes to the cytoplasm, where it activates Rac1 and Cdc42 (238). This suggests that GEF signaling is not only cell-type specific but also disease state specific.

This is the first study to identify Arhgef1 as Plk4 target in cell motility. Arhgef1 depletion antagonized the increased cell migration in Plk4 overexpressing cells. Therefore, Plk4 function in cell motility is at least partially dependent on Arhgef1. Arhgef1 activates Rac1 and RhoA, while Plk4 activates Arhgef1 activity towards RhoA, suggesting another pathway, aside from Arp2/3 regulation and centrosome overduplication, through which Plk4 affects cell motility. Moreover, these pathways may overlap, as Arhgef1 activation of Rac1 likely leads to activation of the Arp2/3 complex.

Based on previous findings and those presented in this chapter I propose a mechanism of Plk4 interaction with Arhgef1 (Figure 4.14). In unstimulated cells, Arhgef1 is located in the cytoplasm and is inactivated by autoinhibitory loop attenuating its GEF activity. When the cell receives a signal to move, Plk4 binds the DH/PH domain of Arhgef1 and phosphorylates the DH domain and possibly the autoinhibitory loop. The presence of the autoinhibitory loop creates an Arhgef1 conformation that brings the Plk4 phosphorylation site in a proper position for Plk4
phosphorylation. Another possibility is that the autoinhibitory loop is necessary for the binding to the kinase domain of Plk4 and therefore for phosphorylation of Arhgef1. Hence, without the autoinhibitory loop Plk4 can still bind the DH/PH domain but not phosphorylate it. Phosphorylation by Plk4 releases the Arhgef1 autoinhibition. Once activated, Arhgef1 relocates from the cytoplasm to the plasma membrane, where it activates RhoA and/or Rac1 (517, 519, 528). In turn, activation of these Rho GTPases results in stimulation of cell migration. Without Plk4 phosphorylation, Arhgef1 is inactive but can still translocate to the PM, since it is the PH domain that targets Arhgef1 to this location (516, 517). I propose that Arhgef1 brings Plk4 with it to the site of protrusions, where Plk4 then affects actin polymerization through Arp2/3 and possibly other mechanisms. One hypothesis for how Plk4 is localized to protrusions is that it may be shuttled from centrosomes to the sites of actin polymerization via microtubules, similar to the centrosomal protein ninein. In epithelial cells ninein shuttles from the centrosome, where it plays an important role in microtubule minus-end anchorage, to the apical side of the cell via microtubules and helps to establish apico-basal polarity (628). Other kinases such as FAK and ILK have also been shown to shuttle between centrosome and cell protrusions but the mechanisms for this are still not clear (629, 630). Consistent with this hypothesis, in murine embryos, Plk4 has been shown to move from spindle poles to the plasma membrane (92). Arhgef1 localization can also be regulated by microtubules during extrusion of apoptotic cells. Cells surrounding the apoptotic cell re-orient their microtubules towards basolateral surfaces, thereby targeting Arhgef1 to that location (631). Arhgef1 then activates RhoA and this defines the direction of extrusion of the apoptotic cell. Importantly, Arhgef1 depleted cells showed delay in actin rearrangement, similar to the depletion of Plk4. This can be explained by the proposed model, where the lack of interaction between Plk4 and Arhgef1 decreases localization of Plk4 to protrusions which leads to less efficient actin rearrangement. This possibility can be a focus for future studies. Additionally, while overexpressed Arhgef1 was distributed throughout the cytoplasm and concentrated in the cell edge/protrusions, it was not concentrated at the centrosome like Plk4 was. This observation argues against the idea that Plk4 effect on cell migration is the result of solely its effect on the centrosome duplication.
Figure 4.15

(a) Unstimulated cell

Centrosome

Cytoplasm

Motility signal

(b) Unstimulated cell

Centrosome

Cytoplasm

No GEF Activation

Plasma Membrane

Motility

* Phosphorylation

Activation
**Figure 4.15. Schematic of Plk4 and Arhgef1 interaction in cell motility.** a) In unstimulated cells Plk4 resides mostly at the centrosome, while Arhgef1 is widely distributed in the cytoplasm and is inactivated by the autoinhibitory loop. b) When the signal to move is received by the cell, Plk4 and Arhgef1 interact via kinase domain (KD) and tandem polo-boxes 1 and 2 (PB1, PB2) of Plk4 and the DH/PH domains of Arhgef1. Plk4 then phosphorylates Arhgef1 on the DH domain and/or the autoinhibitory loop. The phosphorylation activates Arhgef1 GEF activity and Arhgef1 relocates to the plasma membrane, bringing Plk4 to the protrusions site. At the plasma membrane active Arhgef1 activates RhoA and/or Rac1 which initiate protrusion formation and cell movement. c) When Arhgef1 lacks autoinhibitory loop, Plk4 can still bind the DH/PH fragment but it cannot phosphorylate the DH domain, possibly due to incorrect positioning of the phosphorylation site or due to lack of interaction with the kinase domain. Therefore, Arhgef1 stays inactive. Arhgef1 can still translocate to the plasma membrane via its PH domain, bringing Plk4 with it, but because Arhgef1 is inactive, it does not activate RhoA and/or Rac1 efficiently, resulting in a migration defect.
Arhgef1 is overexpressed in breast (554), bladder (554, 555) and prostate (556) cancers, while its overexpression or increased activity lead to cell transformation in vitro (453, 509), suggesting that Arhgef1 may play an oncogenic role. Two inhibitors for Plk4 have been created. One of these inhibitors known as centrinone is highly specific for Plk4 but is not yet orally available (62). Another one, CFI-400945 also inhibits Aurora B kinase and is now in phase 1-2 clinical trials for breast cancer patients (61, 129). However, the mechanisms of how Plk4 regulates tumorigenesis, tumour progression and invasion are only beginning to be uncovered. Results presented in this chapter indicate that Plk4 is promoting cancer cell motility through Arhgef1. Thus, determining the status of Arhgef1 expression could help select patients who can benefit most from the Plk4 inhibitors. To this end, an important future step will be examining the effect of Plk4 inhibition on cancer cells with high vs. low Arhgef1 expression in mouse cancer/xenograft models. In addition, low Plk4 expression also has the potential to be tumorigenic, as loss of Plk4 heterozygosity has been described in human HCC (54). Plk4 deletion in mice is embryonic lethal, while older Plk4 heterozygous mice develop liver, lung and/or soft tissue tumours (52, 60). On the other hand, Arhgef1 null mice are viable and apparently healthy at birth, although they do later exhibit thymic hyperplasia and immune response problems due to decreased motility of cells of hematopoietic and lymphatic origin (532, 533). Nevertheless, the consequences of Arhgef1 inhibition may be less severe than those of Plk4 inhibition, further supporting Plk4 downstream interactors as possible alternative inhibition targets for cancer treatment.
Chapter 5

5 Plk4 may regulate cell spreading via P-Rex2-Rac1 pathway

5.1 Overview

Rho GTPases are among the major regulators of cell spreading and motility as they control the cytoskeleton filaments rearrangement, membrane protrusion and retraction and adhesion formation and turnover. Spreading on a flat surface occurs passively at first but is then followed by Rac1 and Cdc42-driven adhesion formation, actin polymerization and protrusion formation (348). As the cell surface area increases, active Rac1 is localized mostly at the protruding membrane, which allows for increased RhoA activation towards the center of the cell. RhoA then drives adhesion maturation and stress fiber formation. At the later stages of spreading Cdc42 regulates cell polarization. Thus, the balance of the Rho GTPase signaling also determines the final shape of the resting cell, as well as cell shape during motility. Other major factors involved in regulation of spreading and cell morphology include but are not limited to cell membrane rigidity, substrate availability, and substrate rigidity. Thus, cell spreading is a process that utilizes many of the same signaling pathways and cytoskeleton components as mesenchymal cell migration and is often thought of as a simpler way of studying motility. Similarly, cell morphology can be predictive of the type and rate of motility (632, 633).

We have shown previously that Plk4 regulates the activation of Rho GTPases RhoA, Rac1 and Cdc42 (54, 102). Additionally, Plk4 regulates the migration and invasion of MEFs and cancer cells (101). I have shown in the previous chapter that Plk4 physically interacts with two GEFs: Arhgef1 and P-Rex2 and that it regulates cell migration through Arhgef1. Our previous studies also showed that in resting cells Plk4 overexpression induced an arborized phenotype, consistent with enhanced migration, while Plk4 depleted cells were more round and this was consistent with reduced migration of these cells (102). Moreover, we showed that Plk4 enhances cell spreading (101, 102). My aim in this chapter was to determine the effect of the GEFs that physically interact with Plk4 on cell spreading and morphology of the resting cells and compare the results with the effects of Plk4. I show that in spreading and morphology assays Arhgef1 effects are consistent with the effects of RhoA but not Plk4. Effects of P-Rex2 on cell spreading were mostly consistent with those of Rac1 and those of Plk4, indicating that Plk4 may act through P-Rex2 to regulate spreading.
5.2 Introduction

Cell spreading on a flat surface utilizes similar signaling and machinery as mesenchymal cell migration. However, it lacks the added complexity of cell-cell adhesions, polarity and intricate microfilament interactions, and is thus often thought of as a simpler way to study motility. When adherent cells are seeded, their first contact with the surface is passive and the rate of flattening out depends on the rigidity of the cell’s membrane and cytoskeleton (348, 349). Then, integrin-mediated nascent adhesions, reminiscent of those found in lamellipodia, start to form. Rac1 and Cdc42 GEFs are activated at the adhesion sites and in turn activate these Rho GTPases. If the environment of the adhesion surface is favourable, with a lot of ligands, the cell spreads out radially (347, 370). This spreading is regulated by Rac1/WAVE signaling which initiates branched actin polymerization. In the case that ligands are sparse, the cell sends out filopodia-like protrusions to search for better environment and this is mediated through Cdc42/WASP (347, 370). As the cell spreads out further, maturing adhesions as well as increased tension in the center of the cell activate RhoA, which leads to stress fiber formation (348, 349). From this point the cell continues to spread out slowly as it has almost reached its final size. The final morphology of the cell is determined by the balance of the Rho GTPases (more active RhoA vs. Rac1 and vice versa) and the types of cell-matrix adhesions formed inside the cell; and ligand availability, substrate stiffness and signaling molecule types/concentrations outside the cell (348). Thus, studying cell spreading and morphology may provide clues as to which Rho GTPases are activated.

Plk4 is a serine/threonine kinase that is essential for centriole duplication (49, 58), late mitotic progression (52, 53, 60, 62, 100) and post-gastrulation embryonic development (52). It has also been shown to play a role in trophoblast differentiation (589) and it regulates cell motility and invasion (101, 102). Moreover, our laboratory has shown that Plk4 regulates spreading and morphology in MEFs and cancer cells (101, 102). In particular, Plk4 overexpression increases cell spreading, as measured by the cell area at 3h post seeding, while Plk4 depletion decreases it (102). Plk4+/- MEFs are also deficient in cell spreading as compared to the wild types (101). Overexpression of the kinase dead mutant of Plk4 did not enhance spreading, which suggested that Plk4 kinase activity is necessary for the spreading phenotype. In keeping with this, active Plk4 has been shown to localize to the protrusions formed by the spreading MEFs, while its
depletion from the protrusions caused a rounded cell phenotype (101). This suggests that active Plk4 is necessary for cell spreading and protrusion formation. In addition, in resting cells that have not been induced to spread or move, Plk4 depletion caused HeLa cells to adopt a rounded morphology (102). Flag-Plk4 overexpression on the other hand, produced an arborized almost neuron-like phenotype, where cells formed long and thin protrusions (102). However, the mechanisms of how Plk4 affects cell spreading and morphology are still poorly understood.

Our laboratory has shown that Plk4 regulates the activity of Rho GTPases RhoA, Rac1 and Cdc42 ((54, 102) and chapter 1 of this thesis). Rho GTPases cycle between active, GTP-bound and inactive GDP-bound state and are activated by GEFs and de-activated by GAPs. In the previous chapter I identified 12 GEFs that possess Plk4 consensus phosphorylation motif and are potential Plk4 substrates. Of these, Arhgef1 and P-Rex2 localized similar to and co-immunoprecipitated with Plk4, suggesting that they may interact functionally. I then demonstrated that Plk4 regulates cell motility through Arhgef1, while P-Rex2 showed an opposite effect to Plk4 in motility. Arhgef1 is considered to be a RhoA GEF but recent studies and my own work show that it can also activate Rac1 (554, 626). P-Rex2 is known as a Rac1 GEF which has also been shown to weakly activate Cdc42 but not RhoA (560, 561). In this chapter I explore the effect of these GEFs on cell spreading and morphology and compare it to that of Plk4 and other Rho GTPases to determine whether these GEFs may be involved in Plk4-mediated regulation of these processes.
5.3 Results

5.3.1 P-Rex2 but not Arhgef1 affects cell spreading in a manner consistent with Plk4

In our previous studies we have shown that Plk4 overexpression enhances cell spreading, while its depletion decreases it. This was measured by a spreading assay, where the cells were allowed to spread for 3h post seeding and then fixed. The cells were then stained with phalloidin to visualise actin filaments and immunofluorescence pictures were captured. The actin staining was used to calculate the area of each cell by Columbus image analysis software and average cell areas were compared. Cell roundness was also measured by cell axial ratio (length/width, where 1=round). I utilized the same assays to confirm these findings and I show decreased cell spreading and more rounded phenotype in siPlk4 treated HeLa cells (Figure 5.1a). In addition, treatment of the cells with a highly specific Plk4 inhibitor centrinone B also decreased cell spreading in a dose dependent manner (Figure 5.1b). This is in keeping with our previous results that have shown that Plk4 kinase activity is required for the spreading phenotype. However, cell roundness did not change significantly with the centrinone B treatment.

Next, I tested the effect of manipulation of the expression level of Arhgef1 and P-Rex2 on HeLa cell spreading and roundness. Depletion of Arhgef1 by siRNA did not change the cell area but it marginally increased cell roundness (Figure 5.2a). On the other hand, transient overexpression of GFP-Arhgef1 decreased cell spreading but did not affect the cell morphology (Figure 5.2b). Interestingly, GFP-Arhgef1 decreased average cell area by about a 100µm², which is comparable to the decrease in cell area in siPlk4 treated cells. Thus, whereas the cell morphology phenotype is partially consistent with that of Plk4, the effect of Arhgef1 on spreading is opposite to that of Plk4. Depletion of P-Rex2 also did not affect spreading and significantly increased cell roundness (Figure 5.3a). However, unlike overexpression of Arhgef1, transient overexpression of P-Rex2 significantly increased cell area (Figure 5.3b). There was also a trend to V5-P-Rex2 transfected cells being less round than V5 transfected controls, but this difference did not reach statistical significance. Therefore, P-Rex2 enhances cell spreading and causes a less round phenotype in spreading cells, which is consistent with the Plk4 effects on these cellular processes. This suggests that Plk4 may regulate cell spreading efficiency and morphology at least in part through P-Rex2.
3.5h spreading assay

Figure 5.1

![Image of cell spreading assay](image)

**a**

- siLuciferase
- siPlk4

**b**

Centrinone B

- 0µM
- 60µM
- 125µM
- 250µM
Figure 5.1. Plk4 depletion decreases cell spreading and increases cell roundness. a) Representative immunofluorescence images of spreading HeLa cells treated with siLuciferase or siPlk4 at 3.5h post seed (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the spreading cells. b) Representative immunofluorescence images of spreading HeLa cells treated with the indicated amounts of centrinone B for 16h at 3.5h post seed (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the spreading cells. Data are SEM±SE and are representative of at least 3 independent experiments. Bars: 100µm. **p<0.01, ***p<0.001 as compared to siLuciferase or untreated controls.
3.5h spreading assay

**Figure 5.2**

### a

siLuciferase

siArhgef1

![Images of cell staining for siLuciferase and siArhgef1](image1)

**Graphs:**
- Average Cell Area (µm$^2$)
- Average Cell Roundness

### b

GFP

GFP-Arhgef1

![Images of cell staining for GFP and GFP-Arhgef1](image2)

**Graphs:**
- Average Cell Area (µm$^2$)
- Average Cell Roundness
Figure 5.2. Arhgef1 negatively regulates cell spreading and roundness. a) Representative immunofluorescence images of spreading HeLa cells treated with siLuciferase or siArhgef1 for 48h at 3.5h post seed (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the spreading cells. b) Representative immunofluorescence images of spreading HeLa cells transfected for 16h with GFP-Arhgef1 or GFP at 3.5h post seed (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the spreading cells. Data are SEM±SE and are representative of at least 5 independent experiments. Bars: 100µm. *p<0.05 as compared to siLuciferase or GFP controls.
3.5h spreading assay

**Figure 5.3**

**a**

siLuciferase

siP-Rex2

**b**

V5

V5-P-Rex2

Average Cell Area (µm²)

Average Cell Roundness

*
Figure 5.3. P-Rex2 regulates cell spreading and shape. a) Representative immunofluorescence images of spreading HeLa cells treated with siLuciferase or siP-Rex2 for 48h at 3.5h post seed (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the spreading cells. b) Representative immunofluorescence images of spreading HeLa cells transfected for 16h with V5-P-Rex2 or V5 at 3.5h post seed (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the spreading cells. Data are SEM±SE and are representative of at least 3 independent experiments. Bars: 100µm. *p<0.05 as compared to siLuciferase or V5 controls.
5.3.2 Rac1 but not RhoA affects cell spreading in a manner consistent with Plk4

To determine which Rho GTPases may be responsible for Plk4 effect on cell spreading and morphology, I performed cell spreading and phenotype analysis assays on HeLa cells transiently overexpressing constitutively active RhoA and Rac1 mutants. GFP-CA-RhoA overexpression resulted in decreased cell spreading (Figure 5.4a), which is the opposite to the effect of Plk4 on cell spreading. However, overexpression of FLAG-CA-Rac1 enhanced cell spreading (Figure 5.4b), like the overexpression of Plk4. Neither Rac1 nor RhoA significantly altered the roundness of the spreading cells. Since P-Rex2 is a Rac1 GEF, it is not surprising that its effect on cell spreading and roundness mirrors that of Rac1. It is also consistent with the effect of Plk4 on these processes. Therefore, it is plausible that Plk4 may affect cell spreading and morphology through P-Rex2-Rac1 pathway. Although Arhgef1 has been reported to activate Rac1, it is widely known as a RhoA GEF. Consistent with this notion, overexpression of Arhgef1 caused a decrease in cell spreading as did the overexpression of CA-RhoA. However, Plk4 overexpression enhances cell spreading, making it unlikely that Plk4 may act through Arhgef1-RhoA pathway in spreading. A summary of the effects of Plk4, Arhgef1, P-Rex2, RhoA and Rac1 on cell spreading and morphology of the spreading cells can be found in Table 5.1.
3.5h spreading assay

Figure 5.4

A

GFP

Average Cell Area (µm\(^2\))

0.60

0.65

0.70

0.75

0.80

0.85

GFP

GFP-CA-RhoA

Average Cell Roundness

0.60

0.65

0.70

0.75

0.80

0.85

B

FLAG

Average Cell Area (µm\(^2\))

0.60

0.65

0.70

0.75

0.80

0.85

FLAG

FLAG-CA-Rac1

Average Cell Roundness

0.60

0.65

0.70

0.75

0.80

0.85
**Figure 5.4. Constitutively active RhoA and Rac1 regulate cell spreading.** a) Representative immunofluorescence images of spreading HeLa cells transfected for 16h with GFP-CA-RhoA or GFP at 3.5h post seed (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the spreading cells. b) Representative immunofluorescence images of spreading HeLa cells transfected for 16h with FLAG-CA-Rac1 or FLAG at 3.5h post seed (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the spreading cells. Data are SEM±SE and are representative of at least 3 independent experiments. Bars: 100µm. *p<0.05 as compared to GFP or FLAG controls.
Table 5.1. The effect of GEFs Arhgef1 and P-Rex2 and Rho GTPases RhoA and Rac1 on cell spreading and shape as compared to Plk4. Summary of the published effects of Plk4 on cell spreading and roundness and those of Arhgef1, P-Rex2, RhoA and Rac1 described in this chapter. ND= not determined, purple dash means no effect.
5.3.3 Plk4 effect on cell shape is consistent with that of Rac1 but not RhoA, Arhgef1 or P-Rex2

To examine the Plk4 phenotype on the shape of resting cells, we examined HeLa cells that have been transfected for 16h with FLAG-Plk4 or treated with siPlk4 for 48-72h, without re-seeding the cell. We used the same method to calculate average cell area and roundness as for the spreading assay. Plk4 overexpression results in an arborized phenotype in resting cells. However, this phenotype is difficult to capture with the method we have been using to determine cell roundness, due to the software not being able to pick up the very long and very thin protrusions that result from Plk4 overexpression. Nevertheless, depletion of Plk4 by siRNA does result in significant rounding of the cells that can be quantified. I determined the effect of Arhgef1 and P-Rex2 on cell shape to compare their effects to Plk4. Treatment of HeLa cells with siArhgef1 increased cell area (Figure 5.5a), while GFP-Arhgef1 overexpression decreased cell area (Figure 5.5b). Neither depletion nor overexpression of Arhgef1 altered cell roundness (Figure 5.5). Interestingly, siP-Rex2 treatment also increased cell area but significantly decreased cell roundness (Figure 5.6). Moreover, siP-Rex2 treated cells were very thin and long, reminiscent of the arborized phenotype seen in Plk4 overexpressing cells. Thus, neither Arhgef1 nor P-Rex2 effects on cell area and shape are consistent with that of Plk4.

To determine which Rho GTPase may be responsible for the effect of Plk4 on cell shape, I examined HeLa cell area and roundness after 16h transfection with either GFP-CA-RhoA or FLAG-CA-Rac1. Transient expression of GFP-CA-RhoA significantly decreased cell area and increased cell roundness (Figure 5.7a), an effect that is consistent with that of Arhgef1 but is opposite to Plk4. However, transient FLAG-CA-Rac1 overexpression significantly increased cell area and there was a trend to decreased cell roundness, but it did not reach statistical significance (Figure 5.7b). Of note, I did not observe an arborized phenotype with overexpression of either of the Rho GTPases. Taken together, these results suggest that Plk4 does not act through Arhgef1-RhoA pathway to affect cell shape but may act through Rac1 although likely through a different GEF than P-Rex2. A summary of the effects of Plk4, Arhgef1, P-Rex2, RhoA and Rac1 on resting cell size and shape can be found in Table 5.2.
Figure 5.5

a  48h siRNA

siLuciferase

siArhgef1

b  16h transfection

GFP

GFP-Arhgef1
Figure 5.5. Arhgef1 negatively regulates cell size. a) Representative immunofluorescence images of spreading HeLa cells treated with siLuciferase or siArhgef1 for 48h (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the resting cells. b) Representative immunofluorescence images of spreading HeLa cells transfected for 16h with GFP-Arhgef1 or GFP (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the resting cells. Data are SEM±SE and are representative of at least 2 independent experiments. Bars: 100µm. *p<0.05 as compared to siLuciferase or GFP controls.
Figure 5.6

48h siRNA

Average Cell Area (µm$^2$)

Average Cell Roundness

**siLuciferase**

**siP-Rex2**
Figure 5.6. P-Rex2 regulates cell size and shape. Representative immunofluorescence images of spreading HeLa cells treated with siLuciferase or siP-Rex2 for 48h (top). Quantification of the cell area (bottom left) and roundness (bottom right, 1=perfectly round) of the resting cells. Data are SEM±SE and are representative of 4 independent experiments. Bars: 100µm. *p<0.05, **p<0.01 as compared to siLuciferase.
16h transfection

Figure 5.7

A

B
Figure 5.7. Constitutively active RhoA and Rac1 regulate cell size and shape. a) Representative immunofluorescence images of spreading HeLa cells transfected for 16h with GFP-CA-RhoA or GFP (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the resting cells. b) Representative immunofluorescence images of spreading HeLa cells transfected for 16h with FLAG-CA-Rac1 or FLAG (left). Quantification of the cell area (top right) and roundness (bottom right, 1=perfectly round) of the spreading cells. Data are SEM±SE and are representative of 3 independent experiments. Bars: 100μm. *p<0.05 as compared to GFP or FLAG controls.
## Table 5.2

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<th>Expression</th>
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<th>Roundness</th>
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<td>↓</td>
<td>ND</td>
<td>↑</td>
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<tr>
<td></td>
<td>↑</td>
<td>ND</td>
<td>↓</td>
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<tr>
<td>Arhgef1</td>
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<tr>
<td>RhoA</td>
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<tr>
<td>Rac1</td>
<td>↓</td>
<td>ND</td>
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</tr>
</tbody>
</table>

Table 5.2 The effect of GEFs Arhgef1 and P-Rex2 and Rho GTPases RhoA and Rac1 on cell size and shape as compared to Plk4. Summary of the published effects of Plk4 on cell size and shape and those of Arhgef1, P-Rex2, RhoA and Rac1 described in this chapter. ND= not determined, purple dash means no effect.
5.4 Discussion

Cell spreading involves two main processes, attachment and membrane protrusion via actin polymerization. In turn, both of these processes are regulated by the Rho GTPases, the most important of which are RhoA and Rac1. The crosstalk between RhoA and Rac1 during cell motility is very complex and involves mutual inhibitory and activation loops to allow for precise spatial and temporal activation of these Rho GTPases (338). During spreading the antagonistic relationship between RhoA and Rac1 seems to be more clear, where Rac1 regulates cell-surface adhesion and the initial stages of spreading, while RhoA only becomes active later on in the areas with low Rac1 activity (348, 349). Thus, spreading assays present a simpler way of studying Rho GTPase activity and downstream signaling. However, since the signaling is not as complex as during motility, it should be noted that the results of spreading studies are not necessarily directly applicable to motility. For example, although both cell spreading and motility depend on Rho-GTPase mediated adhesion formation, different GEFs and GAPs may be involved or the same GEFs and GAPs might be differently regulated during these processes.

Defects in spreading can be caused either by the inefficient cell adhesion to the surface or by the inefficient actin polymerization and protrusion formation. Here I confirm our previous finding that Plk4 depletion decreases cell spreading (101, 102), which indicates that Plk4 is involved in adhesion formation and/or actin polymerization. Our laboratory has also shown that while Plk4 overexpression enhances cell spreading, overexpression of the kinase dead mutant of Plk4 did not have that effect (102). This indicated that Plk4 kinase activity is necessary for the observed spreading phenotype. I confirmed this finding by using cells treated with centrinone B, inhibitor of Plk4 kinase activity. I show that cells treated with this inhibitor are deficient in spreading and this effect was dose-dependent. Because centrinone B inhibits the endogenous activity of Plk4 (62), this decreases the possibility of spreading defect being an off-target effect caused by the overexpression and/or by the ectopic expression of Plk4. As expected, overexpression of constitutively active Rac1 enhanced spreading (634-636), while overexpression of constitutively active RhoA decreased it (359, 636). My work and previous work from our lab showed that Plk4 activates Rho GTPases Rac1 and RhoA ((54, 102) and Chapter 3). However, in spreading the effect of Plk4 overexpression is consistent with that of Rac1 and is opposite to RhoA, suggesting that in this cellular process Plk4 acts through Rac1.
I show that overexpression of GFP-Arhgef1 decreases cell spreading and this result is consistent with RhoA overexpression and thus with Arhgef1 acting as a RhoA GEF. Therefore, even though Arhgef1 may activate Rac1 (554, 626), in spreading HeLa cells it likely acts as a RhoA GEF. While there are no previous reports of the Arhgef1 effect on cell spreading, it has been reported that Arhgef1 plays a role in maturation of nascent adhesions into focal adhesions and in stress fiber formation, through RhoA activation (368, 637). RhoA activation and the resulting adhesion maturation and stress fiber formation increase cell tension, which in turn reduces Rac1 activity and therefore lamellipodia-like membrane protrusion seen during spreading (638). Hence, my spreading assay results are consistent with Arhgef1 acting as a RhoA GEF during this process. However, given that Plk4 enhances cell spreading, it is unlikely that it acts through activation of the Arhgef1-RhoA pathway to do so.

This was the first study to examine P-Rex2 effect on cell spreading and I show that overexpression of P-Rex2 enhances it. This is consistent with the reported activity of P-Rex2 as a Rac1 GEF (560, 561) and is also consistent with Plk4 effect on spreading. My previous finding that Plk4 physically interacts with P-Rex2 (Chapter 4) further suggests that Plk4 may act through P-Rex2 to affect cell spreading. Since Plk4 kinase activity is necessary for the enhanced spreading, an important next step would be to determine whether Plk4 phosphorylates P-Rex2 and whether this phosphorylation increases its GEF activity towards Rac1.

Depletion of either Arhgef1 or P-Rex2 did not significantly alter cell spreading. This may be due to the wide variety of GEFs for both RhoA and Rac1 that may compensate for the activity of these two GEFs. Alternatively, this could also mean that at least in HeLa cells these GEFs mostly affect spreading when expressed ectopically, such as during transient overexpression. Curiously, depletion of both GEFs resulted in increased cell roundness during spreading, similar to depletion of Plk4. This may indicate that these GEFs and Plk4 are involved in establishing cell polarity during spreading, since unpolarized cells often have a round morphology. The rounder phenotype may also be indicative of an altered mechanotransduction in these cells. Therefore, more studies are necessary to determine the precise mechanism through which Arhgef1 and P-Rex2 affect cell shape during spreading.
Cell morphology and motility depend on similar signaling pathways and cytoskeleton machinery, thus the notion that cell morphology may affect motility is not new (632, 633). Intuitively, in 2D culture, a less round cell shape is indicative of higher motility potential due to increased propensity to polarization and protrusion formation. Indeed, a number of studies have reported lower motility in cells that adopt a rounder morphology, such as those with higher RhoA activity (547, 615) or higher motility in cells with less round morphology (639). Thus, determining the shape of the resting cells that have not been induced to move or spread may provide clues to their motility potential. Plk4 depletion in resting cells increases cell roundness (102). Plk4 overexpression on the other hand results in an arborized phenotype, which could not be captured by the utilized roundness assay (102). I show here that depletion of P-Rex2 significantly decreased cell roundness in resting cells and produced a phenotype reminiscent of Plk4 overexpression. Interestingly, CA-Rac1 overexpression did not alter the morphology of the cells. Therefore, although P-Rex2-Rac1 pathway may be involved in cell spreading downstream of Plk4, it is not likely that Plk4 signals through this pathway in its effect on cell shape. Neither depletion nor overexpression of Arhgef1 altered cell roundness, although Arhgef1 overexpression in breast cancer cell lines was previously shown to increase cell roundness (547). This discrepancy may reflect the cell-type specific effects of Arhgef1 on cell morphology or the differences in methodology to determine cell roundness. As expected, overexpression of CA-RhoA increased cell roundness and as this effect is opposite to Plk4 overexpression, I conclude that Plk4 effect on cell shape is independent of RhoA or that in resting cells Plk4 decreases RhoA activity, possibly by activation of Rac1. It should be noted however, that the roundness assay utilized here is not very sensitive to certain morphological changes, such as missing thin protrusions seen in the arborized phenotype. Thus, a more sensitive assay may be needed to fully elucidate the effect of RhoA and Rac1 Rho GTPases and Arhgef1 and P-Rex2 GEFs on cell shape.

I also tested the effect of RhoA, Rac1 Arhgef1 and P-Rex2 on the size of the resting cells, even though Plk4 overexpression or depletion did not change it. Overexpression of GFP-CA-RhoA and of GFP-Arhgef1 resulted in decrease of cell area. In addition, siArhgef1 treatment led to cell area increase. Therefore, both in spreading and in resting cells Arhgef1 and RhoA negatively regulate cell area, consistent with the two proteins acting in the same pathway. Overexpression
of FLAG-CA-Rac1 on the other hand increased the area of the resting cells. These results are indicative of RhoA-Rac1 antagonism playing a role in determining the cell size. The balance between the activities of these two Rho GTPases thus helps determine the final size and shape of the cell (640), where cells with more active Rac1 are bigger and less round, while those with more active RhoA are smaller and rounder.

Overall, findings presented in this chapter suggest that Plk4 affects cell spreading through Rac1 activation and that this effect may be mediated through P-Rex2. The mechanism of Plk4 effect on the shape of the resting cells remains unclear, but my results indicate that its effect is opposite to that of RhoA. Cdc42 also plays a role in cell spreading and determining the final shape of resting cells (641, 642), and Plk4 has been shown to activate Cdc42 (102). Therefore, Plk4 may act through Cdc42 in these processes and further studies are needed to determine the effect of Cdc42 on cell spreading and morphology. Other mechanisms that mediate the spreading phenotype and cell size and shape may involve the remaining 10 GEFs that have been identified as having Plk4 phosphorylation motif. For example, Trio GEF that can activate both RhoA and Rac1 has been found to enhance cell spreading via Rac1 activation and regulation of lamellipodia dynamics in HeLa cells (643). Importantly, Trio also positively regulated cell motility, similar to Plk4. Other GEFs with Plk4 phosphorylation motif that have been shown to play a role in cell spreading include DOCK3 (494) and DOCK5 (496). RhoA GEF Rgnef on the other hand has been shown to interact with FAK and affect focal adhesion formation (367). DOCK2 (644), DOCK3 (494) and DOCK5 (497) have also been reported to affect cell adhesion.

In addition, microtubule polymerization has also been shown to play a role in spreading, by stabilizing the lamellipodial protrusions (645, 646) and studies are needed to determine how microtubules affect cell spreading and shape in Plk4 depleted or overexpressing cells. Spreading and cell shape assays may therefore be a useful tool to help identify or confirm downstream targets of Plk4 and further develop our understanding of the pathways affected in Plk4-dependent tumorigenesis. In turn, that will help better target Plk4-based therapies to cancer patients and potentially uncover new therapeutic targets.
Chapter 6

6 Discussion

6.1 Overview of the results

Before the work presented in this thesis commenced, we had limited knowledge of Plk4 involvement in cell motility and spreading and little to no understanding of the mechanisms through which Plk4 may affect these processes. During the course of my work, a number of studies have been published which further characterized the role of Plk4 in cell motility, invasion, tumour progression and metastasis formation. A few mechanisms through which this happens have been identified, including Plk4’s role in MTOC formation and regulation of the actin cytoskeleton through interaction with the Arp2/3 complex. Using MEFs and cancer cells I show that Plk4 regulates the activity level of Rho GTPases Rac1 and RhoA. To determine the potential mechanisms involved in regulation of these Rho GTPases by Plk4 I examined a library of 141 GEFs, GAPs and other Rho GTPase interactors for consensus Plk4 phosphorylation motif. Based on this, I found 12 potential Plk4 substrates, all of which are GEFs. I then went on to show that Plk4 physically interacts with GEFs Arhgef1 and P-Rex2 and that Plk4 regulates cell migration through Arhgef1. Importantly, Plk4 increased Arhgef1 activity towards RhoA, while active Rac1 pulldown assays showed that Arhgef1 can also increase Rac1 activity, which suggested that Plk4 regulates both pathways to affect cell migration. I further determined the effect of these two GEFs on cell spreading and morphology and I show that Plk4 may act through the P-Rex2-Rac1 pathway to affect cell spreading. Therefore, the data presented in this thesis describes a novel mechanism of Plk4 regulation of cell migration and deepens our understanding of the pathways that may be involved in Plk4-mediated tumor progression and metastasis formation. It also enhances our understanding of the pathways that may be affected by the Plk4 inhibitors in the clinic. However, whether Plk4 acts through Arhgef1 to affect cell motility \textit{in vivo} remains to be determined. Rho GTPase interplay is complicated, with multiple mutually inhibitory and activating loops which are not fully understood to this date. Thus, how Plk4 fits into this interplay also needs to be explored. In this chapter I will discuss how these findings pertain to our current knowledge in the area and I will suggest further experiments that will enhance our understanding of Plk4 and its role in tumorigenesis and cancer progression.
6.2 Discussion

6.2.1 Plk4, microtubules and actin cytoskeleton

Plk4 is a major regulator of centriole duplication and biogenesis. Moderate Plk4 overexpression leads to the overduplication of existing centrioles, while high Plk4 overexpression can lead to de novo centriole formation (357). Centrosomes, which consist of one mother and one daughter centrioles surrounded by the protein-rich pericentriolar material, are the major microtubule organizing centers, which regulate microtubule nucleation and polarization. In turn, microtubules perform important cellular functions: during mitosis they form mitotic spindle that regulates proper chromosome segregation and cytokinesis; they determine cell shape and polarity by regulation of organelle movement and positioning inside the cell; they act as transport network and regulate intracellular vesicle trafficking; and finally, they are the major structural component of cilia, a sensory organelle that plays an important role in signal transduction and sensing the environment (647). Thus, alterations in centrosome numbers can lead to deregulation of microtubule-mediated processes. Centrosome overduplication is a hallmark of cancer and is often associated with advanced stage and tumour aggressiveness. Increased centrosome number can lead to multipolar spindle formation, improper attachment of microtubules to the kinetochores and thus improper chromosome segregation. In turn, improper chromosome segregation causes chromosome instability, another hallmark of cancer. Cells overexpressing Plk4 form multipolar spindles, while Plk4 depletion can result in monopolar spindle formation which leads to cytokinesis failure (97, 648). Interestingly, cells with overduplicated centrosomes often exhibit centrosomal clustering, where multiple centrosomes are clustered at the two poles of the cell and still create a bipolar spindle, allowing for relatively normal cell division (648, 649). Nevertheless, increased number of centrosomes can still lead to aberrant microtubule nucleation and dynamics. Although Plk4 can affect spindle formation through regulation of centriole number, it has also been shown that Plk4 directly regulates microtubule nucleation and spindle formation in the absence of centrioles (92, 650). For example, the first five cell divisions in mouse embryos occur without centrosomes but bipolar spindle is formed normally. Coelho and colleagues have shown that Plk4 is necessary for microtubule nucleation and proper spindle formation during these divisions (92). Others later showed that Plk4 can regulate acentriolar MTOC formation by recruiting α/β-tubulin and other PCM components (650, 651). Thus, Plk4
regulates microtubule polymerization and stability with or without centrosomes. In turn, microtubules have been shown to regulate cell migration (652). One way through which this happens is through regulation of cell polarity during migration. Once the cell receives a signal to move, the nucleus is pushed away from the stimulus by actin retrograde flow, while Golgi and the MTOC localize in front of the nucleus in the direction of migration. Microtubules are then polymerized and stabilized towards the leading edge, while those at the trailing edge become fewer and are unstable (653). This difference in microtubule dynamics thus helps define the cell leading and trailing edges. Microtubules can also facilitate lamellipodia formation and stability. Even though microtubules do not usually localize to the lamellipodia, some called pioneer microtubules, do extend into protrusions (652). There, they may facilitate membrane extension through the force generated by their elongation. Importantly, microtubule dynamics have been shown to activate a number of GEFs which activate Rho GTPases and thus facilitate actin polymerization and protrusion formation (652). Moreover, microtubules participate in membrane vesicle transport and thus can aid in delivery of Rho GTPases and GEFs to the protrusions of motile cells, as has been demonstrated with Rac1, Cdc42 and βPix GEF (652, 654, 655). Similarly, they can deliver integrins from the retracting rear to protrusions thus facilitating nascent adhesion formation that is necessary for lamellipodia development. Microtubules also regulate adhesion maturation and disassembly of focal adhesions at the cell rear. Both of these processes are regulated by RhoA and microtubule depolymerization has been shown to activate RhoA. For example, stable microtubules at the front of the cell sequester GEF-H1 and do not allow for its translocation to the membrane and RhoA activation (656). However, at the cell rear the microtubules are unstable and GEF-H1 is free to activate RhoA. As RhoA and Rac1 are known to antagonize each other’s activity this also results in high Rac1 activity at the cell front and low activity at the rear. Microtubules further affect focal adhesion turnover through interaction with fascin, an actin-binding and bundling protein which regulates focal adhesion dynamics and migration speed (657). In addition, microtubules interact with dynamin and may deliver it to the cell rear where dynamin regulates focal adhesions disassembly possibly by promoting integrin internalization and turnover (658-660). Given these various motility pathways that microtubules are involved in and the role of Plk4 in microtubule polymerization and stability, it is possible that Plk4 may regulate cell motility via its role in microtubule regulation. In fact, Pellman group has demonstrated that increased centrosome number through
Plk4 overexpression or cytokinesis defects increases cell migration (601). They then show that this is at least in part due to increased microtubule nucleation which leads to increased Rac1 activation. However, our group has shown that Plk4 expression does not always correlate perfectly with the centrosome number (102). For example, Plk4 heterozygous MEFs exhibit centrosome amplification, while they have lower Plk4 expression as compared to the wild type MEFs (54, 102). Yet, heterozygous MEFs still exhibit reduced cell migration and invasion, suggesting that regulation of centrosome number and microtubule nucleation is not the sole mechanism by which Plk4 regulates motility. Indeed, our group went on to show that Plk4 regulates actin rearrangement and protrusion formation in MEFs and cancer cells (101, 102). One mechanism through which it does so is by binding and phosphorylation of the Arp2 from the Arp2/3 actin nucleating complex (102). Here, I show that Plk4 may also regulate actin cytoskeleton through regulation of activity of Rho GTPases Rac1, Cdc42 and RhoA. Furthermore, Plk4 activates RhoA and Rac1 at least in part through interaction with Arhgef1 and enhancement of its GEF activity. I also show that Plk4 interacts with P-Rex2, a Rac1 GEF and that P-Rex2-Rac1 pathway effect on cell spreading is consistent with that of Plk4, suggesting that Plk4 may affect actin polymerization in spreading through P-Rex2 and possibly other GEFs.

Our group has shown previously that Plk4 also interacts with and phosphorylates RhoA GEF Ect2 to affect proper cleavage furrow positioning (54). This suggests that regulation of GEF activity by Plk4 may be a common mechanism of Plk4 regulation of actin cytoskeleton reorganization. To that effect, I identified 10 other GEFs that contain Plk4 consensus phosphorylation motif and whether Plk4 interacts with these GEFs physically and functionally should be the focus for future studies. Interestingly, the location of Plk4 consensus phosphorylation motif was not concentrated in any specific domain of either the Dbl or the DOCK family of GEFs. However, these motifs may be localized in a conserved structural domain of the GEFs and this is also something that can be explored in the future. Overall, there is evidence to suggest that Plk4 may regulate motility and Rho GTPase activity through regulation of both microtubule and actin cytoskeletons. Microtubules and actin cytoskeleton often cooperate to affect cell migration, such as in regulation of adhesion formation, maturation and turnover, in establishing cell polarity and in protrusion formation. This makes it difficult to decipher the precise role of Plk4 in regulation of actin vs microtubules. What further complicates the study of the mechanism of Plk4 in cell migration is the fact that Rho GTPases not only
regulate actin cytoskeleton but also microtubule polymerization and stability. For example, microtubule stabilization at the cell front has been shown to be regulated by the RhoA-mDia pathway (329). In turn, RhoA activation is often Gα12/13 dependent and cells lacking Gα12/13 do not form stable microtubules at the cell front in a scratch-wound assay due to lack of mDia localization to the edge of the wound (661). Thus, Gα12/13-RhoA-mDia signalling is essential for microtubule stabilization, establishment of cell polarity and directed migration. Importantly, Gα13 is also the major regulator of Arhgef1 activity and plasma membrane localization (527, 528). This suggests that through activation of Arhgef1 Plk4 can affect both, actin rearrangement and microtubule stability during cell migration. The latter is supported by the fact that our laboratory has previously shown a polarity defect in Plk4 deficient cells undergoing wound-healing, where the cells often did not re-orient their Golgi in front of the nucleus towards the direction of the wound (102). I have also shown that Arhgef1 depleted cells do not form protrusions in the direction of the wound as efficiently as control cells, suggesting a defect in establishing cell polarity. Further studies are needed to determine whether Plk4 acts through Arhgef1 to affect cell polarity. Others have also shown that Arhgef1 deficient cells show a defect in directional migration, which also suggest a polarity defect (471, 535). In addition, Cdc42 is known as the master regulator of cell polarity that affects both the actin and the microtubule cytoskeleton (662). Our lab has demonstrated that Plk4 regulates Cdc42 activity, suggesting another way through which Plk4 may regulate cell polarity (102). Therefore, cell migration is a highly complex process that requires the cooperation of all the cytoskeletal components and these components can in turn regulate each other. Although we now have a few possible mechanisms through which Plk4 may regulate cell migration its role in the interplay between the cytoskeletal filaments during cell migration remains to be elucidated.

6.2.2 Cell migration vs cell spreading

Cell spreading is often thought of as a simpler way to study mesenchymal cell migration. Indeed, the two processes have many commonalities. One of these is the dependence of both processes on the adhesion formation. First, adhesion formation can activate Rho GTPases thereby initiating actin polymerization which helps drive cell protrusion formation (351). Second, adhesions are necessary for protrusion formation, as otherwise actin retrograde flow would cause membrane retraction (158). In mesenchymally migrating cells, protrusions form at the leading edge, while
in spreading cells they usually form radially in a uniform manner. In both processes nascent adhesions are formed first and they mature as the cell protrudes further and adhesions move away from the cell edge. Adhesion formation for both spreading and migration is regulated by Rac1, while adhesion maturation is regulated by RhoA. Thus, both processes depend on Rho GTPases. Rac1 and Cdc42 regulate actin polymerization, which is the main mechanical force in protrusion formation, making both spreading and migration an active process. Finally, both spreading and migration are substrate-dependent, where different substrate types or different ligand availability determines the rate and the type of spreading and migration. For example, on soft substrates many cell types do not spread out as much and do not form as many protrusions as those on the harder surfaces (196, 663) and in 3D these cells are more likely to move using amoeboid migration (664). However, despite many similarities, there are also differences between spreading and migrating cells. First, the initial spreading of the cell on a flat surface is a passive process that does not involve cytoskeleton filaments polymerization (350). Instead, initially cell spreading is driven by surface tension, similar to a drop of liquid and therefore depends on the rigidity of the cell membrane and cytosol. Thus, the initial rate of cell spreading is not actin polymerization-driven, while protrusion formation in migrating cells always depends on the rate of actin polymerization. Second, spreading cells are not polarized and protrude uniformly in all directions until the very last stage when the cell has almost reached its full size. However, mesenchymally moving cells display a clear front-rear polarity that is mostly established by Cdc42 and mutual inhibition of RhoA and Rac1 activity. Cell polarization involves differential distribution of active Rho GTPases, their GEFs and GAPs, as well as membrane receptors and focal adhesions, which is not usually the case during most of the cell spreading stages. Moreover, spreading mostly mimics the leading edge of the migrating cell and does not exhibit retraction like the one seen at the rear of a migrating cell until the last stage of spreading. Finally, spreading cells do not have cell-cell adhesions like the collectively migrating cells do and therefore are not affected as much by cell-cell signalling. Together this suggests that while spreading and motility share many signalling pathways that regulate them, there are also significant differences between the two processes. From this it follows that while manipulation of these signalling pathways often results in similar effects in spreading and migration, it is not always the case. The research presented in this thesis supports the idea that results obtained in spreading assays are not always predictive of migration phenotype and vice versa. In chapter 4, I
show that Plk4 enhances cell migration through Arhgef1 and activates Arhgef1 activity towards RhoA. However, in chapter 5, I show that although Arhgef1 acts as a RhoA GEF to regulate cell spreading and shape, its effect on these phenotypes is opposite to that of Plk4. Therefore, while Plk4 activates Arhgef1 in cell migration, it is not likely to activate it during spreading. In addition, Plk4 depletion had the opposite effect on cell migration to that of P-Rex2, a Rac1 GEF, which suggested that Plk4 does not act through P-Rex2 activation to affect cell migration. However, Plk4 effect on spreading was consistent with that of P-Rex2-Rac1 pathway. Thus, it is likely that Plk4 activates different GEFs and affects Rho GTPase activity differently during spreading and migration. In addition, Plk4 affects these processes by directly affecting actin polymerization through its interaction with the Arp2/3 complex (102). Differential effects of Rho GTPase regulators and effectors on cell spreading and migration have previously been reported. For example, ROCK, a downstream effector of RhoA signalling has been shown to decrease cell spreading but increase cell migration in a number of cell lines (665-669). ROCK is a major regulator of actomyosin contraction which in spreading results in protrusion inhibition. In addition, ROCK can enhance RhoA activation through phosphorylation and inhibition of the p190RhoGAP (670). In turn, RhoA activation inhibits Rac1 activity, which also inhibits protrusion formation. Thus, in unpolarized and mostly protrusion-driven process such as spreading, ROCK has an inhibitory effect. However, during mesenchymal cell migration the cells are polarized, and ROCK activity is largely restricted to the cell rear, where it facilitates its retraction and also destabilizes microtubules, further contributing to cell polarization (321). As cell rear retraction is an essential part of migration in most cells, ROCK positively regulates it. Moreover, in collective cell migration, ROCK inhibits cell-cell adhesions which also stimulates cell migration (671). Similarly, activity of Arhgef1 may be regulated by localization and may depend on polarization of the cell or the presence or absence of cell-cell adhesions, thus resulting in differing effects on actin cytoskeleton in spreading and migration. Moreover, I have shown that Arhgef1 can also increase Rac1 activity and it is possible that during migration it activates both Rac1 and RhoA, while in spreading it acts mostly as a RhoA GEF, since its effect on spreading is consistent to that of RhoA but is the opposite to that of Rac1. In sum, although spreading is a useful tool to study the signalling pathways involved in actin polymerization and protrusion formation, it is less complex than migration and thus the pathways identified in spreading may be altered in migration.
6.2.3 Plk4 and EMT

During gastrulation the primitive ectoderm cells need to migrate and differentiate into primitive mesenchymal cells. In order to do so, the ectoderm cells lose their cell-cell adhesions and polarity, thus gaining a more motile, mesenchymal phenotype. This process has become known as epithelial to mesenchymal transition (EMT). EMT is important during various stages of embryonic development. In adults, it is necessary for maintaining homeostasis and is required for wound healing and organ tissue regeneration. However, this transition is also often seen in epithelial cancer cells and leads to an increase in their migration and invasion, thereby increasing their metastatic potential, aggressiveness and resistance to therapy. Thus, EMT has been a subject of rigorous study in the field of oncology. Growing amount of evidence suggests that unlike in embryogenesis, metastatic cells often do not undergo complete EMT and usually exhibit both epithelial and mesenchymal markers (Reviewed in (672)). This allows the metastatic cells to escape being targeted by the immune system and helps them avoid anoikis (673, 674), a process whereby anchorage-dependent cells undergo cell death upon detachment. Moreover, besides single-cell migration, cancer cells can also migrate collectively as clusters. In this case the EMT occurs only in the outside cells, while the cells inside the moving cluster mostly retain their epithelial properties, causing heterogeneity that is mostly regulated epigenetically. Numerous changes in the levels of proteins that are involved in adhesion, migration, invasion and proliferation are associated with EMT and it has been shown they can be regulated at transcriptional and post-transcriptional, translational and post-translational levels (675). Several transcription factors that are most frequently upregulated in EMT have been identified and they include basic helix-loop-helix factors TWIST1/2 and zinc-finger factors SNAI1/2 and ZEB1/2 (675). These transcription factors downregulate the expression of E-cadherin and up-regulate the expression of N-cadherin and vimentin thus increasing the motility of cancer cells. Overexpression of these transcription factors has been shown to induce EMT in various cell types and thus their expression is used as a marker for EMT (676, 677). ZEB and SNAIL transcription factors bind the promoter of E-cadherin and recruit DNA methyl transferases and chromatin-remodeling factors to silence the E-cadherin transcription (678, 679). Moreover, miRNAs also play a critical role in in EMT and some of these transcription factors regulate the expression of these miRNAs (680). E-cadherin is the main component of adherence junctions in
epithelial cells and its downregulation is the hallmark of EMT, although whether it is the cause or the consequence of EMT is still debated. Decreased E-cadherin expression causes the cell to detach from the neighbouring cells, which together with changes in the expression of other genes make the cells more motile. N-cadherin, on the other hand, is overexpressed in cells that have undergone EMT. N-cadherin, like E-cadherin is a component of adherence junctions that is mostly expressed in neuronal and cardiac cells but is frequently overexpressed in cancer cells. It is the major adherence junction component holding together the migrating clusters of cancer cells and has also been shown to promote stem-cell like phenotype (681). Vimentin is an intermediate filament that is the major cytoskeletal component of the mesenchymal cells. Thus, it is often overexpressed in cells that have undergone EMT and is used as a marker for this process. Another marker of EMT is overexpression of fibronectin. Fibronectin is a secreted ECM protein that binds to integrins and mediates the interaction of the cell with the ECM, thus regulating cell adhesion, migration and differentiation. In particular, it mediates cell migration by cleaving FAK and therefore facilitating the turnover of focal adhesions (682). Finally, MMPs are also frequently upregulated in the cells that have undergone EMT. MMPs facilitate cell migration not only though degradation of the ECM but also through cleavage of the cell-cell and cell-matrix adhesions. For example, MMP-3 and MMP-7 can cleave E-cadherin, which decreases cell-cell attachment and releases a bioactive fragment that induced EMT and cell migration (181, 683). In addition, increased MMP activity can directly cause the epithelial cells to undergo EMT (684). Changes in the expression of the EMT markers described here can therefore be used to determine whether the cells has undergone EMT.

Our laboratory used a microarray to determine the differences between Plk4 wild type and heterozygous MEFs, which predicted decreased motility in late passage Plk4 heterozygous MEFs (101). Another microarray comparing early passage Plk4 heterozygous MEFs that at late passage became tumorigenic to non-tumorigenic MEFs also predicted decreased motility in Plk4 tumorigenic MEFs (101). In both of these arrays, cells that were predicted to be less motile showed decreased expression of MMP-3 and MMP-13 (101). I confirmed these results using a more sensitive and quantitative method, real-time RT-PCR (chapter 3). In addition, I show that MMP-3 and MMP-13 expression levels are decreased not only in late passage Plk4+/− MEFs, but also in early passage Plk4+/− MEFs. Together with a colleague in the lab I have also shown that
overexpression of Plk4 in Plk4+/− MEFs rescues the MMP-3 and MMP-13 expression, showing the dependence of their expression on Plk4 (101). Moreover, my colleague went on to show that reduction of Plk4 expression leads to decreased MMP-3 and MMP-13 expression, while overexpression of Plk4 increases their expression in HeLa cells (101). This suggested that Plk4 may promote EMT. Indeed, Plk4 depleted HeLa cells exhibited a more epithelial morphology, where they were more rounded and grew in clusters (102). When the expression of the EMT markers was examined in shPlk4 HeLa cells, they showed increased E-cadherin and decreased Snail2, N-cadherin, fibronectin and MMP-2 expression as compared to shLuciferase controls (102). Vimentin expression showed a trend for decreased expression in shPlk4 cells but this was not statistically significant. Similar epithelial-like expression pattern was also detected in Plk4-depleted MDA-MB-231 xenografts as compared to control tumours, which suggests that Plk4 also induces EMT in vivo (102). Others have reported the Plk4-induced promotion of EMT-associated protein expression pattern in neuroblastoma cells (588). Cells that undergo EMT often exhibit reduced polarity and our laboratory has shown that Plk4+/− MEFs exhibit reduced polarity and do not re-orient their Golgi and MTOC in front of the nucleus (101). In addition, Plk4 depleted HeLa cells also showed reduced polarity (102). Together, these results point to the involvement of Plk4 in promotion of EMT. Currently, our laboratory is examining the effect of Plk4 on the expression and activity of all members of the MMP family to determine which MMPs are regulated by Plk4 and how that affects cell migration. So far, we have shown that in addition to MMP-3 and MMP-13, Plk4 also regulates the expression and activity of gelatinases MMP-2 and MMP-9, which have been shown to drive EMT (684). In addition, we are carrying out 3D invasion assays in HeLa cells to more closely mimic the invasion and migration of cancer cells. Cells depleted of Plk4 expression show reduced invasion in a 3D setting and rescue experiments with overexpression of MMPs are underway. Signalling pathways that include TGF-β, Wnt/β-catenin, PI3K/Akt/GSK and Ras/MAPK signalling have been shown to contribute to EMT (675). Signalling from these pathways is also known to induce EMT through activation of Rho GTPases, while I show that Plk4 regulates the activation of Rho GTPases RhoA, Rac1 and Cdc42. Thus, Plk4 may be a downstream target of one of these pathways leading to EMT. Additionally, reduction or enhancement of Plk4 expression or activity induces p53 expression and in turn, p53 regulates the expression of various miRNAs that aid in maintaining the epithelial phenotype (685). Therefore, the involvement of Plk4 in these pathways and how it may regulate
EMT needs to be explored in the future experiments. Also, the precise mechanism of how Plk4 regulates the expression of MMPs and other EMT-promoting genes, as well as cell polarity, remains to be elucidated.

6.2.4 Plk4 and Rho GTPases as therapeutic targets in tumour progression and metastasis

Plk4 role in centriole duplication, spindle assembly, cell cycle progression, and motility, as well as its structure which is the most divergent from other Plk family members makes it an attractive target for small molecule inhibitors that can be used for anti-cancer therapy. Indeed, two Plk4 small molecule inhibitors, CFI-400945 and centrinone, have been generated to date (61, 62). Plk4 was selected as a target for inhibitor design by Laufer et al., based on the RNAi screen of kinases and kinase-related genes that affect breast cancer cell viability, as well as due to its overexpression in most breast cancer cell lines (115, 128). To confirm that Plk4 depletion would decrease tumour growth in vivo, a xenograft model of MDA-MB-468 shPlk4 and control shRNA was established. Tumours formed by the cells with reduced Plk4 expression were significantly impaired in growth compared to controls (61). A screen of ligand-based library of potential kinase inhibitors as well as molecules identified through Plk4-homology model yielded a promising candidate which was then modified to enhance potency and specificity leading to the development of Plk4 inhibitor CFI-400437 (128). This was further modified to increase the drug’s oral bioavailability and activity, resulting in the development of CFI-400945 inhibitor (129). CFI-400945 has been shown to differentiate between Plk4 and other members of the Plk family, however it also inhibits off-target kinases, namely Aurora A (AURKA), Aurora B (AURKB), TIE2, TRKA and TRKB, albeit to a lesser degree than Plk4 (129). Somewhat surprisingly, the treatment of cancer cells (U2OS, MDA-MB-231 and MDA-MB-468) with lower doses of this inhibitor led to centriole overduplication, consistent with overexpression of Plk4 (58, 61). This may be due to only partial inhibition of Plk4. In this case, kinase inactivated Plk4 would bind and stabilize uninhibited Plk4 due to reduced trans-autophosphorylation, similar to overexpression of kinase dead Plk4 (130). This is supported by the fact that at higher dose of the inhibitor, and hence more complete Plk4 kinase inhibition, only one centriole per pole was observed. The centrosome number was not followed for sufficient amount of time to determine
whether prolonged exposure to high doses of CFI-400945 would result in complete centrosome loss. With both doses however, the authors observed multipolar spindle formation that led to cell cycle arrest or cell death (61). In case of higher dose of CFI-400945 the formation of multipolar spindles was due to cytokinesis failure, which the authors attributed to the AURKB inhibition. In a human breast cancer xenograft model in mice, treatment with CFI-400945 resulted in significant reduction of tumour growth at a well-tolerated dose (61). These encouraging results led to the initiation of a clinical trial for this Plk4 inhibitor in breast cancer patients and patients with advanced solid tumours. Further studies carried out by the same group suggest that CFI-400945 may also be used in treatment of pancreatic cancer (120). They show reduced tumour growth in patient-derived pancreatic cancer xenograft models treated with this inhibitor.

Given that CFI-400945 also inhibited AURKA and AURKB, another group set out to create a more specific Plk4 inhibitor to study the role of centrosome overduplication in cancer and in cell proliferation. They started with a pan-Aurora kinase inhibitor VX-680, which targets the ATP-binding site of these kinases and modified it to be Plk4 specific (62, 131). They show high selectivity of the resulting inhibitor centrinone (and less potent centrinone B) towards Plk4, as compared to Aurora kinase family, Plk family and a panel of 422 human kinases. Centrinone inhibited centriole formation in a number of primary and cancer cell lines (62). They confirmed that the loss of centrosomes was due to Plk4 inhibition, by rescuing the centrosome duplication with overexpression of drug-insensitive Plk4 mutant. Interestingly, while primary cells lacking centrosomes arrested at G1, cancer cells were able to continue to proliferate, albeit at a slower rate and with reduced mitotic fidelity. This suggests that while centrosomes are essential for normal cell proliferation, cancer cells have additional mutations that allow them to bypass the G1/S checkpoint. It is important to note that upon centrinone washout, the centrosome number returned to pre-treatment level in cancer cells (62). Together these findings suggest that cancer cells with increased number of centrosomes are not addicted to them, irrespective of the extent of the overexpression, but they do have a certain cell line-specific centrosome number that can be tolerated without triggering cell cycle arrest.

Our laboratory has shown that Plk4 induces cancer cell migration and invasion in vivo (102). When NOD-SCID mice were subcutaneously injected with Plk4 depleted MDA-MB-231 breast cancer cell lines, they formed tumours that did not invade into the peritoneum as efficiently as
controls. Most importantly, these tumours also formed significantly fewer metastases to the lung and those metastases that did form were much smaller compared to the controls. Therefore, this study further validated the use of Plk4 inhibitors to not only reduce tumour growth but also reduce cancer cell invasion and metastasis. However, Plk4 has also been shown to act as haploinsufficient tumour suppressor, where about 50% of Plk4 heterozygous mice form soft tissue tumours later in life (60). Moreover, loss of heterozygosity at Plk4 locus has been identified in 50% of human HCC and correlates with poor survival (54, 107). This raises concerns over the long-term effects of using Plk4 inhibitors in the clinic. Thus, a more targeted approach such as the inhibition of specific pathways regulated by Plk4 may be a better alternative to Plk4 inhibition. Here, I show that Plk4 regulates activity of three Rho GTPases RhoA, Rac1 and Cdc42, which are commonly deregulated in cancer and that Plk4 acts through Arhgef1-RhoA pathway to affect cell migration. Therefore, inhibition of Plk4 downstream targets that lead to increased activity of Rho GTPases is one of the Plk4 pathways that can be targeted to reduce tumour progression and invasion.

According to WHO, cancer is the second leading cause of death worldwide. Yet, about 90% of cancer patients die not as a result of the primary tumour but from the tumour metastasis to organs other than the primary site. Metastasis is a multistage process that involves primary cancer cells of solid tumours gaining the ability to detach, move and invade. The main stages of metastasis in epithelial tumours are: EMT (loss of cell-cell and cell-matrix adhesions, loss of polarity, increased ability to migrate, resistance to detachment-mediated apoptosis), detachment from the primary tumour, migration and invasion through the basement membrane into surrounding connective tissue and ECM, invasion of the lymph or blood vessels, extravasation from the blood and lymph vessels, and finally attachment at the new site. Although some of the important mechanisms that regulate these steps have been identified, much still remains to be learned. Rho GTPases as major regulators of actin cytoskeleton rearrangement, adhesion formation and detachment, polarity establishment and gene expression play a prominent role in all stages of metastasis, making them attractive therapeutic targets. Further supporting this is the fact that Rho GTPases are involved in all types of individual and collective cell migration. This is important since metastasis inhibition strategies that target cell adhesion and mesenchymal migration, such as inhibition of MMPs, often fail due to ability of the cells, especially cancer cells, to switch to
amoeboid migration, which does not require ECM adhesion or matrix degradation. Rho GTPases are overexpressed in many types of solid tumours and in some hematological cancers (686). However, more frequent than overexpression is deregulation of the activity of the Rho GTPases, usually resulting in upregulation of their signalling (687). The increase in Rho GTPase activity can be caused by deregulation of upstream signalling (overexpression of GEFs, inhibition of GAPs or GDIs, or aberrant GPCR/RTK signalling for example) or by mutation. It was previously thought that Rho GTPases are not frequently mutated in cancer, however recent sequencing studies identified cancer-specific mutations in RhoA, Rac1 and Cdc42 (687). While the consequences of many mutations remain to be elucidated, some have been shown to result in increased activity of these Rho GTPases by essentially creating a constitutively active or fast-cycling mutants. In turn, higher activity or expression of Rho GTPases has been linked to increased metastasis formation, resistance to therapy and higher mortality. Thus, a number of strategies have been employed to inhibit Rho GTPase signalling. They include inhibition of their interactions with GEFs, with nucleotide, with effectors (or inhibition of their effectors) and spatial regulation of Rho GTPases (688). As Rho GTPases exhibit high sequence homology in the nucleotide binding domain, it is difficult to create specific Rho GTPase inhibitors and therefore few of these have been developed. The best characterized among these is the EHT1864 Rac1 nucleotide exchange inhibitor. In in vitro studies EHT1864 inhibited cell proliferation, adhesion and migration and reduced transmigration of breast and cancer cells through the brain endothelium, indicating its potential for reduction of metastasis to the brain (689). However, it also impaired the cell-cell adhesions in brain endothelial cells (689) and resulted in off-target effects in platelets at concentrations as low as 100µM, raising questions about its specificity in in vitro studies and especially its use as a therapeutic agent (690). More recently, R-ketorolac, an FDA-approved anti-inflammatory agent given after surgery has been shown to allosterically inhibit the nucleotide exchange of Rac1 and Cdc42 (691). In cell-based assays using ovarian cancer cells, this inhibitor decreased cell adhesion, migration and invasion (691). In MMTV-PyMT murine breast cancer model treatment with R-ketorolac significantly decreased tumour development. Importantly, patients who underwent ovarian or breast cancer surgery and were prescribed ketorolac had a higher 5-year survival than those that didn’t, making R-ketorolac a promising anti-tumour agent (692, 693). Specific inhibitors of Cdc42 have also been created and they reduce fibroblast migration and chemotaxis of ovarian cancer cells in vitro (694, 695).
Moreover, there are inhibitors that target the activity of RhoA, Rac1 and Cdc42 simultaneously, such as Berberine (696). Activity of Rho GTPases can also be inhibited by altering their localization. Rho GTPases are most often activated at the cell membrane and they are attached to the cell membrane via post-secondary lipid modifications of the CAAX box. Thus, elimination of these modifications by geranylgeranyl- and farnesyl-transferase inhibitors leads to inhibition of Rho GTPase activation. One such inhibitor that is specific to Rho, PTX-100 completed phase I clinical trials in patients with advanced solid tumours, where according to the manufacturer (Prescient), its safety and tolerability were demonstrated. It is now in pre-clinical trials for RhoA mutant cancers with focus on hematological malignancies. However, none of the inhibitors of Rho GTPase activity are currently in clinical trials. Rho GTPases play pivotal roles in cell proliferation, migration and gene expression pathways making their inhibition in healthy cells particularly concerning and will likely require a very targeted drug delivery approach.

Some of the negative effects of Rho GTPase inhibition can be avoided by targeting specific pathways that these Rho GTPases regulate. This can be achieved by two strategies: the inhibition of interactions with GEFs or with downstream effectors. Perhaps not surprising, the most common targets for small molecule inhibition are RhoA effector ROCK (at least 14 inhibitors) and Rac1/Cdc42 effector PAK (at least 9 inhibitors), which are the main mediators of actin rearrangement downstream of Rho GTPase signaling (697). Many of the PAK inhibitors prevent its interaction with Cdc42. Of these, only PF-03758309 PAK inhibitor has reached clinical trials for treatment of advanced solid tumours, but the phase I clinical trial was terminated due to the drug toxicity (https://www.clinicaltrials.gov/). A number of ROCK inhibitors are now at stage I or II clinical trials for various conditions, but only one for cancer treatment. AT13148 is at the end of phase I clinical trial for advanced solid tumours (698). Importantly, many of these inhibitors, including AT13148 are not ROCK-specific and inhibit other kinases including AKT, PKA and PKC (698), which raises the possibility of off-target effects leading to serious side effects. For example, a ROCK inhibitor fasudil, has been shown to decrease migration and invasion of breast and other cancer cells in vitro and it is approved for treatment of inflammation and hypertension in Japan and China (699-701). However, the request for FDA approval was terminated due to the kidney failure side effect concerns. This points to the need for target-specific inhibitors of downstream targets of Rho GTPases.
Another promising Rho GTPase inhibition strategy is disruption of interaction of the Rho GTPases with GEFs. For example, ZINC69391, an inhibitor for Rac1 GEF Tiam1 has been shown to inhibit breast cancer cell proliferation and migration in vitro and reduce metastasis to the lung in vivo in a mouse model (702). 1A-116, a more potent analogue of ZINC69391 was then developed and it inhibits Rac1 interaction with the P-Rex2 GEF in breast cancer cell lines (702). In glioblastoma cells which exhibit high Rac1 activity, both of these compounds inhibited cell proliferation and cell motility by interfering with DOCK1-Rac1 interaction (703). As is evident from these studies, this approach is often not very selective and signaling from multiple GEFs or different GEFs in different cell types may be inhibited. On the one hand, this may be beneficial, as inhibition of interaction with multiple GEFs should lead to enhanced inhibition of Rho GTPase signaling. On the other hand, it increases the chance of off-target effects. In order to inhibit a specific GEF, its interaction with the upstream activators instead of Rho GTPases can be targeted. For example, I have shown that Plk4 enhances migration of cancer cells through Arhgef1. Moreover, Plk4 kinase domain and PB1-2 domains physically interact with the DH/PH domains of Arhgef1, Plk4 phosphorylates Arhgef1 and enhances its GEF activity. Like Plk4, Arhgef1 is overexpressed in a number of solid tumours including breast (554), bladder (554, 555) and prostate (556) cancers. It is therefore important to determine the role of Arhgef1 in regulation of the cell motility and metastatic potential in these cancers to uncover the role Plk4-Arhgef1 pathway may play in their progression. This knowledge can then be utilized to specifically inhibit the Plk4-Arhgef1-RhoA pathway to reduce cell motility and metastasis of these cancers by inhibition of the interaction between Plk4 and Arhgef1. However, the challenge with inhibiting a specific GEF is the potential compensation for its activity by other GEFs and it is important to test for that when choosing a GEF target for therapy.

Due to the plasticity and the adaptability of cancer cells, and certain redundancy that exists between the GEFs and GAPs as well as the downstream targets of Rho GTPases, a two-pronged therapeutic approach may be needed. For example, in two mouse models of KRAS driven tumours, combined inhibition of Plk1 and ROCK resulted in decreased tumour growth and significantly prolonged survival (704). My work has shown that combining Plk4 inhibitors with inhibitors of Rho GTPases or their upstream effectors is also a therapeutic avenue to be explored.
6.3 Conclusions

Previous work from our laboratory identified Plk4 as a regulator of normal and cancer cell motility in vitro and in vivo and determined a role for Plk4 in actin rearrangement. I hypothesized that Plk4 does this through the activation of small Rho GTPases. My first aim was to determine the effect of Plk4 on activation of RhoA, Rac1 and Cdc42. The data presented here identify Plk4 as an activator of the two major Rho GTPases: RhoA and Rac1 in MEFs and cancer cells. My second aim was to determine the mechanism through which Plk4 activates these Rho GTPases. By screening for the Plk4 consensus phosphorylation motif, I identified 12 potential substrates of Plk4 among Rho GTPase regulators, all of which are GEFs. I then showed that Plk4 physically interacts with Arhgef1 and P-Rex2 GEFs. Functional characterization of these interactions showed that Plk4 activates Arhgef1 but not P-Rex2 to affect cell migration. Indeed, scratch-wound assays showed that the enhanced cell migration induced by Plk4 is at least in part dependent on Arhgef1. I also show that Plk4 phosphorylates full length Arhgef1 and its L-DH/PH fragment and activates Arhgef1 activity towards RhoA. I further characterized the interaction of Plk4 and Arhgef1 and show that the interaction occurs at the DH/PH domains of Arhgef1 and PB1-2 as well as kinase domain of Plk4. Therefore, Plk4 interacts with, phosphorylates and activates Arhgef1 leading to enhanced cell migration. My third aim was to compare the effect of Plk4 to that of its candidate targets and Rho GTPases on cell spreading and shape. To this end, I examined the effect of Arhgef1, RhoA, P-Rex2, and Rac1 on these phenotypes and I show that Plk4 effect on cell spreading is consistent with the activation of the P-Rex2-Rac1 pathway but not the Arhgef1-RhoA pathway. Thus, this study highlights the differences between the spreading and migration phenotypes, which are often thought of as complimentary. In conclusion, the data presented in this thesis support the hypothesis that Plk4 mediates cell migration and spreading through the regulation of Rho GTPase activation. Plk4 inhibitors are entering clinical trials for advanced solid tumours and this study confirms Plk4 and identifies Arhgef1 as a possible marker of tumour aggressiveness and metastatic potential, which if confirmed, may be used to select patients who would benefit most from the Plk4-targeted therapy.
6.4 Future directions

6.4.1 The mechanism of Plk4 regulation of Rho GTPases

Rho GTPases are the major regulators of cell motility, cell proliferation, cell cycle progression and vesicular transport. They are frequently overexpressed or aberrantly activated in many types of human cancers. Their augmented activity is often correlated with increased cancer aggressiveness, increased metastatic potential and poor survival. Rho GTPases are activated by GEFs, inactivated by GAPs and kept inactive by Rho GDIs, however, signals that lie upstream of these regulators are not fully understood. Moreover, there is a complex interplay between the Rho GTPases, where their downstream signalling results in mutual inhibitory and activation loops, best studied of which is the RhoA-Rac1 mutual inhibition. However, much remains to be learned about the interplay between the Rho GTPases and their activation. In chapter 3, I identify Plk4 is an upstream regulator of the activity of the two best studied Rho GTPases: RhoA and Rac1. Although in my early studies in MEFs I was not able to determine the effect of Plk4 on Cdc42 activation, a colleague and I later showed that Plk4 does activate Cdc42 activity in U2OS cells (Figure 6.1). I describe one of the GEFs through which Plk4 activates RhoA and Rac1 but how it can activate both of these Rho GTPases without them inhibiting each other is an interesting question for the future studies. Moreover, how Plk4 activates Cdc42 remains largely unexplored. A lot of this information can be gathered from studying where and when these Rho GTPases are activated by Plk4. In order to examine this, biosensors that allow to visualize the activation of each Rho GTPase in live cells can be utilized, together with the manipulation of the Plk4 levels. As Plk4 is a cell cycle-regulated protein and Rho GTPases are known to play a role in cell cycle progression, the activation of Rho GTPases at different stages of the cell cycle in cell depleted of or overexpressing Plk4 can also be examined to determine at what stages of the cell cycle Plk4 may activate them. The localization of the Rho GTPase activation will provide clues as to which processes regulated by Rho GTPases Plk4 may be involved in. Moreover, it may aid in identifying the mechanism of their activation, as the detection of Plk4 regulation of RhoA activity at the cleavage furrow led to the discovery of Plk4 physical and functional interaction with RhoA GEF Ect2. This information can then be cross-referenced with the known functions and localization of the GEFs with Plk4 phosphorylation motif to identify the GEFs that may be responsible for the Rho GTPase activation in those processes.
Figure 6.1. Plk4 regulates Cdc42 activity in U2OS cells. a) Level of Plk4 mRNA expression prior to active Cdc42 pulldown in U2OS T-REx YFP-Plk4 cells treated with tetracycline, normalised to untreated controls. b) Representative blot of active Cdc42 pulldown in U2OS T-REx YFP-Plk4 cells treated with tetracycline (0.01µg/ml) or untreated. c) Quantification of 4 independent active Cdc42 pulldown experiments that shows an increase in active Cdc42 levels after induction of Plk4 expression with tetracycline, normalized to untreated controls, where the value for active/total Cdc42 ratio was designated as 100%. All data are representative of at least 3 independent experiments. Data are mean + SEM, **p<0.01.
6.4.2 Further delineation of Plk4 interaction with Arhgef1

I have shown that Plk4 regulates cell migration through Arhgef1. Arhgef1-RhoA pathway is activated by the $G_{a13}$ signalling from the GPCR receptors downstream of angiotensin, LPA and thrombin (515). In turn, Arhgef1-Rac1 pathway has been shown to be activated by $G_{i/o}$ signaling downstream of MCP1 (626). It would be important to find out whether Plk4 activity is also enhanced by these pathways. Upon activation, Plk4 autophosphorylates, thus its kinase activity can be determined by looking at the Plk4 autophosphorylation in an in vitro kinase assay in cells that have been treated with these activators and compared to the untreated controls.

One of the limitations of this study, is that most of the experiments, including localization of the GEFs and Plk4 to the cell edge or protrusions and co-IPs have been done in cells overexpressing both Plk4 and the GEF. Only the siRNA experiments involved the endogenous proteins. Thus, whether endogenous Plk4 interacts with endogenous Arhgef1 is still unclear. Given that overexpression may result in ectopic localization of these proteins, it is important to verify their endogenous interaction and the context in which it occurs. Antibodies for the endogenous Arhgef1 are commercially available and have been used successfully by other labs to detect the Arhgef1 protein. I have used one of these antibodies (Cell Signalling, 3669) for immunofluorescence to determine endogenous localization of Arhgef1 and co-IP experiments with overexpressed Plk4. Although the company website states that this antibody can be used for these purposes, the staining was very weak for immunofluorescence and very little protein immunoprecipitated with this antibody in an attempt for co-IP. More optimization of the protocols may be needed or alternatively, another commercially available antibody can be used. Unfortunately, there are no good endogenous Plk4 antibodies that are commercially available and numerous attempts by our laboratory to raise an antibody against Plk4 have been unsuccessful. The only reliable antibody for detection of Plk4 is the antibody against phosphorylated S305 of Plk4 (37) that detects only the active protein and unfortunately it is not commercially available. If our laboratory can get a hold of this antibody, the localization of endogenous active Plk4 and endogenous Arhgef1 in moving and spreading cells can be examined by immunofluorescence to determine if and where the endogenous proteins interact. Knowing this may aid in answering another question that arises from my work: how and where does Plk4 interact with Arhgef1 to affect cell migration and what stops them from interacting
during cell spreading? Our laboratory has shown that Plk4 localizes to cell protrusions during both spreading and migration, thus I hypothesize that during spreading, Arhgef1 is sequestered elsewhere, which prevents its interaction with Plk4. Additionally, localization of other endogenous GEFs that have Plk4 phosphorylation motif can also be tested in a similar manner to determine whether Plk4 co-localizes and thus potentially interacts with any of them.

A common mechanism of activation of GEF activity is their phosphorylation, and Arhgef1 has been shown to be regulated in this manner (467, 469). I have shown in chapter 4 that Plk4 activates Arhgef1 activity towards RhoA and I have also shown that Plk4 phosphorylates Arhgef1. However, whether Plk4 phosphorylation of Arhgef1 affects its GEF activity remains to be determined. In vitro kinase assays can be carried out to examine whether Plk4 phosphorylates Arhgef1 at the predicted site (S488). This can be followed by a GEF activity assay with wild type Arhgef1, S488A (abolishes phosphorylation) and S488D (phosphor-mimic) Arhgef1 mutants in cells that overexpress Plk4 or not.

6.4.3 The mechanism of Plk4 regulation of cell spreading

Our laboratory has shown that Plk4 localizes to the periphery of spreading cells and induces protrusion formation, thereby enhancing cell spreading (101, 102). We have also shown that this effect of Plk4 is at least in part mediated through its interaction with Arp2 (102). Intriguingly, Arp2/3 complex is one of the major downstream effectors of Rac1 signalling, while Rac1 is the major driver of cell spreading. Although our laboratory has shown that in cell migration, inhibition of Rac1 was not enough to suppress Plk4-mediated migration (102), the results presented in my thesis indicate that cell migration and spreading are not always regulated by the same mechanisms. Moreover, I show that Plk4 increases Rac1 activity in MEFs and cancer cells and that Plk4 effect on cell spreading is consistent with that of Rac1. To determine the dependence of Plk4 spreading phenotype on Rac1 activity, spreading in cells depleted of Rac1 or treated with Rac1 inhibitors, combined with Plk4 overexpression can be examined. Additionally, I show that one of the mediators of Plk4-Rac1 pathway may be P-Rex2 GEF and future studies can test this hypothesis via spreading assays in cells overexpressing Plk4 but depleted of P-Rex2. I also identified 8 other Rac1 GEFs that can be tested for their effect on spreading. In addition, spreading is heavily dependent on cell-matrix adhesion and adhesion formation is also Rac1-
dependent. Therefore, in the future, adhesion assays can be carried out to determine the effect of Plk4 on adhesion formation. Spreading cells can also be stained for the early (paxillin, FAK, talin) and mature (myosin II) adhesion markers to determine if Plk4 may play a role in adhesion maturation and turnover. These findings would also be significant for further delineation of the role of Plk4 in metastasis formation, as metastasis formation depends on primary tumour cell attachment to the secondary site.

6.4.4 The effect of Plk4 on immune cell migration and function

Our group has described the effect of Plk4 on regulation of migration and invasion of cancer cells as well as MEFs and HEK293T cells (101, 102). However, what effect Plk4 may have on the function of immune cells has not yet been determined. Interestingly, Plk4 has been initially reported to be highly expressed in lymphocytes (36). Moreover, Arhgef1, the downstream effector of Plk4 in cell migration, is also most highly expressed in cells of hematopoietic origin (510, 511) and has been reported to affect their migration and function (471, 532-534). Thus, future studies can focus on determining the effect of Plk4 on the proliferation, spreading, migration and function of lymphocytes, macrophages or neutrophils and whether any observed effects are mediated through Arhgef1. To study the motility of the immune cells transwell migration/invasion assays can be used, as well 3D migration assays or assays mimicking the immune cell invasion through endothelium to reach the bloodstream. To study the effect of Plk4 or Arhgef1 on immune cell function, analysis of cell metabolism, antibody production or cytokine secretion can be performed. Moreover, to determine the effect of Plk4 inhibitors on immune cell function in vivo, immune cells from mice treated with these inhibitors can be extracted and subjected to ELISpot assays (ELISA and flow cytometry) which can detect immune cell secreted cytokines or antibodies.

6.4.5 Effect of Plk4 interaction with Arhgef1 on migration and invasion in 3D and in vivo

I have shown that Plk4 regulates cell migration at least in part through Arhgef1 in U2OS cells. However, this was tested only using the 2D scratch-wound assay. Although 2D assays have proven useful in studying cell migration, 3D assays mimic the in vivo environment more closely and many studies have shown that gene expression, proliferation, survival, adhesion dynamics,
mechanosensing, migration and invasion are different in 2D and 3D environments (705-707). For example, whereas actomyosin contractility can be dispensable for motility on 2D surfaces, it is essential for 3D migration (708). Since actomyosin contractility is regulated by RhoA, its activity during 3D migration is increased, while Rac1 activity is decreased as compared to migration in 2D, which allows for increased directionality of migration (709). It is therefore important to determine the effect of the Plk4-Arhgef1 pathway in the 3D and in vivo settings.

Our laboratory has successfully used a spheroid assay to test for the 3D invasion of HeLa cells depleted of Plk4, where we show that Plk4 depletion results in reduced cell invasion into the Matrigel (Lee, in preparation). We can use this assay to study the invasion of cells overexpressing or depleted of Arhgef1 to determine whether manipulation of the Arhgef1 levels have the same effect on invasion in 3D as they do on 2D migration. If the same effect is confirmed, we can then overexpress Plk4 and deplete Arhgef1 to test for the dependence of Plk4 phenotype on Arhgef1. Moreover, we have only studied the effect of Plk4 on mesenchymal cell migration and it is important to determine whether Plk4 and Arhgef1 have any role in amoeboid cell migration. Amoeboid cell migration is mostly driven by RhoA activation and Plk4 was shown to increase Arhgef1 activity towards RhoA. Thus, to examine the role of Plk4 and Arhgef1 in amoeboid migration, cells overexpressing GFP-Plk4 or GFP-Arhgef1 (or depleted of these proteins) can be suspended in the 3D matrix such as Matrigel or collagen and the phenotype of migrating cells can be examined via immunofluorescence for cell roundness and bleb formation, the features of amoeboid motility. Cell migration speed can also be examined, as amoeboid-migrating cells move much faster than mesenchymally-migrating ones. Determining this will further advance our understanding of the role of Plk4 and Arhgef1 in cancer cell migration and metastasis formation, as many cancer cells are known to adopt amoeboid-type migration, especially in harsher conditions such as during hypoxia that is often observed in solid tumours.

Furthermore, it is necessary to determine whether Plk4 enhances cell migration through Arhgef1 in vivo. To do this, the effect of Arhgef1 on cell migration in vivo should be examined first. This can be done by injecting NOD-SCID mice subcutaneously, or in case of breast cancer cells into the mammary pad, with tumour cells depleted of Arhgef1 and examining their invasion though peritoneum and their metastatic potential. Moreover, a xenograft model with Plk4 depleted cells
that our laboratory has previously used (102) can be utilized to determine the GEF activity of Arhegef1 in tumours or metastasis formed by Plk4 depleted vs control cells. Cells from these tumours or metastases can be extracted and GEF activity pulldown assays like the one I used in chapter 4 can be carried out. These assays will help identify whether Arhegef1 may be a suitable, alternate to Plk4 target for therapy in patients with advanced and/or aggressive tumours with high metastatic potential.

6.4.6 Targeting Plk4-Arhegf1 pathway as a therapeutic strategy

Plk4 is overexpressed in breast (115, 117), colorectal (114), pancreatic cancers (115), glioblastoma (115), neuroblastoma (588) and multiple other tumour types and their list keeps growing. Moreover, high Plk4 expression in breast cancer and neuroblastoma is predictive of poor patient survival (115, 117, 588). Together with the recent discovery that Plk4 overexpression and centriole amplification are sufficient to induce tumorigenesis (601), the rationale for the development of Plk4 inhibitors for therapeutic purposes is clear. However, centrinone—the highly specific Plk4 inhibitor is not yet orally available (62). Thus, future studies can focus on generating an orally available version of this drug and once available, it would need to be thoroughly tested in pre-clinical studies. The CFI-400945 inhibitor, on the other hand is orally available and is undergoing phase I clinical trials in patients with advanced solid tumours (61, 129). However, it has also been shown to inhibit Arora B activity (61). Aurora B is a mitotic checkpoint kinase and as such can have significant anti-tumour activity, thereby making it difficult to attribute any anti-tumour effects of the drug to Plk4 alone. An alternative approach to Plk4 inhibition is identifying its key downstream effectors and pathways that drive tumour progression and inhibiting them. Here I identified Arhegf1, a downstream effector of Plk4 that mediates Plk4 effect on RhoA activation and cell migration. Therefore, the role that Plk4-Arhegf1 pathway may play in cancer progression can be examined in future studies. First, the expression and activity of Arhegf1 in patients whose tumours exhibit high Plk4 expression needs to be examined. The evidence presented in this thesis suggests that the activation of Arhegf1 in these tumours may be increased as compared to those that do not overexpress Plk4. If this is the case, the effect on tumour aggressiveness, therapy resistance, propensity to metastasis formation and patient survival of this higher activity of Arhegf1 can then be determined. If Arhegf1 activity status correlates positively with any of these parameters, this would identify Arhegf1 as a
potential therapeutic target. Attempts to inhibit Rho GTPase-GEF interactions so far have led to the inhibitors that are not GEF-specific and inhibit the interactions with multiple GEFs. Therefore, in order to inhibit Arhgef1 and Plk4-mediated motility specifically, its interaction with Plk4 can be targeted. For that, a 3D model of Plk4 interaction with Arhgef1 would be helpful, to determine whether there is a certain pocket or fold involved in the Plk4-Arhgef1 interaction that can be targeted by a small molecule inhibitor.
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253


259


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Section 1.1 and Figure 1.1


Figure 1.2b

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Figure 1.3


Figure 1.4

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Figure 1.6a

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